Item Number: 4 Publisher: 2002.
9610 Description: Molecular and genetic studies into the formation of lateral roots in Eu [manuscript]/Pelosi, A
Line Item Note: AMIS H24/3521 Thesis (Ph.D.)--Monash University, 2002
Vendor Title/Number:
Copies: 1 Cost: 0.00 Adjustments: 0.00 Less Prepay:

AMENDMENTS

Both examiners have recommended that this thesis be passed and the degree of Doctor of Philosophy be awarded without further examination. I am gratefully appreciative of the constructive comments they provided, and to that end, I address the main points raised as follows:-

Table of Contents 2.4.14.1: "digestion" for "digestin".
4.4.2.1: "nucleotide" for "nu.cleotide".
7.2.5: "based" for "dased".

p14 line 23: "...*Aux/IAA* transcripts do not..." should read "...*Aux/IAA* transcripts does not...".

p17 line 10: "... *TobRB7*, *RSI-1* and *LRP1*, is..." should read "... *TobRB7*, *RSI-1* and *LRP1*, is...".

p33 line14: "researchers" for "researches".

p135 Comment: As mentioned in the text, the clones listed in Table 5.3 are possible false positives. This is more likely to be the case for clones showing significant sequence homology to human DNA. The isolation of these sequences may have arisen by amplification of contaminating human DNA.

p164 paragraph 2, line 7: Delete "the" and read "Of these...".
line 17: "give" for "gives".

p184 line 12: "...*Srab1* and *Srab2* from soybean is..." should read "...*Srab1* and *Srab2* from soybean are...".

p195 line 33: Insert "by" and read "...conducted by Sussex".

p240 line 1: Replace "needs" with "need".

p252 line 9: Delete "of" and read "...during LR development...".
line 19: Delete "and" and read "...genes associated with...".
line 22: Delete "and " and read "...achieved by adequate...".

p255 line 18: Replace "random" with "restriction".

p256 Section 7.3.2, paragraph 2: The final sentence did not convey the intended meaning and should be replaced by the following. "However, *Arabidopsis* is not a practicable alternative if searching for genes with specialised functions such as nitrogen fixing in legumes, and genes associated with fibre production in woody species such as *E. globulus*.

PA 24/33 21

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON 20 December 2002

[redacted]
Sec. Research Graduate School Committee

Under the copyright Act 1968, this thesis must be used only under the normal conditions of scholarly fair dealing for the purposes of research, criticism or review. In particular no results or conclusions should be extracted from it, nor should it be copied or closely paraphrased in whole or in part without the written consent of the author. Proper written acknowledgement should be made for any assistance obtained from this thesis.

DEPARTMENT'S RATIFICATION

This is to ascertain that the Department has no objection to the candidate's options regarding access to the Library thesis copy. If so, please sign below and return the completed form to the **Monash Graduate School Research Services, Building 3D, Clayton Campus.**

Supervisor's signature: .....[redacted]..... Date: 30/7/02

(Please print name).....Prof John D Hamill.....

MOLECULAR AND GENETIC STUDIES INTO THE FORMATION OF LATERAL ROOTS IN *EUCALYPTUS* AND *ARABIDOPSIS*

A thesis submitted for the degree of Doctor of Philosophy

Assunta Pelosi
B.Sc. (Hons)

Department of Biological Sciences
Monash University

July, 2002

TABLE OF CONTENTS

ABSTRACT

Formation and proliferation of the root system is important for all land plants and is often a limitation to successful clonal propagation programs involving many species important in horticultural and forestry industries. High frequency of lateral root (LR) formation in many plants can be induced by treatment with exogenous auxin. The main objectives of this study were to use molecular approaches to isolate and characterise genes involved in LR development in *Eucalyptus globulus*, which could then serve as a resource for further studies aimed at a deeper understanding of adventitious and LR formation in commercial eucalypts. An additional aim was to explore the use of *Arabidopsis thaliana* to recover mutants impaired in the root formation process which would also be worthwhile for future studies aimed at understanding the molecular basis for root proliferation and growth.

An optimised method for differential display (DDRT-PCR) was devised and successfully applied in screens for *E. globulus* sequences that are differentially expressed during auxin-induced LR formation. A cDNA subtraction was also employed to supplement and enhance the population of mRNAs represented in these molecular screens. The isolated cDNA fragments (ESTs) were grouped into homologous families based on sequence homologies. A representative clone from each family was then examined further to characterise the temporal and tissue specific expression patterns by Northern blot analysis. This analysis identified 15 cDNA sequences deemed to be differentially expressed during LR development. When searched against the current nucleotide and protein databases, these LR ESTs were found to represent genes involved in an array of cellular and developmental processes including cell wall development; transcriptional activation; plant nutrition; protein synthesis and processing; protein trafficking and secretion. A discussion of the possible roles played by each of the genes represented by the 15 LR ESTs is presented, drawing on the expression and sequence data obtained from this study, together with any relevant published reports in the literature. The efficacy of the two molecular based approaches used to identify these genes was also considered and discussed in the light of more recent technical developments involving genomic analysis using DNA microarray technology.

A genetic investigation was directed towards the isolation of LR mutants of *Arabidopsis* with altered growth responses to exogenously supplied phytohormones. An *in vitro* screening method was devised and employed to identify mutations that affect LR development specifically at the stage of LR emergence and growth. A preliminary phenotypic characterisation and segregation analysis was conducted on two such putative mutants.

Finally, a model was proposed to explain the possible interactions that the genes identified in this study may have during three stages in the development of auxin-induced LRs. In future, these gene sequences may be valuable in breeding programs as selectable markers for rooting in *Eucalyptus*. The knowledge gained from the present study should prove useful in the mid-long term, to help address recalcitrant rooting and poor field establishment in micropropagated eucalypts - two major problems of real importance to clonal forestry programs and essential to a sustainable global pulp and paper industry.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution. To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

Assunta Pelosi

ACKNOWLEDGEMENTS

I would like to extend sincere appreciation to the people who have made an invaluable contribution to the completion of this thesis.

A great many thanks go to my supervisor Professor John Hamill for his guidance, support, and encouragement throughout the course of this project. His willingness to give time and chocolate frogs was very much appreciated.

I am also grateful to Dr David de Little (North Forests Eucalypt Research Centre), Dr Peter Volker (ANM Forest Management) and Mr Phil Whiteman (APM Forests Pty Ltd) for their interest and for arranging financial support to assist the undertaking of this research program.

The friendship and support provided by past and present members of the former Department of Genetics and Developmental Biology was precious. I give particular mention to my little lab buddy Elaine for great conversation, good taste in radio stations and a mutual appreciation of fine chocolate. Thanks also to Angela, Penny, Yupynn, Cecilia, Stan, Gawain, Eugene and Richard for their exchange of ideas, technical support and friendship.

In the world outside, I am immensely thankful to my family for their incredible patience, and to Sean and Scooby for providing the much needed distractions.

I would also like to acknowledge that for the duration of this study I was supported financially by an Australian Postgraduate Award (Priority).

ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-*y*-*y*-dimethylallylaminopurine
aa	Amino acid
AGP	Arabinogalactan protein
AR	Adventitious root
ARP	Adventitious root primordium(a)
BAP	6-benzylaminopurine
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CRT	Calreticulin
DDRT-PCR	Differential display reverse transcription polymerase chain reaction
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
GC-MS	Gas chromatography coupled to mass spectrometry
GS	Glutamine synthetase
HF	Hormone free
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPA	Indole-3-propionic acid
LR	Lateral root
LRP	Lateral root primordium(a)
mRNA	Messenger ribonucleic acid
NAA	α-naphthaleneacetic acid
nt	Nucleotide
ODCI	Ornithine decarboxylase inducible
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFP	Pyrophosphate dependent phosphofructokinase
rabGAP	GTP activating protein of rab-like GTPase
RNA	Ribonucleic acid
rpL2	Ribosomal protein L2
rRNA	Ribosomal ribonucleic acid
SEM	Significant error of the mean

UHQ	Ultra high quality
UTR	Untranslated region
VAMP	Vesicle associated membrane protein

CHAPTER ONE

Introduction:

Lateral Root Formation in Higher Plants

1.1 INTRODUCTION

The root system is of fundamental importance to land plants for anchorage, storage, uptake and transport of water and nutrients from the external soil environment, the synthesis of phytohormones, and their interaction with bacteria and other organisms in the rhizosphere. While the growth and development of root systems have been well described at both an anatomical and physiological level (Esau 1977; Blakely *et al.*, 1982; Dolan *et al.*, 1993), our understanding of the molecular and genetic mechanisms that regulate the fundamental events responsible for lateral root development is still rudimentary. Most notably, although advances have been made in recent years, the processes of initiation and establishment of root apical meristems, cell patterning, cell differentiation, cell division and cell growth, are poorly understood (Malamy and Benfey, 1997a and 1997b). In most plants, the primary root is the first part of the plant to appear after germination (Dolan *et al.*, 1993). Along the length of the *Arabidopsis* root, four phases of differentiation can be distinguished and classified into zones, each with broadly defined cellular activities (Benfey *et al.*, 1993; Dolan *et al.*, 1993). At the root tip is the meristematic zone, where proliferative cell division is maintained, and cell fate and polarity is determined (Fig. 1.1A). The elongation zone is located behind the root tip and is defined as the site of cell division and cell expansion. Cells become specialised and root hair development occurs in the differentiation zone. Dubrovsky *et al.* (2000) reported that LRP are also initiated in this zone, 3-8 mm behind the root tip. The mature region of the root is the area where LRs emerge and new meristems are initiated. All roots that branch from this embryonically derived root are collectively referred to as lateral roots (LRs).

The many advantages of *Arabidopsis thaliana* have made it a reference species for developmental processes, including the study of root development. The roots of *Arabidopsis* have a remarkably simple and consistent root morphology and cellular organisation that is also evident in the LRs (Schiefelbein and Benfey, 1991; Dolan *et al.*, 1993). The uniform, radial pattern in the mature root comprises four cell files, the epidermis, cortex, endodermis and pericycle, which encircles the central vascular cylinder, also known as the stele (Fig. 1.1B). The four files extend vertically along the length of the primary root to their respective meristematic initials in the apical meristem (Dolan *et al.*, 1993). Each file is composed of a single cell layer, with the cortical and endodermal layers invariably consisting of eight cells each throughout root development. The number of cells contained within the pericycle and epidermal layers is more variable, with averages of 12 and 18 respectively. LRs have been reported to possess a higher degree of variability in the number of cells in each layer, ranging from 7 to 11 in the cortex, and 7 to 10 in the

endodermis (Dolan *et al.*, 1993; Malamy and Benfey, 1997b). Dolan *et al.* (1993) suggested that this increased variability may be due to less stringent control of the cell division patterns that initiate the root meristem during vegetative development of the LR. This contrasts with the mechanisms that govern meristem formation in the primary root during embryogenesis which appear to be under stricter control.

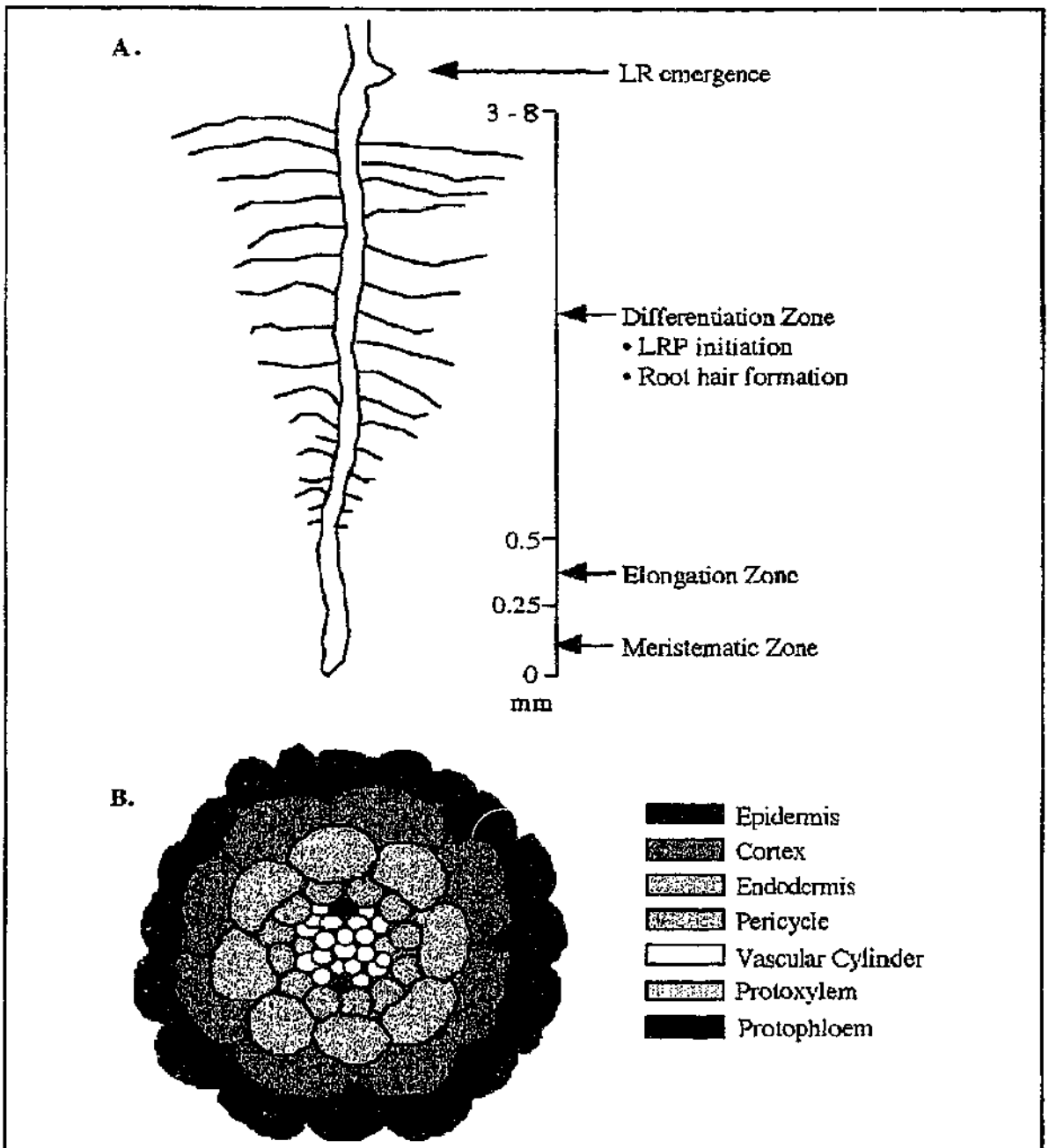


Figure 1.1: Schematic representation of (A) a longitudinal view of the *Arabidopsis* root (adapted from Schiefelbein and Benfey, 1991) and (B) a cross section of the *Arabidopsis* root (adapted from Dolan *et al.*, 1993). Along the length of the root, the meristematic and elongation zones each span 0.25 mm. The differentiation zone extends 3-8 mm from the root tip. LRs emerge in the mature region of the primary root. Encircling the central vascular cylinder from the inside out are the pericycle, epidermal, cortical parenchyma and epidermal cell layers.

1.2 THE PHYSIOLOGY OF LATERAL ROOT DEVELOPMENT

A substantial proportion of the root system in most plants is contributed by the LRs. LR formation is of interest from a plant growth perspective because it involves the regulation of numerous sequential developmental processes, to allow the morphogenesis of a new structural organ arising from differentiated tissues of the parent root. In normal root growth, LRs differentiate at regular intervals along the length of the primary root, requiring the coordinated control of a number of key developmental events including signal transduction, cell division, cell differentiation, the initiation, establishment and organisation of new meristems, cell expansion and maturation.

In primary roots undergoing normal active growth and metabolism, LRs initiate acropetally from the pericycle, the outermost single cell layer surrounding the vascular stele, which contains the phloem, xylem and stelar parenchyma cells (Blakely *et al.*, 1982; Dolan *et al.*, 1993). Initiation of the lateral root primordium (LRP) usually occurs following a series of asymmetric cell divisions in the differentiation region of the primary root (Dubrovsky *et al.*, 2000). Depending on the species, LRP are initiated in competent pericycle cells positioned near the protoxylem or protophloem poles (Blakely *et al.*, 1982; Sussex *et al.*, 1995a). In both *Arabidopsis* and radish, LRP initiate from pericycle cells directly adjacent to the two protoxylem poles (Blakely *et al.*, 1982; Laskowski *et al.*, 1995; Dubrovsky *et al.*, 2000), however not all pericycle cells at this location will initiate a LRP.

Following an anatomical and cytological study of LR formation in radish, Blakely *et al.* (1982) speculated that spontaneous LRP initiate from a subset of pericycle cells that undergo uninterrupted cycling after leaving the apical meristem. In contrast, other pericycle cells are arrested in the G2 phase of the cell cycle. A subsequent study by Casero *et al.* (1995) suggested that in radish, sunflower, corn and carrot, almost all pericycle cells continue dividing in the differentiation zone after displacement from the apical meristem, but only the cells destined to initiate the LRP undergo the formative asymmetric anticlinal division that commits one of the daughter cells to LRP initiation. Pericycle cells that acquire the ability to initiate a LRP are referred to as LRP founder cells and have been observed to divide more frequently than the other cells of the pericycle (Casero *et al.*, 1995). These observations were consistent for each of the plants studied, namely radish, sunflower, and carrot. A more recent study conducted in *Arabidopsis* by Dubrovsky *et al.* (2000) supported these findings by showing that pericycle cells located immediately adjacent to the protoxylem poles undergo continual progression through the cell cycle. However, only some of these cells are committed to the asymmetric divisions that initiate

LRP. Dubrovsky *et al.* (2000) showed that LRP initiation occurs in the young differentiation zone of the root. No new spontaneous LRP were initiated in the older mature root zone, which appeared to be mitotically quiescent. It is still unclear how the fate of the LRP founder cells is determined.

Studies with a number of plants (e.g., *Arabidopsis*, radish, carrot and sunflower) have shown a general consistency in the process of LRP formation, suggesting that the sequence of cell divisions is highly organised and consistent during development of the LRP in dicots (Blakely *et al.*, 1982; Casero *et al.*, 1995; Malamy and Benfey, 1997a and 1997b; Dubrovsky *et al.*, 2000). Pericycle cells committed to the formation of an LRP develop enlarged nuclei and acquire increased cytoplasmic density (Blakely *et al.*, 1972; Blakely *et al.*, 1982; Vuylsteker *et al.*, 1998). In *Arabidopsis*, these LRP founder cells first undergo rapid anticlinal divisions, and then expand radially before dividing periclinally, to generate the dome shaped LRP (Dolan *et al.*, 1993; Laskowski *et al.*, 1995; Malamy and Benfey, 1997b; Dubrovsky *et al.* 2000). Dubrovsky *et al.* (2000) calculated that the formative asymmetric cell division that directs the pericycle cells to initiate an LRP represents the sixth or seventh cell division cycle following displacement from the meristem. In a follow up study, they estimated that a minimum of three founder cells was required to initiate a single LRP in *Arabidopsis* (Dubrovsky *et al.*, 2001).

The LRP increases in length and emerges from the primary root mainly by cell expansion, rather than cell division (Malamy and Benfey, 1997b). At or just after emergence, the LR meristem is generated *de novo* from discrete cells within the LRP (Dolan *et al.*, 1993; Laskowski *et al.*, 1995; Malamy and Benfey, 1997b). From this point the LR meristem is active and growth of the new LR is governed by cell division at the apex (Malamy and Benfey, 1997b). The resulting new LR has an organised cell structure and meristem that is equivalent to that of the primary root (Dolan *et al.*, 1993). The LR elongates and matures, maintaining indeterminate growth and becoming itself capable of differentiating new LRs (Benfey *et al.*, 1993).

1.2.1 The Role of Auxin in Lateral Root Development

Auxin plays a critical role in influencing and modifying a myriad of plant developmental and growth processes including, photo- and gravitropism, cell viability, cell senescence, vascular tissue development, cell expansion, cell wall synthesis and regeneration, morphogenesis, embryo patterning, apical dominance, fruit ripening, deetiolation, root

initiation, root hair formation and lateral branching of shoots and roots (O'Brien *et al.*, 1985; Theologis, 1986; Davies, 1987; Hobbie *et al.*, 1994; Masucci and Schiefelbein, 1996; Eckardt, 2001). Effective levels of auxin are controlled by regulated biosynthesis and degradation, as well as its polar transport to the site of action and its perception at this location (Jones, 1998; Eckardt, 2001). It can act either alone or in combination with other phytohormones to regulate cell division, cell differentiation and cell elongation.

Auxin, of which IAA is the most widely occurring natural form, is mainly synthesised in meristematic regions of the shoot and readily transported to the root predominantly through cells within and next to the stele (Russell, 1977). Recent evidence shows that the cell to cell polar transport of auxin is mediated by influx and efflux carrier molecules acting at the plasma membrane (Geldner *et al.*, 2001). These carriers move the auxin into and out of cells in a directional fashion that is thought to be dependent upon the asymmetric distribution of the efflux carriers (Estelle, 2001). Geldner *et al.* (2001) showed that a putative auxin efflux carrier (PIN1) from *Arabidopsis*, cycles between the plasma membrane and an endosomal site during auxin transport. Within the root itself, polar auxin transport mobilises auxin acropetally to the root apex, and basipetally towards the hypocotyl-root junction (Jones, 1998; Rashotte *et al.*, 2000) to its primary sites of action which appear to be at the plasma membrane (Barbier-Brygoo, 1995).

PINOID (PID) serine-threonine protein kinase, is suspected of enhancing polar auxin transport during organogenesis (Christensen *et al.*, 2000; Benjamins *et al.*, 2001). Originally identified in *Arabidopsis*, plants carrying mutations in *pid* were found to have slightly reduced polar auxin transport (Bennett *et al.*, 1995). Overexpression of *PID* under the control of CaMV 35S promoter often caused collapse of the primary root meristem resulting in enhanced LR proliferation (Benjamins *et al.*, 2001). Meristem organisation and primary root growth in these seedlings was rescued by treatment with the polar auxin transport inhibitor, naphthylphtalamic acid, suggesting that PID may positively regulate polar auxin transport (Benjamins *et al.*, 2001).

Cell elongation and transcriptional activation of specific auxin-induced genes have been well documented as rapid processes implicated in auxin regulated plant growth (Theologis, 1986; Rayle and Cleland, 1992). Auxin is believed to stimulate cell elongation by promoting the activity of a proton pumping ATPase at the plasma membrane (Theologis, 1986; Rayle and Cleland, 1992). This action results in the acidification and subsequent loosening of the cell wall at particular points to facilitate elongation via turgor pressure. Expression of these early auxin responsive genes can occur as rapidly as within 5 min of exogenous application of auxin, well before the onset of cell elongation (Theologis,

1986; McClure and Guilfoyle, 1987). Theologis (1986) proposed that the initiation of cell elongation and the associated cell wall extension is mediated by proteins encoded by the early auxin regulated mRNAs.

It has been suggested that auxin also acts to coordinate such a diverse array of developmental processes by regulating the transcription of specific genes. Selective changes in gene expression in a particular plant cell or tissue can be induced by changes in the local concentration of auxin (Theologis 1986; Guilfoyle *et al.*, 1993; Barbier-Brygoo, 1995; Abel and Theologis, 1996). Presumably, the products of these genes are likely to direct or play a part in most auxin-induced developmental responses during plant growth.

Interaction between the endogenous plant phytohormones is also likely to play a central role in all aspects of normal root development, including LR and adventitious root (AR) formation, with the strongest effects attributed to the interaction of auxin, cytokinin and ethylene (Torrey, 1962; Schiefelbein and Benfey, 1991; Hobbie *et al.*, 1994). However, a clear understanding of the precise mechanisms of auxin action and its interaction with other phytohormones to direct these, and other plant growth and development processes is still lacking.

When auxin is supplied exogenously, pericycle cells at the xylem poles in the mature region of the primary root can be stimulated to commence cell division (Blakely and Evans, 1979). These previously quiescent pericycle cells, arrested at G2 of the cell cycle, can resume active cell division and progress rapidly through the M phase of the cell cycle to divide and enable development of a LRP (Blakely and Evans, 1979).

Schiefelbein and Benfey (1991) proposed that once the auxin signal is recognised by pericycle cells they are stimulated to divide by the activation of an individual cell, which in turn activates others. Alternatively, a number of cells are activated simultaneously. As a result, treatment with an appropriate concentration of auxin can activate a subset of cells in the pericycle that participate in LR initiation. Associated studies have determined that the action of auxin transport inhibitors, elevated levels of cytokinin, and/or inhibitors of RNA and protein synthesis can inhibit the formation of LRs (Wightman *et al.*, 1980; MacIsaac *et al.*, 1989; MacIsaac and Sawhney, 1990; Pelosi *et al.*, 1995a and 1995b). Together, these observations indicate that auxin transport, phytohormone interactions, gene expression and protein synthesis all play important roles in LR formation within the plant.

Physiological studies in a number of plant species have shown that the application of plant phytohormones can greatly affect or modify the normal patterns of LR development, including the processes of LRP induction and LR emergence and outgrowth (Blakely *et al.*, 1982; MacIsaac *et al.*, 1989; Pelosi *et al.*, 1995a and 1995b). Studies in *Haplopappus ravenii* (Blakely *et al.*, 1972), radish (Laskowski *et al.*, 1995), pea (Wightman *et al.*, 1980), lettuce (MacIsaac *et al.*, 1989), tomato (Taylor and Scheuring, 1994), *Eucalyptus globulus* (Pelosi *et al.*, 1995a and 1995b) and chicory (Vuylsteker *et al.*, 1998) have shown that while the frequency of LR initiation can be increased by treatment with high concentrations of exogenous auxin, prolonged exposure to such levels (<24-48 h) can block root growth by inhibiting LR emergence.

These and similar studies suggest that there are three distinct phases in the formation of auxin-induced LRs;

(1) LRP initiation and growth
(2) LR emergence
(3) LR meristem activation to form an independent LR unit.

In normal roots, Phase 1 is thought to occur in the presence of high levels of exogenous auxin in pericycle cells. Upon recognition of the auxin signal, pericycle cells are stimulated to divide and proliferate to form LRP (Celenza *et al.*, 1995). After it has initiated, auxin is no longer required for development of the LRP beyond the 3-5 cell layer stage. This was demonstrated by Laskowski *et al.* (1995) who observed that a LRP contained within a 0.5 mm root explant from radish or *Arabidopsis* continued development into a LR when cultured on hormone free medium.

Phase 2 involves the conversion of LRP to actively growing LRs. As the size of the LRP increases to 8-10 cell layers, its mitotic index declines at the point of LR emergence from the primary root (MacLeod, 1972; Laskowski *et al.*, 1995). Emergence occurs primarily through expansion of the existing cells in the LRP, rather than through cell division (Malamy and Benfey, 1997b). At this point, if exogenous auxin concentration is maintained at a high level, emergence of the LR is inhibited. In the absence of exogenous auxin, the LRP are able to penetrate the cortical and epidermal cell layers, to form a new LR (MacIsaac *et al.*, 1989).

Once the primordium has formed, the LR enters Phase 3 of development. A LR meristem is initiated from a subset of cells undergoing active cell division at the apex of the LRP. Typically, this stage is complete within 24-48 h in most species that have been studied (Laskowski *et al.*, 1995; Sussex *et al.*, 1995a). Upon emergence of the LR, auxin is again required to produce a functional LR meristem. The *alf3 Arabidopsis* mutant

shows arrested growth of both the primary and LRs shortly after emergence (Celenza *et al.*, 1995). When *alf3* plants are cultured on IAA or indole (an IAA precursor), the mutant phenotype is rescued and the meristem remains active. This observation suggests that a continuous supply of auxin is required for the establishment and maintenance of a functional meristem. Arrested growth in newly emerged LRs from 0.5 mm sections noted by Laskowski *et al.* (1995) also suggests this. Malamy and Benfey (1997a) suggested that given the autonomous nature of the early LRP, and the rescue of *alf3* by indole, the auxin required for meristem establishment and maintenance is probably synthesised within or in close proximity to the LRP.

1.2.1.1 Auxin and Cytokinin Interaction During Lateral Root Formation

Like auxin, cytokinin also plays a significant regulatory part in LR initiation and development, which appears to be contingent upon its concentration relative to that of auxin. In early work, Torrey (1962) proposed that an endogenous cytokinin gradient exists at an appropriate level within the primary root that serves to regulate LR induction. In this model, the cytokinin levels at the root tip are present at a concentration completely inhibitory to LRP formation, but which progressively decreases in a basipetal direction along the length of the primary root. Experimental evidence was obtained from chromatographic bioassay and GLC techniques using extracts taken at progressive intervals along the length of the pea root (Short and Torrey, 1972; Wightman and Charbonneau cited in Wightman *et al.*, 1980). In contrast, internal auxin levels are highest at the base of the root and decline progressively towards the root apex (Torrey, 1962). Wightman and Thimann (1980) suggested that the basipetal movement of cytokinin interacts with the acropetally moving auxin to produce a 'window' of auxin/cytokinin which determines the location of the LR initiation zone along the primary root.

Experimentally, treatment with cytokinin can exert responses in root development which appear to be antagonistic to the stimulatory effect of auxin. Generally, application of cytokinin blocks both endogenous and exogenous auxin-induction of LR formation (Wightman *et al.*, 1980; MacIsaac *et al.*, 1989). This inhibitory effect has been observed in a range of species when concentrations of cytokinin greater than ~10^{-6} M were supplied to root explants or whole seedlings, in the presence of auxin levels which would normally induce LRP formation. In these circumstances both LR initiation and/or emergence were suppressed by cytokinin (Wightman *et al.*, 1980; Pelosi *et al.*, 1995a and 1995b; MacIsaac *et al.*, 1989).

1.3 TRANSCRIPTIONAL AND TRANSLATIONAL REGULATION OF LATERAL ROOT FORMATION

Auxin induction of LR and AR initiation is accompanied by detectable changes in translatable RNA, general soluble proteins and some specific proteins associated with cell division such as tubulin. Studies in french bean and mung bean have shown increases in translatable mRNA and protein content during auxin-induced root initiation and development (Kantharaj *et al.*, 1985; Dhindsa *et al.*, 1987). Exposure to the inhibitor of transcription, actinomycin-D, within the first 36-40 h of IBA induction prevented root initiation in french bean (Kantharaj *et al.*, 1985).

Studies in lettuce showed that the protein content in extracts from NAA-treated roots was elevated compared to the levels detected in untreated root extracts (MacIsaac and Sawhney, 1990). The increase in protein levels was first detected 12 h after auxin treatment, reaching maximum levels between 36-72 h. When the translation inhibitor cycloheximide was supplied to roots within 24 h of NAA treatment, both LRP and protein content was suppressed.

In roots of *E. globulus*, auxin-induced LR initiation was totally suppressed following treatment with actinomycin-D or cycloheximide at 1 mM either prior to, or during the initial 48 h of IBA induction (Pelosi *et al.*, 1995a and 1995b). Collectively, these observations indicate that auxin-induced alterations in LR differentiation involves changes in gene expression and most likely requires the coordinated transcription and translation of essential genes.

1.4 MOLECULAR AND GENETIC STUDY OF LATERAL ROOT FORMATION

Studies over the last decade have involved both genetic and molecular approaches to understand the developmental processes that regulate root formation (Schiefelbein and Benfey, 1991; Hobbie and Estelle, 1994; Malamy and Benfey, 1997a). Both approaches have identified an array of genes involved in LR development. Auxin treatment has been employed in molecular approaches geared towards the isolation of genes involved in LR development. Because the LR forming process is normally a random and variable event,

the application of exogenous auxin in these studies has been a useful tool for promoting LR production. As such, it has been reasoned that the cascade of genes necessary for regulating normal LR development is induced by auxin. However, in addition to enhancing LR formation, exogenous auxin treatment promotes cellular changes including a rapid primary growth response (within 15 to 20 min), changes to the cellular pH mediated by H^+ secretion, and alterations to calcium levels (Theologis, 1986; Eckardt, 2001). It is unclear whether any of these auxin-induced cellular changes are associated with LR formation, as rapid changes may be brought about by auxin induced transcription of early auxin-response genes. Later expressing genes, which may be indirectly regulated by auxin, are also induced by auxin treatment and may encode enzymatic components of metabolic pathways, polypeptides required for cell division, differentiation, or meristem formation (Theologis, 1986).

Genetic strategies employed by many researchers have included the recovery of mutants with altered responses to exogenous auxin treatment, when compared to wildtype plants. Such mutants may display root phenotypes exhibiting reduced LR formation, indicating a resistance to auxin, or increased LR proliferation, or elevated endogenous levels of the phytohormone (Boerjan *et al.*, 1995; Celenza *et al.*, 1995; Hobbie and Estelle, 1995; King *et al.*, 1995).

1.4.1 Molecular Analysis of Lateral Root Development

At the molecular level, the cloning and characterisation of genes expressed either preferentially or specifically in LRs has been a useful aid to the study of root development. A common approach to define the molecular controls that direct LR development has been to identify genes that are transcriptionally activated by auxin. However, perhaps due to the myriad roles that auxin plays in plant development, its interaction with LR-forming genes is still relatively undefined.

Some of the genes expressed during auxin-induced LR formation, that are of particular relevance to this present study, are represented in Table 1.1. These genes may not necessarily be expressed exclusively in the developing LR, but nevertheless are activated during LR formation. The molecular characterisation of such genes has enabled researchers to extrapolate putative functions for the encoded gene products at a defined event or period during development of the LR. A more detailed consideration of relevant genes with implicated roles in LR development is presented below.

Gene or Clone	Plant	Encoded Protein	Reference
Early auxin genes			
SAUR gene family	Soybean	Unknown early auxin inducible	McClure & Guilfoyle (1987)
SAUR-AC1	*Arabidopsis*		Gil *et al.* (1994)
GH3 gene family	Soybean	Unknown early auxin inducible	Hagen *et al.* (1984)
Aux/IAA gene family		Short-lived transcriptional regulator	
PSIAA4/5, PSIAA6	Pea		Theologis *et al.* (1985)
			Oeller *et al.* (1993)
IAA1 - IAA14	*Arabidopsis*		Abel *et al* (1995)
Cell cycle genes			
Cdc2	*Arabidopsis*	Catalytic subunit of p34	Hirayama *et al.*, (1991)
			Ferreira *et al.*, (1991)
Cyc1At	*Arabidopsis*	Mitotic cyclin	Hemerly *et al.* (1992)
LR genes			
RPL16A, RPL16B	*Arabidopsis*	Ribosomal protein L16	Williams and Sussex (1995)
HRGPnt3	Tobacco	Hydroxyproline rich glycoprotein	Keller & Lamb (1989)
SbHRGP3	Soybean	Hydroxyproline rich glycoprotein	Ahn *et al.* (1996)
RSI-1	Tomato	Unknown (GAST1-like)	Taylor & Scheuring (1994)
LRP1	*Arabidopsis*	Novel protein similar to the protein kinase C family	Smith & Federoff (1995)
TobRB7	Tobacco	Putative membrane channelling protein	Conkling *et al.* (1990)
			Yamamoto *et al.* (1991)

Table 1.1: A summary of some of the genes expressed during LR formation.

1.4.1.1 Early Auxin Responsive Gene Families

Auxin is thought to activate transcription by either or a combination of two mechanisms. One mechanism involves the small, amphiphillic IAA molecule being transported into the target cell where it enters the nucleus. Here IAA may be recognised by a receptor protein which can interact with an auxin response element (AuxRE), to activate specific auxin regulated transcription (Ballas *et al.*, 1995). Alternatively, the plasma membrane perceives and interacts with the auxin molecule to activate an intracellular signal transduction pathway (Barbier-Brygoo, 1995). This might involve a protein kinase cascade, or the production of second messengers, including Ca^{2+} or inositol triphosphate, which induce the relevant transcription factors required for the expression of regulated genes (Barbier-Brygoo, 1995; Abel and Theologis, 1996).

The expression of early auxin regulated genes has been studied extensively in plant development. The best characterised examples include members of the *Aux/IAA*, *GH3* and *SAUR* gene families (Abel and Theologis, 1996). Evidence gathered from numerous

studies suggest that auxin-simulated expression of these genes occurs rapidly, and in the majority of cases, independently as a result of *de novo* protein synthesis. This initial early response may be necessary for the subsequent transcriptional activation of late genes involved in mediating a variety of secondary auxin responses (Theologis, 1986; Abel *et al.*, 1994). It also implies that the necessary machinery and components are already in place to ensure that the auxin signal reaches the nucleus (Abel and Theologis, 1996).

1.4.1.1.1. *SAUR* and *GH3*

Members of the *SAUR* (*Small Auxin Upregulated RNA*) gene family are regulated transcriptionally by auxin. Originally identified in soybean hypocotyls, *SAUR* transcripts accumulate within 2 to 10 minutes of exogenous auxin application (McClure and Guilfoyle, 1987; Abel and Theologis, 1996). These transcripts are extremely unstable with short half-lives of between 10 to 50 min (Franco *et al.*, 1990). In soybean, the family of *SAUR* genes lack introns, producing small transcripts of 0.5 kb which encode similar polypeptides of 9 to 10 kD (Guilfoyle *et al.*, 1993; Gil *et al.*, 1994; Abel and Theologis, 1996).

The *GH3* gene from soybean and related genes from *Arabidopsis*, also belong to another small family of auxin regulated genes, and encode proteins of about 70 kD (Guilfoyle *et al.*, 1993). Like *SAUR*, *GH3* is a primary response gene with its expression rapidly induced by auxin (Hagen *et al.*, 1984; Hagen and Guilfoyle, 1985; Guilfoyle *et al.*, 1993). Hagen and Guilfoyle (1985) demonstrated that transcription of the *GH3* gene could not be inhibited by treatment with the protein synthesis inhibitor cycloheximide, again suggesting that the transcriptional complex was already in place.

Although the precise biochemical function of the *SAUR* and *GH3* gene products is still unknown, spatial and temporal expression data strongly indicate a role in auxin-induced cell elongation. In roots of untreated soybean seedlings, *in situ* hybridisation studies localised *GH3* transcripts to the inner cortex and protoxylem ridges. Elsewhere, auxin induced transient *GH3* expression was localised to the vascular tissue, epidermis, cortex, and pith of various organs including the hypocotyl, epicotyl, cotyledon and flower (Guilfoyle *et al.*, 1993). *SAUR* transcript accumulation, induced by auxin treatment has also been observed in the epidermis and cortex of elongating hypocotyls, and starch sheath of epicotyls and immature hypocotyls of soybean, *Arabidopsis* and tobacco (McClure and Guilfoyle, 1987; Gil and Green, 1997). These findings suggest a correlation between *SAUR* expression and cell elongation, a well documented auxin growth response.

Protein synthesis inhibitors have also been reported to induce *SAUR* gene expression, just as they do for the *Aux/IAA* genes described below (Franco *et al.*, 1990; Guilfoyle *et al.*, 1993; Gil *et al.*, 1994). When supplied independently, both auxin and cycloheximide induce *Aux/IAA* and *SAUR* gene expression. Supplying auxin and cycloheximide simultaneously resulted in an additive induction of the respective gene transcripts (Franco *et al.*, 1990; Koshiba *et al.*, 1995). It is thought that this is because cycloheximide acts to inhibit either the synthesis or the activation of a short lived protein which functions as a transcriptional repressor. In the absence of this repressor, the *SAUR* or *Aux/IAA* genes are activated by endogenous auxin (Koshiba *et al.*, 1995; Gil and Green, 1997).

Regions conferring auxin inducibility have been identified in the promoters of multiple auxin responsive genes, including members of the *SAUR*, *GH3* and *Aux/IAA* gene families. The *SAUR15A* promoter for example, comprises more than one AuxRE, including a DUE/NDE (near distal element) box (Guilfoyle *et al.*, 1993; Xu *et al.*, 1997). It was postulated by Xu *et al.* (1997) that the diverse effects auxin exerts on plant growth and development may be attributed to the independent or combined action of different AuxREs to confer auxin inducibility to an array of genes.

1.4.1.1.2 *Aux/IAA*

The *auxin/indole-3-acetic acid* (*Aux/IAA*) gene family is comprised of more than 20 related genes from a range of species including pea, soybean and *Arabidopsis*. Each member exhibits a characteristically rapid induction by auxin, with the primary response elicited between 5-60 min after initial exposure of tissue. The resulting increase in the level of *Aux/IAA* transcripts do not involve *de novo* protein synthesis, implying that auxin acts directly to activate gene expression via preexisiting cellular machinery (Abel and Theologis, 1996; del Pozo and Estelle, 1999b).

Members of this large gene family have been identified in dicotyledonous plants including pea, soybean, *Arabidopsis*, *Medicago truncatula*, tomato, tobacco and cotton; the monocotyledons maize and rice; and the gymnosperm *Pinus*. Members of this gene family include *Aux22*, *Aux28* (Abel and Theologis, 1996) and *GH1* from soybean (Guilfoyle *et al.*, 1993), *PS-IAA4/5* and *PS-IAA6* from pea (Theologis *et al*, 1985; Oeller *et al.*, 1993), *Nt-iaa* from tobacco (Dargeviciute *et al.*, 1998) and at least 20 members from *Arabidopsis* (Abel *et al.*, 1995; Guilfoyle, 1998). The *IAA7* and *IAA8* genes from *Arabidopsis* are conspicuous members of the *Aux/IAA* gene family, in that they display a late response to auxin. Both these genes are thought to be involved in a secondary auxin response, as their expression requires protein synthesis (Abel *et al.*, 1995; Abel and Theologis, 1996). Each

member of this gene family encodes small (~20 to 35 kD) short-lived nuclear proteins which share four highly conserved domains (I, II, III and IV) (Oeller *et al.*, 1993; Abel and Theologis, 1996; Eckardt, 2001). The role of domain I is still unknown, domain II functions in rapid protein degradation, and domains III and IV are involved in protein-protein interactions to regulate transcription during auxin signalling (Ulmasov *et al.*, 1999; Worley *et al.*, 2000; Zenser *et al.*, 2001).

Current evidence implicates a regulatory role for the Aux/IAA proteins as transcription factors. This notion is supported by findings that show the presence of two common domains (III and IV) shared between the Aux/IAA proteins and another large family of transcription factors known as the auxin-response factors (ARFs) (Kim *et al.*, 1997; Ulmasov *et al.*, 1997). ARF proteins specifically bind to the AuxRE found in the promoters of many auxin-inducible genes to activate or repress transcription (Ballas *et al.*, 1995; Ulmasov *et al.*, 1997; Ulmasov *et al.*, 1999; Eckardt, 2001). In the presence of elevated auxin levels, these domains function to promote protein-protein interactions enabling Aux/IAA proteins to interact with ARFs or other Aux/IAA proteins, to form homo- or heterodimers (Ballas *et al.*, 1995; Kim *et al.*, 1997). Domain III of the PS-IAA4/5 and PS-IAA6 proteins, contain a putative ßαα motif which resembles the ß-sheet type, DNA-binding domain present in a family of prokaryotic transcriptional repressor proteins (Abel *et al*, 1994; Eckardt, 2001). In addition, expression of the early auxin inducible gene *PS-IAA4/5* was also found to be activated by translation inhibitors (Theologis *et al.*, 1985), implying that this gene may be negatively regulated by a short lived repressor protein (Theologis *et al.*, 1985; Ballas *et al.*, 1995).

The extreme short half-life of many members of the Aux/IAA family (6 to 8 min) indicates that protein degradation is likely to act as a key regulatory mechanism controlling their activity. In *Arabidopsis*, the dominant *axr2* and semi-dominant *axr3* mutations result in pleiotropic phenotypes that suggest a decreased and increased auxin response respectively (Wilson *et al.*, 1990; Leyser *et al.*, 1996). Molecular analysis revealed that both genes encode proteins belonging to the Aux/IAA family (IAA7 and IAA17 respectively). The dominant nature of *axr2* and semi-dominance of *axr3*, along with their respective enhanced auxin responses, indicate that both mutations are gain-of-function mutations (Timpte *et al.*, 1994; Rouse *et al.*, 1998). Both the *axr2* and *axr3* mutations are the result of amino acid substitutions in the domain II motif important for conferring protein instability (Rouse *et al.*, 1998; Nagpal *et al.*, 2000; Worley *et al.*, 2000). To explain this Nagpal *et al.* (2000) suggested that the mutations may act to stabilise the encoded Aux/IAA proteins, thereby increasing their activity. It was hypothesised that the rapid turnover of these proteins is required for normal auxin response (Guilfoyle, 1998;

Worley *et al.*, 2000). This hypothesis was supported by evidence indicating that during the auxin response, domain II destabilises the Aux/IAA proteins and is essential for their rapid proteolysis (Worley *et al.*, 2000; Ramos *et al.*, 2001).

1.4.1.2 Cell Cycle Genes

Numerous cell cycle regulatory genes play an important role in the control of cell division within the pericycle to generate LRP. Amongst these genes is the cell cycle dependent *cdc2* gene (Martinez *et al.*, 1992; Hemerly *et al.*, 1993), and the mitotic cyclin encoding *cyc1At* gene (Ferreira *et al.*, 1994), both well characterised from *Arabidopsis*.

1.4.1.2.1 *cdc2* and *cyc1At*

The *cdc2* gene encodes the catalytic subunit of p34 cyclin-dependent protein kinase and has been implicated to play a critical role in cell cycle progression. Expression of *cdc2* in *Arabidopsis* was found to be constitutive in the meristematic tissue of the root and the shoot. In the root *cdc2* expression was detected throughout the pericycle, including nondividing cells (Martinez *et al.*, 1992; Hemerly *et al.*, 1993). In this species, the accumulation of *cdc2* mRNA was not affected by auxin induction of LR initiation, suggesting *cdc2* expression is associated with cell division competence rather than being a limiting factor *per se* in LR development (Doerner *et al.*, 1996).

In contrast, expression of *cyc1At* , which encodes the regulatory subunit of cyclin-dependent protein kinases (Hemerly *et al.*, 1992), was shown to be induced by IAA treatment, causing transcript abundance to elevate above basal levels by 15-20 fold (Doerner *et al.*, 1996). During LR development, *cyc1At* transcript was localised to early LRP initials and the meristems of the emerging LRs in *Arabidopsis* (Ferreira *et al.*, 1994). *In situ* hybridisation studies, indicated that high levels of *cyc1At* transcript accumulate in cytoplasmically dense pericycle cells immediately prior to cytokinesis, when LRP founder cells are stimulated to recommence active cell division following induction by IAA treatment (Doerner *et al.*, 1996). Expression of the *cyc1At* gene in these dividing cells was specific to both the G2 and mitosis phases of the cell cycle (Doerner *et al.*, 1996). During LR emergence, *cyc1At* expression was restricted to the newly formed meristem (Doerner *et al.*, 1996). Interestingly, transgenic *Arabidopsis* plants showing constitutive expression of *cyc1At* cyclin driven by the *cdc2aAt* promoter, exhibited an increased rate of root growth, including accelerated LR formation, following induction by 10 μM IAA. This observation, along with the strong correlation previously found between *cyc1At* gene activity and cell division, led Doerner *et al.* (1996) to propose that cyclin is a limiting factor involved in the regulation of meristematic activity and indeterminate root growth.

1.4.1.3 Lateral Root Expressing Genes

A number of genes, apparently structurally unrelated, have been detected in differential screens due to their elevated transcript level during LR induction. This is not surprising as the LR forming process is expected to involve the coordinated synthesis of an indefinite number of key proteins. Hydroxyproline-rich structural proteins also exhibit expression profiles that indicate a specific function in LR formation (Keller and Lamb, 1989; Vera *et al.*, 1994; Ahn *et al.*, 1996). This is to be expected in order to accommodate compositional alterations in the cell walls of the developing LR and the surrounding cells, to strengthen them against the mechanical pressure exerted by the LRP as it grows and emerges. Additional LR specific genes identified to date, including *TobRB7*, *RSI-1* and *LRP1*, is indicative of an extensive number of related and independent processes that function coordinately during LR formation. Genes encoding ribosomal proteins, including RPL16 have also been identified in screens for LR genes (Williams and Sussex, 1995; Sussex *et al.*, 1995a and 1995b).

1.4.1.3.1 *Ribosomal protein L16*

Elevated expression of genes encoding ribosomal proteins occurs during the early stages of LRP development (Williams and Sussex, 1995). Sussex *et al.* (1995a and 1995b) isolated clones representing approximately 40 genes from subtracted cDNA libraries of radish, enriched for genes involved in the organisation of the LR meristem. The most abundant class of cDNA clones represented 14 different ribosomal protein genes. A cDNA fragment corresponding to one of the radish ribosomal protein genes was used to identify two members of the *ribosomal protein L16* (*RPL16A* and *RPL16B*) gene family in *Arabidopsis*. *In situ* hybridisation studies showed that mRNA transcripts that encode *RPL16A* and *RPL16B*, accumulated in a range of tissues undergoing rapid proliferation, including LRP, apical meristems of the shoot and the root, developing embryos and expanding flower buds of *Arabidopsis* (Williams and Sussex, 1995).

During auxin-induced LR formation in transgenic *Arabidopsis* seedlings containing the GUS (ß-glucuronidase) construct under the control of either *RPL16* promoter, X-Gluc (5-bromo-4-chloro-3-indolyl ß-D-glucuronide) staining was first observed 16 h after initial exposure to IAA. At this time point activity was mainly localised in the dividing pericycle cells located at the xylem poles in the mature region of the root (Williams and Sussex, 1995). By 48 h, staining was observed throughout the LRP and in adjacent areas of the stele. *RPL16A* driven expression was confined mainly to the pericycle, with lower levels detected within the stele. This staining pattern was only detected in the mature region of the root when cell division was induced by exogenous IAA treatment. GUS expression

from the *RPL16B* promoter was observed in proliferating tissues of the root and shoot meristems. Williams and Sussex (1995) suggested that both the *RPL16A* and *RPL16B* genes may be required for the initiation of new LRs.

1.4.1.3.2 *HRGPnt3* and *SbHRGP3*

The *uidA* gene, under control of the tobacco *HRGPnt3* gene promoter was introduced into tobacco (Keller and Lamb, 1989). In roots, strong expression of *HRGPnt3-GUS* was detected in a small population of epidermal and pericycle cells that had started to divide and would ultimately give rise to the LRP. During the later stages of LR development, expression was detected in cells at the apex of the LRP, as it penetrated the cortex and emerged through the epidermis and of the primary root.

In a subsequent study, *HRGPnt3-GUS* activity was found to be transiently increased during the initial cell division stage of both the LRP and adventitious root primordium (ARP) formation in transgenic tobacco plants (Vera *et al.*, 1994). The occurrence of *HRGPnt3-GUS* expressing sites in the root was found to increase following exposure to IAA for 36-48 h, and was strongly correlated with subsequent LRP formation 5-6 days later (Vera *et al.*, 1994). The induced expression pattern of GUS activity in the early stages of the incipient LRP and ARP, was specifically localised to cells derived from the pericycle that were fated to become the progenitors of the new developing root organ (Vera *et al.*, 1994).

Transgenic tobacco seedlings were exposed to the cell cycle progression inhibitor hydroxyurea for 3-5 h before addition of IAA. Hydroxyurea treatment blocked LRP initiation but did not inhibit *HRGPnt3* driven GUS expression (Vera *et al.*, 1994). This observation suggested that *HRGPnt3* induction occurs before the first cell cycle is completed to initiate the LRP . As such, this gene is considered as one of the first to be activated in a cascade of events that eventually lead to the formation of new lateral and ARs. Clearly, *HRGPnt3* may be valuable as a molecular marker for LR initiation.

The *HRGPnt3* gene encodes a hydroxyproline rich glycoprotein (HRGP; Keller and Lamb, 1989). HRGPs are a class of proteins thought to possess a structural role to reinforce and strengthen the cell wall (Showalter, 1993; Wycoff *et al.*, 1995; Hirsinger *et al.*, 1999). The transient expression observed in cells of the incipient LRP, led Keller and Lamb (1989), to suggest that the primary function of the HRGPnt3 protein may be to strengthen the cell walls of the emerging LR tip. In particular, they speculated that HRGPnt3 helps protect the LR from excess mechanical pressure generated during its penetration of the surrounding tissues (cortex and epidermis) within the parent root. An

additional function in the early response to a signal for LR induction was also suggested consistent with activation prior to pericycle cell division (Keller and Lamb, 1989; Vera *et al.*, 1994) An analogous function has also been proposed in shoots for the bean HRPP4.1 protein, to withstand pressure exerted from an emerging axillary branch (Wycoff *et al.*, 1995).

The soybean *sbHRGP3* gene encodes a different class of HRGPs with two domains, each possessing a distinct repeat unit suggestive of an inter- and intramolecular cross-linking extensin (Ahn *et al.*, 1996). *SbHRGP3* expression was detected in maturing regions of the primary root and hypocotyl, with maximum levels accumulating during maturation of the LRs (Ahn *et al.*, 1996; Ahn *et al.*, 1998). In the maturing primary root of transgenic tobacco seedlings, *SbHRGP3*-GUS expression was observed mainly in the zone from which LRs were initiated (Ahn *et al.*, 1996). GUS activity decreased as a subset of pericycle cells began dividing to initiate a LRP. GUS activity was absent during LR emergence from the primary root, but was reactivated in epidermal cells in the mature region of the new LR.

High levels of HRGPs have been previously associated with the cessation of cell elongation as part of the cell wall rigidification process (Monro *et al.*, 1974; Ito *et al.*, 1998). During rigidification, the cell wall becomes less extensible, probably by increasing the crosslinking of cell wall polysaccharides, in addition to increasing the degree of incorporation of structural proteins such as HRGPs (Carpita and Gibeaut, 1993). Ahn *et al.*, (1998) proposed that the pattern of *sbHRGP3* expression they observed, may reflect a role for the encoded polypeptide in terminating root elongation. While HRGPnt3 has a speculated function in the initiation of LRs, sbHRGP3 may have a structural role in strengthening the epidermal cell walls of the primary root to protect them from possible damage that may otherwise be inflicted by the emerging LR (Vera *et al.*, 1994; Ahn *et al.*, 1996; Ahn *et al.*, 1998).

1.4.1.3.3 *RSI-1*

Taylor and Scheuring (1994) identified a gene from auxin treated tomato seedlings, designated *RSI-1*, for *Root System Inducible-1*. The *RSI-1* gene exhibited a similar pattern of expression in roots as that detected for *RPL16* (Williams and Sussex, 1995) and *HRGPnt3* (Vera *et al.*, 1994). Expression of *RSI-1* is induced by auxin, and was detected very early in the development of the LR, within the first 4 h and throughout the period of LR emergence (Taylor and Scheuring, 1994). In contrast, *RSI-1* transcript levels were very low in the shoot.

The deduced RSI-1 protein has N-terminal characteristics of a signal peptide, suggesting that the encoded product may be transported (Taylor and Scheuring, 1994). Comparisons to protein databases revealed a high degree of homology to the GAST1 protein from tomato. In tomato, GAST1 is induced by gibberellic acid, which like auxin is involved in the regulation of cell division and cell elongation. However, while RSI-1 and GAST1 have structural similarities, the respective expression characteristics of their genes differs with *GAST1* transcript not detectable in roots while *RSI-1* mRNA levels are very low in the shoot (Shi *et al.*, 1992; Taylor and Scheuring, 1994). Sequence similarities indicate that RSI-1 and GAST1 may have similar functions but are independently regulated by auxin and gibberellin respectively. Taylor and Scheuring (1994) proposed that *RSI-1* may be involved in an independent pathway that mediates cell division and elongation by auxin induction of *RSI-1* transcription. Inducibility of *RSI-1* by gibberellic acid was not tested in this study, but should be considered to investigate the possibility that auxin and gibberellic acid may work in parallel pathways to activate *RSI-1* expression to promote LRP formation.

A 1030 bb fragment of the *RSI-1* promoter was attached to the *uidA* reporter gene to delineate the regions of *RSI-1* promoter activity in transgenic tomato seedlings via X-Gluc detection of GUS activity (Taylor and Scheuring, 1994). Histochemical staining revealed that the *RSI-1* promoter produced GUS activity mainly in the early initials of the LRP which persisted until the LR had just emerged from the primary root. At the cellular level, staining was specific to the root cap and vascular tissue of the developing LR. GUS activity was also visualised at comparative stages of AR development in hypocotyls. Only minimal GUS activity was detected in the meristems of the mature LRs and the primary root.

1.4.1.3.4 *LRP1*

The *LRP1* (*Lateral Root Primordium 1*) gene from *Arabidopsis* was identified by Smith and Federoff (1995) using insertional mutagenesis with a promoter trap T-DNA construct, containing a promoterless *uidA* gene. GUS activity was detected in the LRP of transgenic plants containing the inserted T-DNA. The gene and its analogous cDNA were subsequently cloned and characterised. As determined by X-Gluc staining, expression of the *LRP1* gene was activated early in the development of the LRP and switched off before the LR emerged from the primary root (Smith and Federoff, 1995). Comparable expression was also detected during development of ARs.

Analyses by *in situ* hybridisation was also used to define the expression pattern of this gene during LRP formation (Smith and Federoff, 1995). *LRP1* expression was

observed exclusively in pericycle-derived cells involved in the early stages of LRP development. Non-dividing pericycle cells located nearby did not express the *LRP1* gene. *LRP1* expression persisted throughout the forming LRP during cell proliferation, and specifically intensified in the basal cells of the LRP, immediately before the LR emerged. In agreement with the GUS staining experiments, the *LRP1* transcript was absent at the point of LR emergence, indicating expression of this gene was terminated during the developmental switch from LRP to LR. This expression pattern suggested a possible function for *LRP1* in the initiation and/or progressive development of the LRP.

Interestingly, homozygotes containing the insertion did not possess a phenotype or reduced capacity to form LRP. Database searches revealed no significant sequence homology at the DNA and amino acid levels but some similarities to the protein kinase C family. A search of the *Arabidopsis* expressed sequence tag (EST) database found a match with 68% and 75% amino acid and nucleotide identity, respectively. From these results, Smith and Federoff (1995) suggested that *LRP1* was a member of a small homologous gene family with similar expression profiles. The absence of a mutant phenotype in plants carrying two copies of the inserted transposon suggests that expression of the *LRP1* gene is either redundant, or has an overlapping function with other members of its putative gene family.

LRP1 expression is clearly specific for, and confined to the developing LRP in *Arabidopsis*. This expression pattern was distinct from other previously described LR specific genes, such as *HRGPnt3* (Vera *et al.*, 1994) and *RSI-1* (Taylor and Scheuring, 1994), in that *LRP1* is not expressed in the newly forming LR meristem. These findings place *LRP1* before *HRGPnt3* and *RSI-1* in terms of temporal expression patterns in *Arabidopsis*, and Smith and Federoff (1995) suggested that *LRP1* can serve as a useful molecular marker for analysing the early stages of LR formation. To date no reports of *LRP1* homology has been studied in other species with respect to LRP formation.

1.4.1.3.5 TobRB7

A differential screen detected a number of genes in tobacco whose transcript was exclusively located in root tissues (Conkling *et al.*, 1990). A 1.4 kb fragment from the promoter of one of these genes, RB7 was isolated and fused to the *uidA* gene and then transformed into tobacco. Histochemical localisation of TobRB7 promoter driven GUS activity and *in situ* hybridisation of the TobRB7 transcript, localised the transcript exclusively to the root meristem and the immature vascular tissues of the primary root, at the site corresponding to LR initiation (Conkling *et al.*, 1990; Yamamoto *et al.*, 1991). GUS activity in transgenic tobacco plants expressing *uidA* under the control of the

TobRB7 promoter was detected from the time of initiation in the progenitor cells of the LRP, and continued throughout LR development into the root meristem. As the LR matured, GUS staining was also detected in the central cylinder of the developing vascular tissue (Yamamoto *et al.*, 1991).

Yamamoto *et al.* (1990) isolated a full length homologue of TobRB7 from *Arabidopsis* (AtRB7). Sequence searches using the deduced amino acid sequences of both genes revealed a high degree of similarity to a number of proteins with possible roles in membrane channelling, including a tonoplast protein from soybean seed vacuoles (γ-TIP), and a mammalian lens fiber major intrinsic protein (MIP26). This family of proteins plays an important role in water transport into and out of cells (Maurel, 1997). Yamamoto *et al.* (1990) suggest that the TobRB7 and AtRB7 proteins may play key roles in transporting water and nutrients from the root to aerial parts of the plant. In the root itself, it was proposed that the TobRB7 and AtRB7 proteins function in cell-to-cell channelling of water and nutrients taken up from the soil, and photosynthetic products transported from the shoot.

1.4.2 The Isolation and Characterisation of Mutants Defective in Lateral Root Development

In contrast to the molecular biology approaches noted in section 1.4.1, the isolation and characterisation of genetic mutants can provide an alternative, yet complementary approach by which individual steps in a developmental pathway can be deduced. This approach has been utilised to study the process of LR formation in plants, particularly *Arabidopsis*, thereby facilitating an understanding of the root developmental process and shedding light on the mechanisms by which plant phytohormones can regulate root growth. The well known genetic advantages and the simple root structure of *Arabidopsis* has facilitated the study of root development at the cellular level by enabling the isolation and characterisation of a number of single gene morphological root mutants.

The characterisation of phenotypes in mutant *Arabidopsis* plants with aberrant auxin sensitivity, has been valuable to help elucidate possible physiological interactions between gene products and auxin with respect to root development and morphology (Leyser *et al.*, 1993; Celenza *et al.*, 1995; Hobbie and Estelle, 1995; Ruegger *et al.*, 1998). Many root developmental mutants identified in this model system have also been isolated and classified according to their main phenotype. These include abnormal cellular

organisation including *root epidermal bulger (reb)*, *radially swollen 1* (*rsw1*), *rsw2*, *rsw3*, *stunted plant* (*stp1*) (Baskin *et al.*, 1992), *shortroot* (*shr;* Benfey *et al.*, 1993), *pom-pom* (*pom*; Hauser *et al.*, 1995), *wooden leg*, *gollum*, *pinocchio*, *scarecrow*, and *fass* (Scheres *et al.*, 1995); those with abnormal cell expansion, *cobra*, *sabre*, and *lion's tail* (Benfey *et al.*, 1993); a lack of roots, *gnom* and *monopterus* (Mayer *et al.*, 1991); a lack of a functional root meristem, *hobbit* and *bombadil* (Scheres *et al.*, 1996); and altered root hair development (*rhd*) including *rhd1*, *rhd2*, *rhd3*, *rhd4* (Schiefelbein and Somerville, 1990).

However, the number of examples of mutations that exclusively influence the development of LRs are very limited. Instead, pleiotropic phenotypes are common, where defective LR development is but one mutant trait amongst numerous others that are also observed. It has been suggested that this pleiotropy may occur because the genes required for LR development may have related functions in the primary root, or may also participate in various developmental processes occurring elsewhere in the plant. Alternatively, the genes involved in LR development may be members of multigene families that have overlapping functions during plant growth and development (Smith and Federoff, 1995). Characterisation of mutants such as those represented in Table 1.2 have begun to uncover information on how the signals triggering mitosis in the pericycle are produced, and how these cells in turn respond to the auxin stimulus to direct LR formation.

Mutant	Plant	LR Phenotype	Segregation	Physiology	Reference
Dgt	tomato	No LRs	Recessive	Auxin resistant	Zobel (1972) Zobel (1973) Muday *et al.* (1995)
axr1	*Arabidopsis*	Reduced LRs	Recessive	Auxin, ethylene & cytokinin resistant	Estelle & Somerville (1987) Hobbie and Estelle (1994)
tir1	*Arabidopsis*	Reduced LRs	Semidominant	Auxin resistant	Ruegger *et al.* (1997)
aux1	*Arabidopsis*	Reduced LRs	Recessive	Auxin, ethylene & cytokinin resistant	Maher & Martindale (1980) Pickett *et al.* (1990)
axr4	*Arabidopsis*	Reduced LRs	Recessive	Auxin resistant	Hobbie & Estelle (1995)
Rty *alf1* *sur1*	*Arabidopsis*	Excess LRs	Recessive	Auxin overproducer	King *et al.* (1995) Celenza *et al.* (1995) Boerjan *et al.* (1995)
alf3	*Arabidopsis*	Defective maturation	Recessive	Auxin deficient	Celenza *et al.* (1995)
alf4	*Arabidopsis*	No LRs	Recessive	Auxin resistant	Celenza *et al.* (1995)
rgr1	*Arabidopsis*	Reduced LRs	Recessive	Auxin resistant	Simmons *et al.* (1995)

Table 1.2: A summary of some of the mutants described in the literature with altered LR development.

Genetic and physiological studies of mutant plants with abnormal LR development has been particularly valuable in providing an insight into how the signals triggering mitosis in the pericycle are produced, and how these cells in turn respond to the auxin stimulus (Hobbie and Estelle, 1994). Mutants exhibiting phenotypes with specific alterations in LR development include those failing to form LRs, as well as those with excess LRs, and those defective in maturation (Table 1.2). The phenotypic characterisation of phytohormone mutants can provide insight into the function of growth regulators in plant physiology, growth and development. Mutants which exhibit an abnormal response to exogenous auxin treatment, also display altered LR morphologies. Mutants belonging to this category have been described as either auxin-resistant or auxin-sensitive.

Several auxin-resistant mutants, also display altered responses to other plant phytohormones including, cytokinin, ethylene and/or abscisic acid (ABA) (Pickett *et al.*, 1990; Wilson *et al.*, 1990). This cross resistance implies that the encoded wildtype proteins may have roles in the signal transduction pathway of more than one phytohormone. Alternatively, the different phytohormones interact to influence various aspects of plant growth and development. Thus, it would be expected that a defect in a single gene could alter the sensitivity to different phytohormones.

Other auxin-resistant mutants, including *axr4* and *rgr1*, do not appear to show resistance to other phytohormones tested. In each case, the mutant plants were comparable to wildtype plants in their response to cytokinin, ethylene and ABA treatment. Such mutants help decipher which phenotypic traits can be attributed to auxin effects alone, and are therefore invaluable for defining the genes involved specifically in the auxin response pathway.

1.4.2.1 Auxin Resistant Mutants Exhibiting Altered Lateral Root Development

As noted above, genetic screens for root mutants have identified plant lines that exhibit phenotypes reminiscent of an inherent resistance to auxin inhibited growth. Characterisation of these mutant phenotypes has led to the identification of several loci involved in LR development.

1.4.2.1.1 *dgt*

With respect to root morphology in tomato seedlings, *diageotropica* (*dgt*) mutants are phenotypically agravitropic and did not form LRs even when exogenous IAA was supplied (Zobel, 1973). Following exposure to exogenous auxin, the *dgt* root displayed an

increased resistance to auxin mediated growth inhibition, when compared to wildtype (Muday *et al.*, 1995). The *dgt* mutant root was also found to be less sensitive to auxin transport inhibitors and ethylene.

Although the endogenous auxin levels detected in both *dgt* and wildtype plants were similar, sensitivity to this phytohormone was estimated to be about 50 fold less in the *dgt* mutants than in wildtype plants (Muday *et al.*, 1995). This reduced auxin sensitivity could not be attributed to the endogenous auxin levels or auxin uptake. This finding was compatible with the phenotypic observations made for this mutant, including its inability to form LRs, leading Muday *et al.* (1995) to suggest that the *dgt* mutation affected the plant's ability to perceive or respond to auxin. It was proposed that the lesion associated with this mutation in some way affected a primary site of auxin action or perception.

1.4.2.1.2 *axr1* and *tir1*

Genetic studies in *Arabidopsis* suggest that ubiquitin-mediated protein degradation may serve a regulatory role during auxin signalling, as a major participant in the auxin response. It has been postulated that one or several proteins involved in the auxin perception are degraded or modified post-translationally by either ubiquitin or the ubiquitin-related protein RUB1 (del Pozo *et al.*, 1998; Ruegger *et al.*, 1998; del Pozo and Estelle, 1999b). Target proteins, which may include repressors of the auxin response, are directed for proteolysis by the combined actions of RUB1-conjugation and ubiquitin ligase complexes (del Pozo *et al.*, 1998; Leyser and Berleth, 1999).

Plants of the *auxin resistant 1* (*axr1*) *Arabidopsis* mutant display a diminished sensitivity to auxin. As a consequence, a number of auxin regulated process are affected by the mutation. Plants homozygous for the mutant alleles of *axr1* exhibit pleiotropic phenotypes that are assumed to be due to a reduced response to the phytohormone. These phenotypic traits include shortened internodes, altered root hair development, decreased apical dominance, altered photo- and gravitropic growth, leaf wrinkling, and reduced capacity for auxin-induced callus formation (Estelle and Somerville, 1987; Lincoln *et al.*, 1990; Hobbie and Estelle, 1995; Cernac *et al.*, 1997). The *axr1* mutation also causes a deficiency in the transcription of some auxin-induced gene families and confers a reduction in cytokinin sensitivity (Hobbie and Estelle, 1994; Abel *et al.*, 1995; Timpte *et al.*, 1995).

Expression studies conducted on various organs from *axr1* mutant seedlings, revealed a significant decrease in the accumulation of mRNA transcripts corresponding to the auxin regulated *SAUR-AC1* gene (Timpte *et al.*, 1995) and members of the *Aux/IAA* gene family including, the *IAA1* and *IAA2* genes (Abel *et al.*, 1995). This expression

profile suggests a reduction in auxin sensitivity (Abel and Theologis, 1996). In addition, it was suggested that the *axr1* mutation disrupts the expression of genes that mediate the early auxin response and auxin regulation, implying that the wildtype *AXR1* gene product has a central role in the auxin signal transduction process (Timpte *et al.*, 1995).

When compared to wildtype plants, the auxin levels of *axr1* mutants did not differ (Lincoln *et al.*, 1990; Leyser, 1997). The reduced auxin response was observed in both the shoots and roots of mutant plants suggesting that the wildtype AXR1 protein is likely to play a role in aerial parts of the plant as well as in the root (Lincoln *et al.*, 1990).

A search for *Arabidopsis* seedlings exhibiting resistance to auxin-transport inhibitors, resulted in the isolation of the *transport inhibitor response 1* (*tir1*) mutant (Ruegger *et al.*, 1997; Ruegger *et al.*, 1998). Ensuing characterisation studies revealed that the *tir1* plants displayed defects in various auxin responses, including resistance to auxin-induced inhibition of root growth and a deficiency in auxin-dependent elongation of the hypocotyl.

LR formation was also reduced by the semidominant *tir1* mutation (Ruegger *et al.*, 1998). Mutant *tir1-1* seedlings cultured *in vitro* on hormone-free medium, produced only 1.3±0.4 LRs after 10 days growth, compared to 6.3±0.4 LRs in wildtype seedlings. Microscopic examination of the *tir1-1* root, revealed that no extra LRP had formed, indicating that the genetic aberration was at an early step in the LR forming process.

Root explants excised from *tir1-1* seedlings also displayed a diminished response to LR induction by treatment with auxin (IAA or 2,4-D). Although auxin treatment stimulated LR formation in both wildtype and *tir1-1* root explants, the number of newly formed LRs in the mutant was noticeably reduced and very sporadic. In contrast, the wildtype seedlings initiated LRs in an uninterrupted row along the entire length of the primary root (Ruegger *et al.*, 1998).

The amassed phenotypic data provided convincing evidence that the *tir1* defect was not in auxin transport but instead in auxin response. As a consequence, auxin-induced and uninduced LR formation is deficient, suggesting that the TIR1 protein is essential for the initiation of this organ (Ruegger *et al.*, 1998).

Molecular evidence obtained from expression studies designed to determine the temporal expression pattern of the mitotic cyclin gene *cyc1At*, supported the case for *TIR1-1* function early in LR development (Gray *et al.*, 1999). The results suggested that during

LR formation, *TIR1* was required before the transcriptional activation of the *cyc1At* gene. The authors proposed that *TIR1* may be an essential gene involved in promoting the progression of pericycle cells through the mitotic cycle, following arrest in G2, to initiate division of LRP founder cells.

In roots of transgenic *Arabidopsis* seedlings, the *TIR1-GUS* expression was most predominant in the apical meristems of the primary and LRs, implying that the encoded protein has a function required for various aspects of root development, including LR initiation. *TIR1* is also likely to play a key role in mediating auxin-dependent growth processes elsewhere in the plant, with *TIR1* transcript accumulating in rosette leaves, stems, flowers and developing embryos, and TIR1-GUS activity detected in expanding cotyledons, young hypocotyls and the shoot meristem (Gray *et al.*, 1999).

Phenotypic and expression data indicated that both the *AXR1* and *TIR1* genes are required for auxin response (Ruegger *et al.*, 1998; Gray *et al.*, 1999). To determine the possible interactions between these two genes, double mutant plants were created by combining the *axr1-12* and *tir1-1* mutant alleles, and characterising the resulting mutant phenotypes (Ruegger *et al.*, 1998). While growth on medium containing 0.1 or 0.5 μM 2,4-D, conferred resistance in *axr1-12* single mutant plants, *tir1-1* seedlings were not resistant to auxin exposure at this level. When the *axr1-12 tir1-1* seedlings were grown under the same conditions, the double mutant combination displayed a higher level of resistance than that exhibited by either of the single mutants, suggesting that *AXR1-12* and *TIR1-1* interact synergistically in overlapping pathways.

The Role of RUB1 Conjugation and the Ubiquitin-Proteasome Pathway in Mediating the Auxin Response.

The *AXR1* gene encodes a polypeptide with a high degree of sequence similarity in the N_2-terminus of the ubiquitin-activating enzyme, E1 (Leyser *et al.*, 1993). The functional E1 enzyme catalyses the first step in the ubiquitin-proteasome pathway which is responsible for targeted protein degradation through ubiquitin conjugation in plants, animals and fungi (Leyser and Berleth, 1999). Despite this relatedness, obvious and important differences suggested that AXR1 did not have functional E1 activity. Contrary to E1, AXR1 is a much smaller protein which lacks the conserved cysteine residue that forms the active site obligatory for normal E1 activity. Consequently, it was proposed that AXR1 does not have conventional E1 activity in plants, but rather belonged to a novel class of proteins involved in a ubiquitin-related pathway (Leyser *et al.*, 1993). It has been speculated that the *AXR1* gene product has a putative function in the regulation and turnover of short-lived Aux/IAA

proteins involved in auxin signal transduction and auxin response (Abel and Theologis, 1996; del Pozo and Estelle, 1999b). This process may be mediated by mechanisms analogous to those which direct ubiquitin-dependent proteolysis (Abel and Theologis, 1996).

Comparisons with deduced proteins from other species, including human, yeast and fungi indicated that AXR1 belongs to a protein family, members of which may function with a second protein to form an active enzyme. Studies in budding yeast *Saccharomyces cerevisiae*, discovered that an AXR1 homologue Aos1p, interacts with another E1-related protein Uba2p, to form a dimer (Johnson *et al.*, 1997). The Uba2p protein possesses a conserved active-site cysteine residue, within a region of its sequence that shares similarity with the carboxyl (C) terminus of E1 (Dohmen *et al.*, 1995). The Aos1p-Uba2p dimer is capable of activating the ubiquitin-related protein Smt3p, following the formation of a thiolester bond with this protein. In a similar manner Enr2p, a second AXR1 homologue from *S. cerevisiae*, functions to conjugate the Rub1p ubiquitin-related protein, to the cell cycle Cdc53p protein (Ruegger *et al.*, 1998).

Genetic and molecular studies have helped to elucidate an equivalent process in *Arabidopsis* (del Pozo *et al.*, 1998; Ruegger *et al.*, 1998). The molecular data revealed that *AXR1* encoded one subunit of the RUB1-activating enzyme (del Pozo *et al.*, 1998). This enzyme is the first in the RUB-conjugation pathway and functions to activate the ubiquitin-related protein RUB1 (del Pozo and Estelle, 1999b).

Unlike E1, the RUB1-activating enzyme is comprised of two subunits (del Pozo *et al.*, 1998; Liakopoulos *et al.*, 1998). In *Arabidopsis*, the first subunit AXR1 (an Aos1p ortholog), corresponds to the N-terminal half of E1, whilst a second protein ECR1 (an Uba2p ortholog), corresponds to the C-terminus and contains the cysteine residue which forms a thiolester linkage with RUB1 (del Pozo *et al.*, 1998). The AXR1-ECR1 heterodimer functions in the process of conjugating RUB1 to its target protein. Members of the cullin protein family have been identified as targets for RUB1 modification (Liakopoulos *et al.*, 1999). In fact, del Pozo and Estelle (1999a) were able to show that AXR1-ECR1 and a RUB-conjugating enzyme (RCE1) act to modify the *Arabidopsis* cullin, AtCUL1 by covalently linking it to RUB1 *in vitro* and *in vivo*.

In situ hybridisation analyses revealed that *AXR1* expression was confined to the nuclei of actively dividing and elongating cells. This observed pattern of *AXR1* transcript accumulation closely mirrored that detected for the corresponding AXR1 protein using immunolocalisation studies (del Pozo *et al.*, 1998). These studies have provided evidence

to indicate that AXR1 is a nuclear protein and that like the *AXR1* transcript, the AXR1 protein is located specifically in plant cells undergoing division and elongation. These observations suggest that the proteins targeted for modification via RUB1-conjugation are probably nuclear in their location.

The *TIR1* gene was found to encode a protein comprising a series of 16 degenerate leucine-rich repeats and an F-box domain (Ruegger *et al.*, 1998). As a member of the F-box family of proteins, TIR1 is thought to bind with SKP1 to form an E3 ubiquitin-ligase complex, referred to as SCF for SKP1, Cdc53 (or cullin), F-box protein (Ruegger *et al.*, 1998). Like *AXR1*, the sequence and expression data collected for *TIR1* also implied that the auxin response may involve the modification of one or more important regulatory proteins, mediated either by ubiquitin or a related protein (Ruegger *et al.*, 1998). This supported the genetic evidence that indicated a synergistic interaction between the *axr1* and *tir1* mutations, suggesting that the two genes function in the same ubiquitin-related conjugation pathway (Ruegger *et al.*, 1998).

Genetic studies have established that the *AXR1* and *TIR1* genes both have an important function in numerous auxin-affected processes including LR formation, auxin inhibited root elongation, auxin induction of cell proliferation and gene transcription (Lincoln *et al.*, 1990; Timpte *et al.*, 1995; Ruegger *et al.*, 1998). Investigation of RUB1 activation in *S. cerevisiae* suggests that the RUB1 conjugation pathway overlaps with the ubiquitin conjugation pathway at the SCF ubiquitin ligase complex. In *Arabidopsis* this also appears to be the case as the ubiquitin-proteasome pathway represents common links between the activity of AXR1 and TIR1 (Gray and Estelle, 2000).

In *Arabidopsis*, the RUB1-activating enzyme (AXR1-ECR1), has been implicated in the regulation of the SCF which is thought to comprise TIR1 as its F-box protein constituent (Ruegger *et al.*, 1998; Gray *et al.*, 1999). In the ubiquitin-proteasome pathway, the SCF ubiquitin ligase is involved in the transfer of ubiquitin from the ubiquitin-conjugating enzyme (E2) to the substrate protein. Evidence suggests that RUB1-mediated modification of cullins plays an important role in regulating the activity of SCF ubiquitin ligases (del Pozo and Estelle, 1999a).

del Pozo and Estelle (1999a) identified an E2-related protein in *Arabidopsis*, termed the RUB-conjugating enzyme (RCE1), which forms thiolester bonds with RUB1. These authors demonstrated that the combined activities of the RCE1 and the AXR1-ECR1 enzymes act to facilitate the stable conjugation of RUB1 to the *Arabidopsis* cullin AtCUL1 *in vitro*. Cullins characteristically form a subunit of the SCF ubiquitin-ligase complex

(Patton *et al.*, 1998). Correspondingly, AtCUL1 interacts with ASK1 (a SKP1 ortholog) and TIR1 to form the SCF^{TIR1} complex (Gray *et al.*, 1999).

Working with *Arabidopsis*, Gray *et al.* (1999) showed that the ubiquitin-ligase complex SCF^{TIR1} is necessary for normal auxin response. Furthermore, mutations in *AXR1*, *ASK1*, or *TIR1*, have been correlated with either a loss or a reduction in the auxin response (Gray *et al.*, 1999). This suggests that AXR1-dependent RUB1-conjugation may function in the regulation of SCF^{TIR1} activity, to control the modification and/or degradation of proteins that repress the auxin response (Ruegger *et al.*, 1998; del Pozo and Estelle, 1999a). The observed synergistic interaction between *axr1* and *tir1* in double mutants is in agreement with this hypothesis (Ruegger *et al.*, 1998). Since a role for SCF^{TIR1} has been described in the auxin response, the modification of AtCUL1 by the RUB1-conjugation pathway is also likely to play an important part in auxin regulation (del Pozo and Estelle, 1999a; Gray *et al.*, 1999).

Auxin Regulation of the SCF^{TIR1}-Dependent Degradation of AUX/IAA Proteins.

Abel and Theologis (1996), proposed that the transcription of auxin-regulated genes may be negatively regulated by short-lived repressor proteins. Numerous models have been proposed that are both compatible with this suggestion and also indicates a role for protein degradation and/or modification in mediating the auxin response (Ruegger *et al.*, 1998; del Pozo and Estelle, 1999a; Gray and Estelle, 2000; Gray *et al.*, 2001). The initial auxin response model proposed that repressors, or negative regulators of auxin, are degraded by a ubiquitin or ubiquitin-related system. Degradation of these repressors results in derepression of the auxin-response pathway (Abel and Theologis 1996; Gray and Estelle, 2000).

Auxin may act to alleviate transcriptional repression by inducing the AXR1-TIR1 dependent degradation of negative regulators of the auxin response (Ruegger *et al.*, 1998). When the repressor(s) are degraded, transcription of early auxin response genes, such as those belonging to the *Aux/IAA* family, is activated. Gray and Estelle (2000) suggested that such repressor(s) act as the SCF^{TIR1} substrate. Auxin stimulates SCF^{TIR1} function, which acts as a ubiquitin ligase to target repressor protein(s) for ubiquitin-mediated proteolysis. To operate normally, the SCF^{TIR1} requires RUB1 modification of cullin (e.g. AtCUL1), mediated by the AXR1-ECR1 enzyme. Once the repressor(s) is degraded, transcriptional repression of Aux/IAA genes is eased.

Gray and Estelle (2000) also proposed that some members of the Aux/IAA family are themselves repressors, while others act as positive regulators of the auxin response. Newly synthesised positive Aux/IAA regulators may act to modulate the downstream expression of genes involved in auxin response, whilst newly synthesised negative Aux/IAA regulators accumulate to threshold levels which then reinstate basal repression of gene activity. This derepression may serve to regulate the auxin response pathway by controlling the expression of the early auxin response genes. Expression of these primary genes may be required to trigger the transcription of a cascade of downstream, secondary response genes that are involved in auxin-dependent growth and development.

This hypothesis has been supported by evidence emanating from experiments which made use of proteasome-specific inhibitors (Ramos *et al.*, 2001). These studies showed that an Aux/IAA::luciferase fusion protein was degraded via the polyubiquitin-proteasome pathway. Furthermore, Gray *et al.* (2001) were able to show that SCF^{TIR1} binding to domain II of AXR2/IAA7 and AXR3/IAA17 was required for the degradation of these proteins, and that this interaction was stimulated by auxin. Additional supporting evidence was obtained by Zenser *et al.* (2001), who assayed the effect of auxin treatment in transgenic *Arabidopsis* seedlings expressing a translational fusion of Aux/IAA with firefly luciferase under the control of a non-auxin-responsive promoter. They found that elevated levels of auxin were correlated with the increased proteolytic destruction of some Aux/IAA proteins.

The accumulating evidence suggests that there is, at first sight an apparent paradox in the function of the Aux/IAA proteins during the auxin response. On the one hand, auxin up-regulates *Aux/IAA* transcription, yet at the same time auxin also down-regulates the abundance of the Aux/IAA proteins by promoting their rapid degradation. The antagonistic properties may enable Aux/IAA proteins to increase transiently in response to auxin, to a threshold level which serves to activate expression of downstream genes.

Taken together, the evidence has led researchers to elaborate on the model proposed by Gray and Estelle (2000) to explain this paradox. The refined model predicts that when auxin levels are low, auxin-responsive transcription is also low due to the repressor activity of the Aux/IAA proteins. When auxin levels increase, an associated increase in the rate of Aux/IAA proteolysis occurs as protein binding to SCF^{TIR1} and related SCF complexes is promoted. Functional SCF^{TIR1} activity is itself regulated by AXR1-ECR1-dependent RUB1 conjugation to the AtCUL1 subunit of the SCF. Aux/IAA proteolysis results in a drop in the abundance of steady-state Aux/IAA proteins. This activity relieves the transcriptional repression, enabling early auxin-responsive mRNAs to accumulate. As a

consequence of this up-regulated transcription, the Aux/IAA proteins may accumulate to a point where they begin to inhibit their own synthesis when Aux/IAA repressor activity is restored. This model suggests that the auxin induced expression of *Aux/IAA* genes is a negative feedback loop that enforces strict regulation of the auxin response (Gray and Estelle, 2000; Zenser *et al.*, 2001).

From the studies noted above, it is apparent that the auxin response requires the stringent control of numerous proteins. In particular, the regulation of Aux/IAA protein abundance appears to be an important mechanism to control downstream processes involved in auxin response. This seems to be dependent upon SCF^{TIR1} function in the targeting of its substrate for modification or degradation via the combined activity of AXR1-ECR1-dependent, RUB1-conjugation and the ubiquitin-proteasome pathway.

SAR1 *Function in the AXR1-TIR1 Dependent Auxin Response Pathway.*

Genetic evidence indicates that an additional gene, *SAR1* functions in the same pathway as *AXR1* and *TIR1*. The *sar1* (*suppresser of auxin resistance 1*) mutants were identified in *Arabidopsis* following a screen for suppressers of the *axr1* mutation (Cernac *et al.*, 1997). The *sar1* phenotype is distinct from that exhibited by both the *axr1* mutant and wildtype plants. Compared to wildtype, the *sar1-1* plants were shorter, displayed abnormal leaf development, early flowering and a reduced rate of elongation in the primary root. Also to some extent, the *sar1-1* mutation was observed to suppress every distinctive characteristic of the *axr1* phenotype.

The interaction between *sar1* and *axr1* was characterised with respect to LR formation (Cernac *et al.*, 1997). The density of LRs produced along the length of the primary root was equivalent in *sar1-1* and wildtype plants. In contrast, mutants homozygous for either the severe *axr1-12* allele or the weak *axr1-3* allele, formed less LRs/cm of the primary root than either the wildtype or the *sar1-1* seedlings. When in an *axr1-12* background, *sar1-1* did effectively increase the frequency of LR formation, but did not restore the phenotype to wildtype levels. This observation was not replicated when the weaker *axr1-3* allele was considered, however in the *axr1-3 sar1-1* double mutant, the *sar1-1* mutation was not able to overcome the *axr1-3* LR defect. Together these findings demonstrated that the *sar1-1* mutation was capable, to some extent, of suppressing the altered LR phenotype conferred by the *axr1-12* allele, but not the *axr1-3* allele.

Analysis of the aerial phenotype of mature double mutant plants, revealed that when present in the *axr1* background of either allele, the appearance of *axr1-12 sar1-1* and

axr1-3 sar1-1 plants were indistinguishable from that of *sar1-1 AXR1* indicating that *sar1-1* is epistatic to *axr1* (Cernac *et al.*, 1997). The phenotypic characteristics considered in this assessment included flowering time, leaf number, and plant height at the end of flowering. When flowering time was compared for example, wildtype plants flowered at around 34 days, homozygous *axr1-12* and *axr1-3* each flowered at 33 days, and homozygous *sar1-1* at 22 days. The double mutants *axr1-12 sar1-1* and *axr1-3 sar1-1* each flowered at 21 days, virtually the same time as *sar1-1* homozygotes. Due to the combined suppression and epistatic interaction observed in the double mutant studies Cernac *et al.* (1997) proposed that *SAR1* acts after *AXR1* in a common pathway, where *SAR1* functions in a negative capacity to suppress the auxin response.

Together the genetic data indicates that *SAR1*, *AXR1* and *TIR1* operate in the same auxin response pathway where *AXR1* acts to abate repression of auxin response mediated by the downstream *SAR1* gene (Cernac *et al.*, 1997). Because loss of SAR1 seems to reduce the requirement for AXR1 in auxin response, researches in the field have proposed that the encoded *SAR1* product may be a potential substrate of the AXR1-TIR1 dependent RUB1-conjugation pathway (Ruegger *et al.*, 1998; Gray and Estelle, 2000).

1.4.2.1.3 *aux1*

Originally identified by its agravitropic root growth in response to the auxin 2,4-D, the *Arabidopsis aux1* mutation is responsible for a reduced auxin response and causes defects in a variety of auxin-related growth processes (Hobbie and Estelle, 1994). The *aux1* defect is specific for roots with mutant plants displaying defective root morphologies. The *aux1-7* allele has been used for most of the work conducted for this mutation.

Unlike *axr1*, the *aux1* mutation specifically affects the root phenotype, suggesting that the wildtype *AUX1* gene product functions in a pathway for auxin response which operates exclusively or predominantly in roots (Pickett *et al.*, 1990; Bennett *et al.*, 1996). The abnormal recessive mutant phenotype displayed by *aux1* roots includes enhanced growth rates, reduced LR initiation, a decrease in gravitropism and a diminished sensitivity to auxin and ethylene (Pickett *et al.*, 1990; Evans *et al.*, 1994). The cross-resistance exhibited by *aux1* plants towards other phytohormones suggests that the *AUX1* gene product may also function in the signal transduction of these other phytohormones (Timpte *et al.*, 1995). Because expression of the auxin induced *SAUR-AC1* mRNA was reduced in *aux1-7* mutants, a defect in early auxin regulated gene activity was implicated (Timpte *et al.*, 1995). An additional role of the *AUX1* gene product in the signal transduction of other phytohormone signals can not be ruled out.

The encoded wildtype AUX1 polypeptide is a membrane protein that shares similarity with amino acid permeases found in *Arabidopsis* (AAP1), *Caenorhabditis elegans* and fungi (Bennett *et al.*, 1996). Alignment of the AUX1 and AAP1 amino acid sequences revealed 21% identity and 48% similarity, indicating that the two polypeptides are functionally conserved. The structural similarity of IAA to its precursor, the amino acid tryptophan, makes it a probable AUX1 substrate, leading Bennett *et al.* (1996) to speculate that the AUX1 protein plays a role in IAA uptake and transport. The level of sequence conservation suggests that AUX1 functions in the transport of an amino acid-like signalling molecule. Timpte *et al.* (1995) noted that the *aux1* mutation results in a similar level of auxin resistance as that described for *axr1*, the genetic evidence suggests that the two genes participate in distinct auxin-related pathways.

Yamamoto and Yamamoto (1998) showed that the *aux1* mutant was differentially resistant to auxin. Treatment with the synthetic auxin NAA restored the agravitropic growth phenotype of the mutant roots, while 2,4-D and the natural auxin IAA did not. Earlier studies showed that of these three auxins, only the more hydrophobic NAA molecules were able to efficiently enter cells without the assistance of the auxin influx carrier (Delbarre *et al.*, 1996). These results led Yamamoto and Yamamoto (1998) to propose that AUX1 functions as an auxin influx carrier, a hypothesis subsequently supported by Marchant *et al.* (1999) who demonstrated that auxin uptake mediated by the influx carrier is reduced in *aux1* mutants.

Compared to wildtype, the *aux1-7* mutant accumulates a lower level of auxin-induced *SAUR-AC1* mRNA in roots and rosette leaves (Timpte *et al.*, 1995). However, the *SAUR-AC1* transcript levels detected in *aux1-7* tissues were not as low as that found in tissues taken from plants homozygous for the severe auxin resistant *axr1-12* allele or its weaker *axr1-3* allele (Timpte *et al.*, 1995). This observation suggests that although the *aux1* mutation disrupts the expression of genes which mediate the early rapid auxin response and regulation, the AUX1 protein may have an indirect role in the transcription of auxin regulated genes, because the reduced expression level of *SAUR-AC1* was not as severe as that found for the *axr1* alleles. An alternative explanation, also suggested by Timpte *et al.* (1995), is that the AUX1 response may be specific to a group of auxin responsive cells.

1.4.2.1.4 *axr4*

The two mutant auxin resistant alleles that define the *axr4* locus were identified in *Arabidopsis*. The *axr4-1* mutation was induced by T-DNA insertion and *axr4-2* from a screen of γ-ray mutagenised M2 seed. Both mutations were identified by their ability to

confer elongated root growth on medium containing 10^{-7} M 2,4-D. Both mutations are recessive and confer a specific resistance to IAA and 2,4-D, resulting in an abnormal root phenotype consisting of defective root gravitropism and reduced frequencies of LR initiation (Hobbie and Estelle, 1995; Yamamoto and Yamamoto, 1999). Despite an obvious curling of the rosette leaves along their axis, for the most part, the aerial part of the *axr4* plant exhibits a predominantly wildtype form (Hobbie and Estelle, 1995).

The agravitropic phenotype of the *axr4* mutant can be rescued by treatment with NAA, at concentrations ranging from 20-300 nM. In contrast, IAA and 2,4-D treatment did not restore agravitropic growth. As was the case with *aux1*, the differential effects observed from treatment with these three auxins suggests that the *axr4* lesion may cause a deficiency in the auxin influx into cells (Yamamoto and Yamamoto, 1998 and 1999).

Although the *axr4* plants exhibited a reduced sensitivity to auxin, the level of resistance in these mutants, measured by root growth, was only 30-50% that exhibited by *aux1* and *axr1* mutant plants respectively. An assessment of the *axr4* response to other phytohormones revealed that, *axr4* sensitivity to ABA was variable and slight, whilst the response to cytokinin and the ethylene precursor ACC, was very similar to that observed for wildtype plants. The possibility that the *axr4* response to other phytohormones was minor or difficult to detect could not be excluded, making it difficult to determine whether or not the *AXR4* gene product plays a role in an auxin specific response pathway (Hobbie and Estelle, 1995).

When compared to wildtype, the frequency of LR formation was also reduced in the *axr4* mutants, as was the case in both the *aux1* and *axr1* roots. The *axr4 axr1-3* double mutant exhibited a primary root which was nearly devoid of LRs, together with an increased level of auxin resistance as measured by root elongation on medium containing 2,4-D. Based on these observations, Hobbie and Estelle (1995) suggested the interaction between the two mutations was synergistic, implying that the AXR1 and AXR4 proteins may either function in different auxin response pathways, or alternatively possess a very limited level of redundancy in the same pathway.

The axr4 aux1 *Double Mutant.*

An exacerbated inhibition of LR initiation was also observed in *axr4 aux1-7* double mutant plants. However, the level of auxin resistance displayed by these plants did not differ from that typically conferred by the single *aux1-7* mutation. These results indicated that while

aux1-7 was epistatic to *axr4* with respect to auxin resistant growth, the contribution by each mutation to the LR phenotype was additive (Hobbie and Estelle 1995).

These opposing observations may be explained if *AXR4* encodes a second auxin influx carrier located at a different site within the plant (Yamamoto and Yamamoto, 1999). The distribution of the *AUX1* transcript was determined by *in situ* hybridisation, and found to accumulate in the apical tissues of the root (Bennett *et al.*, 1996). This region of the root is considered to play a more important role in root elongation. It was proposed that AXR4 may have a more basal location in the primary root, where LR formation is more prolific (Yamamoto and Yamamoto, 1999). Molecular characterisation of *AXR4* will facilitate experiments to investigate the expression pattern of this gene and test this hypothesis.

Taken together, these experiments indicate that the proteins encoded by *AXR4*, *AXR1* and *AUX1*, each play a role in conferring auxin sensitivity and the gravitropism response of roots, whilst also affecting their capacity for LR formation. The intricate interactions between these genes suggest that mechanisms directing these auxin-mediated processes do not operate in a single auxin response pathway. Hobbie and Estelle (1995) postulated that *AXR4* is likely to function in the same, or an overlapping pathway as either *AXR1* or *AUX1*.

1.4.2.2 Lateral Root Mutants

The characterisation of mutants with specific defects in LR development has facilitated the identification of several steps in the putative developmental pathway leading to LR formation. The altered responses to auxin that is exhibited by these mutants has elucidated possible interactions between the corresponding wildtype gene products and the phytohormone during normal LR development.

Celenza *et al.* (1995) reported the isolation of three non-allelic mutations affecting LR development in *Arabidopsis* by screening M2 EMS mutagenised seed of the ecotype Wassilewskija, and M2 γ-irradiated seed of the Columbia ecotype. The screens were performed to target putative mutants with altered LR position, LR development, or LR number. Three recessive mutations were identified and designated *alf* for *aberrant lateral root formation*, and exhibited pleiotropic phenotypes with *alf3-1* also affecting the primary root, and both *alf1-1* and *alf4-1* also affecting shoot morphology. Following the characterisation of these mutations, Celenza *et al.* (1995) proposed a pathway for LR

formation that requires auxin at two developmental stages, LRP initiation and meristem activation upon emergence of the LR.

1.4.2.2.1 *rty, alf1-1 and sur1*

rty

In *Arabidopsis*, the recessive nuclear mutant *rooty* (*rty*) exhibited hyperproliferation of LRs and ARs from the primary inflorescence shoot (King *et al.*, 1995). Other affected morphological traits were consistent with enhanced auxin effects, including reduced inhibited shoot growth. GC-MS analysis detected a 2-17 fold increase in the internal levels of IAA in *rty* plants compared to wildtype. Moreover, the distribution of IAA within different parts of the plant was also altered. *rty* plants were found to contain higher levels of IAA in the shoots than in the roots, whereas the IAA distribution in wildtype plants was reversed.

Both *rty* and wildtype plants exhibited similar responses to exogenous IAA treatment, suggesting that tissue sensitivity to this phytohormone was unaffected in mutant plants (King *et al.*, 1995). All *rty* phenotypic characteristics were phenocopied in wildtype plants following the application with 10 μM NAA. In addition, a wildtype phenotype of *rty* mutant plants could be partially restored following exogenous treatment with cytokinin. These results indicated that the encoded RTY polypeptide may have an important role in the regulation of endogenous IAA levels during normal plant growth and development.

A double mutant was created by crossing *rty* with an auxin resistant *axr1-3* mutant plant. The *axr1-3* mutation did not completely block the *rty* phenotype. The double mutant combination conferred auxin insensitivity, a reduction in epinastic growth and root proliferation, and enhanced root elongation. These observations led King *et al.* (1995) to suggest two alternative hypotheses to account for the single mutant *rty* phenotype. They proposed that the primary gene defect did not involve auxin biosynthesis *per se* but rather that elevated auxin levels were a secondary effect which may have served to enhance the mutant phenotype. The alternative hypothesis proposes that the auxin levels in *rty* were sufficiently elevated to induce some auxin responses in the otherwise resistant *axr1-3* background.

alf1-1

The *Arabidopsis alf1-1* mutant exhibits hyperproliferation of LRs (Celenza *et al.*, 1995). Along with an increase in the number of LRs initiated along the primary root, an increased rate of AR formation from the hypocotyl was observed. Both the cotyledons and the true leaves are small and epinastic, and the initiation of floral organs from the flowering stalks is infrequent resulting in sterile plants. Given the infertility of *alf1-1* flowers, Celenza *et al.* (1995) identified heterozygous *alf1-1/ALF1* plants from a subset of the original M2 population. The *alf1-1* phenotype appeared in 25% of the heterozygous populations, suggesting that the mutation was recessive. Crosses required for genetic analysis were made using non mutant, Alf1$^+$ plants from an *alf1-1/ALF1* family to strains of interest.

The phenotype described for *alf1-1* closely resembles that described for *rty*. This prompted Celenza *et al.* (1995) to investigate the possibility that the two independently isolated mutations represented defects in the same gene. Phenotypic observations of double mutants, together with allelelism tests demonstrating that the two mutations mapped to approximately the same location, led to the conclusion that the two mutations were allelic.

sur1

In an independent study conducted at around the same time, Boerjan *et al.*, (1995) isolated seven allelic *Arabidopsis* mutants displaying phenotypes indicative of increased auxin production. These recessive mutants were designated as *sur1-1* to *sur1-7* for *superroot* and are allelic to *rty* and *alf1-1*. Like *rty* and *alf1-1*, the *sur1* plants typically produced small, epinastic cotyledons, an elongated hypocotyl, had increased adventitious and LR proliferation, fewer leaves, and lacked an inflorescence.

The LRP of mutant plants exhibited a variety of altered morphological and developmental characteristics. Individual LRP of *sur1* mutant plants were often fused, or formed in very close proximity to one another, and to the root tip. While some LRP were quiescent, others grew into LRs in a similar fashion as wildtype. The *sur1* LRs were often thicker than those of wildtype plants.

Levels of free and conjugated IAA were increased by up to 4 and 7 fold respectively, over wildtype levels in *sur1*. This was consistent with observations made following treatment of wildtype plants with exogenous auxin, which produced a phenocopy of the *sur1* phenotype. These observations indicated that the likely wildtype

role for *SUR1* is to prevent auxin overproduction, possibly via feedback inhibition of auxin biosynthesis (Boerjan *et al.*, 1995).

1.4.2.2.2 *alf3-1*

The LRP that initiate along the length of the *Arabidopsis alf3-1* primary root are arrested in their growth, implying a primary defect in the maturation of the LRs (Celenza *et al.*, 1995). Following initiation, LRP develop as normal in *alf3-1* mutants to the stage of penetrating the epidermis before growth is terminated, preventing outgrowth of the LR.

At an average of 7 LRP/cm of primary root, the number of LRP that form in the *alf3-1* plants was found to be more than double that observed in wildtype (2.9 LRs/cm on average) when cultivated in similar hormone free conditions. Furthermore, the spacing of LRP along the primary root was also disturbed in the *alf3-1* mutants. While LRP are clearly separated by some distance from one another along the length of the wildtype primary root, the *alf3-1* mutant initiates LRP directly adjacent to, or on top of previously arrested primordia (Celenza *et al.*, 1995). The *alf3-1* mutant phenotype was rescued by treatment with exogenous IAA, and also indole, permitting LRP to complete development and mature into LRs. Moreover, the *alf1-1 alf3-1* double mutant, generated an increased abundance of both lateral and ARs, indicating that *alf1-1* suppresses the effects of the *alf3-1* mutation, and hence must act before *alf3-1* in LR development. This would be consistent with elevated levels of auxin in the *alf1-1* mutant.

1.4.2.2.3 *alf4-1*

The *alf4-1* mutant is completely deficient in its ability to initiate LRs from the primary root and ARs from the hypocotyl. In addition, the *alf4-1* mutation also produces short, bushy plants with male-sterile flowers. Unlike the *alf3-1* plants, *alf4-1* does not respond to the stimulated induction of LRs by exogenous IAA treatment. As a result, a normal phenotype can not be produced by exposure of *alf4-1* mutants to this phytohormone (Celenza *et al.*, 1995).

Although *alf4-1* plants exhibited a resistance to auxin induction of LR initiation, mutant plants retained their sensitivity to auxin-mediated inhibition of root elongation. This is in contrast to the response displayed by the auxin resistant mutants (*axr1-3*, *axr2*, and *aux1-7*), which were able to form LRs capable of elongation under the same conditions for auxin treatment (Maher and Martindale, 1980; Estelle and Somerville, 1987). These observations led Celenza *et al.* (1995) to propose the possibility of at least two separate pathways for auxin signal transduction. One of the pathways was suggested to be involved

in auxin-induced LR formation, with the other being responsible for auxin-inhibited root elongation.

Both the *alf1-1 alf4-1* and *alf3-1 alf4-1* double mutants produced a primary root lacking LRP, characteristic of the *alf4-1* single mutant phenotype (Celenza *et al.*, 1995). This suggested that *alf4-1* was resistant to the elevated levels of endogenous auxin produced in the *alf1-1* mutant, just as it was resistant to treatment with exogenous auxin. Because *alf1-1* was found to suppresses the *alf3-1* phenotype, it was deduced that it must act before *alf3-1*. Celenza *et al.* (1995) suggested that *alf4-1* contains an early block in the pathway for LR formation that occurs after *ALF1* regulation of internal IAA levels to induce LRP and before *ALF3* acts to facilitate maturation of the LRP into LR.

1.4.2.2.4 Proposed Role for *alf* Mutants in a Pathway for Lateral Root Formation

The cumulative data collected from the study of the three *alf* single mutations and double mutant combinations, indicated a role for auxin in the initiation, morphogenesis, and viability of LRs. These observations led Celenza *et al.* (1995) to present a model which is consistent with earlier observations that indicate two stages in LR formation are mediated by auxin-dependent developmental events.

In the first of these auxin-dependent steps, auxin induces cell division in the pericycle layer to ultimately form the LRP. Based on the phenotypic evidence, it is assumed that the *ALF1* gene product up-regulates the level of free auxin to initiate development of the LR. It is likely that the role of the *ALF4* gene is to enable pericycle cells to either perceive, or respond to this auxin signal, to initiate LRP formation. In the second auxin-dependent step, LR formation requires auxin levels to be elevated, or maintained within the LRP to levels capable of sustaining cell viability and continued mitosis. It was proposed that the role of *ALF3* is to increase auxin levels in the LRP. The physiological and phytohormone studies conducted in lettuce (MacIsaac *et al.*, 1989) and *E. globulus* (Pelosi *et al.*, 1995a and 1995b) suggested that LR emergence takes place in the absence of high auxin concentrations. Therefore, to be consistent with these observations, the *ALF3* mediated increase in auxin is probably localised to the root apex to initiate the newly formed meristem. When the LR has emerged, *ALF3* activity would ensure the activation and maintenance of a functional LR meristem to maintain growth of the new LR.

1.4.2.2.5 *rgr1*

The phenotype of the recessive *Arabidopsis rgr1* mutant plant includes short roots with fewer LRs and reduced gravitropism compared to wildtype plants (Simmons *et al.*, 1995).

nts also displayed an elevated level of resistance to administered auxin and polar sport inhibitors. However, relative to wildtype, *rgr1* mutants did not alter in nses to ethylene, ABA or cytokinin.

tant *rgr1* plants formed fewer LRs than wildtype, with their development being ed at the stage of LRP initiation and LR emergence. The *rgr1* root was capable an average of only 0.7 LRP and 0.2 LRs per seedling after 17 days growth in of applied auxin. This response was much lower than that of wildtype roots uced an average of 5.7 LRP and 6.5 LRs. Outgrowth of the few LRs that did he *rgr1* mutant was also affected, as mutant LRs were generally shorter than pe counterparts. The pattern of *rgr1* LR formation remained unaffected, with ing in acropetal sequence along the length of the primary root, forming closest hypocotyl transition region. LR formation remained significantly lower in *rgr1* wing culture on medium supplemented with 10^{-7} M 2,4-D, the only auxin-type nsider LR formation. Together these phenotypic comparisons strongly suggest *r1* mutation affects LR formation during both the initiation and outgrowth R development (Simmons *et al.*, 1995).

MARY OF THE GENES IMPLICATED IN LATERAL ROOT CLOPMENT

y and physiology of root systems have been studied extensively by researches ecades. This work has included detailed studies of plant phytohormones and ce on LR initiation and development in a number of plant species, including More recently the identification of mutants, mainly in *Arabidopsis*, and genes morphology and/or auxin signal transduction has begun to provide an insight the fundamental processes involved in the regulation of root development

These studies have highlighted the fact that root development is likely to ge number of complex, overlapping and interrelated pathways which direct the the new organ. Transcription of genes that have a primary function in these y stimulate a cascade of downstream genes with indirect or tangential roles in ent.

Developmental event in LR formation	Gene(s)
1. Auxin signal transduction	*SAUR, GH3, IAA/Aux, RSI-1, DGT, AXR1, TIR1, AUX1, AXR4, ALF4*
2. Regulation of endogenous auxin levels	*RTY/ALF1/SUR1*
3. Pericycle cells divide	*cdc2, cyc1At*
4. LRP initiation	*HRGPnt3, RSI-1, LRP1, TobRB7, RGR1*
5. LRP growth	*RPL16, RSI-1, LRP1*
6. LR emergence	*HRGPnt3, sbHRGP3*
7. Meristem activation	*TobRB7, cyc1At, TIR1*
8. LR outgrowth	*RGR1*

Table 1.3: A summary of the genes with proposed function at various developmental stages of LR formation.

1.6 THE IMPORTANCE OF ROOT FORMATION IN *EUCALYPTUS* FOR PLANTATION IMPROVEMENT VIA MICROPROPAGATION OF ELITE GENOTYPES

It has been estimated that roots contribute between 40 to 85% of the net primary production in ecosystems ranging from forests to grasslands (Fogel, 1985). Thus, an understanding of the mechanisms that control LR formation is just as important from an agronomic and horticultural standpoint, as it is from a fundamental developmental biology perspective. Issues associated with the establishment of clonal forestry programs has sparked particular interest in the study of both LR and AR formation in forest tree species.

There are approximately 800 species and hybrids represented in the genus *Eucalyptus*. Although endemic to Australia and parts of Papua New Guinea, their broad adaptability to different environmental conditions has seen the establishment of eucalypt plantations on all continents except Antarctica. Selected *Eucalyptus* species are currently among the most extensively planted forest trees throughout the world (FAO, 1979; Zobel, 1993; Eldridge *et al.*, 1994).

Desirable traits, such as a fast growth rate and fibre quality, make *E. globulus* in particular an economically important species in industry for timber, pulp and paper

production (Tibbits *et al.*, 1997). An increasing demand for forest products has resulted in the need for greater yields from plantations in order to increase productivity and product quality.

In order to increase yields, genetically improved planting material is essential and clonal propagation is the preferred means of short term capture of genetic gain. The success of large scale micropropagation of elite eucalypt trees for commercial purposes is dependent upon the rooting ability of the clonal material. The formation of ARs followed by the growth of LRs is critical for establishing stem cuttings, or micropropagated clones. A healthy root system is required for essential plant functions such as regular uptake of water and nutrients from the soil, storage of nutrients, association with beneficial organisms in the rhizosphere such as mycorrhizae, phytohormone synthesis, and overall structural support. It follows then, that poor root development is likely to result in inadequate field establishment and economic loss.

Unfortunately, many selected clones of *E. globulus*, and other temperate eucalypts such as *E. nitens*, *E. grandis* and *E. regnans* do not root, or root very poorly in tissue culture (Hartney, 1980; MacRae, 1991; MacRae and van Staden, 1993; Bennett *et al.*, 1994; Sasse and Sands, 1995). This has long been considered to represent a major hindrance to vegetative propagation programs (Hartney, 1980; Sasse and Sands, 1995). The inability to form roots, or unreliable rooting, severely limits the potential genetic gain and the selection of favourable genetic characteristics. In Australia, this problem creates a serious obstacle to clonal propagation for Australian pulp producers, which could result in poor field establishment and economic loss. Consequently, an understanding of the fundamental processes involved in plant root differentiation and growth, the formation of root primordia, and their subsequent development into actively growing roots, has become important for the successful micropropagation of elite eucalypt genotypes in horticulture and silviculture.

1.7 AIMS OF THIS RESEARCH PROJECT

Much information is still to be collected in order to gain a clearer understanding of the mechanisms that govern LR development, particularly in long-lived woody species such as members of the *Eucalyptus* genus. The knowledge gained from the research outlined above, has provided a valuable base for the undertaking of a continuing search to identify

the regulatory mechanisms that control root development. In particular, the genetic and molecular mechanisms that drive LR development including those which mediate cell division and differentiation, as well as LR initiation, emergence and growth, still require further elucidation. The current research study was aimed to identify some of these mechanisms, using the accumulated evidence as a framework upon which to further build our understanding of the developmental processes that regulate LR formation and growth in higher plants. As a means to achieve this aim, there were two principal objectives considered in this current study.

1. At the molecular level, the study was directed towards the isolation and preliminary characterisation of genes responsible for, or closely associated with LR initiation in young seedlings of *E. globulus*. Of particular interest was the identification of genes associated with rooting in *Eucalyptus* for two main reasons. Firstly, to compare *Eucalyptus* with other systems, particularly *Arabidopsis*, and identify commonalties and possible novel gene sequences, some of which conceivably may be unique to eucalypts or woody species. Secondly, the DNA sequences produced may be available for mapping programs in *Eucalyptus*. The DNA sequences identified may be associated with quantitative trait loci (QTLs) such as rooting, and therefore may be valuable for marker-assisted breeding programs.

2. At the genetic level, an aspect of the current study aimed to identify and initiate characterisation of novel root mutants in the model plant, *Arabidopsis*. The long generation time of *E. globulus* makes it unsuitable for genetic analysis aimed to identify and characterise mutant phenotypes, as has clearly been noted. As outlined previously, the genetic advantages and simple root structure of *Arabidopsis* has made it a favoured model species for the identification of a number of morphological root mutants and subsequent genetic analyses.

It is anticipated that many aspects of LR formation, a fundamental process in plant growth and development, will be conserved between different widely divergent plant species, just as floral development appears to be. For example, functional homologues of two genes involved in floral meristem development in *Arabidopsis* (*LEAFY* and *APETALA1*) have recently been recovered and characterised from *E. globulus* (Kyozuka *et al.*, 1997; Southerton *et al.*, 1998). Understanding of the genetics of LR development and growth in *Arabidopsis* is likely to be of direct relevance to understanding the processes in other higher plants, including woody species such as *Eucalyptus*.

CHAPTER TWO

Materials and Methods

2.1 MEDIA AND REAGENTS

All chemicals (Analar grade) and plant tissue culture media were purchased from Sigma-Aldrich, Australia unless specified. Ultra high quality (UHQ) water purified via a Millipore Milli-Q plus Ultra High Quality Water System (18.2MΩcm quality) was used for all buffers and reagents required for tissue culture and RNA work. Single distilled water (dH_2O) was used for all other procedures unless specified.

2.1.1 Plant Culture Media and Solutions

Auxins:

All auxins were purchased from Sigma-Aldrich, Australia and dissolved as stocks as follows:

2,4-D (2,4-Dichlorophenoxyacetic acid): 10 mg/ml stock dissolved in ethanol.

IAA (Indole-3-acetic acid): 10 mg/ml stock solution dissolved in ethanol.

IBA (Indole-3-butyric acid potassium salt): 10 mg/ml stock dissolved in UHQ water.

IPA (Indole-3-propionic acid): 10 mg/ml stock solution dissolved in UHQ water.

NAA (α-Naphthaleneacetic acid): 10 mg/ml stock dissolved in 1 M NaOH.

All Stock solutions were filter sterilised and stored at -20°C.

Cytokinins:

All cytokinins were purchased from Sigma-Aldrich, Australia and dissolved as stocks as follows:

2ip (6-*y*-*y*-Dimethylallylaminopurine): 5 mg/ml stock solution dissolved in 1 M NaOH.

BAP (6-Benzylaminopurine): 10 mg/ml stock solution dissolved in 1 M NaOH.

Kinetin (6-Furfurylaminopurine; N^6-Furfuryladenine): 10 mg/ml stock solution dissolved in 1 M NaOH.

Zeatin (t-Zeatin-riboside): 5 mg/ml stock solution dissolved in UHQ water.

All Stock solutions were filter sterilised and stored at -20°C.

ERM (Eucalypt Rooting Medium): 2% (w/v) sucrose, 4.5 mM KNO_3, 5 mM NH_4NO_3, 20 mg/l myoinositol, 1x MS (Murashige and Skoog, 1962) micro nutrients (132 µM $MnSO_4$, 30 µM $ZnSO_4$, 100 µM H_3BO_3 1 µM Na_2MoO_4, 5 µM KI, 0.1 µM $CuSO_4$, 0.1 µM $CoCl_2$), 0.1 mM FeEDTA, 1x B5 vitamins (8 µM Nicotinic acid, 30 µM Thiamine, 5 µM Pyridoxine), 0.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 6.8 mM

K_2SO_4, 5.4 mM Ca Gluconate, 3 mM Mg Gluconate. Media was adjusted to pH 5.8 and solidified with a final concentration of 0.8% agar.

EGM (Eucalypt Germination Medium): 0.433% MS salts (Murashige and Skoog, 1962), 2% sucrose, 0.2% phytagel (Sigma-Aldrich, Australia), pH 5.8.

MS (Murashige and Skoog, 1962): 0.443% (w/v) MS plant medium powder, 2% (w/v) sucrose, pH 5.8-6.0. Solid media contained 0.2% (w/v) phytagel (Sigma-Aldrich, Australia).

ARAB MS: 0.443% (w/v) MS plant medium powder (Murashige and Skoog, 1962), 3% (w/v) sucrose, pH 5.8-6.0. Solid media contained 0.2% (w/v) phytagel (Sigma-Aldrich, Australia).

MX: 0.443% (w/v) MS plant medium powder (Murashige and Skoog, 1962), 3% (w/v) sucrose, 0.5 mg/l 2,4-D, pH 5.8-6.0.

Arabidopsis Nutrient Solution: 5 mM KNO_3, 2.5 mM KH_2PO_4 (pH 5.5), 2.5 mM K_2HPO_4 (pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.05 mM FeNaEDTA, 1 ml/l micro nutrient stock solution (70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 10 mM NaCl, 0.01 mM $CoCl_2$).

2.1.2 Bacterial Media and Solutions

Luria-Bertani Broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% NaCl; pH 7.5. Solid media contained 1.5% agar.

IPTG: 500 mM stock of isopropyl-ß-D-thio-galactopyranoside in dH_2O, filter sterilised and stored at -20°C.

Transformation and Storage Buffer + Glucose (TSBG): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% NaCl; pH 6.1. To this solution the following reagents are added to final concentrations as indicated: 10% PEG, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

X-Gal: 50 mg/ml stock of 5-Bromo-4-chloro-3-indoyl-ß-D-galactopyranoside in Dimethylformamide and stored at -20°C.

2.1.3 General Buffers and Solutions

Ampicillin: 25 mg/ml stock in dH_2O filter sterilised with 0.2 µm sterile disposable filters (Millipore) and stored at -20°C.

50x Denhardts solution: 1% (w/v) Ficoll type 400, 1% (w/v) PVP, 1% (w/v) BSA.

Ethidium bromide: 10 mg/ml stock in dH_2O.

6x Loading buffer: 15% (w/v) Ficoll, 25% (w/v) Xylene cyanol, 25% (w/v) Bromphenol blue.

10x MOPS (4-Morphilinopropane sulphonic acid): 0.2 M MOPS, 80 mM Na acetate, 10 mM EDTA; pH 8.

Sodium dodecyl sulphate (SDS): 10% (w/v) SDS in dH_2O.

20x SSC: 3 M NaCl, 0.3 M Na_3 citrate; pH 7.5.

10x TBE buffer: 89 mM Tris, 89 mM Boric acid, 25 mM Na_2EDTA; pH 8.0.

TE buffer: 10 mM TrisCl, 1 mM EDTA; pH 8.0.

2.2 PLANT CULTURE

2.2.1 *In Vitro* Plant Culture of *Eucalyptus globulus*

2.2.1.1 Plant Material

Seeds of *Eucalyptus globulus* subsp. *globulus* used in this study were donated by Richard Appleton of APM Forests Pty. Ltd., Gippsland, Vic., Australia, or purchased from the

Tasmanian Seed Centre, Hobart, Tas., Australia. Seeds were surface sterilised for 30 min with agitation in a concentrated commercial bleach, White King (4.2% sodium hypochlorite, 4% available chlorine, 0.9% NaOH). Seeds were then rinsed thoroughly with sterile dH_2O before sowing onto EGM in deep Petri dishes (90 mm x 25 mm). Twenty-five seeds were sown per plate and allowed to germinate at 22°C±2°C in the dark to promote elongation of both the hypocotyls and roots. On the eighth day seedlings were removed from the dark and grown under a 16 h photo period with fluorescent lighting (Sylvania Gro-Lux, Thorn, UK, waveband ~360-720 nm) at a photon flux density of 60 $\mu mol\ m^{-2}\ s^{-1}$.

2.2.1.2 Phytohormone Induction

Root and hypocotyl explants from eight day old seedlings were laid flat on the surface of ERM supplemented with the appropriate phytohormone. Plates containing root explants were oriented in a vertical position. This allowed roots to grow straight down the surface of the medium and avoid becoming entangled with neighbouring explants, making subsequent scoring easier. After the required length of time explants were removed and cleared for LRP or ARP analyses, or snap frozen in liquid nitrogen and stored at -70°C for RNA extraction.

2.2.1.3 Clearing Plants for Root Primordia Observation

To visualise root primordia in roots and hypocotyls of *E. globulus*, explants were cleared in a solution of 2% (w/v) CrO_3 (BDH Chemicals) for 16 h at room temperature. *Arabidopsis thaliana* seedlings were cleared in 1% (w/v) CrO_3 for 2 min. The explants were then rinsed in dH_2O until they were completely rid of CrO_3. Explants were examined with a stereomicroscope (Leica MZ8) placed on top of a light box and scored for the number of root primordia per millimetre of explant length.

2.2.1.4 Growth and Harvesting of Cell Suspension Cultures

Cell suspension cultures were grown in 250 ml conical flasks containing 40 ml MX medium on a orbital shaker at 22°C±2°C. Subculture involved transferring approximately 15 ml of a 4-6 week old cell suspension into fresh MX. Cell Suspensions were harvested by filtering the culture through a funnel lined with blotting paper. Cells were rinsed with sterile dH_2O before the filter paper containing the cells was removed and placed onto a pad of absorbent paper to remove the excess liquid. The cells were then snap frozen in liquid nitrogen and stored at -70°C until required for total RNA isolation.

2.2.2 *In Vitro* Plant Culture of *Arabidopsis thaliana*

The background strain of *Arabidopsis* used for phytohormone induction experiments and mutant isolation screens was Landsberg *erecta*. Seeds were surface sterilised for 10 min with agitation in a 10% (v/v) solution of a commercial bleach, Domestos (5.25% Na hypochlorite, 5% available chlorine 1.25% NaOH, 0.5 g/l alkaline salts), and then rinsed with sterile dH_2O. Seeds were kept in an immersion of water to prevent dehydration before sowing, and incubated at 4°C for two days to synchronise germination.

A sterile filter disc was placed on the surface of solidified MS medium contained in a 90 mm x 15 mm Petri dish. Ten to 15 seeds were sown onto each plate by pipetting onto the surface of the filter disc. Plates were sealed with micropore tape and incubated vertically at 22°C±2°C under a 16 h photo period with lighting conditions the same as those employed for *in vitro* growth of *E. globulus*. When seedlings reached the four leaf stage they were transferred and cultured in 15 cm tall plastic pots with vented caps.

2.2.2.1 Plant Culture in Soil

All plants either from seed or transplanted from *in vitro*, were grown in a 1:1 mixture of perlite and soil (Debco Seed Raising Mix) and watered with *Arabidopsis* nutrient solution. Plants were grown in a temperature controlled, insect free glasshouse at 18-20°C under constant fluorescent lighting (Sylvania Gro-Lux, Thorn, Germany wave band 360-720 nm, photon flux density 150 μmol m^{-2} s^{-1}).

Sown seeds were kept under clear plastic and held at 4°C for two days before transfer to the glasshouse. At this point holes were cut into the plastic which was completely removed upon the appearance of the cotyledons. Plants transferred from *in vitro* culture were transplanted into individual pots containing soil moistened with *Arabidopsis* nutrient solution. Clear plastic was wrapped around the pot to create a tube surrounding the plant. The top of the tube was partially closed off with a paper clip to allow for some ventilation while still maintaining a humid environment. Once the plant had become sufficiently acclimated to the soil conditions, the paper clip was removed in order to completely open the top of the tube. The plastic tube itself was not removed acting as an effective barrier to prevent the introduction of stray seeds from neighbouring plants of different genotypes.

2.2.3 EMS Mutagenesis

Three grams or 150,000 wildtype seeds of *Arabidopsis* from the Landsberg *erecta* background were added to 300 ml of dH_2O. The container was wrapped in aluminium foil to reduce exposure to light, and the mixture stirred with a paper clip driven by a magnetic stirrer. Ethyl methanesulfonate (EMS) was added to a final concentration of 40 mM and the mixture was left to stir for 8 h, and then decanted into a 10 M solution of NaOH and discarded. For rinsing, water was added to the seeds and stirred for about 10 min and then poured off and replaced. This process was repeated about ten times over a period of 2 h. After the final wash, 700 ml of cooled 0.15% agar was added to the seeds. Using a Pasteur pipette, about 50,000 seeds were sown at a density of approximately 200 seeds per punnet (13.5 cm x 8 cm). The sown seeds were covered with plastic wrap and incubated at 4°C for two days before transfer to the glasshouse.

2.3 BACTERIAL TECHNIQUES

2.3.1 Competent Cells and Transformation (adapted from Chung and Miller, 1988)

Escherichia coli DH5α cells were incubated at 37°C with shaking, and grown to early log phase (OD_{600} = 0.3-0.6) in LB. Cells were collected by centrifugation at 3,000 rpm for 10 min at 4°C, resuspended in 0.1 volume of TSBG, and DMSO added to a final concentration of 5% (v/v). The cells were aliquoted into 1.5 ml eppendorf tubes in 100 µl lots and incubated on ice for no less than 10 min. Cells were then snap frozen in liquid nitrogen and stored at -70°C.

For each transformation, 100 µl of cells were thawed and left to cool on ice. To the cells 3-5 µl of a ligation reaction was added and mixed. Cells were returned to the ice for 30 min. Following the addition of 200 µl of TSBG, the cells were incubated at 37°C with shaking for 60 min. 100-200 µl of the cells were spread onto LB agar plates containing 0.5 mM IPTG and 80 µg/ml X-Gal for blue-white selection and 100 µg/ml of ampicillin to select for transformed colonies. Plates were then incubated overnight at 37°C and white colonies checked for containing the introduced plasmid.

2.3.2 Alkaline-Lysis/PEG Precipitation for the Isolation of Plasmid DNA

This method for isolating plasmid DNA was recommended by Applied Biosystems, Inc., for use in automated sequencing. The same isolation procedure was also employed for all other applications requiring mini preparations of plasmid DNA.

Cultures of 10 ml volume were incubated overnight at 37°C with shaking in LB supplemented with the appropriate antibiotic. Between 1-4.5 ml of cells were pelleted by centrifugation for 1 min at 14,000 rpm. The cells were resuspended in 200 µl of Plasmid isolation buffer (50 mM glucose, 10 mM EDTA pH 8.0 and 25 mM Tris-Cl pH 8.0) before the addition of 300 µl of freshly prepared Lysis buffer (0.2 M NaOH, 1% SDS). The contents of the tubes was mixed by inversion until the solution cleared and then incubated on ice for 5 min. 300 µl of 3 M K/5 M acetate pH 4.8 was added to the tubes, mixed and then incubated on ice for a further 5 min.

Tubes were centrifuged for 10 min at room temperature to pellet the cellular debris. The supernatants were transferred to clean tubes and 1.6 µl of RNase A (10 mg/ml) was added before incubating at 37°C for 20 min.

Following RNase A treatment the supernatants were extracted twice with 400 µl of chloroform. For each extraction, the samples were mixed for 30 sec and then centrifuged for 1 min to separate the phases. The aqueous phases were transferred to clean tubes.

Total DNA was precipitated by adding an equal volume of 100% isopropanol and centrifuging immediately for 10 min at room temperature. The pellets were washed with 500 µl of 70% ethanol, dried under vacuum and resuspended in 32 µl of UHQ water. Plasmid DNA was then precipitated by adding 8 µl of 4M NaCl and 40 µl of 13% PEG_{8000}. After thorough mixing the tubes were incubated on ice for 20 min and then centrifuged for 15 min at 4°C to pellet the plasmid DNA. The supernatants were removed and pellets washed in 500 µl of 70% ethanol. The pellets were dried under vacuum and resuspended in 20 µl of UHQ water.

2.4 MOLECULAR TECHNIQUES

2.4.1 Preparation of Polyacrylamide Gels

To make a 6% denaturing polyacrylamide gel, 15 ml of a 40% acrylamide stock solution (38% acrylamide and 2% Bis in 100 ml UHQ water) was mixed with 42 g of pure urea in a final volume of 100 ml 1x TBE. Amberlite beads were added to the solution and stirred for 10 min before the acrylamide mix was filtered using a Whatman 1 Qualitative filter disc. Just before pouring, 36 µl TEMED and 650 µl ammonium persulphate (0.1 g/ml) were added to the acrylamide solution. Urea was omitted for nondenaturing polyacrylamide gels.

The gel was poured between two glass sequencing plates, separated by 0.4 mm spacers and allowed to set overnight. Following polymerisation, the top of the gel was rinsed to remove any unpolymerised acrylamide before a 0.4 mm sharkstooth comb was set in place.

2.4.2 Agarose Gel Electrophoresis of DNA and RNA

DNA samples were mixed with loading buffer and run on a 0.8% (w/v) agarose gel containing 0.04 µg/ml ethidium bromide. Mini gels were run in 1x TBE buffer for approximately 90 min at 60 volts. Larger gels were run overnight at 40 volts. The linearised DNA fragments were electrophoresed alongside 250 ng of a lambda *BstE*II or a ϕX174 *Hae*III ladder. A standard curve of these ladders was generated to determine the size of the DNA fragments of interest.

RNA samples were prepared for electrophoresis by combining 20 µg of total RNA to a solution with final concentrations of 15% (v/v) formaldehyde, 50% (v/v) deionised formamide and 1x MOPS. The samples were heated for 15 min at 65°C and then quickly chilled on ice. Before loading, 2 µl of loading buffer was added to the samples.

Prepared RNA samples were run on a formaldehyde denaturing gel [1.5% (w/v) agarose, 1x MOPS, 5.4% (v/v) formaldehyde, 0.4 µg/ml ethidium bromide]. Gels were pre-run in 1x MOPS running buffer for 15 min. The prepared RNA was loaded into the

gel and run overnight at 40 volts, until the dye front had migrated 8 cm from the wells. Extreme care was taken to ensure all apparatus used for making and running gels was RNase free.

In both cases, DNA and RNA on gels were visualised over ultraviolet (UV) transillumination. Gels were photographed with either a Polaroid camera or a gel documentation system.

2.4.3 PCR Purification

The QIAquick PCR Purification Kit (Qiagen Pty. Ltd.) was used to purify double stranded PCR products to be used for ligations, transformations and direct sequencing. PCR products were purified directly from PCR reaction mixes using QIAquick spin columns to separate the amplified product from primers, nucleotides, DNA polymerase and salts. The protocol was followed exactly as outlined in the QIAquick Spin Handbook, July 1997.

2.4.4 Restriction Enzyme Digestion

Restriction endonucleases used for digesting DNA were supplied by Promega and used according to the conditions specified by the manufacturer. 1-10 µg of DNA was digested with 1 unit/µg of a specific restriction enzyme in the presence of the appropriate 1x enzyme buffer. 2 mM spermidine was sometimes added to facilitate digestion. The digests were incubated at temperatures specified by the manufacturer for anywhere between a minimum of 3 h and maximum of overnight. In cases where digestion had not gone to completion additional enzyme and further incubation was required. In order to inactivate the enzyme the reaction was heated to 65°C for 10 min and chilled on ice.

2.4.5 DNA Fragment Isolation from Agarose Gels by Phenol Freeze

The DNA fragments used in standard molecular biology techniques were recovered from agarose gels following gel electrophoresis of PCR products or restriction enzyme digests.

The bands of interest were identified under UV illumination and cut from the gel using a sterile scalpel blade. After excess agarose was removed, the gel block containing the band to be isolated, was placed into an eppendorf tube and crushed thoroughly before the addition of 500 µl phenol. The samples were homogenised by vortexing and then incubated overnight at -20°C or -70°C for 30 min.

Samples were centrifuged for 30 min at 14,000 rpm. The upper aqueous phase was transferred to a new tube. To the lower organic layer, 200 µl of TE was added and mixed by inversion before centrifuging for a further 15 min. The aqueous phase was again removed and combined with the one taken previously. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the combined aqueous phases, mixed and centrifuged for 10 min at 14,000 rpm. The top layer was removed, and the DNA precipitated with the addition of 1/10 volume of 3 M Na Acetate and 2 volumes of 100% ethanol. After an overnight incubation at -20°C, the DNA was pelleted by centrifugation at 14,000 rpm for 30 min, washed with 70% ethanol, dried under vacuum and resuspended in 10 µl of sterile UHQ water.

2.4.6 T-Vector

PCR products were subcloned into a home-made T-vector prepared by digesting 5 µg of pBluescript SK (+/-) with *Eco*RV. Following complete digestion, two phenol/chloroform extractions were performed. Firstly an equal volume of phenol:chloroform (1:1) was added to the digestion reaction, mixed and centrifuged for 10 min. The aqueous phase was removed to a clean tube and re-extracted with an equal volume of chloroform. The aqueous phase was again separated and removed and precipitated in 1/10 volume 3 M Na Acetate pH 6.0 and 2.5 volumes 100% ethanol. Samples were left to precipitate at -20°C overnight or -70°C for 30 min. DNA was pelleted by centrifugation for 30 min at 4°C. The supernatant was removed and the pellet washed with 70% ethanol, dried under vacuum and resuspended in 31 µl UHQ water.

The resuspended DNA is incubated at 72°C for 3 h in a total reaction volume of 50 µl containing, in final concentrations, 1x *Taq* Polymerase buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 2 mM dTTP and 1.25 units *Taq* DNA Polymerase (Promega). When incubation was complete the T-vector was extracted and precipitated as before and the resulting pellet resuspended at 50 ng/µl.

2.4.7 Ligations

DNA fragments generated by PCR were ligated into a T-vector. Ligation reactions were performed using 50-100 ng of T-vector and an equimolar amount of DNA insert at a ratio of 1:1. Ligations were incubated in 1x ligase buffer (30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) with 3 units T4 DNA ligase (Promega) at 15°C for 3 h followed by 4°C overnight. Following incubation 3-5 µl of the ligation reaction was used to transform competent cells of *E. Coli* DH5α.

2.4.8 Cycle Sequencing

Sequencing reactions were performed using the ABI Prism Big Dye Terminator Cycle Sequencing Reaction supplied by the Perkin-Elmer Corporation. Each reaction contained 6 µl Big Dye Terminator Ready Reaction Mix, 300-500 ng of insert cloned into a pBluescript T-vector, and 15 ng of T3 (5'-AATTAACCCTCACTAAAGGG-3') or T7 (5'-GTAATACGACTCACTATAGGGC-3') primer. The reaction volume was made up to 15 µl with dH_2O and overlaid with a drop of paraffin oil. Thermal cycling was performed in the Perkin-Elmer DNA Thermal Cycler 480 under the following conditions; denaturation at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min. This cycle was repeated 25 times before proceeding with purification of the extension products.

Sequencing reactions were transferred to eppendorf tubes containing 2 µl of 3M Na Acetate pH 6.0 and 50 µl of 100% ethanol. Tubes were vortexed briefly and left at room temperature for 15 min to precipitate the extension products. Tubes were then centrifuged for 20 min at maximum speed, the pellets washed with 70% ethanol and then dried in a vacuum centrifuge. Sequence was determined using an Applied Biosystems ABI373A automated sequencer by the Department of Microbiology at Monash University, Clayton, Victoria.

2.4.9 Total RNA Extraction from *Eucalyptus globulus*

The method used to isolate total RNA from *E. globulus* tissues was modified from a procedure reported by Lester *et al.* (1994). The amount of tissue required for each

extraction depended on the tissue type, 2.0 g for apical shoot tissue, always including the cotyledons, 2.5-3.0 g for hypocotyls and roots, and 5.0-8.0 g of cells harvested from suspension cultures.

Tissue was homogenised in liquid nitrogen with a mortar pestle, and then added to 50 ml Falcon tubes containing 40 ml Extraction Buffer [100 mM Tris pH 9.0, 100 mM NaCl, 1% (w/v) SDS, 1% (w/v) PVP, 1% (v/v) ß-mercaptoethanol, 100 µg/ml proteinase K] and left at room temperature for 5 min. Samples were centrifuged at 3,500 rpm for 10 min to sediment the cellular debris. The supernatant was removed and an equal volume of phenol was added. Samples were mixed by inversion and then centrifuged at 3,500 rpm. The aqueous phase was transferred to a clean tube and re-extracted with an equal volume of phenol:chloroform (1:1). The aqueous phase was again removed and extracted for a third and final time with an equal volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was placed on ice and 1/10 volume 3M NaAc pH 4.8, 1/100 volume 10% SDS, and 1/10 volume of 5 M NaCl were added. Samples were incubated on ice for 2 h or at 0-4°C overnight. To remove the precipitate, tubes were centrifuged at 6,000 rpm for 20 min and the supernatants transferred to clean tubes and mixed with an equal volume of 6 M LiCl and precipitated overnight at 4°C.

The RNA was collected by centrifugation at 6,000 rpm for 30 min. Following resuspension of pellets in 400 µl UHQ water, samples were transferred to eppendorf tubes. To each tube 100 µl of 500 mM NaCl and 1 ml 100% ethanol were added and the RNA left to precipitate overnight at -20°C. After centrifugation at 14,000 rpm for 30 min the resulting RNA pellets were washed with 70% ethanol, dried under vacuum and resuspended in 100 µl of UHQ water.

To remove any remaining carbohydrates, samples were spun for 5 min at 14,000 rpm. The carbohydrate containing pellets were discarded, while the supernatants containing the RNA still in suspension were removed to new tubes. This process of pelleting the carbohydrates and transferring the supernatants was repeated until no pellets formed. The quality of the RNA was checked by electrophoresis and the concentration determined by spectrophotometry.

2.4.10 Northern Blot Analysis

Following electrophoresis and photographing of RNA, the denaturing gels were rinsed twice in 10x SSC for 20 min to remove the formaldehyde. Capillary blots were prepared by first placing a flat perspex plate over a tray filled with blotting buffer (10x SSC) to create a platform. Three sheets of filter paper were placed over the platform to form a wick with both ends immersed in the blotting buffer. The wick was saturated with additional blotting buffer and any trapped air bubbles were removed. The gel was placed upside down onto the wick again avoiding air bubbles, and then surrounded with plastic cling wrap. Placed on top of the gel was a sheet of Hybond-N^+ membrane (Amersham International plc) cut to size. Any air bubbles trapped between the gel and membrane were squeezed out using a glass pipette. Layered on top were three sheets of filter paper followed by a stack of absorbent paper towels approximately 5 cm high. A second flat perspex tray was placed on top to hold a weight of 0.75-1 kg. Transfer of RNA occurred over 48 h with a top up of blotting buffer and a paper towel replacement after 24 h. The transferred RNA was alkali fixed to the Hybond N+ membrane, by laying the membrane, RNA side up on three sheets of filter paper soaked in 0.05 M NaOH for 20 min. The membrane was then rinsed in 5x SSC with gentle agitation for no longer than 1 min, and then dried between two sheets of filter paper.

For radioactive labelling, membranes were prehybridised and hybridised using ExpressHyb hybridisation solution supplied by Clontech Laboratories, Inc. Prehybridisation was in a hybridisation tube with 5 ml of ExpressHyb solution at 65°C for 30 min. Radioactively labelled DNA probe was denatured and added to 3 ml of fresh ExpressHyb and used to replace the prehybridisation solution. Hybridisation was for 1 h at 65°C. Following incubation membranes were rinsed twice for 20 min in wash solution 1 (2x SSC, 0.05% SDS) at room temperature with gentle shaking. These washes were followed by a further two 20 min rinses at 50°C in wash solution 2 (0.1x SSC, 0.1% SDS) with continuous agitation. Throughout the labelling and washing procedure, great care was taken to prevent the membrane from drying out in order to reduce nonspecific background.

Radioactively labelled membranes were autoradiographed and scanned using a phosphoimager to correct for loading differences and calculate the relative strength of signals. Probes were stripped from membranes by washing at room temperature with boiling 0.5% SDS. Membranes were left in the 0.5% SDS until the solution had cooled to

room temperature. Membranes were dried with filter paper and wrapped in plastic for storage.

2.4.11 Preparation of Radioactive Probes

DNA was radioactively labelled using [α-^{32}P]-dATP and the Gigaprime DNA labelling kit both supplied by Geneworks. DNA probes were prepared according to the manufacturer's instructions which accompanied the labelling kit. For each reaction, approximately 100 ng of denatured DNA was labelled and then separated and recovered from the unincorporated nucleotides using a sephadex G-50 drip column.

Columns were prepared by partially plugging a short (14.5 cm) Pasteur pipette at its neck with glass wool and then filling it to about 2 cm from the top with a suspension of sephadex G-50 in TE. The flow rate of the columns was checked as they were equilibrated with TE. A flow rate of about 2 drops per second was optimal.

Following a 15 min incubation at 37°C, labelling reactions were pipetted into the sephadex column and continually topped up with TE until collection of fractions was complete. A total of 12 fractions, each containing eight drops, were collected into eppendorf tubes. The radioactivity contained in each fraction was monitored using a Geiger counter. When labelling reactions were successful, two peaks were detected. The first peak was the one of interest, containing the fractions with the ^{32}P-dATP labelled DNA fragments. The second peak to be detected was that which consisted of fractions containing the unincorporated nucleotides, these fractions were of no use and discarded. The labelled DNA incorporating the ^{32}P was denatured by boiling for 5 min and quickly chilled on ice immediately before Northern hybridisation.

2.4.12 Autoradiography

All ^{32}P labelled membranes and gels were wrapped in plastic and exposed to Fuji X-ray film in an X-ray cassette with one intensifying screen. Cassettes were held at -80°C for up to 7 days depending on the level of radioactivity being emitted. X-ray films were developed using the Agfa-Gevaert Gevamatic 60 X-ray machine.

2.4.13 Purification of mRNA from Total RNA

Purification of polyadenylated mRNA [poly(A)$^+$ RNA] was achieved with the use of Dynabeads® Oligo $(dT)_{25}$ manufactured by Dynal. The supplied protocol was modified to optimise the downstream enzymatic reaction for cDNA synthesis.

The volume of 150 µg of total RNA was adjusted to 200 µl with UHQ water. In order to disrupt the secondary structures the RNA was heated at 65°C for 2 min. During this incubation 400 µl of resuspended Dynabeads® Oligo $(dT)_{25}$ was transferred to an eppendorf tube standing in the Dynal MPC®-E magnet. After 30 sec the supernatant was removed and the beads washed once with 200 µl 2x binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA). The eppendorf tube containing the beads was transferred to a rack and 200 µl 2x binding buffer added with the total RNA. The contents of the tube were mixed gently and then left to hybridise for 10 min. The tube was then returned to the magnet for 30 sec and the supernatant removed. The beads were washed twice with 400 µl washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA) and twice with 400 µl of reverse transcription buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 6 mM $MgCl_2$). Care was taken to ensure the complete removal of supernatant following the final wash.

To elute the mRNA from the beads, 10 µl of 2 mM EDTA (pH 7.5) was added and the tube heated to 65°C for 2 min. The tube was then placed directly into the magnet and the eluted mRNA transferred to a new eppendorf tube. The mRNA was checked by running 1 µl on a 0.8% agarose gel. If ribosomal RNA had not been completely removed, the mRNA was put through a second round of purification. The reused beads were washed once in 400 µl washing buffer and then resuspended in 40 µl 2x binding buffer before adding the mRNA. After a 10 min hybridisation, the mRNA bound beads were washed and the mRNA eluted as described for the first round purification.

The mRNA was again checked by electrophoresis of 1 µl as previously outlined. The mRNA concentration was estimated by spotting 1 µl on an ethidium bromide plate with known concentration standards. When not used immediately, the mRNA was stored at -70°C.

2.4.14 CLONTECH PCR-Select cDNA Subtraction

E. globulus tissue that ultimately gave rise to the tester cDNA population comprised of root explants taken from 8 day old seedlings, and exposed to 10^{-5} M IBA for 6, 12, 18, 24 and 36 h to induce LRP formation. Poly A^+ RNA was independently purified from each time point using Dynabeads® Oligo $(dT)_{25}$. Following mRNA quantification, 400 ng from each time point was combined for tester cDNA synthesis. Poly A^+ RNA used for driver was isolated from untreated shoot apex, hypocotyl and root explants. For driver cDNA synthesis, 800 ng of mRNA from each of the three untreated tissue types was prepared. The root tips were removed from all root explants at the point of tissue harvest, whilst the shoot apical samples included the cotyledons.

Poly A^+ RNA from human skeletal muscle was provided in the CLONTECH PCR-Select cDNA Subtraction Kit for use as a control during the entire procedure. As such, the skeletal muscle control was subjected to the same manipulations as described for the *E. globulus* LRP induced roots and uninduced control samples.

2.4.14.1 *Rsa* I Digestion and Adaptor Ligation

In independent reactions, the double stranded tester and driver cDNAs were incubated with the *Rsa* I restriction endonuclease. To finish preparing the tester cDNA, ligation of an adaptor to the blunt ends created by the *Rsa* I digestion was required.

To perform the adaptor ligation, two different adaptors were employed. This was significant, supplying unique annealing sites for two different PCR primers. The *E. globulus* tester cDNA was divided into two groups, with each group ligated to only one of the two adaptor sequences (refer to Appendix A-1 for adaptor sequences). The pool of tester cDNA ligated with Adaptor 1 became known as Tester 1-1, whilst the second pool formed Tester 1-2 following the attachment of Adaptor 2. An aliquot was taken from each of Tester 1-1 and Tester 1-2 and mixed to form the Unsubtracted tester control 1-c, which was to be utilised as a negative control for the subtraction that followed.

The control skeletal muscle tester cDNA, underwent an extra preparatory step before the same ligation procedure was applied to create Tester 2-1, Tester 2-2 and Unsubtracted tester control 2-c. *Hae* III digested ϕX174 DNA was added to the skeletal muscle tester cDNA, so that each restriction fragment comprised of approximately 0.02%

of the total cDNA. The restricted ϕX174 DNA represented unique sequences which should remain after subrtaction with the skeletal muscle driver cDNA.

2.4.14.2 First Hybridisation

Two separate hybridisation samples were prepared for each *E. globulus* and control skeletal muscle tester cDNA. Each contained 1.5 µl of *Rsa* I digested driver cDNA along with 1.5 µl from one of either Adaptor 1-ligated Tester 1-1 (hybridisation sample 1), or Adaptor 2-ligated Tester 1-2 (hybridisation sample 2). The final volume of each sample was made up to 4 µl with the addition of 1 µl 4x Hybridisation buffer. The samples were then overlaid with a drop of paraffin oil and incubated in the Perkin-Elmer DNA Thermal Cycler 480 for 1.5 min at 98°C. This was immediately followed by a 68°C incubation for 8 h.

2.4.14.3 Second Hybridisation

For the second hybridisation, the two samples taken from the first hybridisation were mixed together without denaturing. The sample was enriched further for differentially expressed sequences with the addition of extra freshly denatured driver cDNA. One µl of driver cDNA was diluted with 1 µl 4x Hybridisation buffer and 2 µl water. From this mixture, 1 µl was transferred to a new 0.5 ml microcentrifuge tube, overlaid with paraffin oil and incubated at 98°C for 1.5 min. This freshly denatured driver along with hybridisation sample 2, were then carefully transferred to the tube containing hybridisation sample 1 and mixed by gentle pipetting. Following an overnight incubation at 68°C, 200 µl of dilution buffer was added to the tube, mixed and incubated at 75°C for a further 7 min. The completed reaction was stored at -20°C until required.

2.4.14.4 Primary PCR

The protocol that accompanied the CLONTECH PCR-Select cDNA Subtraction Kit was modified slightly to optimise parameters for PCR amplification of the subtracted cDNA template from the *E. globulus* hybridised sample. Five reactions were performed in total. These included the subtracted *E. globulus* and control skeletal muscle cDNAs, their corresponding unsubtracted tester controls, 1-c and 2-c respectively, and the PCR Control subtracted cDNA (provided in the kit).

Subtracted and Unsubtracted cDNA: An aliquot of 4 µl was taken from the diluted, subtracted and unsubtracted cDNA samples of *E. globulus* and the skeletal muscle control, for use as templates in the primary PCR.

Positive Control: A single positive PCR control was set up using the PCR Control subtracted cDNA provided in the kit. This template contained a successfully subtracted mixture of *Hae* III-digested ϕX174 DNA. For the PCR template, 1 µl of this cDNA was added to 3 µl water for template.

A PCR Master Mix was prepared by combining, per individual reaction; 2.5 µl of 10x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0 and 0.1% Triton X-100 final concentrations), 1.5 µl of 25 mM $MgCl_2$ (1.5 mM final), 0.5 µl of 10 mM dNTPs (0.2 mM final), 1 µl of 10 µM PCR primer 1 (0.4 µM final) and 14.5 µl water. To each template, 20 µl of the prepared Master Mix was added and then overlaid with paraffin oil. A hot start PCR was essential to reduce nonspecific DNA synthesis during the PCR preparation.

PCR was performed in the Perkin-Elmer DNA Thermal Cycler 480. The samples were heated at 75°C for 1 min before the temperature was lowered to 60°C for the addition of 1 µl *Taq* DNA polymerase (1 unit/µl; Promega), bringing the final reaction volume to 25 µl. The reaction was then incubated for a further 5 min at 75°C. This step was essential for filling in the complementary strand of the overhanging adaptors to produce binding sites for the PCR primers. The adaptor extension was immediately followed by thermal cycling; 94°C for 30 sec, 63°C for 30 sec and 72°C for 1.5 min, over 32 cycles.

2.4.14.5 Secondary PCR

Three µl was taken from each primary PCR mix and diluted in 27 µl water, from which 6 µl of the subtracted and unsubtracted *E. globulus* cDNA was used for template in a second PCR. The control samples required less template for amplification, 1 µl of the diluted primary PCR mix was sufficient for the PCR control subtracted cDNA, and the subtracted and unsubtracted skeletal muscle cDNAs. Two water controls were performed using 1 µl and 6 µl of the corresponding diluted primary PCR.

Added to each template was 23 µl of a new Master Mix which contained for each single reaction; 2.5 µl of 10x reaction buffer (1x final), 1.5 µl of 25 mM $MgCl_2$ (1.5 mM final), 0.5 µl of 10 mM dNTPs (0.2 mM final), 1 µl of 10 µM Nested PCR primer 1 (0.4 µM final), 1 µl of 10 µM Nested PCR primer 2 (0.4 µM final) and 16.5 µl sterile water (refer to Appendix A-1 for primer sequences). After overlaying each sample with a drop of

paraffin oil, the reaction tubes were transferred to the Perkin-Elmer DNA Thermal Cycler 480. Here the samples were incubated at 75°C for 1 min and then held at 60°C for the addition of 1 µl *Taq* DNA polymerase (1 unit/µl; Promega) for a hot start PCR. Thermal cycling immediately followed with 20 rounds of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1.5 min.

2.4.15 Cloning of DDRT-PCR and cDNA Subtracted Bands

Following reamplification, the DDRT-PCR and cDNA subtracted bands were purified using either the QIAquick PCR Purification Kit or the phenol freeze method. The purified bands were ligated into a T-vector which was then transformed into competent cells of *E. coli* DH5α, and subjected to blue-white selection. White colonies were selected and checked for the presence of the introduced plasmid containing the cloned fragment.

Up to 14 white colonies were routinely checked for an insert from the cloning of each band. For each colony tested, two fresh 1 ml LB broths were inoculated with a single white colony and incubated overnight at 37°C with shaking. This was important since multiple cDNAs have been reported to co-migrate in a single band during DDRT-PCR (Averboukh *et al.*, 1996; Zhang *et al.*, 1996).

2.4.15.1 PCR to Check Colonies for Inserts

The presence of a cloned insert of the expected size was assayed by PCR using intact bacterial cells. Joshi *et al.* (1991) demonstrated that chromosomal DNA can be amplified directly from bacterial cells grown in liquid or cell culture. The advantage of this technique over restriction enzyme digestion was that plasmid isolation was not required, making it possible to screen more colonies for cloned inserts in less time, and with fewer manipulations.

Three controls were included in each experiment. To ensure the procedure was working, 50 ng of the purified DNA fragment used in the ligation reaction, was used as a positive control. This control also served to help identify inserts of the correct size. Untransformed DH5α was used as the negative control, while a water control was required to detect possible contamination that may have been introduced during the PCR set up.

Each bacterial broth was diluted 5 µl into 95 µl sterile water. PCR was completed in a final volume of 40 µl containing 10 µl of the diluted bacterial cells. The PCR conditions varied slightly for the DDRT-PCR and cDNA subtraction derived inserts.

PCR for DDRT-PCR inserts: The same primers used to identify each band, were employed to check colonies for the corresponding insert. The PCR was performed in 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 20 µM of each dNTP, 1 µM anchor primer, 0.2 µM arbitrary 10mer and 1 unit *Taq* DNA polymerase. Thermal cycling was conducted for 40 cycles at 94°C for 30 sec, 42°C for 2 min and 72°C for 30 sec. PCR was completed with a final 5 min incubation at 72°C.

PCR for subtracted inserts: PCR was carried out in 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM Nested PCR primer 1 and 0.4 µM Nested PCR primer 2. A hot start cycle was performed initially, 75°C for 1 min, followed by a 60°C incubation while 1 unit *Taq* DNA polymerase (Promega) was added to each individual reaction. Thermal cycling then followed for 30 cycles at 94°C for 1 min, 68°C for 1 min and 72°C 1.5 min.

Following amplification a 10 µl aliquot from each reaction was run on a 0.8% agarose gel and checked for the presence of an amplified product representing the cloned fragment. Bacterial cultures were discarded when colonies showed no evidence of an insert. On the other hand, bacterial cultures grown from colonies testing positive for a cloned insert were stored at -70°C after mixing 70% broth with 30% LB:glycerol (1:1). Glycerol stocks were available to recover cloned fragments for sequencing or use as probes, by either PCR or restriction enzyme digestion following plasmid purification.

CHAPTER THREE

Optimisation of Differential Display Procedures for *Eucalyptus globulus*

3.1 INTRODUCTION

In 1992, Liang and Pardee introduced differential display as an effective approach for isolating genes that are differentially expressed in various cells or in response to altered conditions (Liang and Pardee, 1992). The method was originally developed to identify and isolate differentially expressed genes involved with changes in growth, development and environmental responses in mammalian cell populations (Liang and Pardee, 1992). Also referred to as the differential display reverse transcription polymerase chain reaction (DDRT-PCR; Bauer *et al.*, 1993), the method has since been successfully applied to other eukaryotic systems, including plants (Oh *et al.*, 1995).

Initially, the technique was based on the premise that in higher organisms about 15,000 genes are expressed in each cell, with every distinct mRNA capable of being reverse transcribed and amplified by PCR (Liang and Pardee, 1992; Liang *et al.*, 1993). The main perceived advantage of DDRT-PCR over more conventional procedures such as subtractive hybridisation and differential library screening are;

(a) the relative speed and ease in which differentially expressed messages can be analysed in any eukaryotic cell;
(b) the relatively small amount of total cellular RNA or mRNA required as the starting material and;
(c) the ability to simultaneously screen mRNA from different sources to identify up-regulated as well as down-regulated gene expression (Liang and Pardee, 1992; Liang *et al.*, 1993; Oh *et al.*, 1995).

At the time of using DDRT-PCR in this study, the technique was still new. Modifications to improve the reliability and efficacy of the technique were frequently reported in the literature. Today DDRT-PCR kits are readily available and adaptable to most species. However, preparation of the cDNA template and PCR parameters are still critical to ensure the displayed patterns are consistent and false positives are minimised.

3.1.1 A Brief Description of the Differential Display Method and Principles

The DDRT-PCR method is a technique involving numerous steps. The basic concepts and principles of this procedure are outlined in Figure 3.1. The general strategy (Liang and Pardee, 1992) is to use a set of oligonucleotide primers to amplify cDNA sequences from

subsets of mRNAs by reverse transcription and PCR in order to identify tissue, developmental, or treatment-specific gene expression.

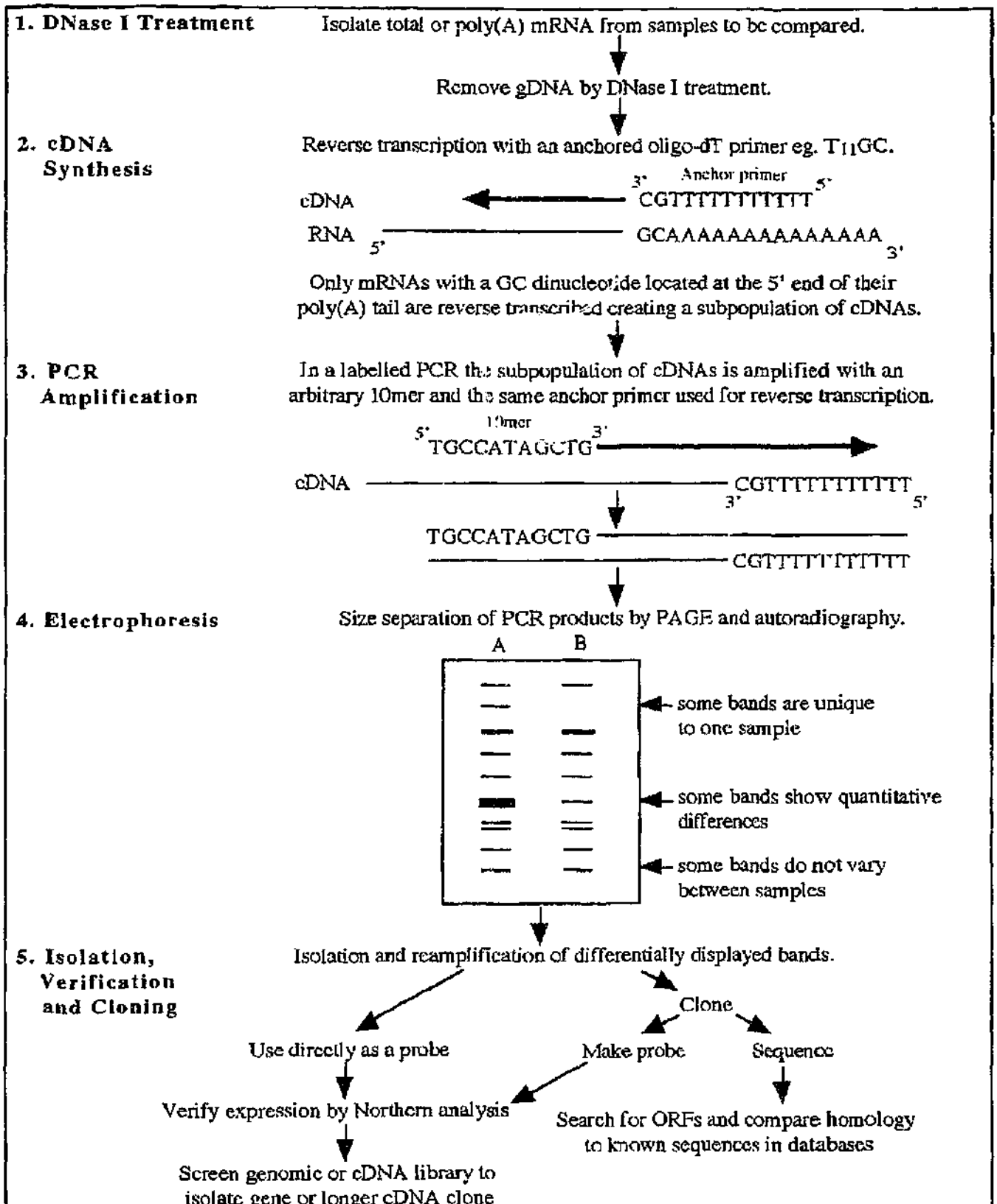


Figure 3.1: The DDRT-PCR Method. Reverse transcription and PCR are used to generate a population of radioactive cDNAs representing mRNA species from different sources for comparison. Following separation of the labelled PCR products by polyacrylamide gel electrophoresis (PAGE), differences in gene expression can be detected between the samples by identifying polymorphisms in banding patterns.

A 3' 'anchor primer' (5'-T_{11}MN-3', where M = A, C, or G & N = A, C, G, or T) exploits the fact that the vast majority of mRNA transcripts in the eukaryotic cell contain a poly(A) tail. It consists of 11 deoxythymine residues that anchor the primer at the 3' end of the mRNA, while the two additional 3' bases provide specificity. There exist 12 possible combinations of these last two bases (omitting T as the penultimate base), making it feasible to subdivide the total mRNA population from any one sample into 12 different fractions.

In independent reactions, mRNA is reverse transcribed using any one of the 12 possible anchor primers, effectively reducing the pool of candidate cDNAs. An anchor primer such as T_{11}GC for example, would anneal only to those mRNA molecules with a CG located just upstream of their poly(A) tails producing a population of first strand cDNAs. These first strand cDNAs are amplified by PCR in the presence of a radioactively labelled isotope such as, [α-^{35}S]-dATP, using the same anchored oligo-dT primer with a 5' arbitrary decamer. This 10mer consists of a random sequence of nucleotides with a G+C content of 60-70% and has no self complementary ends to avoid the possible formation of secondary structures. The 5' random oligo primer anneals at different positions along the cDNA relative to the 3' anchor T_{11}MN primer, producing PCR products of various sizes, up to about 500 bp in length.

Any primer pair has a statistical probability of identifying a number of target sequences amongst the reduced cDNA population. So by using different combinations of anchored oligo-dT primers and random 10mers, it should be theoretically possible to analyse virtually all mRNA species from particular tissues, developmental stages or treatments in a relatively short period of time (Liang and Pardee, 1992; Liang *et al.*, 1993).

After PCR amplification, the radioactively labelled cDNAs from two or more RNA samples are resolved side by side on a 6% denaturing polyacrylamide sequencing gel. Gels are vacuum dried onto Whatman paper without fixing and exposed to X-ray film. Following autoradiography, an array of bands corresponding to differentially expressed mRNAs in the different samples tested can be observed. The patterns displayed are compared to identify any polymorphic bands showing either a quantitative or a qualitative variation in expression. Once identified, bands of interest can be recovered from the dried gel, purified and the DNA reamplified for cloning, sequencing, and Northern and Southern analyses, to further characterise and verify the expression profile of the isolated fragment. Screening cDNA and genomic libraries to isolate full length clones and their corresponding genes is also possible.

3.1.2 Modifications

Since its introduction, DDRT-PCR has undergone many modifications to optimise efficiency and consistency (Bauer *et al.*, 1993; Liang *et al.*, 1993; Callard *et al.*, 1994; Averboukh *et al.*, 1996). Particular attention has focused on improving the resolution of DDRT-PCR products to ultimately facilitate the screening and cloning of differentially expressed bands (Appel *et al.*, 1999). Substantial effort has also been directed towards decreasing the redundancy of the anchor primers used in reverse transcription (Liang *et al.*, 1993; Oh *et al.*, 1995). This reduces the artificial complexity of banding patterns (Bauer *et al.*, 1993), improving reproducibility and sensitivity (Liang *et al.*, 1994) and minimising the number of false positives obtained (Callard *et al.*, 1994).

Due to its exquisite capacity to amplify rare cDNA sequences, the DDRT-PCR technique is extremely sensitive to changes in experimental conditions. This in turn can lead to inconsistent banding patterns and cause difficulties in reproducing results. Variable displays can be avoided if great care is taken to standardise important factors such as, the reagents, the master mixes, and the apparatus used in each experiment. Annealing temperature (Liang *et al.*, 1993) and dNTP concentration (Liang and Pardee, 1992; Song *et al.*, 1995), are two additional components that greatly effect the specificity of cDNA amplification. Changes in the batch of *Taq* DNA polymerase used (Haag and Raman, 1994), the amount of template (Liang *et al.*, 1993; Oh *et al.*, 1995), the size of the reaction volume (Oh *et al.*, 1995), the brand of thermal cycler used (Colonna-Romano *et al.*, 1998), or even the tubes used for the amplification reaction (Sompayrac *et al.*, 1995) also can contribute markedly to the degree of consistency in banding patterns between replicates and within experiments.

3.1.2.1 Anchor Primers

Work by Liang *et al.* (1993) suggests that the penultimate base (M) of the $T_{11}MN$ anchor primer is to a large extent degenerate during priming in reverse transcription. However, the last base (N) is critical for providing most of the specificity to anchor the primer to the beginning of the mRNA poly(A) tail. Therefore, it is possible to substitute the 12 $T_{11}MN$ primers for four degenerate ones that differ only in the last base ($T_{12}VN$, where V = an equimolar mix of A, C and G), thereby reducing the number of reverse transcription reactions necessary while continuing to maintain specificity.

Oh *et al.* (1995) developed this idea further by comparing the results obtained from the use of degenerative and specific anchor primers to amplify the same cDNA template in DDRT-PCR. Following reverse transcription using a degenerate primer, cDNA was amplified with either the same degenerate primer employed for cDNA synthesis or the corresponding set of specific anchor primers. In all cases, the same arbitrary 5' primer was used. They found that the degenerate oligonucleotide primed amplification gave significantly poorer resolution making identification of differentially expressed genes difficult. The specific anchor primer however, gave rise to extra bands of interest that did not appear in the degenerate primer derived displays.

This indicates that the degenerate primer does not efficiently amplify all cDNAs. Oh *et al.* (1995) speculated that this might be due to competition for amplification of certain sequences. If this is the case, the relative abundance of cDNAs or the level of homology between template and the random primer used may determine which sequences are preferentially amplified.

Although the efficiency of DDRT-PCR was improved simply by employing degenerative primers during reverse transcription, the use of specific anchor primers were essential during amplification to maximise the yield of differentially expressed products, by producing clearer display patterns (Oh *et al.*, 1995).

Liang *et al.* (1994) simplified the method further by describing the use of three one base anchored primers (H-T_{11}M, where H = *Hind* III restriction enzyme site). This allows for a further reduction in the number of reverse transcription reactions required to represent the entire mRNA pool of any given sample. Lengthening both the anchored and arbitrary primers at their 5' ends with the addition of a restriction enzyme site improved the efficiency of cDNA amplification by increasing specificity. This also served to improve reproducibility, minimise false positives and aided in the cloning of differentially displayed products.

3.1.2.2 Arbitrary Primers

The requirements imposed by Liang and Pardee (1992) when choosing the 5' primer were that it be short to permit frequent and random annealing along the cDNA. In combination with the anchor primer, the arbitrary primer should also allow amplification of products up to 500 bp in size that represent the majority of mRNAs from the samples to be compared. Theoretically a 6 or 7 bp primer should have been the most suitable, but in practice was too short to yield any amplification products under the PCR conditions imposed. Instead,

when using *Taq* DNA polymerase at its optimal temperature of 72°C, 10mers were found to work best, providing specific amplification at the lower dNTP concentration (2 μM) and the low annealing temperature (42°C) used in DDRT-PCR.

The arbitrary 10mers also produced more amplification products (50-100) within the desired range (<500 bp) than was initially predicted (<1). This difference can be attributed the occurrence of 5' mismatches during the low temperature annealing of the arbitrary primer. Although the 10mers anneal best to perfect matches along the cDNA during PCR amplification, infrequent mismatches do occur. Published studies (Liang and Pardee, 1992; Bauer *et al.*, 1993; Liang *et al.*, 1993) report that at least six nucleotides at the 3' end of the arbitrary 10mer must be perfectly matched for amplification. Using a computer simulation allowing for zero to four mismatches between primer and template, Bertioli *et al.* (1995) found that mismatching increased the number of potential binding sites much more efficiently than using an equivalent shorter primer. A 7 bp primer for example, was expected to have 1,098 binding sites as opposed to 55,102 sites from a 10mer with three mismatches. However, different binding sites vary in their priming efficiency, depending on the number of mismatches, and where they occur. Binding sites with fewer mismatches, particularly at the 3' end of the primer will be more effective.

An adverse effect of 5' mismatching is that it can result in the appearance of nonreproducible bands on the display gels (Diachenko *et al.*, 1996). To address this problem, longer arbitrary primers, with or without an attached restriction enzyme site at the 5' end, have been employed (Liang *et al.*, 1994; Zhao *et al.*, 1995; Diachenko *et al.*, 1996; von der Kammer *et al.*, 1999). These longer oligoprimers make it possible to increase both the dNTP concentration and the annealing temperature of the PCR reaction. By making the PCR conditions more stringent, amplification specificity and reproducibility should be enhanced.

3.1.2.3 Polyacrylamide Gel Electrophoresis

Bauer *et al.* (1993) found that the use of denaturing sequencing gels to display PCR samples produced deceptively complex banding patterns. This can be attributed to the presence of bands that vary slightly in form, but are derived from the same cDNA species. Individually these bands may represent truncated PCR products (Averboukh *et al.*, 1996), or a single cDNA molecule with numerous polyadenylation sites (Liang *et al.*, 1992), or even a difference in the electrophoretic mobility of the two separated strands of a cDNA molecule, with or without extra adenosine nucleotides attached to their 3' ends by *Taq* DNA polymerase (Bauer *et al.*, 1993; Liang *et al.*, 1993). Such bands were observed to migrate as doublets, triplets, or in sets of four (Liang *et al.*, 1993).

In an attempt to improve gel resolution by reducing the complexity of the banding pattern, Bauer *et al.* (1993) successfully introduced the use of nondenaturing gels to separate PCR products. Because these gels were capable of resolving double stranded fragments as single bands, fewer bands were produced resulting in a less crowded and less complicated display pattern.

3.1.2.4 Detection of Band Patterns

Alternative detection methods have also emerged in an attempt to improve the level of sensitivity offered by the original procedure. Since Liang and Pardee (1992) described the use of ^{35}S-dATP for the detection of amplification products, ^{33}P-dATP has gained popularity among researchers as a safer isotope that requires a shorter exposure time and provides better band resolution than ^{35}S-dATP (Liang and Pardee 1995; Song *et al.*, 1995; Tokuyama and Takeda, 1995).

The use of nonradioactive detection methods such as silver staining (Lohmann *et al.*, 1995) and chemiluminescence (An *et al.*, 1996) have also been applied to DDRT-PCR. Both these alternative procedures claim to maintain the same levels of sensitivity offered by the radioactive detection system. In order to increase output and accelerate the entire procedure, the DDRT-PCR method has also been adapted for an automated recording system using an automated DNA sequencer (Bauer *et al.*, 1993). Primers and PCR conditions were modified for the inclusion of fluorescent dyes in the protocol.

3.1.3 Limitations

Although DDRT-PCR is conceptually straightforward and appears technically easy to undertake, in practice difficulties have been encountered with this procedure by most users. The DDRT-PCR procedure has undergone a number of modifications since its original description, but several technical flaws inherent in the method still need to be resolved. In the literature, the most common areas receiving criticism and attention for improvement are,

(i) the high level of false positives generated by the method;
(ii) the complicated process of verifying differential expression;
(iii) the length and type of fragments produced; and
(iv) the detection of rare transcripts (Appel *et al.*, 1999).

3.1.3.1 False Positives

The technique's greatest limiting factor lies in its potential for isolating a high proportion of false positives. A false positive can be defined as a candidate polymorphic band isolated from a display that gives a hybridisation signal in both or neither of the samples being compared on a Northern blot, or an alternative procedure. Even if only a small percentage of the putative differentially expressed bands isolated are false positives, they can significantly increase work load and complicate subsequent analyses.

False positives can have their origins at any one or more steps of the DDRT-PCR procedure. For a successful DDRT-PCR the chance of isolating false positives must be eliminated, or at the very least minimised. Appel *et al.* (1999) identified the three main areas that contribute to false positives as, the quality of the starting material, PCR artefacts, and the reamplification and cloning of candidate bands.

An important consideration for high quality DDRT-PCR is to ensure the total or poly(A) RNA samples used as the starting material, are not degraded and are DNA free (Liang *et al.*, 1993). RNA samples can often be contaminated by genomic DNA (gDNA) carried through during the isolation procedure. Subsequent amplification of the contaminating chromosomal DNA instead of the intended cDNA can provide a major source of false polymorphic bands. If the gDNA out competes cDNA as the preferred template, rare mRNAs may go undetected. To address this problem all RNA samples to be used in DDRT-PCR should be treated with RNase free DNase I before reverse transcription.

False positives caused by PCR artefacts can be mainly attributed to the low stringency conditions and the short primers employed for DDRT-PCR (Zhao *et al.*, 1995). Modifications made to the primer design, the reaction conditions, electrophoresis and methods of detection have demonstrated a higher level of specificity and reproducibility. In so doing, the rate of false positives due to PCR artefacts should be reduced.

Crowded display patterns can make isolation of candidate fragments difficult. If great care is not taken to realign gels to autoradiographs, the incorrect band may be inadvertently excised. An additional complication is that displayed bands are often comprised of a heterogeneous mix of comigrating fragments (Bauer *et al.*, 1993). In such cases, targeted fragments may become hidden by the others of no interest which may be reamplified and/or cloned more efficiently.

Whatever the source, the incidence of these undesirable false positives may be reduced by running samples in duplicate or repeating samples that may display putative bands of interest (Liang *et al.*, 1993). Candidate bands should be reproducible and dependent upon reverse transcription.

3.1.3.2 Verification of Differential Expression

Following the identification and isolation of candidate bands it is essential to confirm that they do actually represent a differentially expressed mRNA. This has proven to be a considerably challenging and time consuming process. Following recovery and reamplification, there are two alternative strategies available for screening and analysing candidate bands to confirm the expression profile. The first is to use the bands directly as probes for Northern blot analysis or similar procedures, whilst the second option is to clone the bands and characterise then before using as probes.

Both strategies may be complicated by the occasional contamination of reamplified fragments by unrelated sequences. The inadvertent isolation of these contaminating products can occur when what may sometimes appear on an autoradiograph as a distinct band representing a single cDNA species, is instead a mixture of a number of different cDNA fragments that have comigrated during electrophoresis of the DDRT-PCR products (Averboukh *et al.*, 1996; Zhang *et al.*, 1996). Studies conducted by Li *et al.* (1994) found that each band recovered from 6% polyacrylamide gels contained at least three different cDNA fragments. Callard *et al.* (1994) proposed that such a heterogeneous mix can be generated by any one or a combination of the following; (i) the presence of gDNA

contamination in total RNA samples used for cDNA synthesis; (ii) the comigration of overlapping bands either identical or similar in size to unique differentially expressed fragments; and (iii) poor separation by gel electrophoresis leading to copurification of nearby bands. Consequently, the direct use of these heterogeneous PCR products as probes for Northern blot analysis or library screening may lead to the detection of multiple transcripts or clones. Therefore, identifying the correct differentially expressed fragment can become a difficult task (Zhang *et al.* 1996).

An added problem associated with using a reamplified fragment directly as a probe, is the possibility of failing completely to detect a mRNA transcript on Northern blots (Liang and Pardee, 1992; Liang *et al.*, 1993; Li *et al.*, 1994). This may be attributed to a number of factors such as the inefficient recovery of the fragment following reamplification, the length of the probe is too short, or gene expression is too low to be detected by Northern blot analysis (Liang *et al.*, 1993). If this problem is encountered further analysis is required to distinguish whether these fragments represent low abundance mRNAs or are simply PCR artefacts.

The alternative strategy of cloning the reamplified band before use as a probe also has its problems (Zhang *et al.*, 1996). Depending on the extent of DNA contamination, cloned inserts may also be heterogeneous (Callard *et al.*, 1994). Although inserts are of the expected size, not all will represent the differentially expressed gene of interest (Xu *et al.*, 1997b). It then becomes a tedious and time consuming process to purify and screen each insert by Northern blotting, until that which shows the differential expression pattern of interest is identified.

Attempts have been made to overcome this problem by screening the isolated or cloned fragment by dot blot analysis (Callard *et al.*, 1994), Northern blot affinity capturing (Li *et al.*, 1994), or reverse Northerns (Zegzouti *et al.*, 1997). Although all three approaches allow many putative differentially expressed fragments to be screened in a short time, the results may still be inconclusive. Dot blots require that all bands be cloned including false positives before probing with a possibly heterogeneous DDRT-PCR product, which itself may contain false PCR contaminants. Northern blot affinity capturing is impractical if RNA is limited because one Northern blot is required for every clone to be screened.

Among the advantages of using a reverse Northern approach are that it requires small amounts of RNA and it is sensitive and fast. In this method, reamplified fragments are run on duplicate agarose gels and then blotted. The bands are then screened with a

cDNA probe made from each of the samples or conditions being tested. If a signal is detected with cDNA probes from all the conditions trialed, it may be possible to assume that the fragment represents a false positive. Given the heterogeneous nature of some reamplified PCR products, it is not possible to discount the alternative possibility, that this observation may actually represent a weakly expressed differential sequence which is masked by a more abundant and constitutively expressed cDNA (Zegzouti *et al.*, 1997). The wrong conclusion may inadvertently result in the elimination of low abundance transcripts. A possible variation on this idea may be to clone the reamplified bands and purify the inserts for blotting. Although more time consuming, uncertain or ambiguous results could be avoided.

3.1.3.3 Length of Clones

Another factor affecting the applicability of DDRT-PCR is that fragments that are produced tend to be no longer than 500 bp (Liang and Pardee, 1992; Averboukh *et al.*, 1996). Due to the use of oligo dT primers to produce cDNA, the amplified fragments may comprise mostly of 3' untranslated regions located 100-500 bp upstream of the poly(A) tail of mRNA (Haag and Raman, 1994). Because these regions tend to be variable between organisms, and even between different genes within a gene family, finding homologies in sequence databases to known characterised genes can be difficult (Sompayrac *et al.*, 1995). In addition to providing more sequence information, longer cDNAs generally make for better probes in Northern analyses, hybridising to target mRNA with greater affinity (Averboukh *et al.*, 1996).

To address this problem, Haag and Raman (1994) replaced the anchor primer with a secondary arbitrary primer during the amplification step of DDRT-PCR. In this way, they were able to display fragments along the entire length of the cDNA. The two arbitrary primers also enhanced gel resolution by improving specificity during amplification. Longer fragments can also be generated by increasing the elongation time during the PCR cycling conditions (Averboukh *et al.*, 1996) or using *Pfu* DNA polymerase instead of *Taq* (Diachenko *et al.*, 1996).

3.1.3.4 Rare Transcripts

As noted by the proponents of the technique (Liang and Pardee, 1992) one of the attractive features of the DDRT-PCR approach over alternative methods such as subtractive

hybridisation and differential library screening, was that in principle, it could facilitate the isolation of low abundance sequences, by permitting amplification of rare transcripts. Such transcripts may represent important genes in development or facilitate a response to environmental stimuli.

Liang *et al.* (1993) cautioned that bands, which were not reproducibly amplified in duplicate samples, could not always be attributed to the background noise level of the method. Instead these bands may actually represent rare mRNAs which were not present in sufficient quantities to be amplified reliably to detectable levels. In order to target rare transcripts they recommended the use of different amounts of RNA from each of the samples undergoing comparison.

Liang and Pardee (1992) demonstrated the potential for isolating rare transcripts when they were able to identify thymidine kinase (TK) mRNA, which is present at about 30 copies per cell, in tissue from mice. To do this the 5' primer used was perfectly matched to the isolated sequence at all ten bases. Given that arbitrary 10mers will tolerate mismatches, further experiments were recommended to properly determine the technique's sensitivity towards rare transcripts.

The sensitivity of DDRT-PCR was investigated further by Bertioli *et al.* (1995), who used perfectly matched primers designed to detect rare transcripts in tobacco. The first was to target mRNAs encoding the pathogenesis related (PR-1) proteins, which have been reported to occur at levels of about 1% in leaves infected with the tobacco mosaic virus (Pfitzner and Goodman, 1987), and <0.01% in uninfected tissue (Hooft van Huijsduijnen *et al.*, 1985). The second target was the TobRB7 mRNA, which is expressed constitutively and specifically in root tissue and is present at about 0.3% (Conkling *et al.*, 1990). Both attempts failed to detect the targeted mRNAs.

Bertioli *et al.* (1995) continued their analysis to address the question of whether messages known to be expressed at low levels can still be detected using 5' 10mers that are mismatched at up to four bases. A simpler system was employed to detect the abundant α and ß-globin mRNAs from rabbit. When globin RNA was mixed with heterologous total RNA to mimic the conditions of DDRT-PCR, no primer-target combination tested could detect α or ß-globin at levels below 1.2% of mRNA (equivalent to an intermediate/abundant mRNA), even when primers were perfectly matched.

They concluded that DDRT-PCR strongly favours the isolation of more abundant mRNAs. They also suggested that sensitivity towards less abundant mRNAs could be

improved by reducing competition for limiting reagents during PCR and improving the priming specificity. This idea was supported by Benito *et al.* (1996) who suggested that the degree of amplification of a given cDNA is determined not only by its level of abundance, but also the degree of template complexity as well as the priming efficiency.

Competition is arguably greater in DDRT-PCR than in standard PCR reactions. Under the conditions required by DDRT-PCR, competition becomes an important factor in determining the sensitivity of the technique (Bertioli *et al.*, 1995). It has been proposed that an increase in sensitivity towards low copy number mRNAs can be achieved with longer primers which facilitate more efficient and selective binding to templates by making it possible to use higher annealing temperatures (Bertioli *et al.*, 1995; Diachenko *et al.*, 1996).

3.2 AIM

At the time of commencing experiments to explore the usefulness of DDRT-PCR in this study, examples of its application in plants was limited in the literature, and its use for isolating genes in *Eucalyptus* had not been reported. The available protocols varied slightly, as modifications and improvements to the original procedure progressed in the literature.

The experiments outlined here were designed to optimise conditions for DDRT-PCR to improve reproducibility and gel resolution. The effect imposed on the quality of the displayed banding patterns was examined when modifications were made at each of the individual steps of DDRT-PCR namely, DNase I treatment of total RNA, reverse transcription of mRNA, amplification by PCR and separation of the resulting products by polyacrylamide gel electrophoresis.

3.3 OPTIMISING THE DIFFERENTIAL DISPLAY PROTOCOL FOR *EUCALYPTUS GLOBULUS* TISSUES

3.3.1 Areas for Improvement Identified

An initial trial was conducted to identify any problem areas that may compromise the efficiency of the DDRT-PCR method when applied to the *E. globulus* system. Before an extensive screen for differentially expressed genes involved in LR formation could be initiated, a high level of reproducibility and gel resolution had to be ensured. Fulfilling these two requirements would greatly minimise any technical difficulties brought about by poor separation and false positives.

After reviewing the reports in the literature (Liang and Pardee, 1992; Liang *et al.*, 1993; Oh *et al.*, 1995; Song *et al.*, 1995), it was decided to subject total RNA to be used for DDRT-PCR to the standard 30 min incubation with 10 units DNase I (RQ1, Promega) to remove any contaminating gDNA.

Following treatment, 0.2 µg total RNA from *E. globulus* shoot apices was reverse transcribed using 1 µM of the 5'-T_{11}AT-3' anchor primer, in the presence of 1x First Strand Buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTPs, 20-40 units RNase inhibitor (RNasin, Promega) and 200 units reverse transcriptase (SUPERSCRIPT, BRL). The reaction was completed at 37°C for 1 h.

The amplification step of DDRT-PCR was performed as described by Liang and Pardee (1992), incorporating some of the modifications made by Oh *et al.* (1995). The resulting cDNA population was diluted 1:4 (1 part cDNA in 4 parts water) and amplified in triplicate using 1 µM of the same anchor primer and 0.2 µM OPAP-14 (Kit AP - Operon Technologies; Appendix A-2) as the random 10mer. In a reaction volume of 10 µl, final concentrations of 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 20 µM dNTPs, 1.5 mM $MgCl_2$, 5 µCi [α-^{35}S]-dATP and 1 unit *Taq* DNA polymerase were included. DDRT-PCR was performed at 94°C for 30 sec, 42°C for 2 min, 72°C for 30 sec for 40 cycles, followed by 72°C for 5 min before holding at 4°C.

To test for the persistence of gDNA contamination, RNA controls were performed in parallel. Treated total RNA was used as a template under the same conditions as those employed for DDRT-PCR, except that the cDNA synthesis step was omitted.

The amplified products were resolved side by side on a 6% denaturing polyacrylamide gel. The resulting display showed poor reproducibility between the patterns obtained from the triplicate cDNA samples (Fig. 3.2, Lanes 1-3). Also of concern was the presence of bands in the RNA control lanes (Fig. 3.2, Lane 4). The fact that these bands were produced independently of reverse transcription suggests that the RNA samples used were still contaminated by gDNA even after DNase I treatment. It is likely that the persistence of this gDNA may have also been a contributing factor to the poor reproducibility observed.

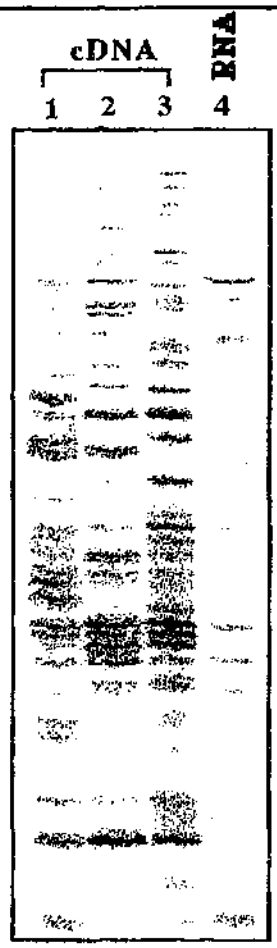


Figure 3.2: gDNA contamination of RNA samples used for DDRT-PCR results in inconsistent banding patterns. *E. globulus* shoot apical cDNA derived from total RNA, was treated for 30 min with 10 units of DNase I and amplified in triplicalte during the PCR step of DDRT-PCR (Lanes 1-3). DDRT-PCR on the treated RNA control was performed without reverse transcription (Lane 4).

This preliminary trial identified the important need to modify the conditions before undertaking a DDRT-PCR approach to search for genes involved in LR formation in *E. globulus*. The high level of gDNA contamination even after DNase I treatment was a major problem, adversely affecting reproducibility and complicating banding patterns. Improvement in these areas would undoubtedly enhance gel resolution and minimise the chances of isolating false positives.

3.3.2 DNase I Treatment

If DDRT-PCR was to be implemented successfully, it was essential that total RNA used for cDNA synthesis was completely free of chromosomal DNA contamination. This would serve to limit the potential for obtaining false polymorphic bands resulting from PCR amplification of gDNA instead of cDNA.

The presence of gDNA was typical among the total RNA samples isolated from *E. globulus* tissues. The persistence of gDNA even after DNase I treatment has also been reported elsewhere (Liang *et al.*, 1993; McKendree *et al.*, 1995). McKendree *et al.* (1995), suggest that the method for DNase I treatment may require optimisation depending on the tissue type to be used. This may include modifying the treatment time and/or the concentration of DNase I to be used.

In the current study, conditions for DNase I treatment of *E. globulus* tissues were investigated in order to obtain DNA free total RNA without adversely affecting its quality. The preliminary DDRT-PCR experiment (Fig 3.2) suggested that the routine practice of incubating total RNA for 30 min with 10 units DNase I enzyme was not adequate in completely removing the contaminating gDNA. The possibilities of optimising conditions by increasing the incubation time or the amount of DNase I enzyme used were examined.

3.3.2.1 Treatment Length

Initially, a comparison between the standard 30 min treatment and longer incubation times of up to 3 h was considered. Total RNA (50 µg) isolated from the shoot apices of *E. globulus*, including the cotyledons, was incubated with 20-40 units RNase inhibitor (RNasin, Promega) and 10 units RNase free DNase I (RQ1, Promega) at 37°C for 30 min, 1, 2 and 3 h.

Following treatment, RNA was extracted with phenol:chloroform (3:1), then again with an equal volume chloroform:isoamyl alcohol (24:1), before precipitating in 0.1 volume 3 M Na acetate (pH 4.8) and 2.5 volume 100% ethanol at -20°C overnight. The RNA was collected by centrifugation for 30 min at 14,000 rpm, rinsed in 70% ethanol and resuspended in 20 µl UHQ water.

A 1 µl aliquot from each time point was run on an agarose gel to check the integrity of the RNA (Fig. 3.3). Staining the agarose gel with ethidium bromide suggested the RNA remained undegraded after the 30 min treatment. The longer incubations slightly compromised the RNA quality with the 3 h sample showing visible signs of partial degradation. At this limited level of sensitivity however, no conclusions could be made regarding the total eradication of the contaminating gDNA. Liang *et al.* (1993) reported that even the smallest amount of gDNA can remain undetected on ethidium bromide stained agarose gels, yet may be sufficient to act as a template in the amplification step of DDRT-PCR, resulting in the production of false positives.

Figure 3.3: Integrity of the 28S rRNA band after incubation with 10 units of DNase I. Total RNA isolated from shoot apices of *E. globulus* was DNase I treated for 30 min, 1 h, 2h, and 3 h. The level of RNA degradation increased with the length of treatment.

To determine the level of persistence of gDNA contamination, the four treated RNA samples were subjected to a DDRT-PCR, omitting the reverse transcription step from the reaction. This would permit amplification only if gDNA was present. The conditions used for the amplification step where kept constant, as outlined above.

Results show that all four RNA samples produced PCR products independent of reverse transcription (Fig. 3.4). This observation indicated that the gDNA was either resistant to digestion regardless of the length of DNase I treatment or was present at a level too high to be effectively removed. In addition the pattern of banding observed in the RNA lanes is not consistent, indicating the general difficulty in reproducing bands between samples when gDNA is present.

Conclusion:

Together these results showed that gDNA could not be eliminated from total RNA samples by increasing the length of DNase I treatment above 30 min. Instead the longer treatments appeared to contribute to an increase in the level of RNA degradation, rendering samples as totally unsuitable or suboptimal for cDNA synthesis by decreasing yields, or introducing inconsistencies in the DDRT-PCR patterns.

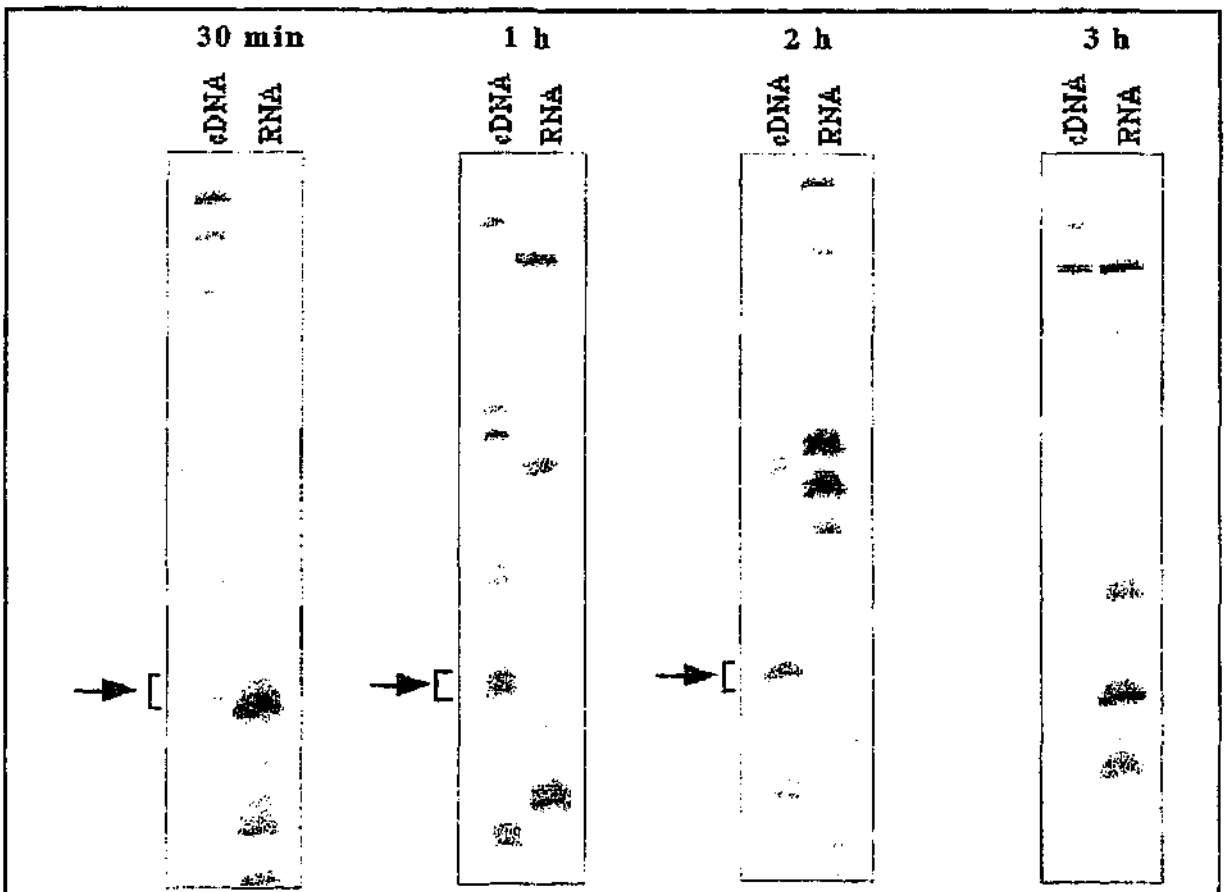


Figure 3.4: DDRT-PCR following DNase I treatment of total RNA for varied lengths of time. cDNA derived from treated RNA were run as positive controls alongside the treated RNA samples without reverse transcription. The arrows indicate equivalent bands in each of the cDNA controls.

3.3.2.2. Amount of DNase I

An alternative to increasing the treatment time was to increase the amount of DNase I enzyme used during the incubation. Total RNA (50 µg) was subjected to a 30 min treatment with 13.5 units DNase I instead of the 10 units normally used. The resulting purified RNA sample showed no visible signs of degradation when checked on an agarose

gel (Fig. 3.5A). Two reactions were set up for DDRT-PCR. In the first, cDNA was synthesised from 0.2 µg of RNA for use as a positive control. In the second, the RNA was not reverse transcribed so that if gDNA were present, amplification would occur from this source only. DDRT-PCR was then carried out using the same primers and conditions as before.

The cDNA positive control displayed fragments following electrophoresis on a 6% denaturing polyacrylamide gel (Fig. 3.5B). The treated RNA however, failed to produce any bands indicating that residual gDNA had been removed successfully from the sample. This analysis would also seem to indicate that amplification from RNA only is not possible. Removal of the contaminating gDNA also contributed a higher degree of consistency in banding patterns between the duplicate samples.

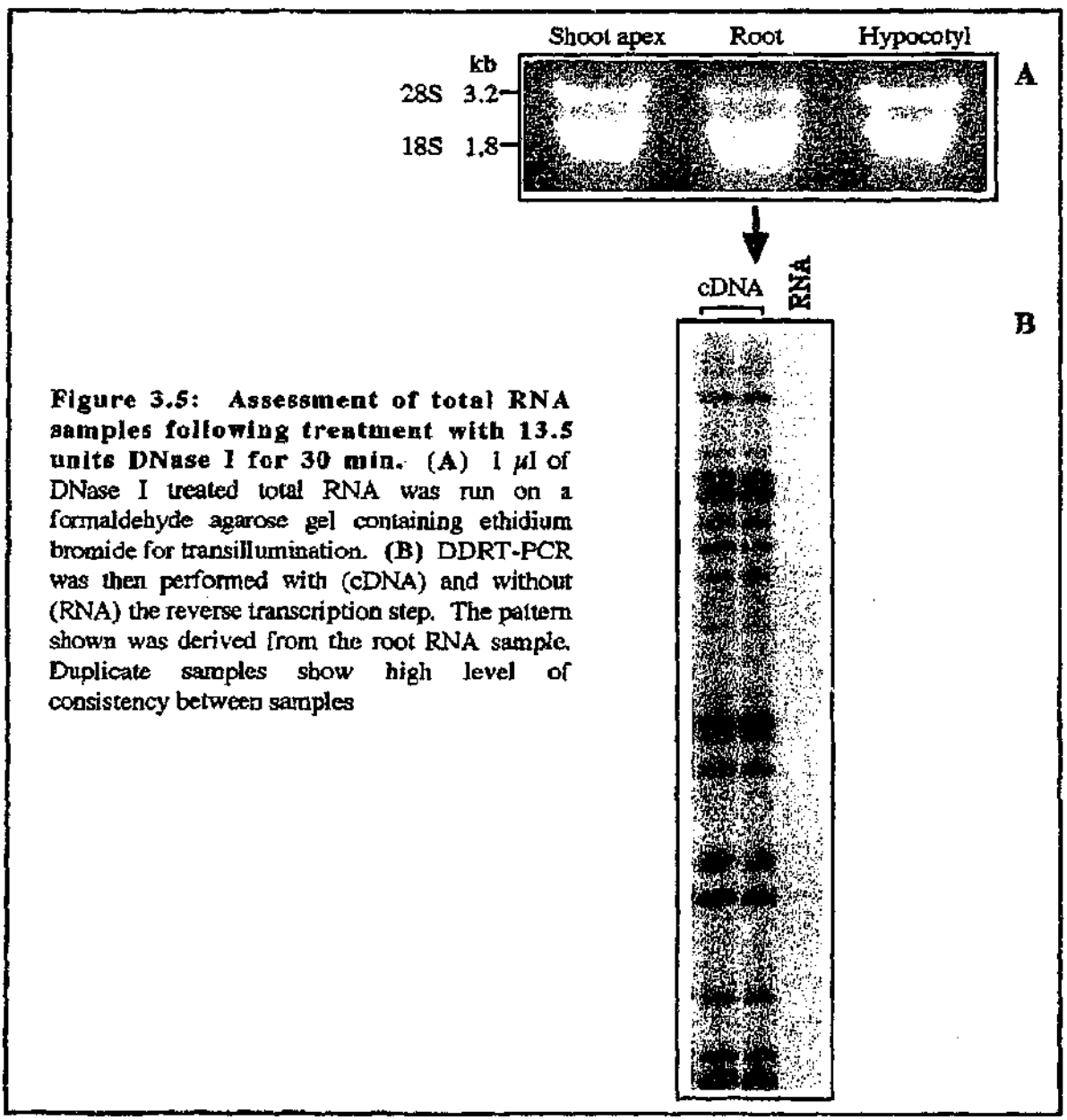


Figure 3.5: Assessment of total RNA samples following treatment with 13.5 units DNase I for 30 min. **(A)** 1 µl of DNase I treated total RNA was run on a formaldehyde agarose gel containing ethidium bromide for transillumination. **(B)** DDRT-PCR was then performed with (cDNA) and without (RNA) the reverse transcription step. The pattern shown was derived from the root RNA sample. Duplicate samples show high level of consistency between samples

Conclusion:

Varying the amount of enzyme used in the DNase I treatment of total RNA proved to be more beneficial than lengthening the incubation period of the reaction. A 30% increase in the level of DNase I was extremely effective in providing DNA free total RNA without compromising its quality. Given that the potential still exists for the incomplete removal of contaminating gDNA, this group of experiments has highlighted the importance of using RNA template controls in all DDRT-PCR screens. These controls serve to help identify products resulting from the amplification of persistent gDNA instead of the intended cDNA, even after DNase I treatment. Comparison between the cDNA banding patterns and any fragments that may be produced in the RNA controls should help reduce the chances of isolating false positives.

3.3.3 cDNA Synthesis

The second important step in DDRT-PCR is the synthesis of first strand cDNA from the DNase I treated RNA. The amount of reverse transcriptase and the use of degenerate anchor primers were the parameters that were investigated in this step.

3.3.3.1 Amount of Reverse Transcriptase

In the original protocol, Liang and Pardee (1992) used 300 units of reverse transcriptase for cDNA synthesis. In subsequent studies the amounts vary from 50 to 400 units. Innis *et al.* (1990) suggested that excess enzyme in cDNA synthesis may bind cDNA inhibiting downstream PCR. This prompted Oh *et al.* (1995) to use 50 units instead of the 300 units originally described, claiming that the six fold reduction had no noticeable effects on DDRT-PCR.

This was not the case in the *E. globulus* system. DNase I treated RNA from the shoot apices of *E. globulus* was reverse transcribed in the presence of the $T_{11}AT$ anchor primer and either 50, 100, or 200 units of M-MLV RNase H$^-$ Reverse Transcriptase (SUPERSCRIPT, BRL). Following DDRT-PCR amplification with $T_{11}AT$ and OPAP-14, differences were evident in the resulting banding patterns (Fig. 3.6). The 200 unit sample generated more bands producing a dense and more complicated array. It is possible that the unique bands displayed in this sample may represent rare transcripts that require high concentrations of reverse transcriptase to be transcribed. The only obvious advantage in using 50 units over the higher amounts seemed to be a slight reduction in the amount of background smearing.

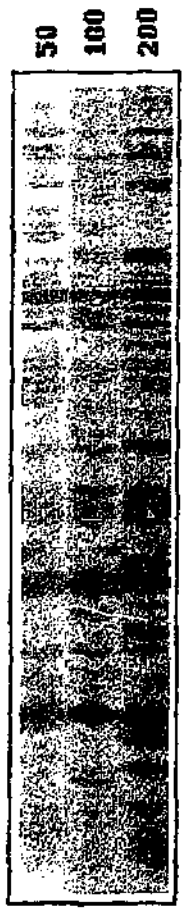


Figure 3.6: The effect of altered amounts of reverse transcriptase on DDRT-PCR. Total RNA was reverse transcribed using either 50, 100, or 200 units of SUPERSCRIPT RT.

Conclusion:

A high level of background smearing may mask the presence of fainter bands produced by DDRT-PCR. The lesser amount of reverse transcriptase (50 units) improved gel resolution, increasing the chances of identifying polymorphisms among the fainter or less abundant products, which may sometimes represent rare mRNAs. In addition to running the appropriate RNA controls, it was found that the isolation of false positives can be minimised further by performing DDRT-PCR on duplicate cDNA samples. This additional control was necessary to ensure that the display results obtained are real and not artefacts produced by the procedure. Only polymorphic bands showing reproducible differences in the duplicate samples while absent in RNA controls, would be targeted for isolation.

3.3.3.2 Degenerate Primers

The use of degenerate anchor primers in the cDNA synthesis step of DDRT-PCR gained attention with the advantage of reducing the number of cDNA fractions to be screened for each sample from 12 to four (Liang *et al.*, 1992; Liang *et al.*, 1993; Oh *et al.*, 1995). This

idea was examined further to assess the validity of substituting the twelve 5'-T_{11}MN-3' anchor primers for a degenerate system. Two separate reverse transcription reactions were performed from the same DNase I treated RNA sample. The first reaction was set up with T_{11}AA as the single anchor primer, while the second reaction was performed using a degenerate primer mix containing equal amounts of T_{11}AA, T_{11}CA, and T_{11}GA. The amplification step of DDRT-PCR then followed using the OPAP-01 and T_{11}AA primers only (Operon Technologies; refer to Appendix A-2). A comparison of the resulting display patterns showed a higher degree of reproducibility was achieved when the single T_{11}AA anchor primer was used in reverse transcription (Fig. 3.7). In addition to banding inconsistencies between the duplicates, the anchor primer mix also gave more background smearing.

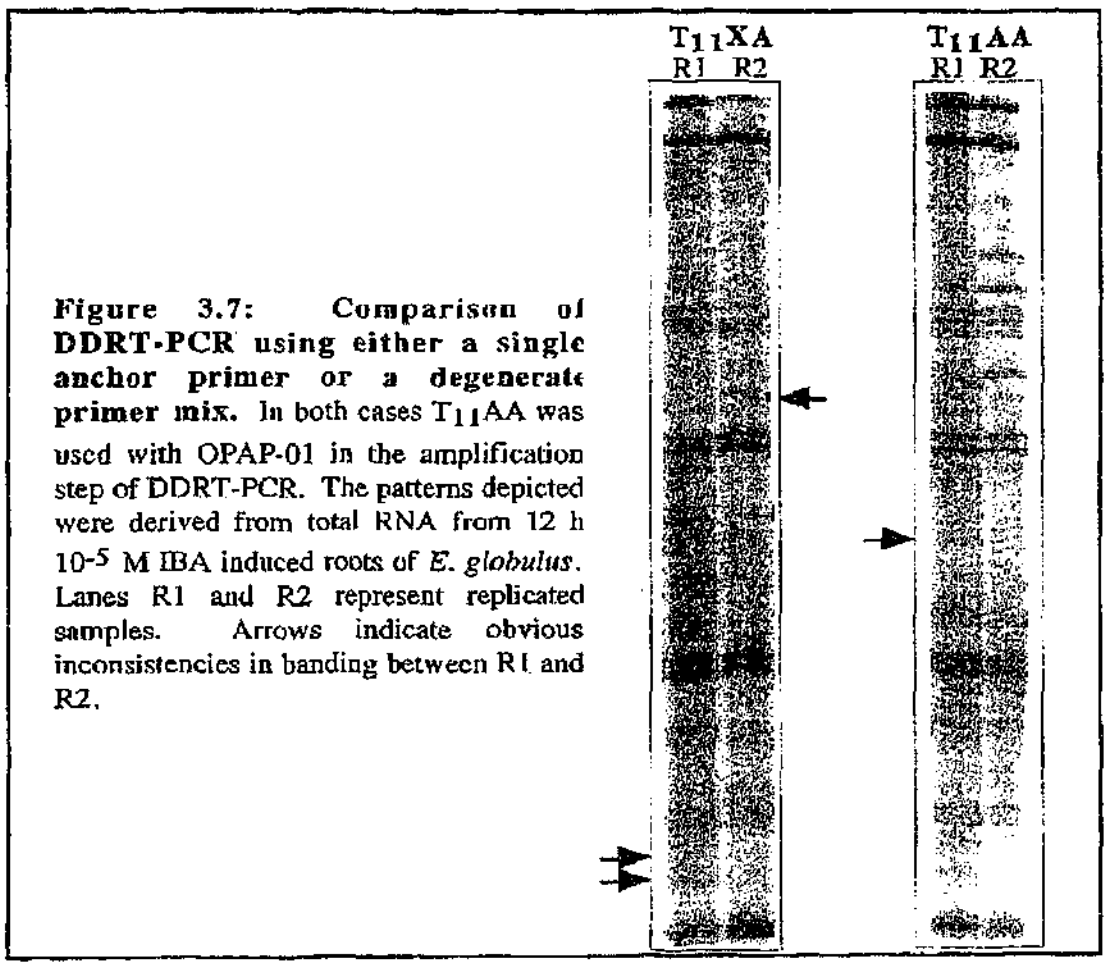


Figure 3.7: Comparison of DDRT-PCR using either a single anchor primer or a degenerate primer mix. In both cases T_{11}AA was used with OPAP-01 in the amplification step of DDRT-PCR. The patterns depicted were derived from total RNA from 12 h 10^{-5} M IBA induced roots of *E. globulus*. Lanes R1 and R2 represent replicated samples. Arrows indicate obvious inconsistencies in banding between R1 and R2.

nclusion:

·ptimum consistency and reproducibility between replicates, the single anchor primer led better results. The degenerative primer mix produces a cDNA pool that is much omplex than when the single specific primers were used. Bertioli *et al.* (1995) s that greater template complexity contributes to an increase in competition during PCR. Consequently, the level of priming specificity that is required for high

quality reproducible displays, may be compromised by the degenerative primer system. The increased specificity and selectivity provided by the single primer over the degenerative mix was deemed essential for better gel resolution, facilitating more accurate screening of samples for comparison.

3.3.4 Amplification

In order to ensure the success of DDRT-PCR, it was important to identify the parameters that would facilitate reliable and reproducible amplification of the cDNA subpopulations. In doing so, attention was also given to optimising gel resolution by keeping background smearing to a minimum.

3.3.4.1 cDNA Template

Following reverse transcription, the cDNA was diluted 1 part to 4 parts UHQ water. Typically 2 µl of this dilution was used as template in a DDRT-PCR reaction volume of 10 µl, as used by Oh *et al.* (1995). On occasions when the level of diluted cDNA was increased from 2 µl to 3 µl, background smearing increased to a point where the fainter display bands were difficult to see. Although not formerly tested, observations were made during DDRT-PCR screens regarding the age and the amount of cDNA template used. It was noticed that reproducibility progressively worsened with each experiment conducted to screen for differentially expressed sequences. When cDNA stocks up to 72 days old were replaced with a freshly synthesised batch, the problem disappeared. From this point cDNA was stored at -70°C in single use aliquots to avoid repetitive freeze thawing which may have caused damage to DNA strands thereby leading to difficulties in amplification.

Conclusion:

The efficiency of DDRT-PCR is greatly dependent upon properties of the template. It is advantageous to use the same cDNA pools for all screens to maintain consistency. Reproducibility seems to be greatly determined by the template quality while gel resolution is affected by its quantity.

3.3.4.2 Primer Concentration

Another difference between the original protocol (Liang and Pardee, 1992) and the modified version reported by Oh *et al.* (1995) is the concentration of primers used during DDRT-PCR amplification. The two conditions were trialed and compared in the *E.*

globulus system. The primer concentrations used originally were 2.5 µM anchor and 0.5 µM arbitrary 10mer. In the subsequent modification these levels were lowered to 1 µM and 0.2 µM respectively. In *E. globulus* the original combination resulted in a 61% increase in the number of bands produced, and provided better DDRT-PCR gel resolution (Fig. 3.8). The modified concentrations however produced poorer clarity and reproducibility. This could be explained by the possibility that the lower primer concentration may have imposed conditions whereby competition during amplification was high due to the limited amount of primer available.

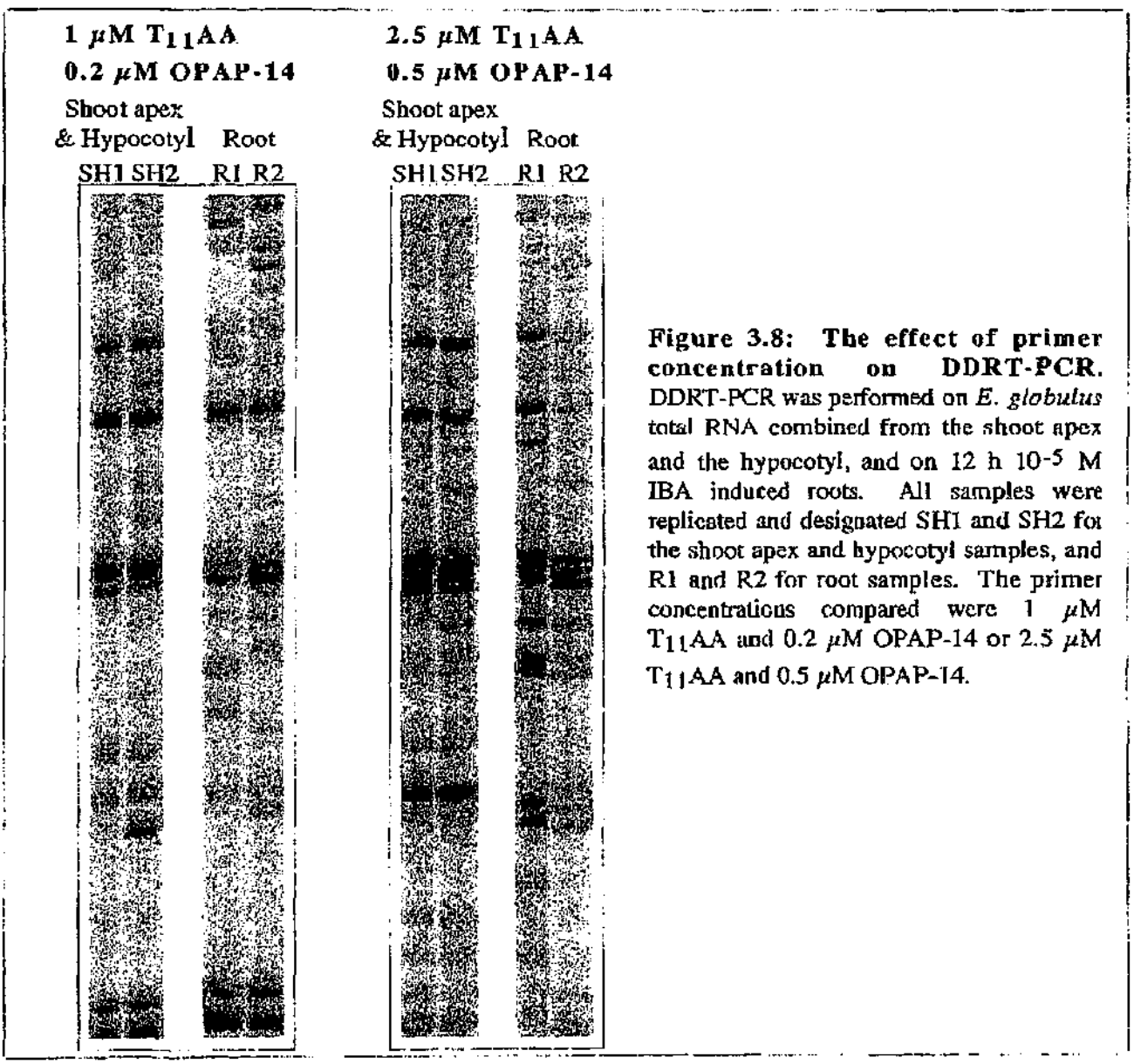


Figure 3.8: The effect of primer concentration on DDRT-PCR. DDRT-PCR was performed on *E. globulus* total RNA combined from the shoot apex and the hypocotyl, and on 12 h 10^{-5} M IBA induced roots. All samples were replicated and designated SH1 and SH2 for the shoot apex and hypocotyl samples, and R1 and R2 for root samples. The primer concentrations compared were 1 µM T_{11}AA and 0.2 µM OPAP-14 or 2.5 µM T_{11}AA and 0.5 µM OPAP-14.

Conclusion:

The higher primer concentration contributed to less competitive DDRT-PCR reaction conditions, producing an increase in the number of bands displayed. This is potentially desirable if amplification of rarer transcripts is facilitated.

3.3.4.3 Arbitrary Primer

Haag and Raman (1994) observed that faint bands consistently occupied virtually all positions on the display gel. A further area of concern was that the nature of the oligo-dT anchor primer is such that it allows amplification exclusively from the 3' end of the mRNA sequence. In order to facilitate priming along the entire length of the cDNA they replaced the anchor primer with a second arbitrary 10mer in the amplification step of DDRT-PCR. In doing so, background was reduced resulting in more distinct bands. They hypothesised that the observed improvement in gel resolution was due to an increased specificity imposed by the greater sequence complexity of the arbitrary primers.

These observations held true in the *E. globulus* system (Fig. 3.9) The background smearing common to display patterns generated by the use of an anchor and 10mer combination almost completely disappeared. Fewer bands (45% less) were also generated which is probably indicative of the enhanced specificity of the two arbitrary primer approach. The added advantage of this approach is that it may overcome the problem of isolating the 3' untranslated region of a potential differentially expressed sequence of interest.

Conclusion:

Other modifications described greatly contributed to a decrease in the level of background smearing, such that it no longer presented a real obstacle for accurately comparing samples when screening for differences in banding. On this basis, although the two arbitrary primer approach improved gel resolution further, it would not substitute for the original anchor-10mer approach. Rather, it would supplement it.

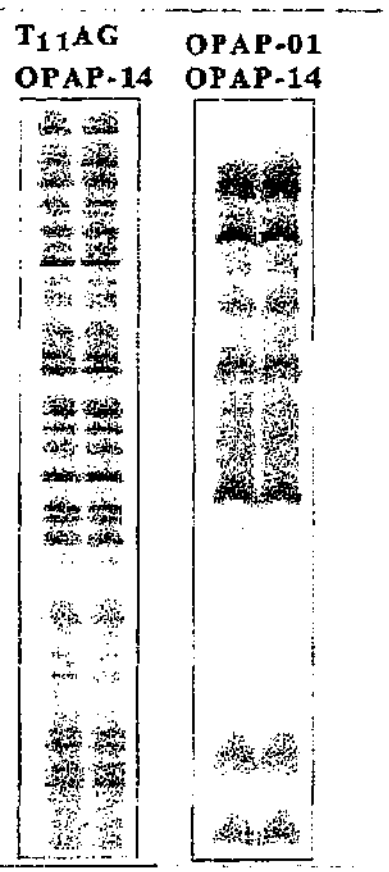


Figure 3.9: Comparison of DDRT-PCR using two arbitrary 10mers instead of the conventional anchor-10mer combination. Following reverse transcription with 1 μM $T_{11}AG$, amplification was performed using either 2.5 μM $T_{11}AG$ and 0.5 μM OPAP-14, or 0.5 μM of each OPAP-01 and OPAP-14. The patterns shown were derived from total RNA isolated from *E. globulus* roots which had undergone an 18 h 10^{-5} M IBA treatment.

3.3.4.4 ^{35}S Vs ^{33}P

Inconsistencies in the level of gel resolution produced between experiments were still evident even when all optimised conditions had been employed. The only parameter to be considered that might alter was the batch of [α-^{35}S]-dATP (Geneworks). Poor quality display patterns could be directly attributed to the ^{35}S used. When a batch was exhausted and a new one obtained the quality of gel resolution was also observed to change.

The potential hazard of using ^{35}S was also of concern. It has been observed that [α-^{35}S]-dATP forms high levels of radioactive volatile decomposition products when heated during PCR (Liang and Pardee, 1995; Trentmann *et al.*, 1995). These radioactive products can escape from the tops of the PCR tubes and contaminate thermal cyclers.

The possibility of consistently generating high quality gels by replacing ^{35}S with [α-^{33}P]-dATP (Geneworks) was investigated. Two identical DDRT-PCR experiments were conducted with the only variable being the radioactive isotope used.

The ^{33}P did not alter the displayed pattern but did give much clearer and more distinct bands (Fig. 3.10). This significant improvement in sensitivity and band resolution was also observed by Liang and Pardee (1995). Progressive experiments showed that the quality of banding produced by the use of ^{33}P remained constant regardless of changes made to the batches used. An added advantage of ^{33}P was that a shorter exposure time was required (12-18 h compared to 24-48 h for ^{35}S).

Conclusion:

Good gel resolution is essential for accuracy in detecting real polymorphisms representing differentially expressed sequences. Consistently high quality displays with more intense bands were provided by ^{33}P without altering banding pattern. Although ^{35}S was also suitable, on occasion inconsistent quality of [α-^{35}S]-dATP batches consumed valuable time and resources. ^{33}P is recommended for future experiments.

3.3.5 Size Separation of Amplification Products

Although clearer and discrete banding was achieved using the optimised conditions described above, excision of candidate bands was sometimes complicated by overcrowded displays, brought about by poor separation. In an attempt to simplify the display patterns produced, separation of DDRT-PCR products on nondenaturing gels instead of the conventional sequencing gel was investigated.

3.3.5.1 Denaturing Vs Nondenaturing Gels

Denaturing gels emphasise slight differences between strands, leading to doublets or triplets with no functional significance. However, Colonna-Romano *et al.* (1998) maintain that nondenaturing gels should not replace denaturing ones because heteroduplexes, and incomplete annealing of single stranded cDNAs that represent the same gene, will still have altered electrophoretic mobility on nondenaturing gels. The reported advantage of separating DDRT-PCR fragments on nondenaturing gels as opposed to denaturing gels is that banding pattern complexity is greatly reduced (Bauer *et al.*, 1993). This is achieved by the fact that fewer bands are produced in the displayed pattern as double stranded fragments migrate as single bands. However, in the *E. globulus* system, the use of nondenaturing gels provided very poor gel resolution. A very high level of background smearing was observed consistently, making fainter bands particularly difficult to see (Fig. 3.11). Potentially interesting differentially expressed sequences could be missed if obscured by the high background.

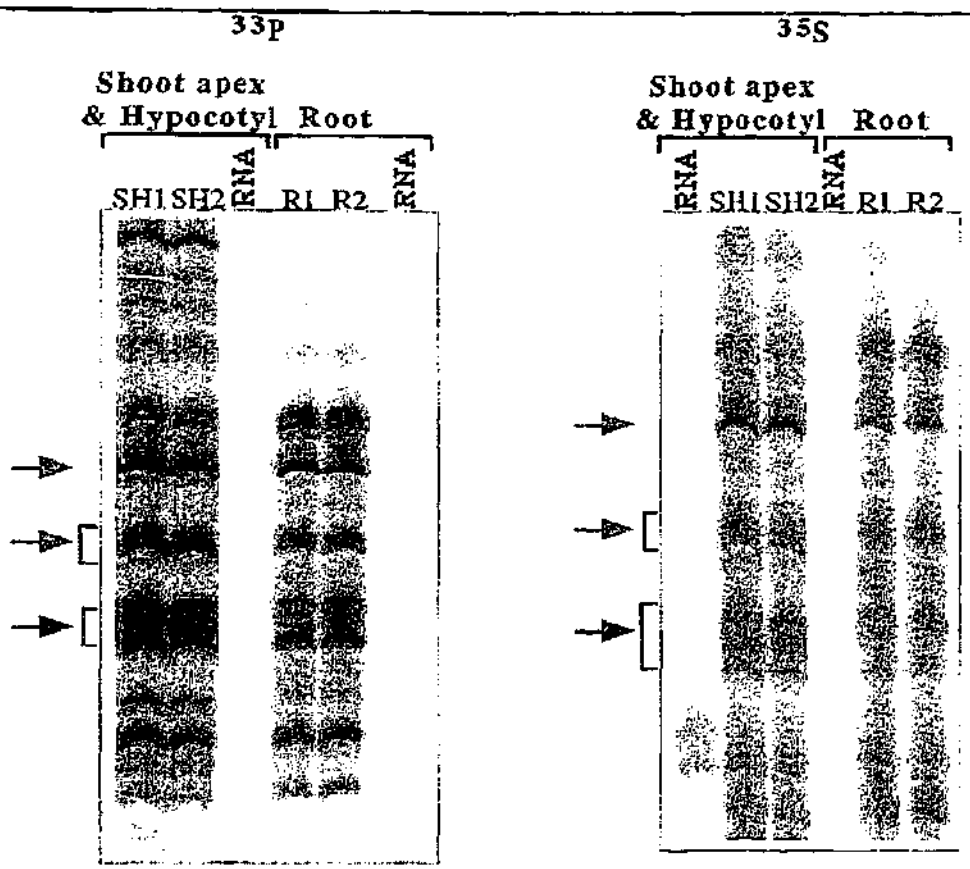
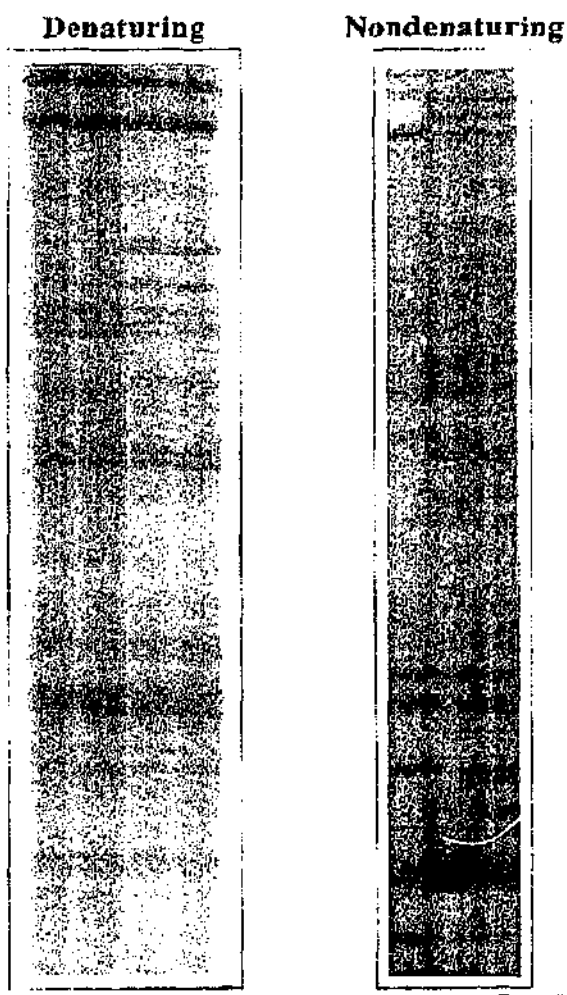


Figure 3.10: Differences in the detection of DDRT-PCR products using [α-^{33}P]-dATP compared to [α-^{35}S]-dATP. The patterns presented here were derived from total RNA isolated from combined shoot apices and hypocotyls, and root samples of *E. globulus*. In each case, 1 μM T_{11}AA was used in reverse transcription and 0.5 μM of each of OPAP-02 and OPAP-01 were used for amplification. All cDNA samples were replicated and designated SH1 and SH2 for the shoot apex and hypocotyl samples, and R1 and R2 for the root samples. The different coloured arrows indicate equivalent bands between gels.

Figure 3.11: Separation of DDRT-PCR products on denaturing versus nondenaturing gels. Following DDRT-PCR using 2.5 μM T_{11}AA and 0.5 μM OPAP-01, products were electrophoresed on 6% denaturing and nondenaturing gels. The patterns represent total RNA from 12 h 10^{-5} M IBA induced roots of *E. globulus*.



Conclusion:

These experiments indicated that in the *E. globulus* system, the use of denaturing gels should be maintained as the preferred method for separating DDRT-PCR products.

3.4 AN OPTIMISED DIFFERENTIAL DISPLAY METHOD

The final optimised method used to screen for differentially expressed genes involved in LR formation in *E. globulus* by the DDRT-PCR approach (Chapter 4), is a hybrid version of the original published protocol (Liang and Pardee, 1992) and a subsequent modified version (Oh *et al.*, 1995).

Collectively the optimisation experiments involved looking at the effect on banding patterns when alterations were made to a number of DDRT-PCR parameters. Once conditions for DNase I treatment of total RNA were determined, the amount of reverse transcriptase and the use of degenerate primers were investigated for cDNA synthesis. For amplification, the quantity, and quality of cDNA template, the type of radioactive label, and the concentration of primers used were investigated. Separation of DDRT-PCR products on denaturing versus nondenaturing gels was also assessed.

Further examination was performed in an attempt to improve the efficiency of the optimised method. For cDNA synthesis the possibility of substituting single anchor primers for degenerative ones was evaluated, while for amplification the use of two arbitrary 10mer primers instead of the conventional anchor-10mer combination was compared.

Each of the factors tested was found to influence the reproducibility of banding patterns obtained by DDRT-PCR. Consequently, observed variations in banding patterns between duplicate samples have been reduced although not completely eliminated. This highlighted the importance of running the proper controls, including RNA controls alongside the duplicate cDNA samples. This should at least reduce substantially the number of false positives that may be obtained.

3.4.1 DNase I Treatment: Preparation of RNA Samples for DDRT-PCR

Optimum conditions for DNase I treatment of total RNA isolated from *E. globulus* seedling tissues were determined based on the experiments previously described. The following procedure was employed for the DNase I treatment of all RNA samples to be used in all subsequent DDRT-PCR experiments.

- Total RNA (50µg) was incubated with 13.5 units DNase I (Promega) at 37°C for 30 min.

- The sample was then extracted with an equal volume of phenol:chloroform (3:1), then again with an equal volume of chloroform:isoamyl alcohol.

- The treated RNA was precipitated with 0.1 volume 3 M Na acetate (pH 4.8) and 2.5 volume 100% ethanol at -20°C overnight or -70°C 30 min. RNA was pelleted by centrifugation for 30 min at 14,000 rpm, then rinsed with 70% ethanol.

- The RNA was resuspended in 20 µl UHQ water and quantified by spectrophotometry. A 1 µl aliquot was run on an agarose gel to check for degradation. Treated RNA was stored at -70°C.

3.4.2 DDRT-PCR: cDNA Synthesis

The procedure employed for cDNA synthesis in DDRT-PCR screens was as follows:

- In a volume of 11 µl, DNase I treated total RNA (0.2 µg) was incubated with 1 µM anchor primer (Poly T Primers Kit 1, Operon Technologies) at 65°C for 10 min and then quickly chilled on ice.

- Reverse transcription was then undertaken in a 20 µl final volume, in the presence of 1x First Strand Buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.5 mM dNTPs, 20-40 units RNase inhibitor (RNasin, Promega) and 50 units reverse transcriptase (SUPERSCRIPT, BRL).

- The reaction was incubated at 37°C for 1h.

• cDNA was diluted in UHQ water (1:4) and stored in single use aliquots at -70°C.

3.4.3 DDRT-PCR: Amplification

• 2 µl of the diluted cDNA was amplified in a final volume of 10 µl. Included in each reaction were final concentrations of 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 1.25 mM $MgCl_2$, 2 µM of each dNTP, 2.5 µM anchor primer, 0.5 µM arbitrary primer (Kit AP, Operon Technologies), 0.5 µCi [α-^{33}P]-dATP and 1 unit *Taq* DNA polymerase (Promega). Although [α-^{33}P]-dATP was the preferred isotope, [α-^{35}S]-dATP was used in some initial screening experiments.

• For PCR, a Corbett FTS-320 Thermal Sequencer was used for all reactions. PCR was performed at 94°C for 30 sec, 42°C for 2 min, 72°C for 30 sec for 40 cycles. This was followed by 72°C for 5 min, before holding at 4°C.

3.4.4 Denaturing Polyacrylamide Gel Electrophoresis and Autoradiography

• A 6% polyacrylamide gel (Chapter 2.4.1) was pre run in 1x TBE buffer at 1450 volts for 30 min.

• 2 µl of loading buffer was added to 3 µl of each amplified DDRT-PCR sample and heated at 85°C for 2 min just before loading.

• The gel was run at 1450 volts until the second dye front had reached the bottom of the gel.

• The gel was vacuum dried onto Whatman and then taped to X-ray film. The gel and film were then carefully marked by cutting out wedges along the edges to facilitate accurate realignment for band recovery. The film was exposed for up to 48 h at -80°C.

3.4.5 Recovery and Reamplification of Fragments of Interest

• Autoradiographs were aligned to the dried gel using the wedges as reference points. The film and gel were taped together and viewed over a light box, gel side down. The bands of interest were visible through the Whatman paper and were cut from the gel using a sterile scalpel blade without damaging the film.

• The excised gel slices (still attached to the Whatman paper) were placed in a 1.5 ml microcentrifuge tube and soaked in 100 µl UHQ water for 20 min at room temperature.

• The tubes were vortexed briefly then sealed with parafilm and boiled for 15 min to elute the DNA from the acrylamide gel slice. The tubes were again vortexed and then allowed to cool to room temperature. After centrifugation at 14,000 rpm for 3 min, the supernatant was transferred to a fresh tube. DNA was precipitated at -20°C overnight or -70°C for 1 h, in 0.1 vol 3 M Na acetate, 50 µg glycogen and 4.5 vol 100% ethanol. To pellet the DNA, the tube was spun at 14,000 rpm for 30 min at 4°C. The supernatant was removed and the pellet washed with 70% ice cold ethanol, vacuum dried and resuspended in 10 µl UHQ water.

• To reamplify the band of interest, 4 µl of the eluted DNA was used as template per PCR. Reaction conditions were identical to those used for the DDRT-PCR amplification except that the radiolabelled nucleotides were omitted and the reaction volume was increased to 40 µl.

• To check for successful amplification, 10 µl of the resulting PCR samples were run on a 0.8% agarose gel and stained with ethidium bromide.

• If no band was visible the PCR reaction was taken through a second round of reamplification. This was necessary when a low quantity of DNA was eluted from the polyacrylamide gel. When an additional round of reamplification was necessary, a 1 µl aliquot was taken from the initial PCR reaction and diluted in 99 µl UHQ water. From this, 4 µl was then reamplified as previously described.

• The remaining reamplified DNA was then purified by either the QIAquick PCR Purification Kit (Chapter 2.4.3), or the phenol freeze method (Chapter 2.4.5). The DNA fragments were then ligated into a T-vector (Chapter 2.4.6 and 2.4.7).

3.4.6 DDRT-PCR: Application of Optimised Conditions

An initial DDRT-PCR trial was performed before undertaking an extensive screening program to test the optimised conditions. DDRT-PCR was conducted using total RNA isolated from untreated shoot apices, hypocotyl and root explants of 8 d old seedlings of *E. globulus*. The DNase I treated RNA controls showed no banding pattern indicating that amplification did not result from gDNA contamination. Seven differentially displayed bands were identified from the cDNA samples for isolation (Fig 3.12A). Five bands showed specific amplification from untreated roots while absent in untreated hypocotyl and shoot apical tissues. While another two bands showed specific amplification in untreated hypocotyls.

The bands were excised and eluted from the polyacrylamide gel, and successfully reamplified after one round of PCR (Fig 3.12B). The reamplified products were purified and cloned into a T-vector. A more detailed analysis of these bands is described in Chapters 4 and 5.

3.5 CONCLUSION

Modifications made to the original DDRT-PCR protocol have maximised its efficiency, sensitivity and simplicity, making it a powerful technique for identifying differentially expressed genes. Its main advantages are that it enables mRNA from different cell types or altered biological or physiological conditions, to be screened simultaneously from a small amount of starting material.

The high level of sensitivity offered by this method can be offset by its potential for isolating false positives. The efforts described here have been concentrated towards improving reproducibility and gel resolution, in order to minimise the chances of obtaining false positive results.

When optimised conditions for DNase I treatment were employed, gDNA contamination was largely removed and, as a result, the reproducibility improved. However, given the sensitive nature of DDRT-PCR, if undigested chromosomal DNA fragments survive treatment (McKendree *et al.*, 1995; Zhao *et al.*, 1995) they can become

plified during PCR, contributing false bands to the display pattern obtained. To avoid possibility of isolating these false signals, it is essential that RNA controls be formed by omitting reverse transcription from the DDRT-PCR procedure.

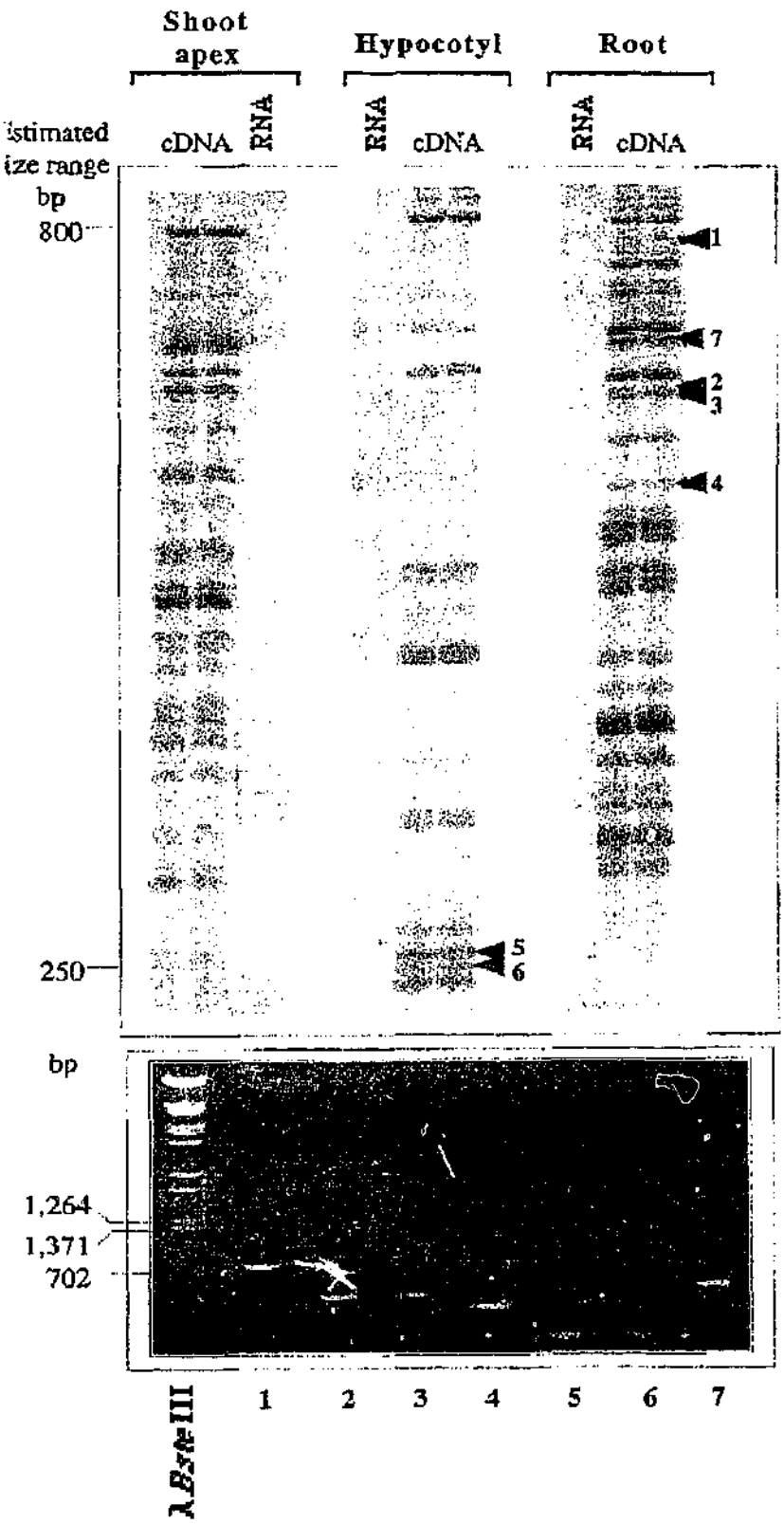


Figure 3.12: (A) Banding patterns were compared between the shoot apex, hypocotyl and root cDNA samples. DNase I treated RNA controls in each reaction did not yield products indicating that gDNA had been removed by DNase I digestion. The five root specific and two hypocotyl specific bands excised for further processing are indicated by the arrows and numbered 1-7. (B) Following purification the excised bands were reamplified to yield single PCR products.

The complete removal of gDNA may not necessarily guarantee the elimination of false positives. Random technical errors or inconsistencies in RNA and cDNA quality, pipetting, reaction components, PCR master mixes and reaction tubes, may all result in the amplification of additional nonreproducible bands. To overcome the possible misinterpretation of such bands as true polymorphisms between patterns, reactions should be performed in duplicate. Only bands consistently appearing in duplicated samples, whilst absent in RNA controls will be targeted for isolation.

The optimised conditions presented here for DNase I treatment, reverse transcription, PCR and gel electrophoresis have improved the consistency and quality of banding patterns generated by DDRT-PCR. The method is both a reliable and practical approach to identify differentially expressed genes involved in LR formation in *E. globulus*.

CHAPTER FOUR

The Isolation and Cloning of Sequences Representing Putative Differentially Expressed Lateral Root Genes in *Eucalyptus globulus*

4.1 INTRODUCTION

In the absence of applied auxin, LR formation is normally a stochastic and sometimes infrequent event which can make the molecular targeting of genes involved in the process unnecessarily difficult. By treating root explants with the appropriately high level of auxin, the frequency of LR initiation is increased creating a larger population of cells expressing genes involved in LR development. This stimulatory auxin effect can be exploited to facilitate the isolation and molecular characterisation of differentially expressed LR genes.

4.1.1 Auxin Induced Lateral Root Primordia and Lateral Root Formation in *Eucalyptus globulus*

It was anticipated that LR development would involve the coordinated and/or regulated expression of a wide variety of genes at different stages throughout the entire process of LR formation. Work carried out prior to, and in the early stages of this study, aimed to identify the developmental steps involved in the pathway for LR formation and their timing in roots of *E. globulus*.

Treatment of axenic root explants with exogenous application of auxin, has been previously found to increase the frequency of LR initiation in 8 d old seedlings (Pelosi *et al.*, 1995a and 1995b). In order to define the time frame for LRP initiation, root explants were exposed to 10^{-5} M IBA for various lengths of time (Fig. 4.1). These published findings report that at least 30 h was required for the formation of visible LRP. The rate of greatest induction began at 24 h and continued until maximum levels were observed at 42-48 h (1.4±0.1 - 1.4±0.2 LRP/mm). Longer treatments did not increase LRP numbers beyond this level indicating that at some time between 0-48 h of IBA treatment, a saturation point had been reached.

When roots were maintained on the auxin containing medium, the conversion of LRP to LRs was inhibited. However, transfer to hormone free (HF) medium, following auxin treatment was essential to permit the LRP to emerge from the primary root to form a new LR (Pelosi *et al.*, 1995a and 1995b).

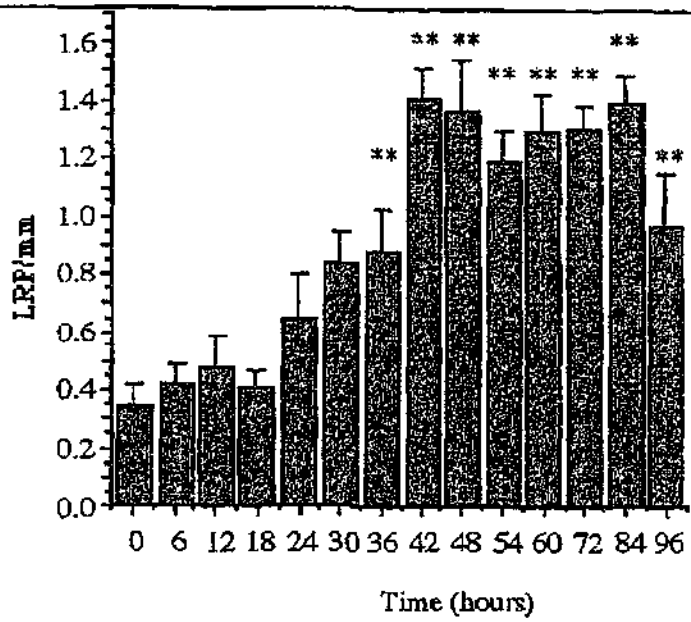


Figure 4.1 : Time course of LRP induction in root explants of *E. globulus* seedlings. Roots were treated with 10^{-5} M IBA for the times indicated. Data represent means of 9-40 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from time 0 h at $p<0.01$ are indicated by **. (Figure adapted from Pelosi *et al.*, 1995a and 1995b.)

4.2 AIM

This section of the study was divided into two parts in order to best describe the molecular isolation of DNA fragments that represent genes involved in LR development. The aim of Part A was to expand upon the *E. globulus* data presented in the published reports (Pelosi *et al.*, 1995a and 1995b), to incorporate shorter exposure times, and to study the process whereby LRP emerge from the primary root to form actively growing LRs. The observations made, formed the basis for an attempt to identify individual steps in a putative pathway for auxin induced LR formation in *E. globulus*.

It was expected that auxin induced alterations to the growth and development of the root would involve changes in gene expression. To gain a better understanding of the molecular mechanisms associated with LR differentiation, mRNA populations in auxin treated root explants from *E. globulus* were examined using DDRT-PCR and cDNA subtraction (Part B) procedures. The differentially expressed DNA fragments were cloned, sequenced and organised into homologous families. A critical evaluation and assessment of the performance of the two alternative cloning strategies is also discussed.

4.3 PART A: FURTHER CHARACTERISATION OF THE EFFECTS OF IBA ON LATERAL ROOT DIFFERENTIATION IN *EUCALYPTUS GLOBULUS*

4.3.1 Results

Before initiating a screen, further characterisation was required to help define the treatment conditions for DDRT-PCR and subtractive hybridisation. The IBA induction experiments described here served to expand upon the results reported by Pelosi *et al.* (1995a and 1995b). Collectively, the information gained would help elucidate the timing of key events involved in LR formation, so that the accumulation of mRNA identified by an isolated cDNA clone, may be correlated with a specific developmental stage during the LR formation process in *E. globulus*.

Events occurring during the initial 0-30 h of treatment remained unclear, given that an obligatory 30 h delay was required for the formation of LRP. This period was assessed here in greater detail, when LRP were allowed to progress through development into newly formed LRs. This made it possible to determine the minimum length of auxin exposure required to stimulate the process of LR initiation and define the time required to induce LR formation to the point of saturation (Fig. 4.2). Transfer of root explants to HF medium for a further 7 d culture, following IBA induction, showed that as little as 30 min (0.4±0.1 LR/mm) was sufficient to induce a small but statistically significant increase in the number of LRs above the 0 h control level (0.2±0.4 LR/mm). Maximum rates of LR formation (1.1±0.2 LR/mm) were achieved after a minimum 18 h treatment.

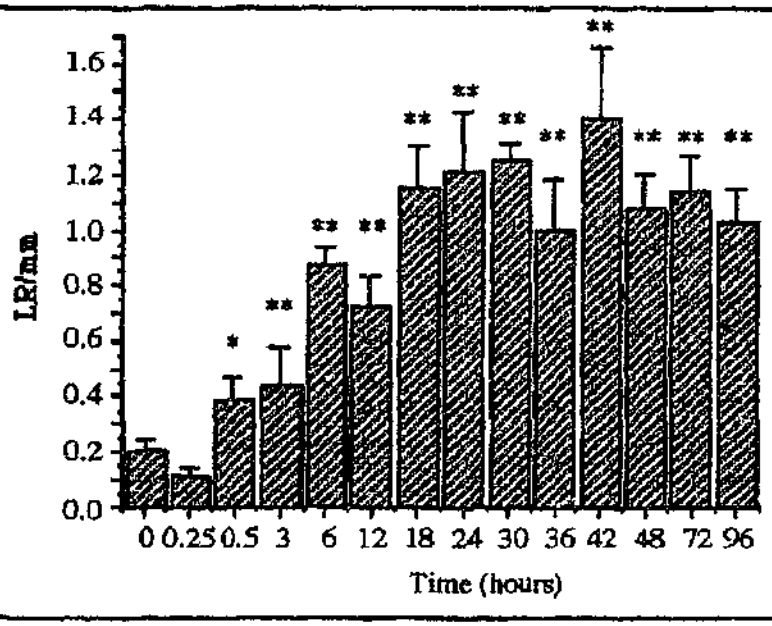


Figure 4.2: LR formation in root explants of *E. globulus* seedlings. Roots were transferred to HF medium for 7 d following treatment with 10^{-5} M IBA for the times indicated. Data represent means of 9-32 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from time 0 h at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively. (Time points 6-24 h and 48-96 h were reported and adapted from Pelosi *et al.*, 1995b.)

To further investigate the timing of events involved in LR formation, the conversion of LRP to LR was examined in more detail. Root explants were induced with 10^{-5} M IBA before transfer to HF medium for the remainder of a 96 h total treatment period. This enabled the culture time on HF to be varied following LRP induction to ascertain the timing for LR emergence, whilst also permitting roots to be assessed at a constant age. When roots were maintained on IBA medium for the whole 96 h treatment period (96 h IBA+0 h HF) the conversion of LRP (0.9±0.2/mm) to LRs (0.5±0.1/mm) was significantly inhibited (Fig. 4.3A). In fact the data suggests that following IBA induction, LRP were not converted to LRs by an additional HF culture period of 48 h or less. However, after 72 h of HF culture (i.e., 24 h IBA+72 h HF), LRs (1.6±0.2/mm) began to out number LRP (0.9±0.4/mm). By 88 h most LRP had emerged as the proportion of LRs (1.2±0.2/mm) reached a significantly higher level than LRP (0.1±0.2/mm) (Fig. 4.3A and B).

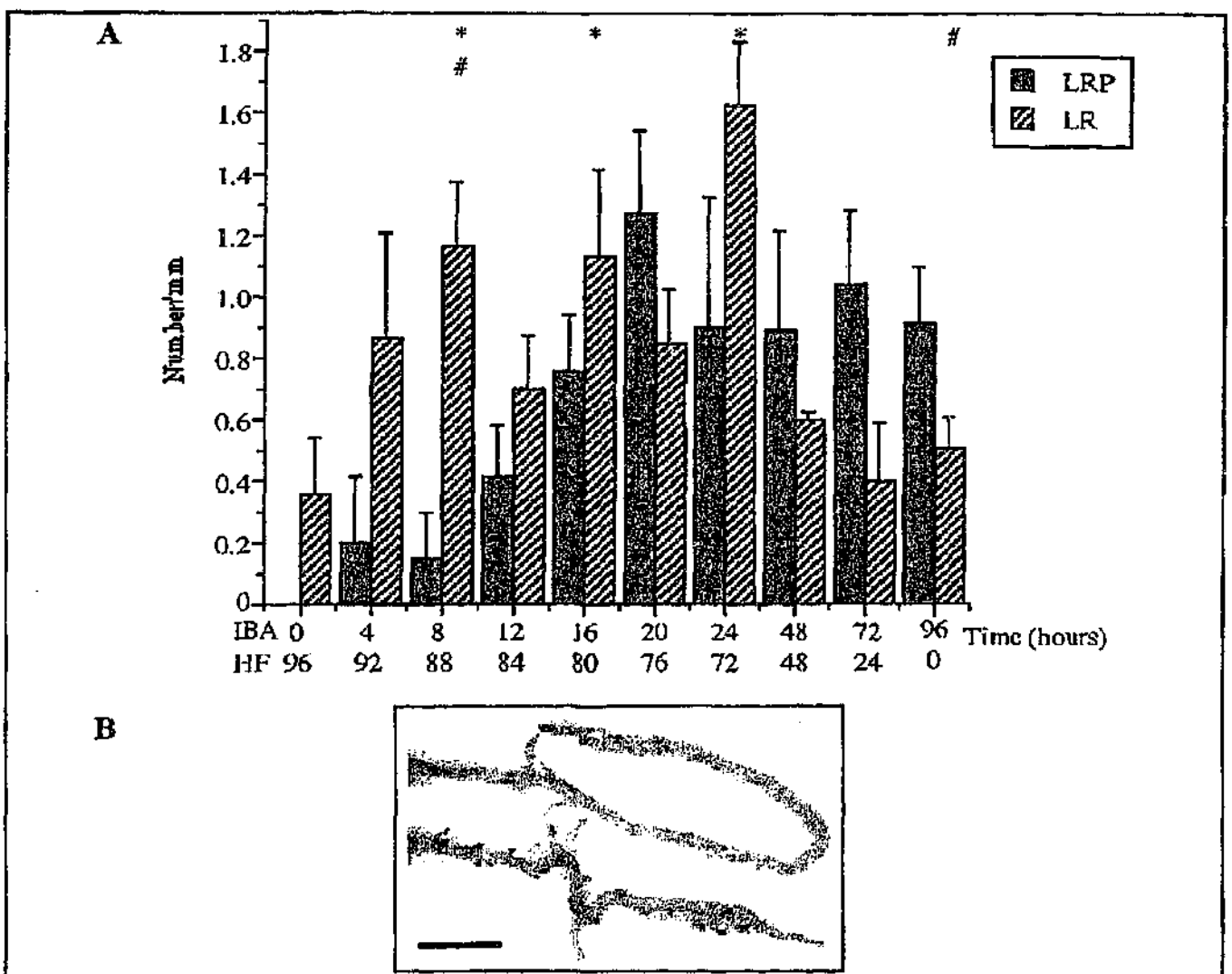


Figure 4.3: Conversion of LRP to LRs. (A) Root explants were exposed to 10^{-5} M IBA then cultured on HF medium for the remainder of a 96 h treatment period. Data represent means of 4-6 replicates ± SEM. Significance values are derived from t-tests on unpaired samples. Significant differences in LR numbers from HF (0 h IBA + 96 h HF) and IBA (96 h IBA +0 h HF) controls are indicated by * ($p<0.05$). Significant differences between LRP and LR numbers for each treatment are indicated by # ($p<0.05$). (B) Top: Inhibited LR emergence in root explants cultured on 10^{-5} M IBA for 96 h. Bottom: Transfer to HF medium for 88 h following 8 h 10^{-5} M IBA promoted LR emergence. Bar represents 2.3 mm.

From these studies, a tentative pathway for auxin induced LR formation in *E. globulus* seedlings is proposed (Fig. 4.4). The observations made here indicate that the application of exogenous auxin triggers a response in root explants of *E. globulus*, leading to the initiation of LRP 30 min after the initial exposure.

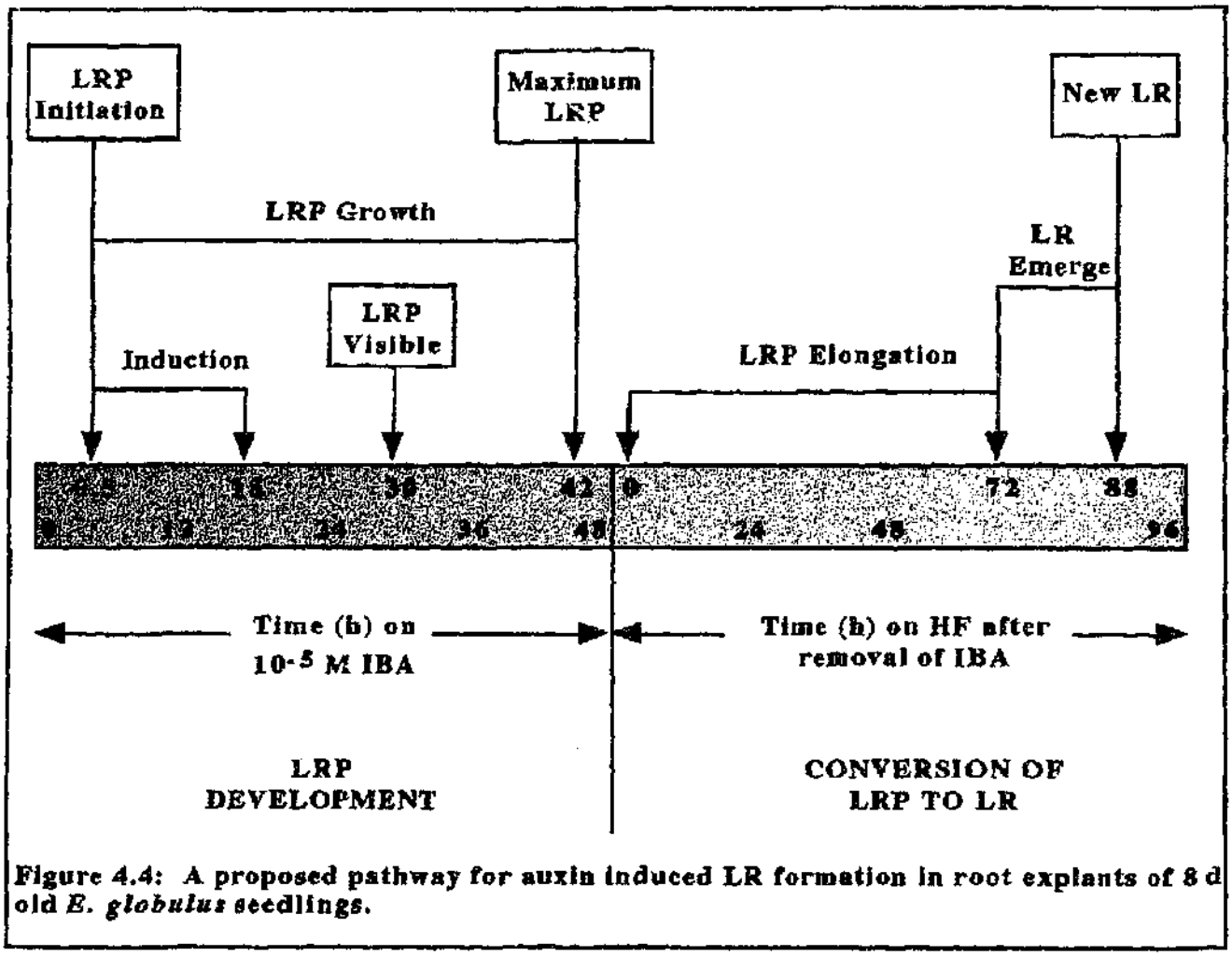


Figure 4.4: A proposed pathway for auxin induced LR formation in root explants of 8 d old *E. globulus* seedlings.

In *E. globulus* roots, it is proposed that while auxin remains present, LRP induction and initiation continues until it reaches a saturation point, 18 h after the initial exposure. Each LRP continues to develop and grow until it is visible at maturity, approximately 30 h. By 42-48 h maximum levels of LRP formation is complete. The conversion of LRP to LR occurs independently of exogenous auxin. After the removal of auxin, a further 72-88 h is required for the LRP to elongate and penetrate the cell wall of the primary root, emerging as a new and actively growing LR.

4.4 PART B: THE ISOLATION OF DIFFERENTIALLY EXPRESSED DNA FRAGMENTS

The DDRT-PCR and cDNA subtraction strategies were designed to target sequences representing mRNAs that accumulate during various stages of LRP development, from initiation through to maturity. Root explants from *E. globulus* were exposed to 10^{-5} M IBA for 6, 12, 18, 24 and 36 h. The 6, 12 and 18 h time points were selected to identify transcripts that may be involved early in the development of the LR, namely initiation and induction of the LRP. The inclusion of the 24 and 36 h time points would incorporate mRNAs that may be involved specifically in the growth of the LRP to maturity, before emerging from the primary root as a new LR.

4.4.1 Materials and Methods

4.4.1.1 Preparation of Total RNA Samples for Comparison by DDRT-PCR

Following IBA treatment to stimulate LR initiation, the root explants from each of the chosen treatment periods were harvested and the root tips removed in order to eliminate the pre-existing meristem. Total RNA extracted from these auxin induced root tissues became the 'treated' samples for DDRT-PCR.

The control samples included total RNA isolated from untreated shoot apices (including the cotyledons), hypocotyls and root explants (with the root tips removed). An unorganised cell suspension of *E. globulus* was developed to be used as an additional control to try and ensure that only root specific genes associated with LRP initiation were recovered rather than genes which show stimulation by auxin, but which are not specifically involved in root differentiation.

4.4.1.2 Establishing Cell Suspension Cultures

To develop the *E. globulus* cell suspension cultures, callus was induced on shoot apex, hypocotyl and root explants of 9-10 day old seedlings, and subjected to a number of different combinations of auxin (0.2 or 1 mg/l IAA or IBA) and cytokinin (0.5, 1 or 2 mg/l kinetin or BAP) contained in MS solid medium. The most friable calli, best suited for cell suspension culture, were produced from hypocotyl explants using 1 mg/l IBA in

conjunction with 1 mg/l kinetin after a 14 day culture. Other tissues and phytohormone combinations tested showed less prolific callus production, but some were also sufficiently adequate. The calli were transferred and cultured in liquid MX containing 0.5 mg/l of the auxin 2,4-D (2,4-Dichlorophenoxyacetic acid), with constant orbital shaking at 80 rpm allowing the callus to break up to form a suspension. The cells were harvested for RNA extraction when the culture was in its active phase of growth. In order to determine when this occurred, growth curves were constructed for the fastest growing and finest cell suspension cultures (Fig 4.5). The period of active growth varied for each cell line measured. Consequently, the time of harvest also differed. For example, the 15H cell line which was used for most experiments, was harvested at 5-6 weeks after subculture, while 6C was used at 2 weeks. Microscopic observation revealed that no organised structures were visible in the suspensions which consisted in the most part, of small groups of cells with 10-20 cells in each cluster.

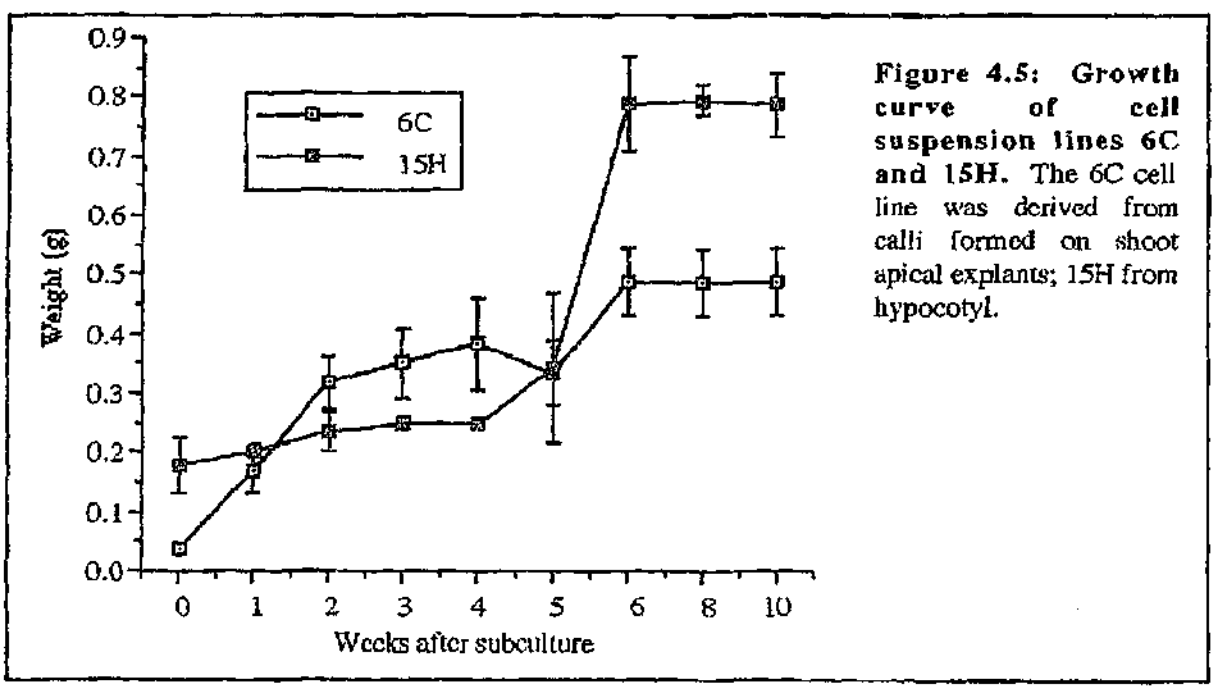


Figure 4.5: Growth curve of cell suspension lines 6C and 15H. The 6C cell line was derived from calli formed on shoot apical explants; 15H from hypocotyl.

4.4.1.3 The DDRT-PCR Procedure

The total RNA isolated from all the control and treated samples was subjected to a DDRT-PCR analysis using the optimised method described in Chapter 3.4. All samples were performed in duplicate to ensure that only reproducible differences were considered for isolation.

The 3' anchor primers $T_{11}AA$, $T_{11}AG$ and $T_{11}AC$ (Poly T Primers Kit 1, Operon Technologies) were used in all possible combinations with three 5' arbitrary 10mers

OPAP-01, OPAP-02 and OPAP-14 (Kit AP, Operon Technologies) (refer to Appendix A-2 for primer sequences). DDRT-PCR was also performed with paired 10mer combinations for amplification of cDNAs derived from reverse transcription using any one of the three anchor primers listed. These 12 primer pairings alone yielded a sufficient number of differentially displayed bands for further characterisation.

When the DDTR-PCR products derived from each sample were run side by side on a denaturing polyacrylamide gel, changes in banding patterns representing possible alterations to gene expression during the development of LRP were noticeable. The displays generated by each of the individual treated samples were compared in an attempt to identify mRNAs which may be specific to a particular stage of LRP formation. The bands of interest were those present in duplicate samples of one or more of the treated patterns, yet absent in all the controls. Only these bands were considered to be representative of putative differentially expressed genes, and hence were targeted for isolation and further analysis.

4.4.1.4 Isolation of cDNA Clones by cDNA Subtractive Hybridisation

cDNA subtractive hybridisation is a powerful technique that allows for the comparison of two mRNA populations, to identify clones representing genes that are expressed in one population but not in another. A cDNA subtraction was performed using the CLONTECH PCR-Select cDNA Subtraction Kit (CLONTECH Laboratories, Inc.). The procedure was completed essentially as described in the accompanying User Manual. Minor modifications were made to PCR parameters where required, to optimise conditions for the *E. globulus* system. A detailed description of the PCR-Select cDNA subtraction procedure undertaken in this study appears in Chapter 2.4.14.

A schematic illustration of the principles which define this method is summarised in Figure 4.6. Briefly, double stranded cDNA was synthesised from the two different mRNA populations undergoing comparison. The cDNA pool containing the differentially expressed transcripts of interest was referred to as 'tester', whilst the reference or control cDNA was termed 'driver'. The tester and driver cDNAs underwent two rounds of hybridisation, with hybrid sequences removed after each round. The remaining unhybridised cDNAs should be representative of genes which are expressed exclusively in the tester population. These cDNA sequences were then targeted for amplification by PCR. All primer and adaptor sequences used for this procedure are listed in Appendix A-1.

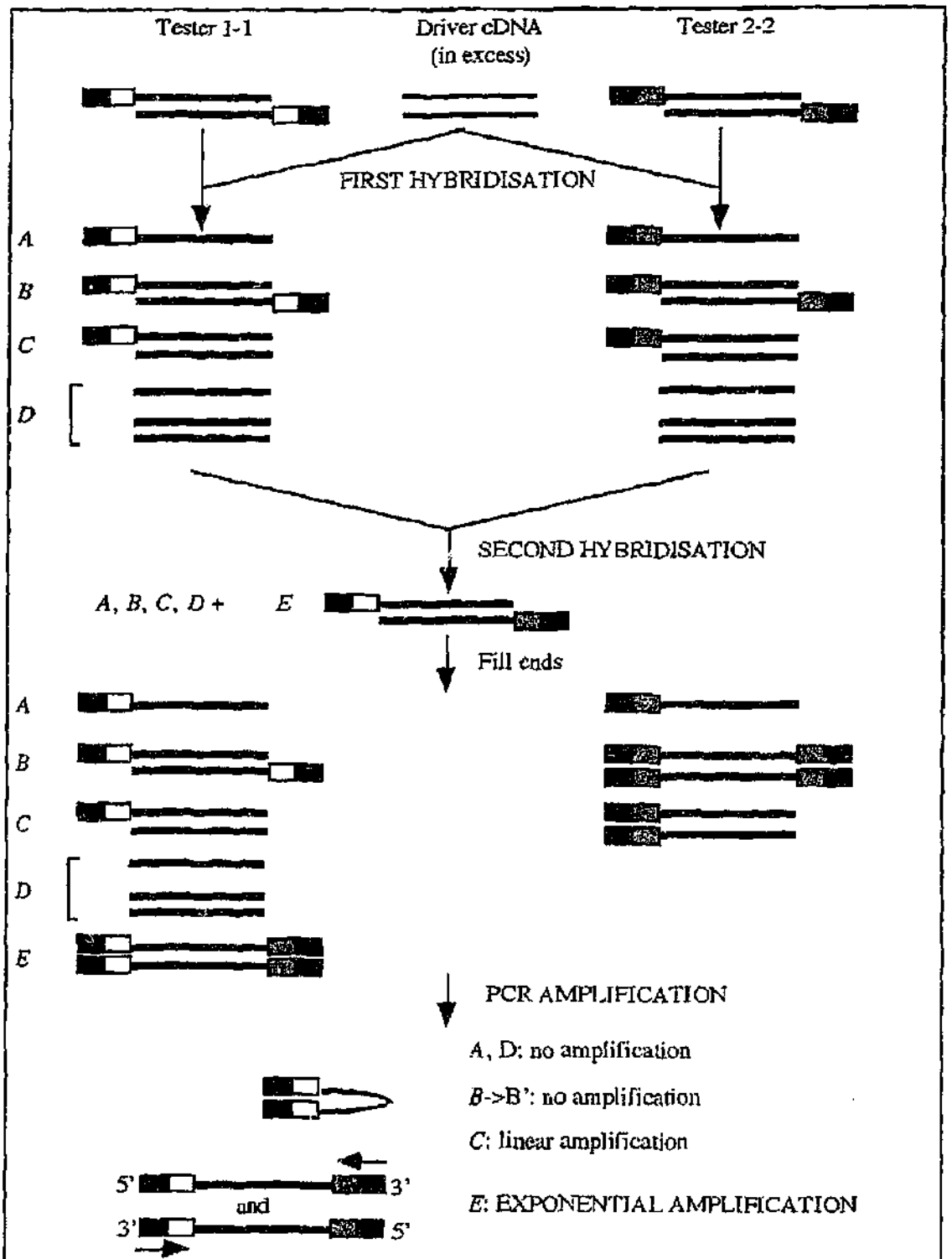


Figure 4.6: A schematic representation of the PCR-Select cDNA subtraction procedure. The solid lines represent the tester and driver cDNAs. The boxes represent the Adaptors 1 and 2, with the black sections corresponding to the PCR primer 1 binding site, and the white and red sections corresponding to the binding sites of Nested primer 1 and 2 respectively. Type *E* molecules will form only if the sequence is up-regulated in the tester cDNA sample alone. (This diagram has been adapted from the CLONTECH PCR-Select cDNA Subtraction Kit User Manual p. 6.)

4.4.1.4.1 Application and Principles of the CLONTECH PCR-Select cDNA Subtraction

Tester and Driver cDNAs

Root explants were taken from 8 day old *E. globulus* seedlings and treated with 10^{-5} M IBA for 6, 12, 18, 24 and 36 h to induce LRP formation. Poly A^+ RNA was purified from each time point and used to synthesise first strand tester cDNA. In contrast, the driver cDNA population was derived from untreated shoot apex, hypocotyl and root explants. Poly A^+ RNA from human skeletal muscle was used as a control to ensure the kit components were satisfactory and experimental manipulations were performed correctly.

Both the tester and driver cDNA populations were digested with a four base cutter, *Rsa* 1 to produce molecules which were shorter in length to optimise the downstream hybridisation steps. Adaptors were ligated to the ends of the tester cDNA to ultimately facilitate PCR amplification of the resulting subtracted molecules. The adaptors were designed such that they lacked a phosphate group permitting only a single strand from each adaptor attaching to the 5' end of the tester cDNAs.

Before ligating adaptors to the skeletal muscle tester control, the cDNA sample was spiked with *Hae* III digested ϕX174 DNA. This served to introduce unique sequences which would be left behind following subtraction against the skeletal muscle driver cDNA. Amplification of bands corresponding to these control fragments in the final PCR, could then be used to indicate the successful elimination of common molecules during the subtraction procedure.

cDNA Hybridisation

In essence, the first hybridisation involved combining an excess amount of driver cDNA to each of the two tester cDNA pools. The samples were then heat denatured and allowed to anneal, resulting in the type *A*, *B*, *C* and *D* molecules illustrated in Figure 4.6. An important consideration taken into account by this procedure, is that reannealing proceeds more rapidly for more abundant molecules due to the second order hybridisation kinetics (James and Higgins, 1985). On this basis, the concentration of both the high and low abundance sequences amongst the remaining single stranded type *A* molecules was equalised after annealing. Furthermore, because the non-target sequences present in both the tester and driver cDNA pools form type *C* hybrids, the single stranded type *A* molecules were enriched considerably for the targeted differentially expressed sequences.

The second hybridisation reaction allowed the remaining equalised and subtracted single stranded tester cDNAs to anneal and form the new type *E* hybrids (Fig. 4.6). The desired, differentially expressed sequences represented by these double stranded tester cDNAs were targeted for amplification by PCR, which made use of the different adaptor sequences attached to each end of the type *E* hybrid.

PCR Amplification

In order to amplify the differentially expressed sequences, the hybridised *E. globulus* and skeletal muscle control samples were subjected to two rounds of PCR amplification. A PCR Control subtracted cDNA provided in the kit was included as a positive control for PCR amplification. In the first, exponential amplification occurs exclusively for the double stranded cDNAs that have a different adaptor on their 5' and 3' ends. The second PCR utilises nested primers, to reduce background and enrich for the sequences representing differentially expressed mRNAs.

Both type *A* and *D* molecules (Fig 4.6) can not be amplified, lacking a primer annealing site at their 5' and/or 3' end. The type *B* molecules succumb to the suppression PCR effect, forming a pan-like structure that prevents their exponential amplification. The suppression PCR effect (Siebert *et al.*, 1995) inhibits the exponential amplification of these molecules. During the annealing step of PCR, the complementary adaptor sequences at each end of the denatured type *B* molecule, hybridise to form a pan-like structure, preventing the annealing of primer. If a primer does happen to anneal and extend, the same inverted terminal repeats will also be present on the new synthesised strand, causing the formation of another pan-like structure, thereby suppressing PCR amplification. This process effectively ensures that PCR amplification is specific for the cDNA molecules that have a different adaptor at each end. Type *C* molecules can only undergo linear amplification with a single primer annealing site. The type *E* molecules however, have a different adaptor on each end providing annealing sites for exponential PCR amplification.

Analysis of PCR Products

The completed secondary PCR mixture should be enriched for differentially expressed transcripts, which should theoretically all be present in equal proportions. In order to determine whether or not the subtraction had been successful, 8 µl of each completed reaction was electrophoresed on a 2% agarose gel and the PCR products examined (Fig 4.7A).

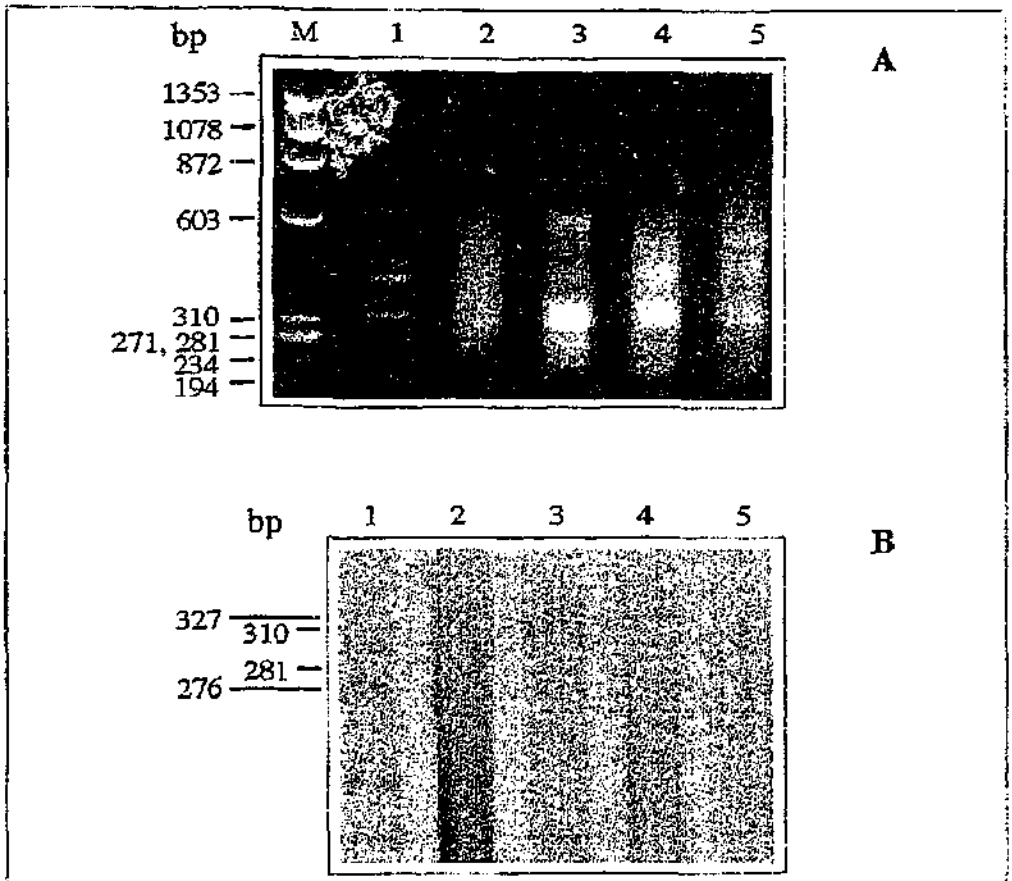


Figure 4.7: Analysis of the secondary PCR products on a 2% agarose gel (A) or by PAGE (B). Lane M: ϕX174/*Hae* III-digested DNA size marker. **Lane 1:** secondary PCR products of the subtracted *E. globulus* tester cDNA. **Lane 2:** secondary PCR products of the unsubtracted *E. globulus* tester cDNA. **Lane 3:** PCR control subtracted cDNA. **Lane 4:** secondary PCR products of the unsubtracted skeletal muscle tester cDNA containing 0.2% ϕX174/*Hae* III-digested DNA. **Lane 5:** secondary PCR products of the subtracted skeletal muscle tester cDNA containing 0.2% ϕX174/*Hae* III-digested DNA.

As expected, amplification of the PCR control subtracted cDNA produced bands corresponding to the ϕX174/*Hae* III digest (Fig 4.7A; Lane 3). The control skeletal muscle subtraction yielded similar results, with most of the bands produced also equivalent to the ϕX174/*Hae* III fragments (Fig 4.7A; Lane 5). The adaptor sequences on both ends of these fragments accounted for the increase in size of these PCR products compared to the *Hae* III-digested ϕX174 DNA marker (Lane M). Two additional PCR fragments were also observed. The kit's User Manual indicated that this was a possible outcome but of little concern when only a few extra bands appeared.

The *E. globulus* subtracted PCR products appeared as distinct bands (Fig 4.7A; Lane 1). Electrophoresis on a 2% agarose gel showed that these bands ranged in size from

around 160 to 870 bp. Contrary to this, the unsubtracted samples which served as negative controls for the subtraction, produced a smeared secondary PCR product for both the *E. globulus* and skeletal muscle tester cDNAs (Fig 4.7A; Lanes 2 and 4 respectively). The fact that the banding pattern of the unsubtracted cDNAs was different from that obtained for the subtracted samples, was indicative of a successful subtraction.

The subtracted PCR mixture should at this point be enriched for sequences corresponding to differentially expressed genes involved in LR formation. In addition, the variation in the relative abundance of differentially expressed transcripts within the initial mRNA sample, should no longer exist. Instead the subtraction procedure should have ensured that all differentially expressed transcripts were present in approximately equal proportions.

Targeting the Subtracted cDNAs

The individual bands generated by PCR amplification of the subtracted *E. globulus* tester cDNA were targeted for isolation. These bands were then reamplified and cloned into a T-vector, to facilitate the analysis of individual clones by sequencing and Northern blotting.

Direct excision of these typically small subtracted PCR bands (160 to 870 bp) was complicated by the inadequate band separation provided by agarose gels. As an alternative, the secondary PCR products were subjected to polyacrylamide gel electrophoresis, which offered greater capabilities for resolving low molecular weight DNA.

Secondary PCR was completed in the presence of 0.4 µCi [α-^{35}S]-dATP and dried down to 2 µl in the Savant Speedvac SC100. Half of the PCR sample was mixed with 1 µl of loading buffer (15% Ficoll, 25% Xylene cyanol, 25% bromophenol blue) and loaded onto a 6% nondenaturing polyacrylamide gel, and electrophoresed at 1450V until the second dye front had reached the bottom of the gel. The gel was dried under vacuum onto Whatman paper and autoradiographed for 8 days at room temperature. The banding patterns observed corresponded to those obtained from agarose gels, in that the subtracted secondary PCR products appeared as faint bands, while the unsubtracted products were represented by a smear (Fig. 4.7B). Distinct bands were excised from the gel, eluted and reamplified.

Reamplification of the Isolated cDNA Subtracted Bands

DNA purified from the isolated subtracted bands were reamplified in a 40 µl reaction, using the same PCR conditions employed for the nested secondary PCR (Chapter 2.4.14.5). One round of amplification was sufficient to successfully reamplify most bands (Fig.4.8). In cases where a second PCR was required, 4 µl of a 1:50 dilution of the first round PCR mix was used as template in a 40 µl reaction.

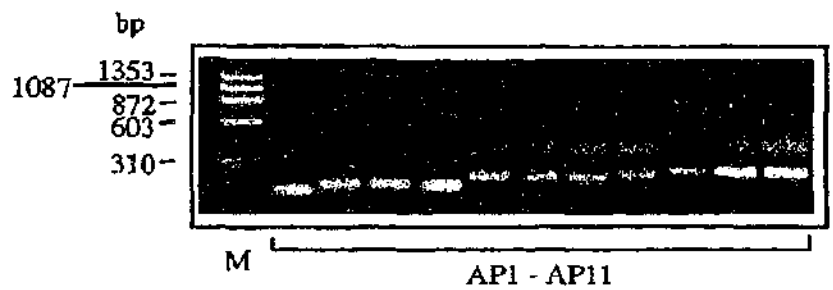


Figure 4.8: An example of reamplified bands (AP1-AP11) following elution and purification from polyacrylamide gels. (Lane M: ϕX174/*Hae* III-digested DNA size marker.)

4.4.1.5 Cloning of DDRT-PCR and cDNA Subtracted Bands

Given that both the DDRT-PCR and cDNA procedures were based upon PCR and PAGE, the possibility existed that the bands isolated by either approach were heterogeneous in nature. On this basis it was decided to adopt a clone and sequence strategy over the direct probing alternative. Although this was a time consuming and tedious task, it was important to ensure that just one DNA fragment was dealt with when determining or verifying differential expression profiles. Cloning individual fragments followed by sequencing would guarantee that this was the case. Ultimately, this strategy did prove to be worthwhile. The sequencing results reported in a later section of this chapter (Chapter 4.4.2.1), revealed that some of the excised bands contained up to four different unrelated DNA sequences. Such heterogeneous bands would have been unsuitable to use directly as probes for Northern blot analyses, complicating results by the detection of multiple unrelated signals (Diachenko *et al.*, 1996), or low abundance transcripts may have been masked by the presence of more abundant ones (Bauer *et al.*, 1993). Analysis of the complete set of cloned bands was not possible, instead an assortment of clones derived from each DDRT-PCR and cDNA subtraction was chosen for further processing.

4.4.2 Results

From approximately 6,400 cDNAs generated by 12 primer pairings in DDRT-PCR, and a mean of 59.2 bands per treated sample, 50 bands were excised from polyacrylamide gels which appeared to show specific amplification from one or more of the auxin induced root samples (Table 4.1). Ikonomov and Jacob (1996) reported similar findings when they isolated 36 cDNAs from ten primer combinations. Bands isolated from treated root samples were absent in all the untreated control tissues, as well as the RNA controls. These bands were labelled with a 'DD' prefix in order to distinguish them as having been identified by the DDRT-PCR screening procedure.

Sample	Excised Band(s)	Total
Treated Root - 6 h	DD197.5; DD197.6; DD197.7; DD197.8; DD208.1; DD211.1; DD216.1; DD224.3; DD228.2; DD230.1; DD230.2; DD230.3; DD243.1	13
Treated Root - 12 h	DD144.1*; DD150.1A*; DD150.1B; DD150.2*; DD192.1*; DD192.2*; DD192.3; DD197.3; DD197.4; DD202.1; DD208.2; DD228.3	12
Treated Root - 18 h	DD112.1*; DD196.1; DD197.1; DD197.2; DD202.2; DD202.3; DD206.1; DD208.3; DD224.2; DD232.1; DD243.2	11
Treated Root - 24 h	DD208.4; DD216.2; DD216.3; DD216.4; DD218.1; DD224.1; DD228.1	7
Treated Root - 36 h	DD196.2; DD196.3; DD208.5; DD216.5	4
Treated Root - 6, 12 h	DD235.1	1
Treated Root - 18, 24, 36 h	DD235.2	1
Treated Root - 24, 36 h	DD243.4	1
Untreated Root	DD101.1*; DD101.2*; DD101.3*; DD101.4*; DD101.7*	5
Untreated Hypocotyl	DD101.5*; DD101.6	2

Table 4.1: The total number of bands recovered from each tissue sample screened by DDRT-PCR. Bands processed further are indicated by *.

Added to these were the seven bands isolated from the initial trial conducted to test the optimised conditions devised for DDRT-PCR (refer to Chapter 3.4.6). These fragments were designated as DD101.1 through to DD101.7, and would continue to be processed to ensure all procedures were working efficiently, including cloning, sequencing and Northern analyses. This would act as a safeguard against the needless waste of the reamplified bands isolated from screens conducted on the auxin induced root samples. Therefore, in total 57 DDRT-PCR bands were isolated. For practical reasons a more detailed study of 12 of these targeted bands was conducted.

From the 26 subtracted bands purified from the polyacrylamide gels (AP1-AP26), reamplification of 11 bands (AP13-AP19, AP21-AP23 and AP26) produced multiple PCR products, ranging from two to four fragments of different sizes. In addition, only three bands, AP20, AP24 and AP25, could not be reamplified (Table 4.2).

Excised band	Number of reamplified PCR products
AP1	1
AP2	1
AP3	1
AP4	1
AP5	1
AP6	1
AP7	1
AP8	1
AP9	1
AP10	1
AP11	1
AP12	1
AP13	4
AP14	4
AP15	3
AP16	2
AP17	2
AP18	2
AP19	2
AP20	0
AP21	2
AP22	2
AP23	2
AP24	0
AP25	0
AP26	2
TOTAL	39

Table 4.2: A summary of the number of PCR products produced following reamplification of the eluted cDNA subtraction bands.

Reamplification produced a total of 39 bands ranging in size from approximately 60 to 530 bp. This size range is at the lower end of the scale that was observed following agarose gel analysis of the subtracted secondary PCR products, where bands of up to 870 bp were clearly visible (Fig 4.7A).

In total, 12 DDRT-PCR and 30 cDNA subtracted bands were successfully cloned. In some cases, a number of colonies representing the cloning of an individual band, produced inserts of varying sizes. Cloning of band DD101.2 for example, revealed fragments of two different sizes (Fig. 4.9). Colonies 1, 2, 4 and 5 contained an insert of the same size (ca. 500 bp) as that indicated by the positive control. The insert from colony 6 however was clearly smaller (ca. 400 bp). This result was indicative of the isolation of a heterogeneous DDRT-PCR product. On this basis, cloned fragments differing in size from the expected, such as that represented by colony 6, could not be omitted from the subsequent analyses, until it could be determined which of the fragments showed true differential expression. The expression analyses reported in Chapter 5, did indeed demonstrate that colonies 4 and 6 contained cloned inserts representing two independent, differentially expressed genes of interest. This indicated that the exclusion of clones from further characterisation based on unexpected insert sizes can be inappropriate.

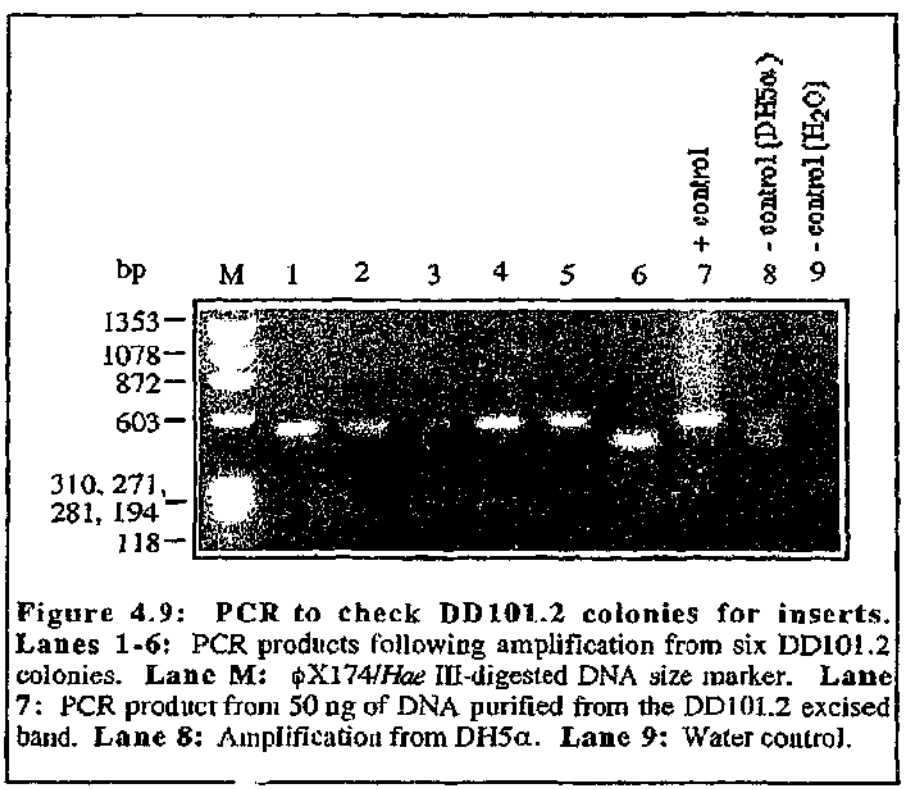


Figure 4.9: PCR to check DD101.2 colonies for inserts. **Lanes 1-6:** PCR products following amplification from six DD101.2 colonies. **Lane M:** ϕX174/*Hae* III-digested DNA size marker. **Lane 7:** PCR product from 50 ng of DNA purified from the DD101.2 excised band. **Lane 8:** Amplification from DH5α. **Lane 9:** Water control.

4.4.2.1 Test for Heterogeneity and Redundancy by Nucleotide Sequencing

Sequence data was obtained to check the level of heterogeneity and redundancy amongst the 42 clones. Each clone was then allocated into a group based on the identified sequence homology to other different clones. Inserts were sequenced from up to nine colonies for each independently cloned band. The resulting data exposed the existence of sequence heterogeneity among bands originally excised from polyacrylamide gels following either DDRT-PCR or cDNA subtraction.

4.4.2.1.1 DDRT-PCR Clones

Sequence analysis revealed the presence of arbitrary primer sequences at both ends of the DDRT-PCR inserts. This is interesting considering that these particular fragments were generated from DDRT-PCR using 3' anchor primers in combination with 5' 10mer primers. von der Kammer *et al.* (1999) suggest that such sequences should not be excluded from further analysis, as the use of arbitrary primers without anchor primers has been employed successfully for both DDRT-PCR (Haag and Raman, 1994) and RNA fingerprinting (McClelland *et al.*, 1995).

Overall, five of the 12 DDRT-PCR bands were identified as having originally been heterogeneous in nature (ie., DD101.2, DD101.4, DD144.1, DD192.1 and DD192.2). The number of distinct DNA species per excised band ranged from 1-4 (Table 4.3). For example, DNA sequence data obtained from the five different colonies representing DD101.2 revealed the presence of four different sequences. Although colonies 1, 2, 4 and 5 contained inserts of the same expected size (Fig. 4.9) only inserts from colonies 1 and 5 were identical in DNA sequence. Sequenced inserts from colonies 2 and 4 were different from 1 and 5, as well as from each other. As expected, the colony 6 insert also differed in DNA sequence from all the other DD101.2 cloned fragments.

Bands	Colonies	Sequenced inserts	Insert size (bp)	Representative clone for processing
DD101.1	4,7,8,12,14,16	4=7=12=14	786	DD101.1:12
DD101.2	1,2,4-6	1=5	504	DD101.2:1
		2	505	DD101.2:2
		4	504	DD101.2:4
		6	402	DD101.2:6
DD101.3	1-7	1=2=3	439	DD101.3:2
DD101.4	1-3,7,12,13	1	402	DD101.4:1
		2	433	DD101.4:2
		13	431	DD101.4:13
DD101.5	1-8,10-12,15	1=4=12	249	DD101.5:12
DD101.7	1-14	4=7=9	569	DD101.7:4
DD112.1	2-5,8	2=5=8	547	DD112.1:3
DD144.1	1B-6B,4C-6C,1D,4D,6D	1B=2B=6B	435	DD144.1:2B
		4C=5C=6C=4D	467	DD144.1:4C
		1D=6D	474	DD144.1:6D
DD150.1A	1,4,6,8	1	501	DD150.1A:1
DD150.2	1,4	4	229	DD150.2:4
DD192.1	1-3,5,6	1=5	439	DD192.1:1
		2=3	227	DD192.1:2
		6	333	DD192.1:6
DD192.2	2-4	2=3	298	DD192.2:2
		4	295	DD192.2:4

Table 4.3: Summary of the level of sequence heterogeneity among the bands excised from the DDRT-PCR gels. Cloned fragments were grouped according to sequence homology. One clone was chosen to represent each of the excised bands. Bands which were not successfully cloned have been omitted from the table.

In addition to the DD101.2 example, sequencing of three DD101.4 and nine DD144.1 colonies revealed three distinct DNA species apiece. Five DD192.1 colonies represented three sequences, while data obtained from three DD192.2 colonies identified two different sequences. These results are further testimony to the potential heterogeneity that has been reported to exist in bands produced by the DDRT-PCR approach. In order to avoid future confusion a system of clone nomenclature was devised. A clone (e.g. DD101.2:1) can be traced back, or distinguished from other clones as follows:

DD101 = the name given to a particular DDRT-PCR gel;
DD101.1 = indicates one band excised from gel DD101;
DD101.2 = indicates a second independent band isolated from gel DD101;
DD101.2:1 = Identifies a single clone (derived from excised band DD101.2) representing and corresponding to a unique nucleotide sequence;
DD101.2:6 = Identifies a different clone derived from excised band DD101.2.

At this stage it was not possible to determine which of the clones derived from the five heterogeneous bands represented true differentially expressed transcripts. Consequently, with the addition of the 15 clones chosen to represent these heterogeneous bands, the total number of clones corresponding to the 12 bands originally excised, was increased to 22. DNA sequence comparisons conducted between these 22 clones revealed 18 different homologous groups, each representing a unique sequenced insert (Table 4.4). One clone from each of the 18 groups, designated DD-A to DD-R, was chosen to undergo further processing to confirm differential expression profiles.

Group	Homologous clones
DD-A	DD101.1:12*
DD-B	DD101.2:1*
DD-C	DD101.2:2*
DD-D	DD101.2:4*
DD-E	DD101.2:6*; DD101.4:1
DD-F	DD101.3:2; DD144.1:2B; DD192.1:1*
DD-G	DD101.4:2*
DD-H	DD101.4:13*
DD-I	DD101.5:12*
DD-J	DD101.7:4; DD144.1:6D*
DD-K	DD112.1:3*
DD-L	DD144.1:4C*
DD-M	DD150.1A:1*
DD-N	DD150.2:4*
DD-O	DD192.1:2*
DD-P	DD192.1:6*
DD-Q	DD192.2:2*
DD-R	DD192.2:4*

Table 4.4: Grouping of homologous DDRT-PCR clones. Clones chosen to represent each homologous group in further analyses are denoted by an asterix (*).

4.4.2.1.2 cDNA Subtraction Clones

The sequenced inserts varied in size from 63 to 528 bp, confirming the isolation of molecules from the lower end of the size range detected for the subtracted cDNAs. The data also exposed sequence heterogeneity among 11 of the 30 cloned bands examined. The five colonies assayed for band AP2 for example, represented two entirely different sequences (Table 4.5). Colonies 1, 3, 4 and 5 contained a 129 bp insert of identical DNA sequence, but which differed from the 117 bp insert contained by colony 2. This level of heterogeneity indicated 41 independent cloning events requiring further processing.

The subtracted and reamplified bands, and their corresponding clones, were assigned a label accordingly:

AP = All cDNA subtracted bands and resulting clones were prefixed with 'AP' in order to distinguish them from the DDRT-PCR bands;

AP13 = Each band isolated from the polyacrylamide gels and reamplified were assigned a number from 1 to 26;

AP13A = When multiple PCR products were observed following the reamplification of a particular subtracted band, such as AP13, each individual fragment was labelled with the letter A, B, C or D;

AP13A:2 = Indicates a single clone or sequenced insert derived from the band AP13A.

As expected, some clones proved to be redundant, indicating that the subtracted band from which they were derived had been isolated on more than one occasion. Perhaps this is a reflection of an increased abundance in the level of transcript represented by these clones. Although the cDNA subtraction method was designed to equalise the abundance of different mRNAs, it is possible that some variation may still exist in the *E. globulus* subtracted sample.

For each subtracted band, a representative clone was chosen to simplify the allocation of clones into different homologous groups for further analyses (Table 4.6). Heterogeneous bands, such as AP2, were allocated one representative clone per divergent sequence, AP2:1 and AP2:2. The level of redundancy amongst the sequenced clones revealed 21 different homologous groups, designated SUB-A through to SUB-U. Five groups comprised of two or more redundant clones, while the remaining 16 groups were represented by a single clone of unique sequence. The largest group, Group SUB-B, contained representative clones derived from ten independently isolated bands.

Bands	Colonies	Sequenced inserts	Insert size (bp)	Representative clone for processing
AP1	1,3,6	1=3=6	104	AP1:1
AP2	1-6	1=3=4=5	129	AP2:1
		2	117	AP2:2
AP3	1-6	3=4=5	172	AP3:3
AP4	1-6	4=5=6	129	AP4:4
AP5	1-6	1=3=5	169	AP5:1
AP6	1,2,4-6	1=2=5	169	AP6:1
		4=6	177	AP6:4
AP7	1-3,5	2=3=5	198	AP7:3
AP9	3,4	3=4	172	AP9:3
AP10	5,9	5=9	223	AP10:5
AP11	1,3-8,10-15	1=6=7=8=13=14	229	AP11:7
AP12	1,3,6,9	1=9	227	AP12:1
		3=6	228	AP12:3
AP13A	2,3,5,9	2=3=5=9	129	AP13A:2
AP13B	3	3	229	AP13B:3
AP13C	5	5	129	AP13C:5
AP13D	5,6	5=6	102	AP13D:5
AP14A	4,6	4=6	165	AP14A:4
AP15B	2,3	3	129	AP15B:3
AP15C	1,2,5,6	5	102	AP15C:5
		1=2=6	129	AP15C:1
AP16A	3	3	102	AP16A:3
AP16B	1,2,4,5,6	1=4	129	AP16B:1
		6	63	AP16B:6
AP17A	3,5	3	129	AP17A:3
		5	116	AP17A:5
AP17B	1,4,5,6	1=6	102	AP17B:6
		4	177	AP17B:4
		5	129	AP17B:5
AP19A	1-3,5	1	96	AP19A:1
		2=5	94	AP19A:2
AP19B	2,3	2	93	AP19B:2
		3	276	AP19B:3
AP21A	1,2-5	1	60	AP21A:1
		2=5	513	AP21A:5
		4	528	AP21A:4
AP21B	1,2,4-6	1=2=5	276	AP21B:1
		4	327	AP21B:4
		6	348	AP21B:6
AP22A	2	2	276	AP22A:2

Table 4.5: Summary of the level of sequence heterogeneity among the bands excised from the cDNA subtraction gels. Cloned fragments were grouped according to sequence homology. One clone was chosen to represent each of the excised bands. Bands which were not cloned due to time constraints were omitted from the table.

Group	Homologous clones
SUB-A	AP1:1*; AP13D:5; AP15C:5; AP16A:3; AP17B:6
SUB-B	AP2:2*
SUB-C	AP2:1; AP3:3*; AP4:4; AP13A:2; AP13C:5; AP15B:3; AP15C:1; AP16B:1; AP17A:3; AP17B:5
SUB-D	AP5:1*; AP6:1; AP14A:4
SUB-E	AP6:4*
SUB-F	AP7:3*
SUB-G	AP9:3*
SUB-H	AP10:5; AP11:7; AP12:1*; AP13B:3
SUB-I	AP12:3*
SUB-J	AP16B:6*
SUB-K	AP17A:5*
SUB-L	AP17B:4*
SUB-M	AP19A:1*
SUB-N	AP19A:2*
SUB-O	AP19B:2*
SUB-P	AP19B:3; AP21B:1; AP22A:2*
SUB-Q	AP21A:1*
SUB-R	AP21A:5*
SUB-S	AP21A:4*
SUB-T	AP21B:4*
SUB-U	AP21B:6*

Table 4.6: Grouping of homologous cDNA subtraction clones. Clones chosen to represent each homologous group in further analyses are denoted by an asterix (*).

4.5 DISCUSSION

In summary, 83 bands were isolated by the two alternative molecular screening methods (Table 4.7). Further processing was undertaken for 38 of these bands, involving their reamplification and cloning into a T-vector. Forty-two bands were successfully cloned and the inserts sequenced to reveal 63 independent cloning events. Sequence comparison conducted between these 63 clones indicated 39 different homologous groups, 18 derived from DDRT-PCR and 21 from cDNA subtraction.

Screening method	Excised bands	Processed	Reamplified	Cloned	Independent colonies	Homologous groups
DDRT-PCR	57	12	12	12	22	18
cDNA subtraction	26	26	39	30	41	21
TOTAL	**83**	**38**	**51**	**42**	**63**	**39**

Table 4.7: A summary of the total number of bands isolated and processed by both molecular screening methods employed.

4.5.1 Comparative Analysis of the Cloned Fragments Isolated by Each Technique

At this level of analysis, the cloned fragments generated by either DDRT-PCR or cDNA subtraction can be compared with respect to size, the degree of heterogeneity and the level of redundancy. These points will be addressed here. Characterisation of the clones by Northern blot analysis and a more detailed examination of the sequences will provide information regarding the level of false positives, sensitivity, and sequence structure offered by each technique. These issues are discussed in Chapters 5 and 7.

4.5.1.1 Size of the Cloned Inserts

The size of the bands recovered from the cDNA subtraction were on average 180 bp in length, substantially shorter than those isolated by DDRT-PCR, which averaged at 430 bp. However, larger fragments (>200 bp) were observed following the secondary PCR of the subtracted tester cDNA (Fig. 4.7). Perhaps these sequences could be more successfully targeted by optimising the band separation and improving the resolution of larger fragments on polyacrylamide and/or agarose gels, or alternatively by shotgun cloning the entire secondary PCR mix.

4.5.1.2 Degree of Heterogeneity

Both techniques yielded heterogeneous products. The incidence was only slightly higher in DDRT-PCR clones at 41.67% compared to 36.67% amongst the cDNA subtraction derived clones. This occurrence is most likely to be attributed to the comigration of similar sized fragments to the same point on the polyacrylamide gel, or the limited separation capabilities provided by the gel electrophoresis.

4.5.1.3 Level of Redundancy

Although DDRT-PCR generated a higher degree of heterogeneity, the level of sequence redundancy observed amongst these clones was less than that found for the cDNA subtracted clones. Table 4.4 shows that of the 22 DDRT-PCR clones analysed, 15 contained inserts representing unique sequences (68.18%). This compares favourably to the cDNA subtracted clones where only 16 from 41 inserts (39.02%) were found to correspond to a unique sequence (Table 4.6).

4.5.2 Future Directions

The selected 39 clones identified by DDRT-PCR and cDNA subtraction, were investigated further by Northern blot analysis to confirm and examine the differential expression profile of the corresponding genes. In addition, sequence information obtained from the clones of interest was used to search current nucleotide and protein databases to identify possible homologies to known genes and their encoded products. These results are reported in the following chapter.

A further 38 bands excised from DDRT-PCR gels remain to be processed. Cloning and sequencing each band will prove to be a time consuming task. The number of bands for cloning may be reduced if false positives can be detected early in the system. Screening by means of a reverse Northern approach may facilitate this process by confirming root specific expression. The reamplified bands can be electrophoresed on an agarose gel, blotted and probed with cDNA probes made from control and treated tissues. A positive signal detected by the control probes may help eliminate any false positives, and thereby potentially exclude the DDRT-PCR band from needless cloning. However before undertaking such a screen, the limitations of this approach will need to be considered in light of the real possibility that the DDRT-PCR bands are heterogeneous.

The results reported here have uncovered that five of the 12 DDRT-PCR bands processed to date contained heterogeneous sequences. If this level of heterogeneity (42%) is maintained over the entire set of DDRT-PCR bands isolated, then a further 15-16 bands from the remaining 38 may also be heterogeneous. This high incidence may complicate the screen as a weakly expressed differential sequence may go undetected if it is masked by a more abundant constitutive one, leading to the possibility that the band is wrongly eliminated. For the purposes of analysing the bands isolated here, reverse Northerns may be helpful in categorising the bands or prioritising them for cloning, rather than excluding them from further study.

In theory the subtracted PCR mix generated by the cDNA subtraction, can also be used as a hybridisation probe to screen a full length cDNA, or genomic DNA library of *E. globulus*, in order to isolate the differentially expressed sequences of interest. Another option may be to create a subtracted cDNA library by ligating the secondary PCR products into an appropriate vector. This library could then be differentially screened using cDNA probes to target sequences corresponding to a particular time point, or developmental stage during LR induction and development. For instance, a search for genes involved in the

early events preceding LRP initiation may be specifically targeted by using probes derived from tissue subjected to very short auxin treatments. Alternatively, manipulating the phytohormone conditions to promote LRP conversion to LR, may serve to target later events such as LR emergence. This could be achieved through the use of probes derived from the transfer of root material to either HF or low cytokinin conditions following auxin induction.

These considerations may also be applied to DDRT-PCR. To target a specific event or stage in LR development using this approach, one needs only to alter the treated samples to be screened. In addition, the primer combinations used in this study were by no means exhausted. Future screens may use different primer pairings to uncover more differentially expressed genes using either the same or different treatments for comparison to the appropriate controls.

CHAPTER FIVE

The Molecular Characterisation of Sequences Representing Genes Involved in Lateral Root Development in *Eucalyptus globulus*

5.1 INTRODUCTION

Comparison of the DDRT-PCR products generated by the auxin-treated root samples against the untreated tissues and cell suspension, was designed to specifically target the isolation of sequences that represent differentially expressed genes involved in LR development. However the previously reported high incidence of false positives using this technique (Appel *et al.*, 1999) meant current data must be critically evaluated.

Likewise, cDNA subtraction of the auxin induced root tissue (tester) against the untreated tissues (driver) was designed to ensure an enrichment of differentially expressed LR genes. However, as it is possible that the subtraction was not completely efficient, some cDNAs corresponding to transcripts common to both the original tester and driver samples, may have persisted to the final subtracted sample. As such, the possible occurrence of false positives amongst the clones isolated by this procedure also required investigation.

It was decided that examination of the expression characteristics would help distinguish false positives from clones representing genes involved in the development of LRs in *E. globulus* seedlings. Further analysis would focus on these clones alone to determine the identity of the corresponding genes and their encoded products.

5.2 AIM

The aim of this section of the study was to characterise selected clones, representing each of the homologous families, using expression and sequence analyses. Firstly, the expression characteristics of each clone was investigated by Northern hybridisation to verify differential expression during LR development, and eliminate false positives. Secondly, when differential expression profiles were confirmed, an analysis of the sequence data was performed to enable database comparisons and help elucidate a possible role for the corresponding gene and its encoded product during LR development.

5.3 MATERIALS AND METHODS

5.3.1 Expression Analysis

Differential expression profiles for each of the individual clones representing 36 of the 39 homologous groups, were investigated by Northern hybridisation analysis. This procedure also helped determine the size and the number of mRNA transcripts represented by each clone. Because auxin was used to induce LRP formation, genes associated with an auxin response but not necessarily involved in LR formation may also have been isolated. In an attempt to distinguish such sequences a cell suspension control was incorporated into each Northern blot when confirming differential gene expression represented by each isolated clones. Note that the SUB-Q, SUB-R and SUB-S homologous families remain to be characterised in a follow up research project.

Each cloned fragment was ^{32}P-labelled and used as a probe to hybridise Northern blots containing total RNA isolated from *E. globulus* cell suspension 15H, untreated shoot apex, untreated hypocotyl and untreated root explants. Alongside these samples, RNA was included from root explants treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h.

To correct for loading differences, all Northerns were stripped and reprobed with a ~1 kb fragment of pTA71 containing rDNA gene sequences from wheat (Gerlach and Bedbrook, 1979). (pTA71 was chosen because preliminary work showed homology with *E. globulus* RNA.) When used as a probe for Northern blots, the pTA71 fragment detects the 28S rRNA transcript, giving a measure of total RNA loaded per well. An IMAGEQUANT computer program was used to perform volume quantitation on all the signals detected on a single Northern blot. When used as a probe the quantitated volume detected by each clone was standardised against that obtained by the pTA71 probe to give a relative expression value for each sample.

5.3.2 Sequence Analysis

DNA and deduced amino acid sequences of the cloned inserts were searched against the NR nucleic and protein databases using the FASTA search program. The matching alignments were evaluated through the use of the *E* value. This statistical score is a measure of the expected number of database sequences that would obtain a given score by chance (Brenner, 1998). Significant homology was implied when matching alignments

achieved E values below 0.01, as scores below this set criteria were expected to occur by chance very rarely.

5.4 RESULTS

The untreated root tissue determined the basal level of expression. Although LRP numbers were significantly less in untreated roots compared to their treated counterparts, obviously they were never completely absent as is described in Chapter 4 (Fig. 4.1). Therefore it was expected that a low level of expression would be detected in these samples. The untreated shoot apex and hypocotyl samples helped to define whether gene expression was root specific. As the shoot apical sample also included the meristem, it was expected that expression associated typically with cell division could be distinguished. Induction of RNA from the cell suspension was critical to distinguish cases where gene expression was altered by auxin stimulation but not necessarily specific to LRP formation.

When a hybridisation signal was detected by Northern blot, the number of transcripts observed for each clone varied from one to four. The expression profiles of the analysed clones fell into five categories;

(i) auxin independent expression, specific to roots with stimulated formation of LRP,
(ii) auxin enhanced expression specific to roots with stimulated LRP production,
(iii) expression related to cell division in roots with stimulated LRP formation and in the shoot apex,
(iv) no differential expression, and
(v) no detected transcript.

(i) *Auxin independent expression specific to roots with stimulated formation of LRP*

The LRP specific expression profile was typically characterised by an equal or higher basal expression level in the untreated roots compared to the cell suspension. This was allied with transcript accumulation in the root, at a specific point during LRP formation. Clones representing seven of the homologous families were classified into this group, DD-M, DD-N, DD-O, DD-P, SUB-G, SUB-T and SUB-U (Table 5.1). Each of these families also represents differential expression during ARP formation in hypocotyls treated with 10^{-4} M IBA (data not shown).

Homologous family	Probe	Size of cloned Fragment	Number of transcripts	Transcript size
DD-M	DD150.1A:1	501 bp	1	2.2 kb
DD-N	DD150.2:4	229 bp	1	1.9 kb
DD-O	DD192.1:2	227 bp	1	2.3 kb
DD-P	DD192.1:6	333 bp	1	1.65 kb
SUB-G	AP9:3	172 bp	2	0.8, 1 kb
SUB-T	AP21B:4	327 bp	3	1.2, 2.7, 3.45 kb
SUB-U	AP21B:6	348 bp	2	1.6, 3.9 kb

Table 5.1: The number and size of transcripts represented by the clones detecting expression specific for LR formation.

The expression pattern observed in the control tissues indicated that these clones were root primordia-specific as opposed to being simply auxin related. Typically there was an almost complete absence of transcript in the cell suspension RNA. This observation also suggests that induction of gene expression was dependent upon the presence of an organised root (or hypocotyl) structure. The virtual absence of any mRNA transcript in the shoot apex samples lends support to the idea that these clones represent genes involved specifically in root differentiation. Examples of the expression pattern exhibited by this category of clones are illustrated in Figures 5.1, 5.4, 5.20, 5.29, 5.33, 5.36 and 5.40, located in the text below that considers each clone in detail.

The presence of transcript at low abundance in the untreated root control, is in accordance with the formation of a small number of LRs observed for these samples. Expression in the untreated hypocotyl was detected at slightly lower levels than that found in the untreated root controls and may be attributed to the formation of root primordia at the base of the hypocotyl.

(ii) *Auxin enhanced expression specific to roots with stimulated formation of LRP*

These clones were characterised by a markedly higher level of detected transcript in the cell suspension sample compared to the untreated control tissues, suggesting that the isolated clone represents a gene which is regulated by auxin. However, transcript accumulation appeared to be specifically timed, associated with a particular stage of LRP (and ARP) formation. Clones representing eight homologous families belonged to this

category, DD-D, DD-E, SUB-A, SUB-B, SUB-C, SUB-D, SUB-I and SUB-M (Table 5.2). In the text below, Figures 5.7, 5.9, 5.12, 5.14, 5.17, 5.24, 5.27 and 5.38 represent examples of the expression profile detected by clones in this category.

Homologous family	Probe	Size of cloned fragment	Number of transcripts	Transcript size
DD-D	DD101.2:4	504 bp	1	2.65 kb
DD-E	DD101.2:6	402 bp	1	1.65 kb
SUB-A	AP1:1	104 bp	3	1.6, 2.3, 3.3 kb
SUB-B	AP2:2	117 bp	4	3.4, 2.35, 1.5, 0.41 kb
SUB-C	AP3:3	172 bp	2	1.5, 1.8 kb
SUB-D	AP5:1	169 bp	1	1.4 kb
SUB-I	AP12:3	228 bp	3	1.55, 1.9, 2.6 kb
SUB-M	AP19A:1	96 bp	1	1.5 kb

Table 5.2: The number and size of transcripts represented by the clones detecting enhanced LR formation specific expression.

(iii) *Expression associated with cell division in the shoot apex and in roots with stimulated LRP formation*

This class of clones showed signs of transcript accumulation during LRP formation. However, expression was also detected in the untreated shoot apex tissue and cell suspension, indicating that gene expression is likely to be associated with cell division and not specific to the process of LRP formation. Clones from three homologous families belonged to this category, DD-A, SUB-E and SUB-J.

(iv) *No differential expression*

Three homologous families from the DDRT-PCR (DD-B, DD-L and DD-Q) and five from the subtracted (SUB-F, SUB-H, SUB-L, SUB-P and SUB-O) did not exhibit a differential expression profile. The representative clones from these families were deemed to be false positives and further examination was discontinued.

(v) *No transcript detected*

Clones from eight DDRT-PCR homologous families (DD-C, DD-F, DD-G, DD-H, DD-I, DD-J, DD-K and DD-R) and two subtracted families (SUB-K and SUB-N) failed to detect a signal on Northern blots. Apparently this is not uncommon, similar observations having been reported previously for fragments isolated by DDRT-PCR (Liang and Pardee, 1992; Liang *et al.*, 1993). The failure to detect a signal could be attributed to a number of reasons. If a clone represents a real differentially expressed gene, the corresponding mRNA transcript may be in low abundance and outside the detectable limits of Northern hybridisation. Alternatively, a clone may represent a real false positive. When no signal was observed, Northerns were repeated using new probes derived from the same clone or another member of the same homologous family. It was not possible to distinguish between the alternative possibilities within the scope of the analyses presented here. Further investigation is required using more sensitive techniques (e.g., RT-PCR procedures or Northern hybridisation using mRNA) before these clones can be discarded as false positives with confidence.

Homology searches of the current nucleotide and protein databases were conducted to determine whether these clones represented previously characterised genes. Clones representing families DD-C, DD-G, DD-I, DD-J, DD-K, DD-R, SUB-K and SUB-N were significantly matched to uncharacterised sequences (Table 5.3). Of these, only families DD-R and SUB-N have homology to *Arabidopsis* genes. No matches were made to clones representing families DD-H, DD-F and SUB-N.

Clone	nt			aa		
	Species	% homology	*E*	Species	% homology	*E*
DD-C	Human	99	2.7e-134	no match		
DD-G	Human	99	47e-76	Human	57	0.00018
DD-I	Human	83	6.5e-25	*Maca fascicularis*	66	7.8e-09
DD-J	Human	100	5.7e-107	no match		
DD-K	Human	99	7.3e-104	no match		
DD-R	*Arabidopsis*	56	0.00044	no match		
SUB-K	Human	100	1.4e-23	Human	100	2.2e-13
SUB-N	*Arabidopsis*	59	3.7e-11	*Hordeum vulgare*	37	5.5e-18

Table 5.3: Highest scoring matches made following searches of nucleic and protein databases for significant homology.

5.4.1 Characterisation of Clones Detecting Specific Expression for Lateral Root Formation

Clones detecting auxin-independent and auxin-regulated expression associated with LR formation were studied in more detail (expression profile categories I and II). Together, the expression and sequence data was useful in identifying some of the developmental pathways and cellular processes involved in LR formation in *E. globulus*. Clones were found to correspond to genes involved in cell wall development, transcriptional activation, protein synthesis and processing, protein trafficking and secretion, and genes encoding enzymes in metabolic pathways involved in plant nutrition (Table 5.4). Clones representing novel or previously uncharacterised genes were also identified when no homology was found following searches of the nucleic and protein databases.

Clone	Putative encoded protein
	Cell Wall Proteins
AP9:3	ß-expansin
AP21B:6	arabinogalactan
AP1:1	extensin
	Transcription, Protein Synthesis and Processing
AP2:2	RNA polymerase II
AP3:3	ribosomal protein L2
AP5:1	calreticulin
AP12:3	polyubiquitin
	Protein Transport and Secretion
DD192.1:2	synaptobrevin/vesicle associated membrane protein
DD101.2:4	rab GTPase activator protein
DD101.2:6	ornithine decarboxylase inducible-like
	Plant Nutrition
DD150.2:4	glutamine synthetase 1
DD192.1:6	pyrophosphate dependent phosphofructokinase ß-subunit
	Novel genes
DD150.1A:1	unknown
AP19A:1	unknown
AP21B:4	unknown

Table 5.4: A summary of the isolated clones characterised in this study. Clones denoted in red show auxin independent expression specific to roots forming LRP (Category i). Clones denoted in blue are those exhibiting auxin enhanced expression specific to LRP forming roots (Category ii).

5.4.2 Clones Representing Cell Wall Protein Genes

5.4.2.1 AP9:3: ß-expansin (*E* value = 1.6e-05)

Expression characteristics

The 0.8 kb AP9:3 transcript was first detected above control levels 6 h after initial exposure to IBA, and reached a maximum at 24 h, during the period of LRP growth, following induction (Fig. 5.1). This was followed by a decline in the detected level of this mRNA during the remaining time points sampled. The low level of this transcript in the cell suspension control implies that accumulation of this transcript was not induced by auxin. A second, 1 kb transcript was detected at a relatively high level in the cell suspension and shoot apex control samples, suggesting a possible correlation with auxin induction and cell division. Expression of the larger mRNA in the 10^{-5} M IBA treated roots was generally in the order of 7 fold lower than the 0.8 kb message, with peak expression at 24 to 48 h.

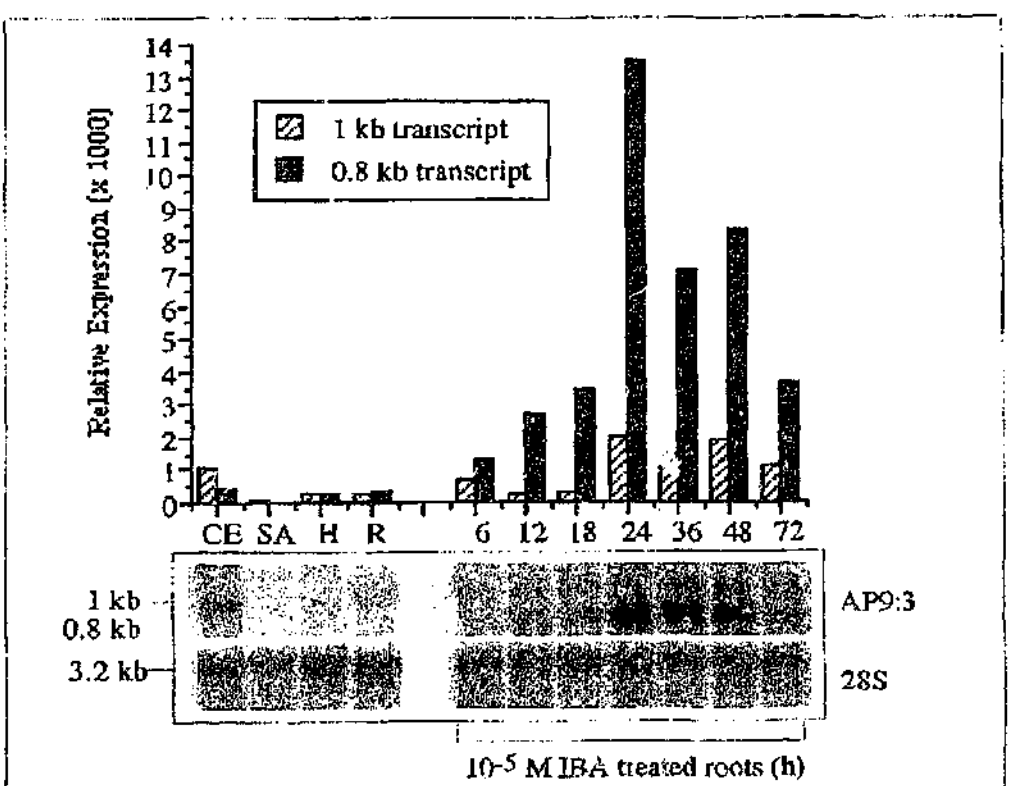


Figure 5.1: Expression profile detected by AP9:3 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

Although the 172 bp sequence of the AP9:3 clone is not significantly homologous to any nucleotide sequence in the current NR nucleic database, a significant match was found to exist at the amino acid level in the NR protein database. A predicted partial polypeptide consisting of 56 amino acids encoded by AP9:3 (Fig. 5.2), exhibited 52.9% amino acid identity (E = 1.6e-05) to an mRNA encoded sequence (*Al-EXP1*) for ß-expansin from the plant *Atriplex lentiformis* (GenPept accession number BAB20817). The *Al-EXP1* gene and protein have not been characterised with respect to structure and function, however some inferences can be made from comparisons to other published expansins. Moreover, homology was also detected with unpublished sequences of two clones from *Arabidopsis*, which both show sequence similarity to a *Holcus* major pollen allergen.

Since the expansin encoding cDNA clones *CsEXP1* and *CsEXP2* were first isolated from cucumber, homologues have been predominantly recovered from the rice and *Arabidopsis* Expressed Sequence Tag (EST) collections (Shcherban *et al.*, 1995). Additional examples come from cDNA clones isolated from pea petals (Michael, 1996), ripening tomato fruit (Rose *et al.*, 1997), tomato apical meristems (Reinhardt *et al.*, 1998), rice internodes (Cho and Kende, 1998), cotton fibres (Shimizu *et al.*, 1997) and auxin induced pine hypocotyls (Hutchison, 1999).

The mature form of the predicted protein deduced from these clones exhibit a high degree of amino acid similarity, in the order of 70 to 90% (Cosgrove, 1997; Shieh and Cosgrove, 1998; Hutchison *et al.*, 1999). This not only suggests that the protein structure of different expansins is conserved, but that the inferred protein interactions with the cell wall are also similar. Shcherban *et al.* (1995) recognised a lower yet still significant degree of sequence similarity between expansins and a group of proteins described as the major pollen allergens of grass pollen. Homologues to these group I allergens have been reported to exist in vegetative tissues of rice, *Arabidopsis* and soybean, suggesting a function for these proteins beyond the pollen tube, stigma and style. These observations led to the formation of a second multigene family of expansins termed the ß-expansins, which included both the group I allergens and their vegetative homologues (Cosgrove *et al.*, 1997). The original family of expansins have since come to be known as the α-expansins.

```
  1  CCAAAGTGTCTCGAGAATCACATGGCGCTTCTTCAATCTCCATCGATTTG
     P  K  C  L  E  N  H  M  A  L  L  Q  S  P  S  I  C   17
 51  CTTCCCTCTCGCCCTTCTCCTTTCCCTCCTCATAAACTCGTCCAACTGCT
      F  P  L  A  L  L  L  S  L  L  I  N  S  S  N  C  F  34
101  TCAACCCCAAATCGCTTAACATCTCGAAAGTTCAGTCCAACGGTGATTGG
       N  P  K  S  L  N  I  S  K  V  Q  S  N  G  D  W   50
151  TCGCCGGCAGGTGCGACGTGGT
     S  P  A  G  A  T  W                                 56
```

Figure 5.2: Nucleotide and deduced amino acid sequence of the 173 bp AP9:3 clone.

The ß-expansins have been previously reported to be variable in amino acid sequence, sharing about 25% overall identity with the α-expansins (Shcherban *et al.*, 1995; Cosgrove *et al.*, 1997). The Al-EXP1 ß-expansin, exhibited a slightly higher range of similarity, ranging from 28% to 35%, when compared by GAP analysis to α-expansins from pea (PsEXP1), cucumber (CsEXP1 and CsEXP2), rice (OsEXP1 and OsEXP3) and *Arabidopsis* (AtEXP1 and AtEXP5) (Table 5.5A).

A

α-expansins	% aa identity to Al-EXP1	% na identity to AP9:3
CsEXP1	35.0	26.5
CsEXP2	32.1	21.5
PsEXP1	30.9	36.8
OsEXP1	33.8	21.1
OsEXP3	33.8	33.8
AtEXP1	32.4	32.4
AtEXP5	28.0	28.0

B

ß-expansins and Group I allergens	% aa identity to Al-EXP1	% aa identity to AP9:3
locus AAB61709.1	41.9	41.9
At2g20750	40.8	40.8
At2g45110	47.3	47.3
EXPB3	43.2	26.5
EXPB5	47.5	25.9
PPAL	48.3	25.0
cim1	56.0	41.2
Phl pI	42.4	21.3
F5I14.21	50.9	44.0
F1E22.6	52.2	44.0
Lol pI	40.1	21.3

Table 5.5: The degree of similarity exhibited by the predicted Al-EXP1 and AP9:3 of amino acid sequences to **(A)** the α-expansins and **(B)** the ß-expansins and group 1 pollen allergens. The GenPept accession numbers are as follows CsEXP1 (AAB37746), CsEXP2 (AAB37749), PsEXP1 (CAA59470), OsEXP1 (AAB38074), OsEXP3 (AAB38075), AtEXP1 (AAB38070), AtEXP5 (AAB38071), locus AAB61709.1 (AAB61709), At2g20750 (AAD20920), At2g45110 (AAD32826), EXPB3 (AAK15453), EXPB5 (AAF72986), PPAL (AAG52887), cim1 (AAA50175), Phl pI (CAA55390), F5I14.21 (AAB60916), F1E22.6 (AAF23829) and Lol pI (AAA63279).

Comparisons to the ß-expansins and group I allergens yielded slightly higher results, with the greatest identity revealed to a cytokinin group I pollen allergen (cim1; Crowell, 1994) from soybean (Table 5.5B). Other proteins compared in this group included, clones encoding putative ß-expansins from *Arabidopsis* (locus AAB61709.1, At2g20750, At2g45110) and rice (EXPB3 and EXPB5), and clones encoding putative group I pollen allergens from *Nicotiana tabacum* (PPAL), *Phleum pratense* (Phl pI), *Arabidopsis* (F5I14.21 and F1E22.6) and *Lolium perenne* (Lol pI).

When the same comparisons were conducted using the deduced amino acid sequence from the AP9:3 clone, a slightly lower degree of sequence identity was detected. This can be attributed to the limited length of the AP9:3 clone, which represents the 5' end of the encoded ß-expansin sequence. It appears that this region of the protein is the most variable amongst the ß-expansins.

Despite the limited protein sequence similarity between the two expansin families, they are conserved in size and share a number of common properties which attest to the structural, evolutionary and functional relation of the α- and ß-expansins (Shcherban *et al.*, 1995; Cosgrove *et al.*, 1997). At the amino terminus, the expansin protein has a hydrophobic signal sequence to direct protein secretion into the cell wall via the ER-Golgi secretory pathway. Located in the middle of the mature form of both the α- and ß-expansins are six conserved cysteines that could form intra-molecular bridges, and a His-Phe-Asp (HFD) domain (Fig. 5.3). These features are common with the catalytic domain of family-45 glycosyl hydrolases, and are presumed to form the active site of the protein. In the expansin carboxyl terminus are four conserved tryptophans. The positioning of these residues is analogous to that found in the cellulose binding domains (CBD) of some cellulases, where they function to anchor the enzymes to cellulose (Din *et al.*, 1994). A similar binding role has been extrapolated for the conserved tryptophans in expansin binding to cellulose or other wall glycans (Shcherban *et al.*, 1995).

Discussion

The expandable walls that shape growing plant cells undergo high rates of polysaccharide synthesis and turnover. They are made up in part of, cellulose microfibrils which are noncovalently bound to a matrix of hemicellulose polymers, of which xyloglucan is the most predominant (Carpita and Gibeaut, 1993). In dicotyledonous plants, the cellulose and hemicellulose components each constitute about 30% of the cell wall complex, with the remainder comprising of approximately 35% pectin polymer and 1-5% structural protein. Cell wall expansion is generally associated with the synthesis, secretion and deposition of

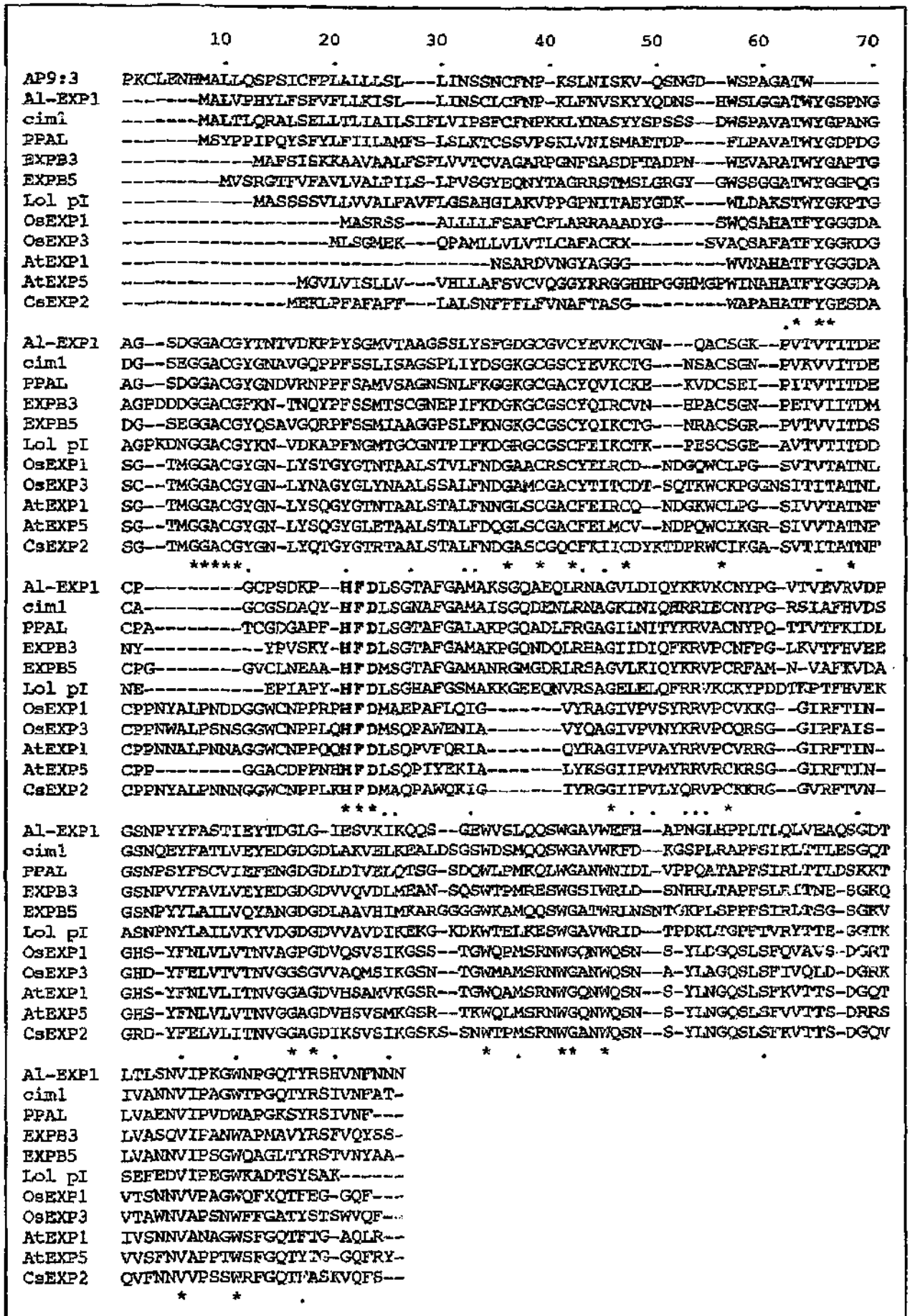


Figure 5.3: An amino acid sequence alignment of α- and ß-expansins and group I allergens. Sequence identity is represented by an asterix (*) and similarity by a dot (.). The HFD domain is highlighted in **bold** type, while the six conserved cysteines and four conserved tryptophan residues are represented in blue and red respectively. See text for functional significance of the conserved regions.

new polymers. Unlike the cellulose microfibrils which are synthesised in the plasma membrane and then deposited in the cell wall, the hemicelluloses and pectins are synthesised in the Golgi apparatus and delivered to the wall by secretory vesicles (Cosgrove, 2000). The rate of incorporation of these polymers into the cell wall was found to be highest in the growing regions of the plant (Carpita and Gibeaut, 1993; Cosgrove, 1997).

The growing wall is able to withstand the high tensile stress applied by cell turgor, through a combination of stress relaxation and polymer creep, which allows the wall to expand irreversibly and maintain a constant tension (Cosgrove, 1993). Cell enlargement occurs as a localised event and regulated in a cell specific manner. Cells of the xylem vessels for example, will enlarge to a greater extent than the adjacent cells of the parenchyma. In fact, the cell wall is capable of enduring a vast degree of expansion without compromising its mechanical integrity, and usually without becoming thinner. This can be directed through the mechanism of pH dependent wall loosening, referred to as acid growth (Cosgrove, 1997). This process is considered to be mediated by expansins, a group of novel cell wall proteins (Cosgrove, 1997; Shieh and Cosgrove, 1998).

It has been postulated that expansins may act to disassemble the load bearing linkages of the cell wall complex, perhaps by disrupting the hydrogen bonds between the cellulose microfibrils and the hemicellulose matrix (Shieh and Cosgrove, 1998). In so doing, the network of polymers are permitted to slide, so that the wall is able to yield to the mechanical pressure exerted by the enlarging plant cells, before the microfibril-hemicellulose matrix is restored (Shieh and Cosgrove, 1998). In addition to their primary role in wall loosening, increasing evidence implicates possible roles for expansins in drought response (Wu *et al.*, 1996), fruit ripening (Rose *et al.*, 1997), pollen tube growth (Cosgrove *et al.*, 1997), vascular cell differentiation (Cho and Kende, 1998), cotton fibre growth (Shimizu *et al.*, 1997) and leaf organogenesis (Reinhardt *et al.*, 1998).

A loss in the ability of the wall to expand has been correlated with the cessation of cellular growth. It has been postulated that this reduction in wall extensibility may be mediated via an increase in the cross linking of the wall polymers, perhaps by the action of the cell wall structural proteins such as extensins (Cosgrove and Li, 1993). Alternatively, the composition of the cell wall may be modified, rendering it more rigid in structure, and thereby less susceptible to expansin mediated wall loosening (Cosgrove and Li, 1993). Irrespective of the processes employed, the cessation of cell growth can not usually be reversed.

It has been well documented that auxin treatment can have a subtle influence in the cell wall properties for viscoelasticity and stress relaxation, to promote wall loosening and cell elongation without increasing turgor pressure (Gray *et al*, 1998). However, the precise biochemical and molecular mechanisms by which auxin induces cell wall expansion is poorly understood, despite the knowledge that auxin influences multiple cellular, biophysical and biochemical processes associated with the cell wall.

The expansins display patterns of differentially regulated gene expression which is specific to different organs or cell types undergoing rapid growth and in response to a number of environmental and hormonal stimuli. This is best illustrated in tomato where a highly abundant expansin transcript found in the ripening fruit is barely detected in any other organ of the same plant (Rose *et al.*, 1997). Similar findings have been reported for rice seedlings where expansin genes expressed during organogenesis and differentiation in the root and the shoot (Cho and Kende, 1998). In non-growing tissues, expansin expression can be induced by the application of exogenous auxin (Hutchison *et al.*, 1999). These observations suggest that the different expansin isoforms, may have distinct roles in cell growth, organ differentiation and plant development.

The 0.8 kb AP9:3 transcript is most abundant during the growth phase of LRP development, 24 h after initial induction. This correlates well with the high level of expression detected for expansin genes in growing cells. The LRP grows as the cells contained within it increase in size. The walls of these enlarging cells are subjected to considerable pressure. Under these circumstances, activated expansin gene expression would be expected, in order to provide a sufficiently extensible wall to accommodate the expanding cells of the LRP. Cho and Kende (1998) detected high levels of expansin mRNA in developing LRP and ARs, predominantly in the epidermal cells and differentiating cells of the vascular cylinder of the root tips.

Expansins have previously been suggested to play a role in oganogenesis. Fleming *et al.* (1997) found that application of expansin to the apical meristem of tomato, induced local expansion and leaf primordia morphogenesis. Moreover, Cho and Kenede (1998) showed that expansin transcripts and proteins were distributed in the organ initials of rice. It is possible that the ß-expansin represented by the AP9:3 clone has an equivalent role in the differentiation of the LRP.

Whether auxin induced acid growth is mediated through expansin activity is still unclear. Treatment of tomato and pine hypocotyls with exogenous auxin application is reported to up-regulate the expression of expansins (Hutchison *et al.*, 1999; Catalá *et al*, 2000). Although this may be true in *E. globulus* for the larger 1 kb AP9:3 transcript, it was not the case for the 0.8 kb transcript. Accumulation of this smaller mRNA appears to occur in response to a particular event, or stage during the development of the LRP. Although LR induction has been influenced by the presence of auxin, accumulation of the 0.8 kb transcript appears to occur independently of the phytohormone. This idea is supported by the finding that expression in the cell suspension sample was very low, detected at similar levels to that found in the untreated root and hypocotyl tissues.

5.4.2.2 AP21B:6: Arabinogalactan (*E* value = 3.3e-10)

Expression characteristics

Two transcripts were detected, 3.9 and 1.6 kb. The smaller 1.6 kb AP21B:6 transcript was elevated above control levels after 12 h treatment with 10^{-5} M IBA (Fig. 5.4). Maximum levels accumulated during growth of the LRP at 24 h after induction, after which levels decreased noticeably. The larger 3.9 kb transcript was expressed at a low and relatively constant level in all tissues sampled. Like the smaller AP9:3 transcript, the AP21B:6 transcript was not induced by auxin, being present at very low levels in both the cell suspension and untreated control tissues.

Sequence analysis

A FASTA search of the NR protein database was performed using the deduced amino acid sequence derived from the 348 bp AP21B:6 clone (Fig. 5.5). The search resulted in a match to an arabinogalactan protein (AGP) encoded by a cDNA clone, designated as AGP*Na*2, from *Nicotiana alata* (Mau *et al.*, 1995). Although the degree of homology between the two sequences was low at 42.6% identity over 115 amino acids, it was assigned a significant score (*E* = 3.3e-10).

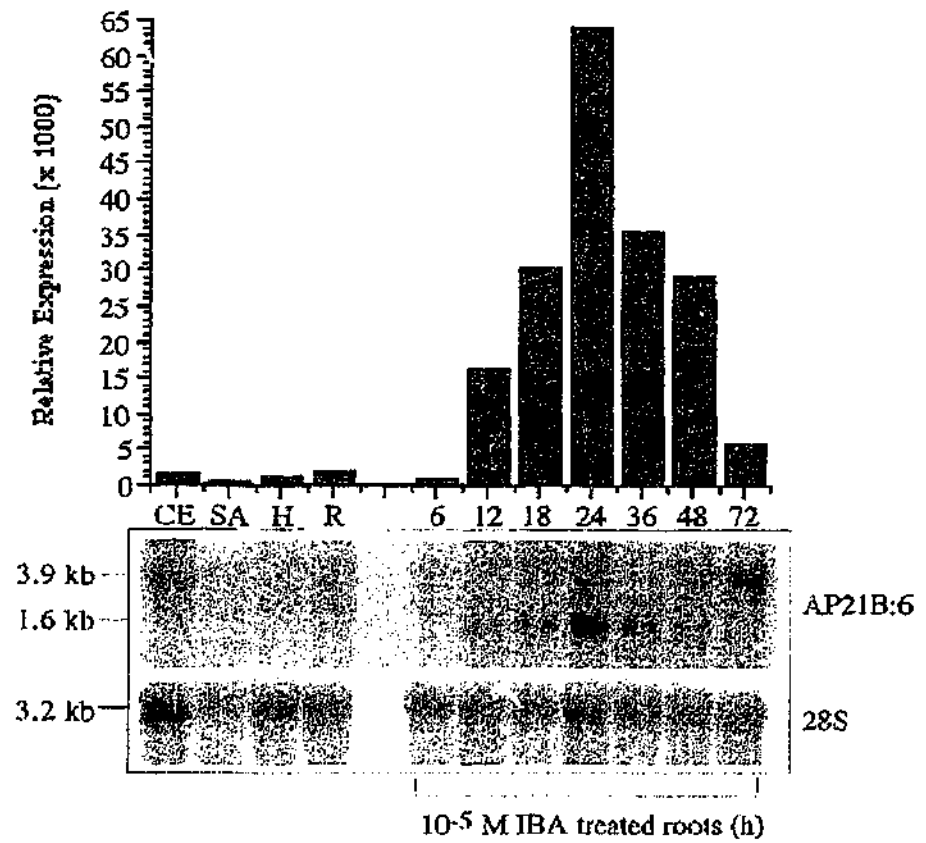


Figure 5.4: Expression profile detected by AP21B:6 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

```
  1  ACACTCCCGTGACCACCACCACCACCGACGTCAACAAGAGGXAAACCACC
       T  P  V  T  T  T  T  T  D  V  N  K  R  X  T  T     16
 51  GGCACTCCGGCCACTGCCACCACCGCTAAGGACGACAAATTCGAGGAAAA
       G  T  P  A  T  A  T  T  A  K  D  D  K  F  E  E  K  33
101  ATTCGCGCGTGAAGAGCTCAGCGGTGAAAGCTTCCAGACGGGCTACACGA
       F  A  R  E  E  L  S  G  E  S  F  Q  T  G  Y  T  N  50
151  ACAACAACAACAACTACAACAACGACAATGGCTACTCCAACAACGAGAAC
       N  N  N  N  Y  N  N  D  N  G  Y  S  N  N  E  N     66
201  TACAACAACAATGGCTACTCCAACAATGAGAACTACAACAACAACAACAA
       Y  N  N  N  G  Y  S  N  N  E  N  Y  N  N  N  N  N  83
251  TGGCTACTCCAACAACAACAAATACAATGGCTACGCGAACACCAGGGAAA
       G  Y  S  N  N  N  K  Y  N  G  Y  A  N  T  R  E  T 100
301  CCAACGGATACGCCAACAACTACAAACCCGACACCTTCACAAGCACGT
       N  G  Y  A  N  N  Y  K  P  D  T  N  T  S  T     115
```

Figure 5.5: Nucleotide and deduced amino acid sequence of the AP21B:6 clone.

Evidently, this low level of sequence homology is not unusual for AGPs. The amino acid composition of AGPs is thought to vary depending on the plant species and tissue from which it is isolated (Cassab, 1988). Attempts to align the deduced amino acid sequences of cDNA clones encoding the AGP protein core from a number of different plant species, have shown a very low level of homology amongst them (Li and Showalter, 1996; Cassab *et al.*, 1998; Schultz *et al.*, 2000). Moreover, Mau *et al.*, (1995) reported that comparisons between cDNAs encoding the protein backbones of AGPs secreted in pear cell suspension cultures (AGP*Pc*1 and AGP*Pc*2), *N. alata* cell cultures (AGP*Na*1) and styles (AGP*Na*2), did not identify any conserved protein motifs. Accordingly, it seems that the AGPs identified, belong to a gene family of divergent sequences (Li and Showalter, 1996).

AGPs are a class of developmentally regulated high molecular mass cell surface proteoglycans. They represent a major group of the superfamily of plant glycoproteins which also includes extensins, proline/hydroxyproline-rich glycoproteins and solanaceous lectins (Nothnagel, 1997; Sommer-Knudsen *et al.*, 1998). Most AGPs differ from these other glycoproteins in that they are typically comprised of between 1% and 10% protein, and of >90% carbohydrate by weight. The protein backbone is neutral to acidic, and the carbohydrate moiety consists primarily of arabinose and galactose with minor amounts of other sugars (Sommer-Knudsen *et al.*, 1998).

AGPs have been identified throughout the plant kingdom in almost all plant tissues including, leaves, stems, trunks, roots, floral organs and seeds (Sommer-Knudsen *et al.*, 1998). They are found predominantly as components of the extracellular matrix, cell secretions, cell walls, plasma membranes and multi-vesicular bodies (Li and Showalter, 1996; Nothnagel, 1997; Youl *et al.*, 1998; Gao *et al.*, 1999), and are thought to play a role in various aspects of plant growth and development including embryogenesis and cell proliferation (Nothnagel, 1997).

The protein moieties of 'classical' AGP core proteins are, by definition, rich in Pro/Hyp, Ser, Ala, and Thr, with common Ala-Hyp or Hyp-Ala repeats (Li and Showalter, 1996; Nothnagel, 1997; Cassab, 1998; Sommer-Knudsen *et al.*, 1998). In addition, classical AGPs encode polypeptides with at least three discrete domains, including an N-terminal secretion signal sequence which is not present in the mature protein. The central domain is predicted to be *O*-glycosylated, and is where the Pro/Hyp residues are predominantly found. The C-terminal domain is hydrophobic and may function as a membrane anchor (Youl *et al.*, 1998).

Mau *et al.* (1995) identified and characterised two AGP cDNA clones, showing them to be exceptions to this definition. Both these 'non classical' AGP clones, one isolated from pear (AGP*Pc*2) and the other from *N. alata* (AGP*Na*2), encoded proteins comprised of Asn-rich domains, in addition to the N-terminal secretion signal sequence and the Hyp/Pro-rich domain similarly found in the classic form. It was proposed that the Asn-rich domains are proteolytically processed, and as a consequence are not found in mature AGPs. It has also been suggested that the Asn residues act as attachment sites for carbohydrate side chains (Mau *et al.*, 1995). Regions high in cysteine residues have also been identified among the clones found to correspond to the non classical group of AGPs. However, to date all the characterised non classical AGPs were found to lack the C-terminal hydrophobic domain (Gao *et al.*, 1999).

Predominant amino acid residues	Proportion in AP21B:6	Proportion in Pro-rich domain of AGP*Na*2	Proportion in Asn-rich domain of AGP*Na*2
Classical AGPs			
Pro	2.6%	21.5%	0.6%
Ser	5.2%	9.3%	9.3%
Ala	5.2%	8.6%	2.0%
Thr	16.5%	10.7%	1.6%
Non classical AGPs			
Asn	27.8%	3.5%	39.0%

Table 5.6: Key amino acid content of the derived amino acid sequences of AP21B:6 and the Pro- and Asn-rich domains of the AGP*Na*2 cDNA. The proportion of each amino acid residue is expressed as a % of the total amino acids of the respective sequences. The AGP*Na*2 data was taken from Mau *et al.* (1995).

Although the putative polypeptide encoded by AP21B:6 is incomplete, asparagine residues are the most predominant feature of the deduced 114 amino acid sequence, comprising 27.8% of the total number of residues within the sequence (Table 5.6). On the other hand, the amino acid residues which are typically abundant in the classical AGPs (Pro, Ser, Ala and Thr), occur infrequently in the AP21B:6 sequence. The amino acid content of the AP21B:6 clone is most comparable to that found for the Asn-rich domain of AGP*Na*2 cDNA. This is not surprising as AP21B:6 shows a higher degree of homology to this domain of AGP*Na*2 (36.8% amino acid identity and 49.1% similarity when the sequences were aligned for maximal homology by GAP analysis, Figure 5.6A). When compared to the Pro-rich domain, AP21B:6 has only 20.7% amino acid identity and 36.0%

similarity (Fig. 5.6B). The presence of an Asn-rich region provides evidence to support placing AP21B:6 into the same category of non classical AGPs.

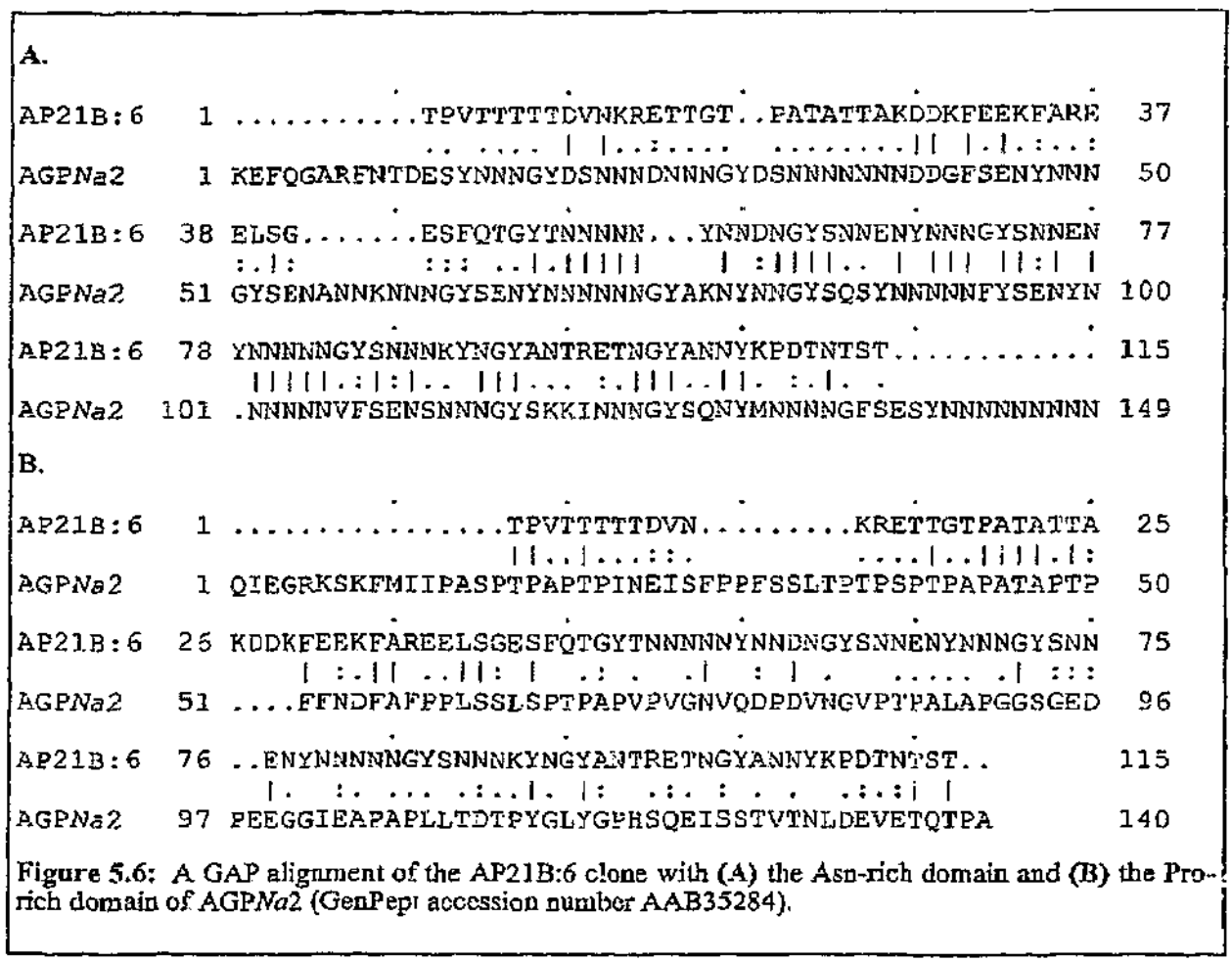


Figure 5.6: A GAP alignment of the AP21B:6 clone with **(A)** the Asn-rich domain and **(B)** the Pro-rich domain of AGP*Na2* (GenPept accession number AAB35284).

Discussion

In *N. alata*, AGP*Na2* detected a 1.7 kb and a 1 kb transcript, with the size and the hybridisation intensity dependent upon the different tissue types tested (Mau *et al.*, 1995). The 1.7 kb AGP*Na2* transcript exhibited strongest hybridisation to suspension cultured cells. This transcript was also found in leaves, stems and roots, but at a comparatively lower level. The smaller 1 kb transcript was expressed only in pollen and styles. In *E. globulus*, two distinct transcripts were also detected by AP21B:6. In this case, the larger 3.9 kb AP21B:6 transcript was found in tissue types similar to those observed for the 1.7 kb AGP*Na2* transcript. However, the level of expression was uniform across the cell suspension, shoot apex and hypocotyl tissues sampled. This level of uniformity was maintained during LRP formation in the auxin treated roots. In contrast, the smaller 1.6 kb AP21B:6 transcript was barely detected in RNA from the same control tissues, and

differentially regulated during LRP formation. In roots, expression of this 1.6 kb transcript was highest during the period when LRP are increasing in size and number, 24 h after induction.

These results, together with those observed from expression studies conducted in other plant systems, appear to indicate that AGPs are expressed in an organ, tissue and cell specific fashion. Although definitive functions for AGPs have not been established, evidence implicates important roles in development. AGPs isolated from *N. alata* and *Pyrus communis* were found to possess glycosylphosphatidylinositol (GPI) membrane anchors in the C-terminal domain (Youl *et al.*, 1998). GPI anchors are thought to be important for conferring a role in cell signalling (Schultz *et al.*, 1998) including, cell-cell interactions and cell-cell recognition (Cassab, 1998). Other suggested functions for AGPs include roles in plant-pathogen interactions (Cassab, 1986), wound healing (Pincher and Stone, 1983), cell proliferation (Langan and Nothnagel, 1997), cell differentiation, somatic embryogenesis (Egertsdotter and van Arnold, 1995), cell wall expansion (Zhu *et al.*, 1993), programmed cell death (Gao and Showalter, 2000), and as glues, lubricants and humectants (Nothangel, 1997).

LR formation requires the coordinated regulation of many developmental processes that could conceivably involve the activity of AGPs. It is possible that the AGP encoded by AP21B:6 plays an critical role in cell signalling events during the development of LRs in *E. globulus*. Upon induction of the LRP, a signal is received by one or more cells in the pericycle which then begin to divide. Competent cells are recruited, presumably through cell-cell interaction and recognition. It is probably at this point when AGP gene expression is switched on in *E. globulus*. At this time, approximately between 6-12 h after induction, AP21B:6 detected expression, is up-regulated in the root. The cells continue to divide and differentiate to form a discrete LRP. This period of LRP growth, coincides with peak AP21B:6 expression, suggesting that AGP is particularly important at this stage of LRP development. Presumably, cell wall expansion is required as the LRP grows and elongates before emerging as a new LR (Cosgrove, 1997). In *E. globulus* roots, AGP expression declines but may still be required at this stage, 36-48 h after induction, when AP21B:6 transcripts levels are still elevated above that of the uninduced control roots.

5.4.2.3 AP1:1: Extensin (*E* value = 6.6e-07)

Expression characteristics

The AP1:1 probe hybridised to three different sized mRNA transcripts in the cell suspension control. The larger 2.3 and 3.3 kb transcripts were exclusive to this sample alone, while the 1.6 kb transcript was also detected in the 10^{-5} M IBA treated roots. Accumulation of this smaller transcript was down-regulated in roots treated for 6 h (Fig. 5.7). After 24-48 h treatment transcript levels were twice that observed for the untreated root control. Peak levels were reached at 72 h, where a substantial 14.3 fold increase over the untreated root control was detected. This expression profile indicates that the gene represented by this clone is up-regulated when formation of the LRP is well completed and ready to emerge from the parent root.

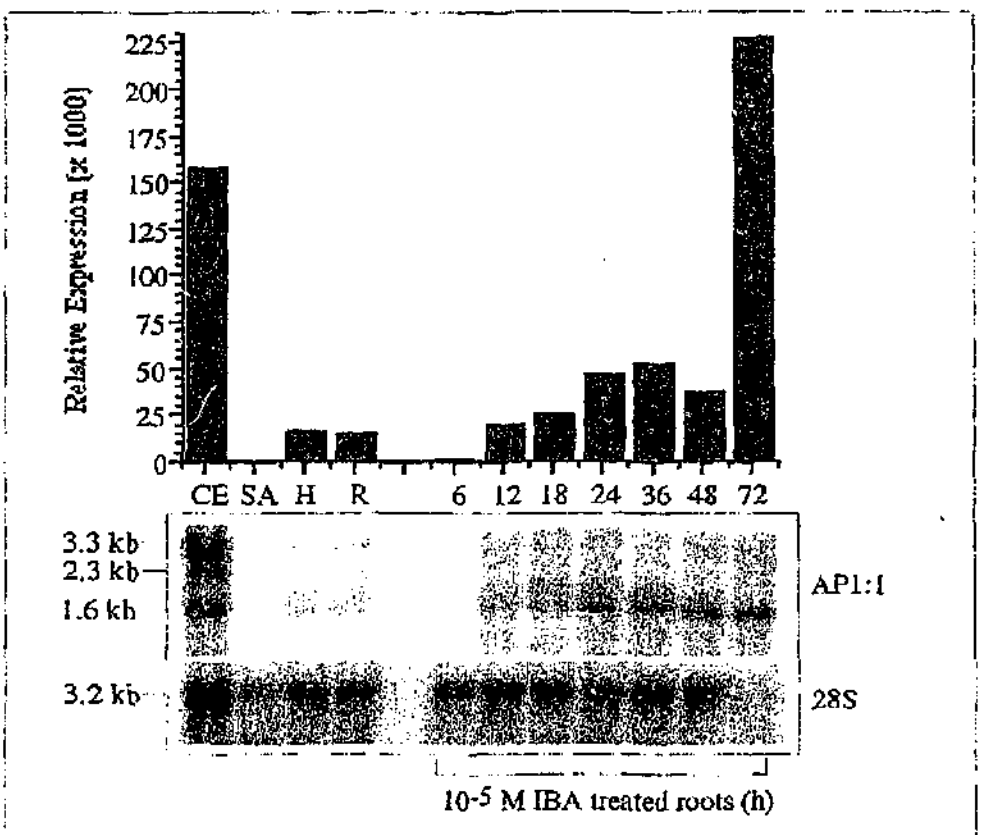


Figure 5.7: Expression profile of the 1.6 kb transcript detected by AP1:1 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

The 104 bp AP1:1 clone encodes a predicted amino acid sequence that forms part of an extensin polypeptide. This sequence shares between 62.5% (E = 0.01) and 85.3% (E = 6.6e-07) homology with extensins from a number of different plant species including, *Daucus carota* (carrot), *Phaseolus vulgaris* (bean), *Glycine max* (soybean), *Pisum sativum* (pea) *Lycopersicon esculentum* (tomato) *Nicotiana sylvestris*, *Manihot esculenta* (cassava), *Brassica napus* (rape), *Arabidopsis*, *Petroselinum crispum* (parsley), *Vigna unguiculata* (cowpea), *Cicer arietinum* (chickpea), *Heliantheae annus* (sunflower), *N. tabacum*, *Prunus amygdalus* (almond), and *Catharanthus roseus*. The five most significant homologies defined by FASTA searches of NR protein databases are represented in Table 5.7.

Gene or Clone	Plant	% Identity	Length of aa overlap	E value	Reference
pDC5A1	Carrot	85.294	34	3.1e-07	Chen and Varner (1985a) Chen and Varner (1985b)
HRGP4.1	Bean	79.412	34	1.4e-05	Wycoff *et al.* (1995)
SbHRGP3	Soybean	79.412	34	1.5e-05	Ahn *et al.* (1996)
Ext	Pea	78.125	32	1.7e-05	Unpublished
clone w6	Tomato	78.125	32	1.7e-05	Showalter *et al.* (1991)

Table 5.7: The five highest scoring hits from a FASTA search of the NR protein database using the deduced amino acid sequence of AP1:1 as the query. The accession numbers are as follows pDC5A1 (SWISS-PROT: P06599), *HRGP4.1* (GenPept: AAA87902), *SbHRGP3* (GenPept: AAB53156), *Ext* (GenPept: AAF73291), clone w6 (GenPept: CAA39214).

Of the polypeptides represented in Table 5.7, characterisation of the soybean SbHRGP3 extensin was the most detailed, providing evidence for a structural role in LR development. The nucleotide sequence for the corresponding gene has one ORF which encodes a novel protein of 432 amino acids with a molecular weight of 49,190. Apart from the signal peptide at the N terminus, all the extensins listed in Table 5.7 are entirely hydrophilic. The SbHRGP3 protein is comprised of two different domains, each containing a different repeated motif, which is organised into an ordered pattern within each domain (Ahn *et al.*, 1996). The repeat units Ser-Pro$_4$-Val-His and Ser-Pro$_4$-Val-Tyr-Lys-Tyr-Lys are found in domain 2 of the SbHRGP3 protein, and is also present in the predicted amino acid sequence of AP1:1 (Fig. 5.8). The latter, highly glycosylated repeat unit confers the rod-like conformation of the extensin molecule (Kieliszewski and Lamport, 1994), while the Tyr-Lys-Tyr-Lys block is known to be involved in

intramolecular IDT bridge formation (Kieliszewski and Lamport, 1994). Also present in the SbHRGP3 and AP1:1 deduced amino acid sequences, is the Pro-Val-Tyr-Lys unit, which is thought to participate in intermolecular Tyr-Lys cross-linking (Kieliszewski and Lamport, 1994). These characteristic repeat units are also found to be identical, or at least very similar, in the other homologous extensins listed in Table 5.7.

```
  1  ACAAGTCCCCCCCTCCTCCTGTGCACTCCCCGCCTCGGCCGGTCTACAAGTA
       K  S  P  P  P  P  V  H  S  P  P  R  P  V  Y  K  Y     17
 53  CAAGTCCCCCCCTCCTCCGGTGCACTCCCCCCCTCCACCAGTCTACAAGTAC
       K  S  P  P  P  P  V  H  S  P  P  P  P  V  Y  K  Y     34
```

Figure 5.8: The nucleotide and deduced amino acid sequence of the 104 bp clone AP1:1. The Ser-Pro4-Val-Tyr-Lys-Tyr-Lys repeat unit is highlighted in red, the Pro-Val-Tyr-Lys unit in blue, the Ser-Pro4-Val-His unit in green, and the Tyr-Lys-Tyr-Lys blocks are underlined.

Like the carrot and *N. sylvestris* extensins, there is a strong indication of codon bias along the limited length of the AP1:1 sequence (Chen and Varner, 1985a; Parmentier *et al.*, 1995). In the case of AP1:1, CCT and TCC were preferred 46.7% and 100% of the time, to specify proline and serine respectively.

Discussion

Several major classes of abundant cell wall proteins have been identified in plants, these being hydroxyproline-rich glycoproteins (HRGPs), glycine-rich proteins (GRPs), proline-rich proteins (PRPs), solanaceous lectins, and AGPs (Showalter, 1993; Sommer-Knudsen *et al.*, 1998). Extensins are a family of HRGPs that are thought to have a structural role in the cell wall, as part of the glycoprotein network (Showalter, 1993; Sommer-Knudsen *et al.*, 1998).

Extensins have been found in dicotyledonous plants where they are most abundant, but are also present in monocotyledonous plants and gymnosperms (Ludevid *et al.*, 1990; Fong *et al.*, 1992 Avila *et al.*, 2001). In dicots, extensins represent the major protein component of the plant cell wall and have been identified in a number of plants, including carrot, bean, tobacco, tomato and soybean (Showalter, 1993). They have been detected in most plant tissues and are encoded by complex gene families (Showalter, 1993).

Extensins of both monocots and dicots contain characteristic repeats of Ser-Pro_4 pentapeptides, with slight variations between the two classes of plants (Chen and Varner, 1985a; Cassab, 1998; Showalter, 1993; Sommer-Knudsen *et al.*, 1998). The majority of proline residues undergo post-translational hydroxylation, before most of the hydroxyproline and serine residues are glycosylated (Kieliszewski and Lamport, 1994). Short arabinoside chains of one to four units in length become attached to the hydroxyproline residues, while single galactosyl residues are attached to some of the serine residues. As a result extensins are normally comprised of 50-60% (w/w) carbohydrate (Sommer-Knudsen, 1998). It has been suggested that these carbohydrates serve to maintain the linear, rod-like structure of the extensin molecule (Kieliszewski and Lamport, 1994).

Extensins are synthesised as soluble precursors on the ER. They are glycosylated in the Golgi apparatus, then deposited into the cell wall where they become insolubilised in response to developmental or environmental signals (Cassab, 1998). This occurs through the intermolecular cross-linking of tyrosines to form isodityrosine (IDT) intermolecular bridges with other cell wall carbohydrate monomers (Fong *et al.*, 1992; Kieliszewski and Lamport, 1994).

Evidence in the literature suggests that the process of extensin synthesis, deposition and cross-linking, provides a means of increasing the mechanical strength of the cell wall (Keller and Lamb, 1989; Wycoff *et al.*, 1995; Ito *et al.*, 1998; Hirsinger *et al.*, 1999). In tobacco for example, *HRGPnt3* and *Ext 1.4* are expressed in a specific and limited group of cells during LR initiation (Keller and Lamb, 1989; Hirsinger *et al.*, 1999). It was speculated that proteins encoded by these genes function to reinforce the walls of the emerging tip, enabling it to withstand mechanical pressure as it penetrates the cortical and epidermal cell layers of the primary root. Supporting evidence comes from the study of extensin genes from bean (*HRGP4.1*) and oilseed rape (*extA*). The *HRGP4.1* and *extA* genes were expressed to high levels in cells at the stem node to withstand the force exerted by the weight of a developing axillary branch, such as a petiole and its leaf (Wycoff *et al.*, 1995; Hirsinger *et al.*, 1999).

Genes encoding extensin are not constitutively expressed, instead transcriptional activation is developmentally regulated and appears to be organ and cell specific. Numerous examples suggest that extensins are synthesised to reinforce the shape and rigidity of cell walls in specific areas of the plant which are being subjected to physical damage. Apart from the structural functions provided by these proteins, extensins are also thought to participate in the control of cell proliferation, signalling, plant defence and adaptation to stress (Showalter, 1993; Wycoff *et al.*, 1995; De Tullio *et al.*, 1999).

Accumulation of extensin mRNAs can often be induced by phytohormone treatments and stress in stems, petioles, seed coats, floral organs, hypocotyls and roots. Ethylene, cytokinin, auxin, pathogen attack, wounding, hypoxia, and cold treatment, have all been previously associated with an increase in the expression of extensin genes (Sommer-Knudsen *et al.*, 1998).

In association with the structural role described for extensins, in some cases extensin gene expression has been correlated with cell division. Ito *et al.* (1998) suggested that the *cyc15* and *cyc17* extensin genes of *Catharanthus roseus* served two major roles. The first was a possible involvement during progression through the cell cycle, whilst the second was in strengthening the cell wall following the completion of cell division. Expression of *cyc15* and *cyc17* appeared to be induced when cell proliferation had ceased, with corresponding mRNA accumulating during the G1 phase of arrested cells, and again in the S/G2 phases during the cell cycle progression. A role in cell cycle progression has also been cited for extensins found in carrot and maize. In these plants, the respective gene products have been localised to the surface of dividing cells of the carrot central root cap and root meristem (Smallwood *et al.*, 1994), and in the root tip cell walls of maize (Ludevid *et al.*, 1990). In the latter example, an associated mRNA transcript was also present in the dividing cells of meristematic and embryogenic calli.

Extensins are also thought to participate in plant defence, to repair damage caused by wounding and/or protect against pathogen infection. To serve this purpose, extensins may function as a microbial agglutinin (Mellon and Helgeson, 1982) to immobilise pathogens in the cell wall, may act directly as a structural barrier, may provide sites for lignin deposition (Hammerschmidt *et al.*, 1984) to create a more impenetrable cell wall barrier.

Northern blot analyses conducted by Ahn *et al.* (1996 and 1998) in soybean seedlings, revealed a 1.6 kb *SbHRP3* transcript in mature regions of the hypocotyl and root, while in mature plants expression was specific to the maturing regions of the primary and secondary roots. Furthermore, they were able to localise this expression in the primary root by histochemical analyses of transgenic tobacco plants carrying the *SbHRGP3* promoter fused to the ß-glucuronidase reporter gene (*SbHRGP3-GUS*). From these studies, Ahn *et al.* (1996) were able to show that *SbHRGP3-GUS* expression was activated just prior to LR initiation in the epidermis, specifically at the site destined to originate a new LR. Expression was then inactivated as a group of pericycle and endodermal cells began to divide to initiate the LR. No *SbHRGP3-GUS* expression was detected when the LR emerged from the primary root. This is in contrast to the findings reported for other

promoter-GUS fusions made with promoter fragments of extensin genes from bean *HRGP4.1* (Wycoff *et al.*, 1995) and tobacco *HRGPnt3* (Keller and Lamb, 1989) and *Ext 1.4* (Hirsinger *et al.*, 1999). These gene fusions exhibited similar patterns of GUS activity in a subset of pericycle and endodermal cells of the parent root just prior to LR initiation. However, expression of each of these gene fusions persisted in the apical cells of the new LR as it broke through the cortex of the parent root. During a later stage of development, weak expression was confined to the root tip of the emerging LR.

The specific nature of expression exhibited by the various extensin genes implies that the encoded proteins have varied roles in LR development, yet in each case, the function is specialised and precise. The findings suggested that SbHRGP3 deposition may confer strength to the mature regions of primary root, reinforcing the epidermal cell walls to minimise the damage caused by the emerging LR (Ahn *et al.*, 1996 and 1998). This proposed function is distinct from that of *HRGPnt3* and *HRGP4.1*, which are considered to be involved in the hardening of cell walls in the LR tip. This would serve to endow the root tip with mechanical strength necessary to enable emergence from the primary root, and growth through the soil (Keller and Lamb, 1989; Wycoff *et al.*, 1995). Both these genes are illustrative of the differentially regulated expression that occurs in select cells to modify the conformation and architecture of the cell wall during formation of the LR.

Accumulation of the individual 1.6, 2.3 and 3.3 kb AP1:1 mRNAs seems to occur in a tissue- or treatment-specific manner. This suggests that gene expression is selectively activated and regulated to meet particular requirements during LR development. The presence of different sized AP1:1 transcripts is analogous to the observations made in carrot and bean, where development and stress differentially regulated the abundance of individual transcripts. The two transcripts observed in carrot were found to be the products of alternative 5' transcription start sites within the one gene (Chen and Varner, 1985a and 1985b). Corbin *et al.* (1987) proposed that a similar double promoter mechanism was responsible for generating the four *Hyp4.1* transcripts, but do not exclude the possibility of alternative splicing. As it has been well documented that extensins belong to a multigene family, the third possibility is that the three transcripts are derived from different AP1:1-like genes.

It is clear that different extensins fulfil different, yet precise roles in the plant cell wall. Peak expression of the 1.6 kb AP1:1 transcript in *E. globulus*, coincides with the presence of mature LRP, at maximum numbers. At this point, 72 h after initial IBA induction, the LRP are ready to penetrate the primary root to emerge as new, actively growing LRs. Considering the findings reported for the *cyc15* and *cyc17* extensin genes (Ito *et al.*, 1998), it is tempting to speculate that the increase in the 1.6 kb AP1:1 transcript

may coincide with deposition of the encoded extensin molecules at the end of cell division and cell growth within the LRP. Whether accumulation of this transcript serves to reinforce cell walls of the primary root (as in *SbHRGP3*) or the emerging LR (as in *HRGPnt3* and *HRGP4.1*) can not be determined without further analysis. Since the 2.3 kb and 3.3 kb transcripts did not accumulate significantly during LRP formation to the levels observed in the cell suspension culture, it may be possible that these two transcripts have specific roles in development, in other regions of the plant.

5.4.3 Clones Representing Genes Involved in Transcriptional Activation, Protein Synthesis and Protein Processing

5.4.3.1 AP2:2: RNA polymerase II (*E* value = 2e-08)

Expression characteristics

The AP2:2 clone hybridised to four mRNA transcripts measuring 3.4, 2.35, 1.5 and 0.4 kb. Only the 1.5 kb transcript exhibited a pattern of accumulation representative of auxin enhanced regulation during LRP formation (Fig. 5.9). This mRNA was not detected after a 6 h auxin treatment, with levels detected below that of the untreated root control. By 12 h however, expression was markedly up-regulated and increased steadily from 18 to 36 h. The most substantial increase occurred between 36 and 48 h, when the detected levels doubled to reach a maximum at 48 h, before decreasing at 72 h. This expression profile corresponds to activated gene expression mid way through the LRP induction phase (12 h), increasing as LRP grow, until maximum LRP numbers are reached and formation is completed (48 h).

The 3.4 and 2.35 kb AP2:2 transcripts were most abundant in the cell suspension but barely detected in all the other tissues sampled. In treated roots, the smallest 0.4 kb transcript was down-regulated at 6 h, but present at a uniform level at all other time points sampled.

Sequence analysis

The 117 bp AP2:2 clone appears to comprise a partial ORF with a short 17 amino acid translated region and 3' UTR (Fig. 5.10). Database searches revealed that the deduced amino acid sequence of the AP2:2 translated region is highly homologous to metallothionein-I gene transcription activating protein (MEE6.8) from *Arabidopsis*

thaliana ($E = 2e\text{-}08$). This is an essential component of RNA polymerase II. AP2:2 also has significant homology with RNA polymerase subunits from yeast (rpb12) and human (ABC10-α). Together, it seems clear that AP2:2 represents a component of the *E. globulus* nuclear gene transcriptional machinery.

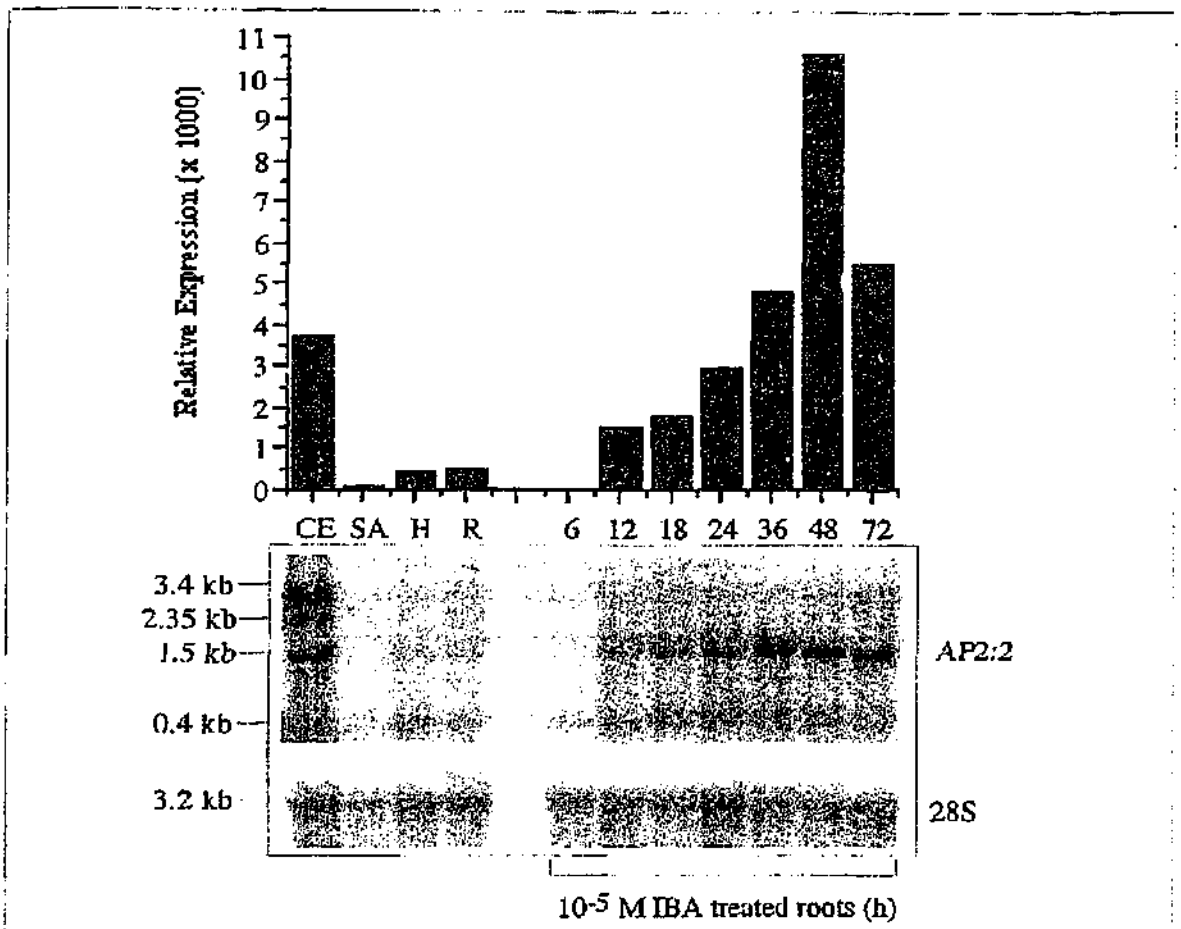


Figure 5.9: Expression profile of the 1.5 kb transcript detected by AP2:2 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

```
  1  ACCGCATCCTCTACAAGAAGCGCACCCGCCGAATCATTCAGTATGAGGCC
       R  I  L  Y  K  K  R  T  R  R  I  I  Q  Y  E  A    16
 51  CGTTGAaagagcttgaggatgtgggcttcaatttccatacagacttattt
     R  stop                                             17
101  gtcattgtgactggggt
```

Figure 5.10: Nucleotide sequence of the AP2:2 clone. The underlined portion of the sequence corresponds to the putative translated region, with the deduced amino acids indicated below. The 3' UTR is represented in lowercase.

A sequence alignment of these three polypeptides shows that the 17 amino acid residues predicted for AP2:2, corresponds to the C-terminal end of the compared protein sequences (Fig. 5.11). A feature of this RNA polymerase II subunit is the high level of amino acid conservation and an invariable $CX_2CX_{12}RCX_2CGXR$ motif which represents a Zn-binding domain (Shpakovski *et al.*, 1995).

```
                    10        20        30        40
                     .         .         .         .
AP2:2               ----------------------------------------
Arabidopsis         ------------MDPAPEPVTYVCGDCGQENELKSGDVIQ
S. pombe            MNHPTSTGGTAFNPPRPATMIYLCADCGRRNTIQAKEVIR
H. sapiens          -----MDTQKDVQPPKQQPMIYICGECHTENEIKSRDPIR

AP2:2               ------RILYKKRTRRIIQYEAR
Arabidopsis         CRECGYRILYKKRTRRVVQYEAR
S. pombe            CRECGHRVMYKMRTKRMVQFEAR
H. sapiens          CRECGYRIMYKKRTKRLVVFDAR
                          *..** **.*.. ..**
```

Figure 5.11: An alignment of the 17 amino acid residues encoded by the AP2:2 clone and the protein sequences of the DNA-directed RNA polymerase II subunits identified in *Arabidopsis* (GenPept accession number BAB09703), *S. pombe* (GenPept accession numbers AAA80487), and *H. sapiens* (GenPept accession number AAH00806). Identical residues and conserved substitutions are denoted by an asterix (*) and dot (.) respectively. The Zn-binding domain is highlighted in red.

Discussion

In eukaryotes, the synthesis of mRNAs is catalysed by DNA-dependent RNA polymerase II. Studies in yeast have discovered that this enzyme is assembled from at least 10 different subunits, with molecular weights ranging from 220 to less than 10 kDa (Sakurai *et al.*, 1996). MEE6.8, rpb12 and ABC10-α represent homologous subunits present in all three nuclear RNA polymerases (Shpakovski *et al.*, 1995, Sakurai and Ishihama, 1997). While RNA polymerase II is responsible for the regulated transcription of protein coding genes to yield mRNA precursors, RNA polymerase I synthesises the ribosomal RNA precursor, and RNA polymerase III transcribes genes responsible for transfer RNA (tRNA) and 5S rRNA. Strains of *S. cerevisiae* carrying a null allele for the Sc10α subunit were found to be nonviable, indicating that it is necessary for transcription (Shpakovski *et al.*, 1995).

Since transcription of a large number of genes most likely contributes both directly and indirectly to the formation of LRs, the significant amino acid homology detected to RNA polymerase subunits from *Arabidopsis*, yeast and human strongly suggests a role for the AP2:2 encoded product as a component of the transcription machinery required during auxin-induced LR formation.

5.4.3.2 AP3:3: Ribosomal Protein L2 (*E* value = 1.3e-15)

Expression Characteristics

The AP3:3 probe detected a 1.5 kb and 1.8 kb transcript in all the tissues sampled, and appear to be subject to slightly different mechanisms of regulation (Fig. 5.12). Both transcripts were most abundant in the cell suspension, with the 1.8 kb transcript accumulating to a level double that detected for the 1.5 kb transcript. Weakest levels were found in the shoot apex sample, where the 1.5 kb transcript was relatively more abundant. Both transcripts accumulated to similar levels in the untreated root and hypocotyl controls. The expression profile exhibited in these control tissues is suggestive of auxin influenced transcript accumulation. This is characterised by transcript abundance following 6 h 10^{-5} M IBA treatment below that detected for the untreated root control. Half-way through the induction phase (12 h), transcription began to increase until the 1.5 kb mRNA was most abundant during LRP growth. Maximum levels of the 1.8 kb transcript coincided with maximum numbers of mature LRP at 48 h.

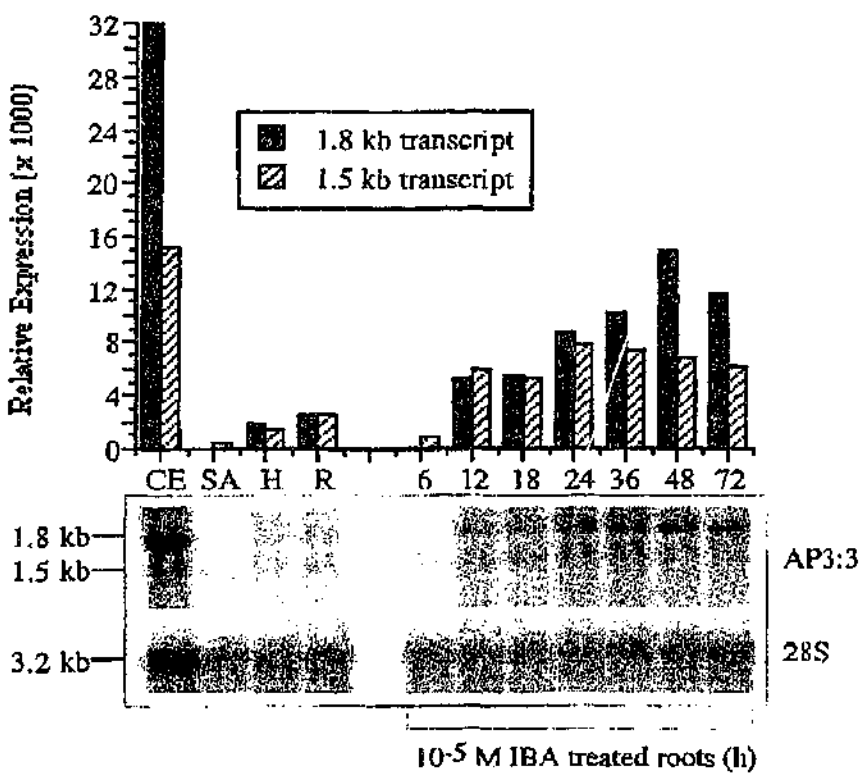


Figure 5.12: Expression profile of the 1.8 and 1.5 kb transcripts detected by the AP3:3 probe on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

The translated 172 bp AP3:3 clone, encodes a predicted sequence of 56 amino acid residues (Fig. 5.13). A FASTA search of the NR protein database clearly showed that this deduced amino acid sequence is part of the cytoplasmic 60S ribosomal protein L2 (rpL2). The significant search results ranged from 91.5% identity (E = 1.3e-15) in *Arabidopsis*, to 41.7% identity (E = 0.0039) in the archaebacterium, *Pyrococcus horikoshii*, over a 47 and 48 amino acid overlap, respectively. In fact, sequence identity was highest in plants including, *L. esculentum* (tomato), *G. max* (soybean) and *N. tabacum* (tobacco), while lower but still significant matches were made to *Xenopus laevis*, human, mouse, *Drosophila melanogaster*, *S. pombe* and *Caenorhabditis elegans*.

```
   1  TAGCGTGGTCGCGGCCGAGGTACCTCAAGGGCGTCGTCACCGAGATCGTG
        A  W  S  R  P  R  Y  L  K  G  V  V  T  E  I  V    16
  51  CACGACCCGGGCCGCGGTGCCCCGCTCGCCCGCGTCGCCTTCCGCCACCC
        H  D  P  G  R  G  A  P  L  A  R  V  A  F  R  H  P  33
 101  CTTCCGCTACAAGAAGCAGAAGGAGCTCTTCGTCGCCGCTGAGGGCATGT
        F  R  Y  K  K  Q  K  E  L  F  V  A  A  E  G  M  Y  50
 151  ACCTGCCCCGGGCGGCCGCTCGA
        L  P  G  R  P  L                                    56
```

Figure 5.13: The nucleotide and predicted amino acid sequence of the 172 bp AP3:3 clone.

The translation of mRNA into amino acids during protein synthesis occurs on the ribosome, a cellular organelle comprised of two subunits. In eukaryotes, the ribosome contains four rRNAs transcribed by RNA polymerases I and II, and over 80 structural proteins encoded by mRNAs and transcribed by RNA polymerase II (Sollner-Webb and Mougey, 1991). The role of the large (L) subunit is to bind tRNA and control peptidyl transfer, while the small (S) subunit is responsible for mRNA binding, decoding and fidelity during translation. In prokaryotes, the large subunit is a 50S particle, and the small subunit a 30S particle. This is in contrast to the eukaryotic cytosolic ribosome, which is composed of a large 60S and small 40S subunit.

The rpL2 belongs to a highly conserved family of ribosomal proteins, and is a key component of the large ribosomal subunit. Studies in bacteria have found that rpL2 has peptidyl transferase activity (Cooperman *et al.*, 1995) and is the primary 23S rRNA binding protein (Cooperman *et al.*, 1995). This protein has been suggested to play an important role in protein synthesis (Cooperman *et al.*, 1995). The high degree of rpL2

sequence conservation between species supports this notion (Müller and Wittmann-Liebold, 1997).

In plants, ribosomes also exist in choloplasts (Hess *et al.*, 1994) and mitochondria (Kubo *et al.*, 1996), where the structure is very similar to the prokaryotic ribosome, comprising of 30S and 50S subunits (Marty and Meyer, 1992). The predicted 56 amino acid sequence of the AP3:3 clone was compared to rpL2 from cytoplasmic, chloroplastic and mitochondrial origins to determine the level of conservation. Pairwise GAPs revealed that at the protein level, the AP3:3 clone has significantly greater homology, up to 82.1% identity, to the cytoplasmic rpL2 group (Table 5.8). The level of homology detected to the chloroplastic and mitochondrial protein forms was low in both cases, ranging from only 25% to 28.3%.

Plant	% identity to AP3:3	% similarity to AP3:3
Cytoplasmic		
Arabidopsis	82.1	91.1
Tobacco	75.0	82.9
Tomato	78.6	87.5
Chloroplastic		
Arabidopsis	28.3	49.1
Tobacco	25.0	44.6
Soybean	25.0	46.4
Mitochondrial		
Arabidopsis	25.5	43.1
Rice	27.3	45.5

Table 5.8: Homology of various cytoplasmic, chloroplastic and mitochondrial forms of rpL2 to the deduced amino acid sequence of the AP3:3 clone. Accession numbers are as follows; for the cytoplasmic proteins - *Arabidopsis* GenPept: AAK32778, tobacco SWISS-PROT: RL2_TOBAC and tomato SWISS-PROT: RL2_LYCES. The chloroplast proteins - SWISS-PROT: RK2_ARATH, RK2_TOBAC and RK2_SOYBN for *Arabidopsis*, tobacco and soybean respectively. The mitochondrial proteins - GenPept: CAA69740 and BAA11350 for *Arabidopsis* and rice respectively.

Discussion

LR formation is likely to involve an increase in RNA and protein synthesis, amongst numerous other coordinately regulated processes associated with the differentiation of a new organ. Evidence from other plant studies suggests that the accumulation of rpL2 mRNA in young tissues appears to be correlated with rapid cell proliferation (Köhler *et al.*, 1992; Marty and Meyer, 1992). In barley for example, Sab2 transcripts were found preferentially in apical meristems of both the shoot and the root (Köhler *et al.*, 1992). Similarly, expression of a cytoplasmic *rpL2* gene in tobacco was strongly correlated with active cell division and growth, as the corresponding transcript accumulated in growing cell suspension, germinating seeds and young tissues including flower buds, leaves and roots (Marty and Meyer, 1992).

In the present study, accumulation of the AP3:3 mRNAs was most abundant in the cell suspension, however only weak expression was detected in the shoot apex. This indicates that expression of the corresponding gene is not directly associated with cell division.

The observed drop in mRNA abundance after a 6 h treatment of root explants with 10^{-5}M IBA, suggests that the transcript levels are not regulated solely by the presence of auxin. Despite the initial drop, transcript levels were elevated during the late induction and growth phases of LRP formation. Thus, transcription of the rpL2 encoding gene represented by AP3:3, appears to coincide with the period of greatest cell proliferation and growth during LR development. This supports earlier reports which suggest that the accumulation of mRNAs encoding ribosomal proteins occurs very early in the development of the LRP (Strafstrom and Sussex, 1992; Williams and Sussex, 1995).

The presence of two different sized transcripts in *E. globulus* may indicate that the AP3:3 probe is not only detecting a rpL2 transcript, but may also be cross hybridising to a similar ribosomal protein transcript. Alternatively, the two transcripts may represent different isoforms of rpL2 in *E. globulus*. If this was the case, functionally distinct ribosomes may result to control the translational specificity or the rate of protein synthesis (Etter *et al.*, 1994).

5.4.3.3 AP5:1: Calreticulin (*E* value = 3.2e-22)

Expression characteristics

In the auxin treated roots, AP5:1 correlated gene expression was not detected in root tissue from 6 h IBA treatment, with transcript levels below that detected for the untreated root control (Fig 5.14). Levels increased at 12 h and continued to accumulate until transcript levels were most abundant at 24 h. The AP5:1 message then declined to levels similar to those observed at 12-18 h. Expression at this level was maintained for the remaining time points analysed. The comparatively high expression in the cell suspension sample over the untreated tissue controls suggests that accumulation of this transcript is regulated by auxin. This clone appears to represent down-regulated gene activity during early auxin induced LRP induction (6 h), followed by an increase in expression during growth of the LRP.

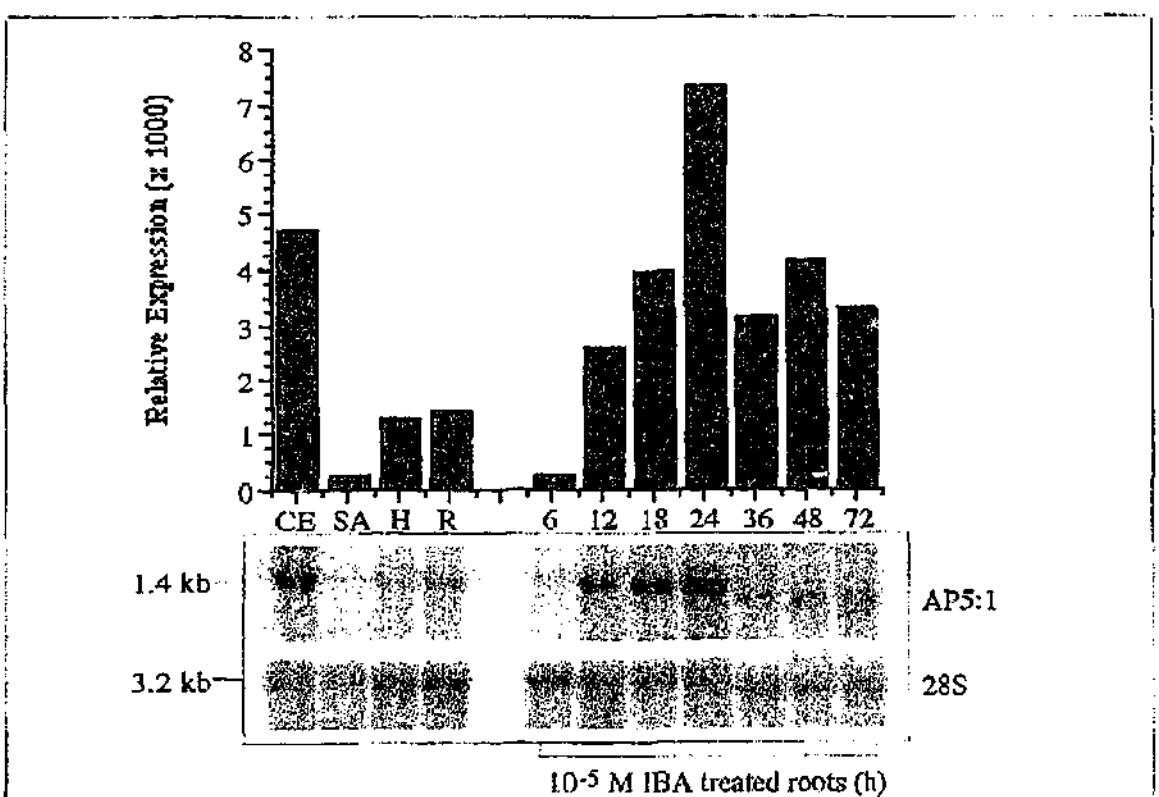


Figure 5.14: Expression profile detected by AP5:1 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

The predicted amino acid sequence encoded by the AP5:1 clone was deduced from the 169 bp nucleotide sequence (Fig. 5.15), and each was compared with those of the current NR nucleic and protein databases. The resulting FASTA searches revealed that the AP5:1 clone has up to 83.4% identity at the DNA level (E = 4.2e-28, over 169 nucleotides) and 92.9% identity at the amino acid level (E = 3.2e-22, over 56 amino acids) to calreticulin from *Arabidopsis*. In fact, significant matches were identified to calreticulin from a number of different organisms, including 14 different plant species, eight vertebrates and 13 invertebrates.

```
1     ACCAAGAAAGTTCATGCCATTCTCACTTATAATGGAACTAATCAACTGAT
      T  K  K  V  H  A  I  L  T  Y  N  G  T  N  Q  L  I   17
51    CAAAAAGGATGTTCCTTGTGAGACAGATCAACTTACTCATGTTTACACTT
       K  K  D  V  P  C  E  T  D  Q  L  T  H  V  Y  T  F  34
101   TTGTCCTCCGTCCCGATGCTACCTATACTATCTTGATTGATAATGTTGAG
        V  L  R  P  D  A  T  Y  T  I  L  I  D  N  V  E    50
151   AAACAAACTGGTAGCTTGT
      K  Q  T  G  S  L                                    56
```

Figure 5.15: The nucleotide and deduced amino acid sequence of the 169 bp AP5:1 clone.

Calreticulin (CRT) has three structural and functional domains which have been best described in humans but are highly conserved across different taxa (Borisjuk *et al.*, 1998; Labriola *et al.*, 1999; Corbett and Michalak, 2000). The N-terminus (N domain) is the most conserved and largest region of the protein. It contains putative phosphorylation and glycosylation sites, and a signal sequence that targets the protein to the ER lumen. Sequence alignments of CRT proteins from different plants reveals four highly conserved consensus sequences in this section of the protein (Fig 5.16). Of the these N domain motifs, DCGGY and MFGPDICG are also found in animal CRT, while RWVKSEWKKD and AGEWKHT are only conserved in plants (Benedetti and Turner, 1995). This is followed by the central proline-rich P domain which in plants contains a putative nuclear targeting signal PK(X)IKDP(S/E) (Fig 5.16). This short signal is presumably analogous to the nuclear localisation signals (NLS) or nuclear export signals (NES) found in mammalian CRT sequences. Typically these sequences are comprised of proline residues with 3 to 4 basic amino acid residues. Next are the repeat motifs KKPEDWD(D/E) (repeat A) and (D/K)GXW(T/K)(A/P)(P/K)XI (repeat B), or slight derivatives, each represented three times. Together these repeats confer the high affinity and low capacity binding of calcium respectively (Baksh and Michalak., 1991) and gives CRT its lectin-like behaviour, permitting it to bind carbohydrates (Vassilakos *et al.*, 1998).

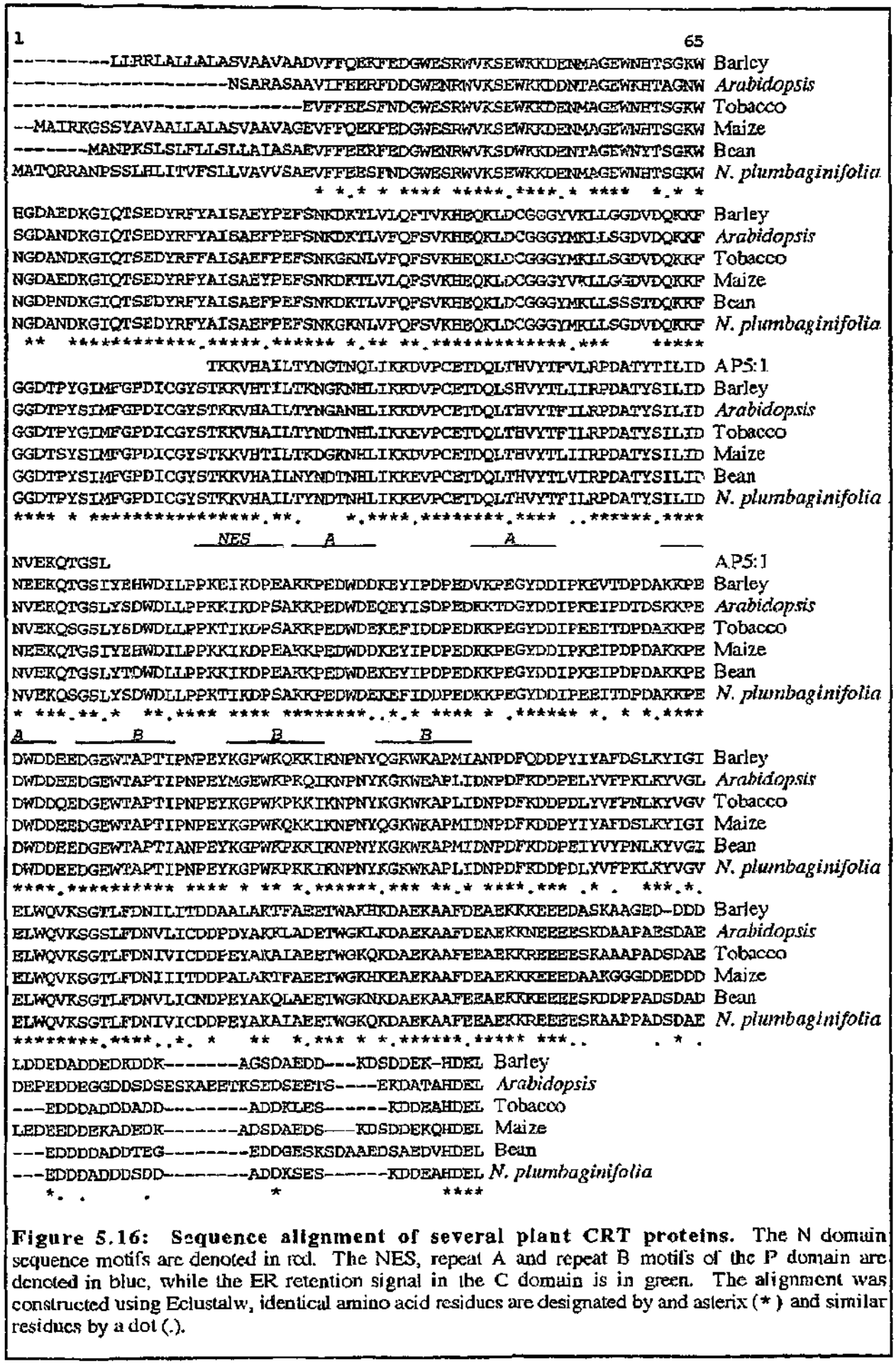


Figure 5.16: Sequence alignment of several plant CRT proteins. The N domain sequence motifs are denoted in red. The NES, repeat A and repeat B motifs of the P domain are denoted in blue, while the ER retention signal in the C domain is in green. The alignment was constructed using Eclustalw, identical amino acid residues are designated by and asterix (*) and similar residues by a dot (.).

The C-terminal (C domain) is less conserved but highly acidic, with many aspartate (D) and glutamate (E) residues. This domain has low affinity and high capacity Ca^{2+} binding sites (Baksh *et al.*, 1991) and is terminated by an ER retention signal sequence (Pelham, 1990). While KDEL (Lys-Asp-Glu-Leu) is the preferred signal in many species, the HDEL (His-Asp-Glu-Leu) variant was found in the plant proteins analysed.

The deduced amino acid sequence for the AP5:1 clone shares 92.9% to 80.4% identity and 96.4% to 89.3% similarity with the corresponding proteins of *Arabidopsis* (Benedetti and Turner, 1995), tobacco (Denecke *et al.*, 1995), maize (Dresselhaus *et al.*, 1996), barley (Chen *et al.*, 1994), bean (Coughlan *et al.*, 1997) and *Nicotiana plumbaginifolia* (Borisjuk *et al.*, 1998) when compared by pairwise GAP. Lower homology was found to animal CRTs, up to 59% identity and 75% similarity (Table 5.9). Since the AP5:1 clone represents an incomplete amino acid sequence, the analysis was repeated using the deduced full length amino acid sequence for the *Arabidopsis* CRT cDNA clone, AtCRTL (Benedetti and Turner, 1995), which has the best homology to AP5:1 as determined by the FASTA results. As expected, comparisons using this sequence yielded similar results.

Discussion

The ER is the organelle responsible for synthesising and processing polypeptides which are targeted for secretion, for the plasma membrane, and for transport to other organelles during endo- or exocytosis (Crofts *et al.*, 1998). Newly synthesised polypeptides are folded within the lumen of the ER by the activity of molecular chaperones and numerous folding factors, which are able to regulate the rate and the efficiency of this process (High *et al.*, 2000). Amongst these ER folding factors is CRT, a member of a class of soluble proteins, known as the reticuloplasmins (Denecke *et al.*, 1995; High *et al.*, 2000).

CRT is an abundant calcium binding protein (Krause and Michalak, 1997), which is thought to function as a molecular chaperone (Denecke *et al.*, 1995; Danilczyk *et al.*, 2000) with lectin-like properties, in the folding and oligomerisation of newly synthesised glycoproteins in the ER (Vassilakos *et al.*, 1998).

CRT interacts transiently with unfolded and partially folded nascent polypeptide chains to rapidly fold them into their native structure (Danilczyk *et al.*, 2000). This process begins with glucosidase I and II mediated removal of two glucose moieties from the N-linked oligosaccharide side chain ($Glc_1Man_9GlcNac_2$) on the newly synthesised glycoprotein, to produce a monoglucosylated form. This permits CRT to specifically bind

the monoglucosylated glycoprotein at the P domain lectin binding site, and assist in its folding (Vassilakos *et al.*, 1998). This process of CRT binding of glycoproteins is dependent upon the level of free Ca^{2+} in the ER (Vassilakos *et al.*, 1998). When Ca^{2+} levels are high, CRT binding of carbohydrate is promoted, when levels are low binding is reduced.

When folding is successful, the native glycoprotein exits the folding cycle, and is free to be transported through the secretory pathway to various intra and extra cellular locations. A single terminal glucose residue is reattached to the incorrectly or partially folded glycoproteins for another cycle of CRT binding and release (Labriola *et al.*, 1999; Corbett and Michalak, 2000). If proteins fail to reach their native configuration they are degraded (Klausner and Sitia, 1990). This process prevents the aggregation of polypeptides in the lumen of the ER and protects nonglycosylated proteins from denaturation (Saito *et al.*, 1999). As such CRT forms part of the ER quality control machinery (Danilczyk *et al.*, 2000).

Organism	% identity to AtCRTL	% similarity to AtCRTL	% identity to AP5:1	% similarity to AP5:1
Plants				
Arabidopsis	100.0	100.0	92.9	96.4
Tobacco	83.3	90.2	89.3	96.4
Maize	76.1	85.1	80.4	91.1
Barley	74.1	83.0	80.4	89.3
Bean	83.2	90.5	87.5	96.4
N. plumbaginifolia	83.8	89.6	89.3	96.4
Invertebrates				
C. elegans	54.9	69.8	53.6	69.6
Leishmania dinovani	37.8	57.2	41.8	58.2
Trypanosoma cruzi	39.7	58.2	39.3	53.6
Schistosoma mansoni	50.8	68.4	50.0	71.4
D. melanogaster	54.9	68.7	53.6	67.9
Vertebrates				
Zebrafish	55.0	70.8	58.9	75.0
Frog	53.5	68.5	46.4	62.5
Human	55.1	69.2	53.6	73.2
Bovine	53.3	69.8	51.8	73.2

Table 5.9: Homology of plant and animal CRT proteins to the partial amino acid sequence deduced for the AP5:1 clone and the corresponding full length sequence from the *Arabidopsis* AtCRTL clone. The accession numbers are as follows, for the plants, *Arabidopsis* GenPept: AAA80652, tobacco GenPept: CAA59694, maize GenPept: CAA61939, barley GenPept: AAA32948, bean SWISS_PROT: CRTC_RICCO and *N. plumbaginifolia* SWISS-PROT: CRTC_NICPL. The invertebrates, *C. elegans* SWISS_PROT: CRTC_CAEEL, *L. dinovani* GenPept: AAB17728, *T. cruzi* GenPept: AAD45370, *S. mansoni* PIR: A48573 and *D. melanogaster* SWISS_PROT: CRTC_DROME. The vertebrates, zebrafish GenPept: AAF13700, frog GenPept: BAA11425, human SWISS_PROT: CRTC_HUMAN and bovine SWISS_PROT: CRTC_BOVIN.

CRT is also proposed to have a role in calcium homeostasis (Krause and Michalak, 1997). This function implicates the involvement of CRT in numerous cell functions including, Ca^{2+} storage, Ca^{2+} signalling, cell adhesion, secretion, contraction and relaxation, motility, metabolism, protein biosynthesis, maturation and folding, gene expression, cell cycle progression, apoptosis, disease and stress responses, and protein-protein interaction (Crofts and Deneke, 1998). By mediating Ca^{2+} signalling and the transport of Ca^{2+} across the ER membrane, CRT can indirectly control calcium dependent processes that occur outside the ER (Corbett and Michalak, 2000).

CRT has been localised beyond the ER, to the nuclear envelope and the nucleus (Denecke *et al.*, 1995), the cytoplasm (Holaska *et al.*, 2001), the plasma membrane, the Golgi cisternae (Borisjuk *et al.*, 1998), in the microtubule arrays at the phargmoplast, and associated with the spindle apparatus in dividing cells (Denecke *et al.*, 1995). This suggests that different forms of this protein may exist (Holaska *et al.*, 2001). Moreover, it has been suggested that the level of cytosolic CRT may be regulated by Ca^{2+} binding or phosphorylation (Krause and Michalak, 1997). Recent biochemical studies have shown that cells contain cytosolic, as well as ER luminal pools of CRT (Holaska *et al.*, 2001). However, the molecular mechanism by which cytosolic CRT is generated remains unclear, as DNA sequencing and RNA expression studies are yet to produce evidence for a CRT isoform lacking an ER targeting sequence.

The findings reported by Holaska *et al.* (2001) suggest that CRT purified from human HeLa cell cytosol, can function as a receptor for nuclear export, to mediate transport between the nuclear and cytoplasmic compartments. This export function for cytosolic CRT was found to be regulated by the Ran group of GTPases. Experimental evidence was collected to support a model whereby, CRT recognises the specific hydrophobic binding domain of the NES, located on the target protein cargo. CRT binds at the NES by a high affinity interaction which requires RanGTP, to form a stable trimeric export complex. Following this assembly reaction, which is thought to occur in the nucleoplasm (Richards *et al.*, 1997), the trimeric export complex is translocated through the nuclear pores. Having reached the correct cytoplasmic compartment, RanGAP is thought to hydrolyse RanGTP to its inactive RanGDP state, resulting in the disassociation of the trimeric complex (Kehlenbach *et al.*, 1999). CRT and RanGDP are then returned to the nucleus for the next round of export.

In plants, expression of genes encoding CRT has been associated with embryo development, cell proliferation and events directly proceeding fertilisation (Chen *et al.*, 1994; Dresselhaus *et al.*, 1996; Nelson *et al.*, 1997). Moreover, their is evidence to suggest

that this expression may be regulated by auxin (Borisjuk *et al.*, 1998). The protein is particularly abundant in roots, young leaves, germinating seeds, floral organs and the vasculature of vegetative tissues (Benedetti and Turner, 1995; Denecke *et al.*, 1995; Coughlan *et al.*, 1997; Nelson *et al.*, 1997). In some instances CRT activity has also been correlated with secretory systems and signal transduction by elicitors (Denecke *et al.*, 1995; Coughlan *et al.*, 1997; Nelson *et al.*, 1997)

The role of CRT in LR formation may be related to its role in regulating calcium homeostasis. It has been suggested that regions of the root undergoing high rates of cell division and cell elongation require increased levels of Ca^{2+} (Kiegle *et al.*, 2000). In controlling the level of free Ca^{2+}, CRT influences the rate at which newly synthesised glycoproteins are folded before transport along the secretory pathway for deposition into cellular compartments such as cell walls undergoing differentiation or expansion during the growth phase of LRP formation. It is perhaps not coincidental that the genes encoding CRT, DNA-directed RNA polymerases, rpL2 and rabGAP, represented in *E. globulus* by the clones AP5:1, AP2:2, AP3:3, and DD101.2:4 respectively, are maximally expressed during active growth phase of the LRP formation.

Ca^{2+} is an important second messenger in signalling pathways. By modulating its levels, CRT has the capacity to affect a number of molecular mechanisms important in LRP development. These may include upstream processes, such as transcriptional activation (e.g., AP2:2/DNA-directed RNA polymerases) and protein synthesis (e.g., AP3:3/rpL2), as well as downstream events, including GTPase mediated protein transport and exocytosis (e.g., DD101.2:4/rabGAP). These processes are essential to ensure that LRP specific or related genes are expressed to generate proteins targeted for secretion, such as arabinogalactan (AP21B:6) and extensin (AP1:1) which reach and interact with their specific targets to contribute to the normal development of the LRP.

5.4.3.4 AP12:3: Polyubiquitin (*E* value = 2.7e-27)

Expression characteristics

When used as a probe, the AP12:3 clone hybridises to three transcripts, 1.55, 1.9 and 2.6 kb. All three transcripts show similar patterns of differential accumulation. In the control tissues, the transcripts are most abundant in the cell suspension sample and either absent or very low in the shoot apex indicating accumulation may be regulated by auxin but not enhanced by cell division (Fig. 5.17). In the auxin treated root samples, the transcripts

accumulate to maximum levels when formation of the LRP is well completed, and numbers have reached the point of saturation along the entire length of the primary root. Transcript levels observed for auxin treatments under 72 h were not elevated above that detected in the untreated root control suggesting that auxin induction occurs in roots over a prolonged treatment.

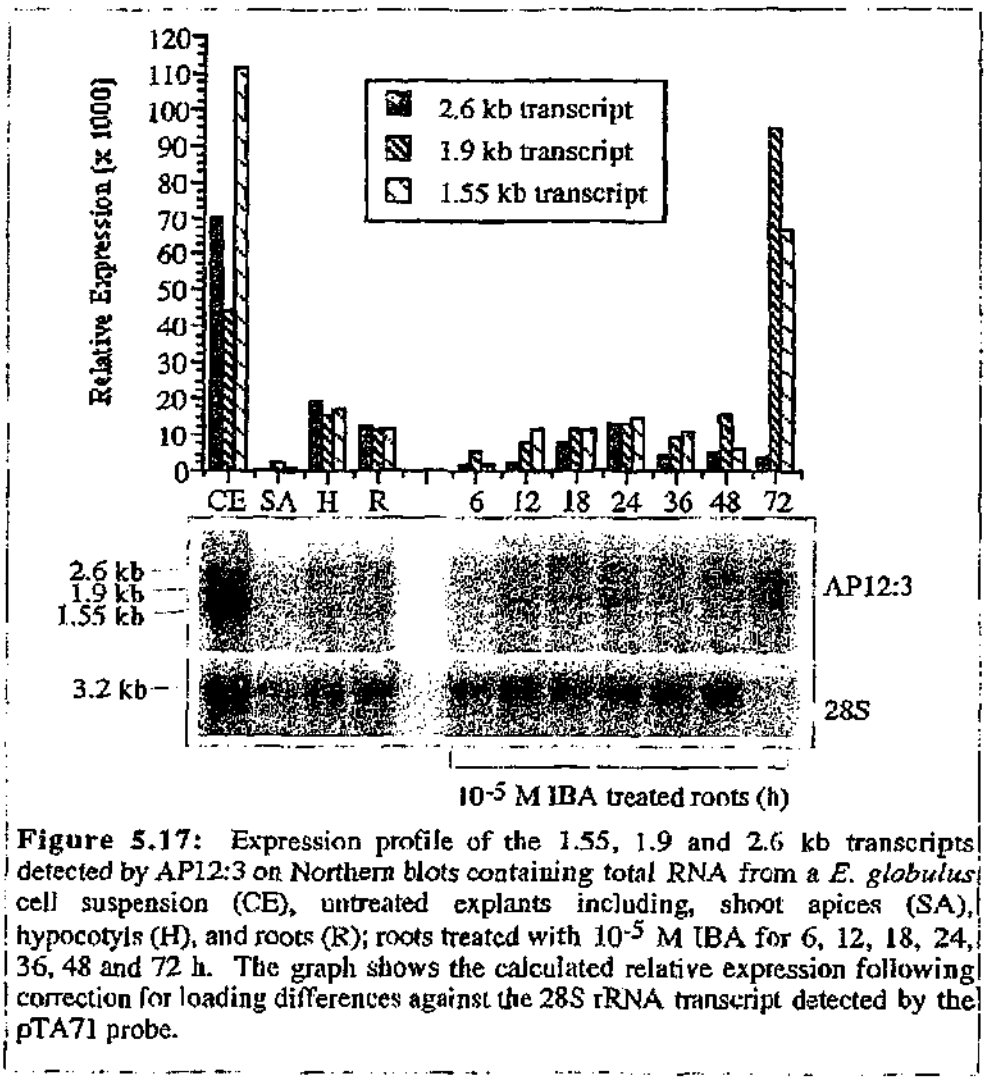


Figure 5.17: Expression profile of the 1.55, 1.9 and 2.6 kb transcripts detected by AP12:3 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

Database homology searches using FASTA, found a high level of homology between the 228 bp coding sequence of AP12:3 and its derived amino acid sequence (Fig. 5.18), with polyubiquitin from a variety of different plant species including rice (Wang *et al.*, 2000). Significant sequence identity was detected at 100% (E = 2.7e-27) at the amino acid level, and up to 90.351% (E = 1.1e-43) at the nucleotide level. The differences in the nucleotide

sequence can be attributed to conservative substitutions in the third base wobble position of the codon.

```
1     ACCCTCGCCGACTACAACATACAGAAGGAGTCCACCCTCCACCTTGTCCT
      T  L  A  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L   17
51    CCGTCTTCGTGGAGGTATGCAGATCTTCGTCAAGACCCTCACCGGCAAGA
       R  L  R  G  G  M  Q  I  F  V  K  T  L  T  G  K  T  34
101   CCATCACCCTCGAGGTCGAGAGCTCCGACACCATCGACAACGTCAAGGCC
        I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A   50
151   AAGATCCAGGACAAGGAAGGAATTCCCCCGGACCAGCAGAGGCTGATCTT
      K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  67
201   CGCTGGAAAGCAGCTTGAGGACGGCCGT
       A  G  K  Q  L  E  D  G  R                         76
```

Figure 5.18: Nucleotide and deduced amino acid sequence of the 228 bp AP12:3 clone.

Ubiquitin is a 76 amino acid protein widely distributed in all eukaryotic cells. This highly conserved protein differs by only 1 to 3 amino acid residues between unicellular organisms and higher plants and animals (Schlesinger and Bond, 1987), suggesting an important common function for this protein. Ubiquitin is encoded by a small multigene family which comprises two different types of genes, the polyubiquitin genes and the ubiquitin fusion genes (Schlesinger and Bond, 1987).

The coding region of the polyubiquitin gene contains multiple long tandem, head-to-tail repeats of 228 bp in length (Hegde *et al.*, 2000; Wang *et al.*, 2000). Each repeat corresponds to a single ubiquitin coding unit, with the number of repeats varying from three repeat units in *Phytophthora infestans* (Pieterse *et al.*, 1991), four units in flax (Agarwal and Cullis, 1991), *Avena fatua* (Reynolds and Hooley, 1992) and *Nicotiana tabacum* (Genschik, 1994), at least five units in *Nicotiana sylvestris* (Genschik *et al.*, 1992), maize (Liu *et al.*, 1995), strawberry (Aguilar *et al.*, 1997), pea (Xia and Mahon, 1998), and *Sporobolus stapfianus* (O'Mahony and Oliver, 1999), at least six units in sunflower (Binet *et al.*, 1991), *Arabidopsis* (Callis *et al.*, 1995), *Aplysia* (Hedge *et al.*, 2000) and rice (Wang *et al.*, 2000), at least eight units in *Schizosaccharomyces pombe* (Okazaki *et al.*, 2000) and up to 52 units in *Trypanosoma cruzi* (Swindle *et al.*, 1988).

The polyubiquitin represented in *E. globulus* by the AP12:3 clone corresponds to two incomplete ubiquitin coding units (Fig. 5.19A). In polyubiquitin, a single 76 amino acid ubiquitin unit begins with a Met-1 and ends with a Gly-76 (Fig. 5.19B). In AP12:3, the 22 amino acid residues extending from Thr-1 to Gly-22 represents the C-terminal end of one unit, where Gly-22 corresponds to Gly-76 of the ubiquitin monomer. The next 54 amino acid residues of the AP12:3 sequence represents the N-terminal end of the next ubiquitin coding unit, where Met-23 corresponds to Met-1 of the ubiquitin monomer.

A.

```
  1  TLADYNIQKESTLHLVLRLRGGMQIFVKTLTGKTITLEVESSDTIDNVKA
 51  KIQDKEGIPPDQQRLIFAGKQLEDGR
```

B.

```
  1  MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIFAGKQL
 51  EDGRTLADYNIQKESTLHLVLRLRGG
```

Figure 5.19: (A) The amino acid sequence of the AP12:3 clone. Residues 1-55, denoted in blue, represent the C-terminal end of one ubiquitin coding unit. The Met residue denoted by the **bold** type represents the first residue of a new ubiquitin repeat unit, denoted in red. (B) The 76 amino acid sequence of a ubiquitin monomer.

The ubiquitin fusion gene encodes one ubiquitin coding unit linked to the N-terminus of a ribosomal protein, either 52 or 76-81 amino acids in length (Nishi *et al.*, 1993). The two types of ubiquitin genes are translated as polyprotein precursors, which are posttranslationally cleaved to release monomeric ubiquitin (von Kampen *et al.*, 1996).

Discussion

Polyubiquitin functions in controlling the intracellular levels of important regulatory proteins (Kornitzer and Ciechanover, 2000). Consequently, polyubiquitin is able to regulate a number of diverse biological processes that require a rapid turnover of proteins, or are influenced by rapid changes in protein levels. As a result, this pathway has important roles in the regulation of transcription, oncogenesis, synapse formation, ribosome biosynthesis, chromatin structure, cell cycle progression, cell division, signal transduction, auxin response, desiccation tolerance, DNA replication, DNA repair and apoptosis (Chung and Baek, 1999; O'Mahony and Oliver, 1999; Gray and Estelle, 2000; Kornitzer and Ciechanover, 2000).

The polyubiquitin-proteasome pathway is the primary system whereby unfolded or misfolded proteins in the ER, denatured and short-lived proteins, such as those encoded by the *Aux/IAA* gene family, are degraded (Komitzer and Ciechanover, 2000). During protein degradation, the C-terminal glycine residue of ubiquitin is covalently linked to the ε-amino group of the lysine residue of the target substrate destined for proteolytic attack by the 26S proteasome.

This event is defined as protein ubiquitylation, and is mediated by three enzymes, the ATP-dependent ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2), and the ubiquitin ligating enzyme (E3), to form a polyubiquitin chain which acts as the signal for protein degradation (del Pozo and Estelle, 1999b; Gray and Estelle, 2000). Upon recognition of the polyubiquitin chain by the 26S proteasome, the tagged protein is degraded and free ubiquitin is released. Alternatively, ubiquitinated proteins may be specifically targeted to the plasma membrane for internalisation by endocytosis, and trafficking to vacuoles or lysosomes for degradation via a proteasome-independent pathway (Chung and Baek, 1999).

Auxin induces changes in an extensive range of developmental processes such as cell division, cell expansion and cell differentiation, to regulate diverse events during the development and growth of plants (O'Brien *et al.*, 1985; Theologis, 1986; Davies, 1987; Hobbie *et al.*, 1994; Masucci and Schiefelbein, 1996; Eckardt, 2001).

Characterisation of the *AXR1* and *TIR1* genes in *Arabidopsis* has provided evidence to suggest that auxin signalling is mediated through the ubiquitin-proteasome pathway. The findings of these studies were reported in Chapter 1.4.2.1.2. In short, the model explains a role for ubiquitination in the auxin response pathway by proposing that repressors, or negative regulators of auxin, are degraded by the ubiquitin-26S proteasome system, causing derepression of the auxin response pathway (del Pozo and Estelle, 1999b; Gray and Estelle, 2000). This would result in the activated transcription of auxin induced genes, including those involved in auxin regulated plant processes.

Although the expression of polyubiquitin coding genes has been observed in all plant organs tested (Wang *et al.*, 2000), transcription of these genes can be highly induced in response to various stimuli. Differential expression of polyubiquitin genes has been reported in response to stresses, such as wounding, pathogen infection, heavy metal treatment and heat shock (Genschik *et al.*, 1992; Wang *et al.*, 2000), as well as in response to phytohormone treatments (Wang *et al.*, 2000).

Accumulation of the 1.9 kb AP12:3 transcript was generally in agreement with that reported for RUBQ2 in rice (Wang *et al.*, 2000). In each case the transcript was detected in all the tissues tested but accumulated to highest levels in roots. In *E. globulus*, accumulation of all three mRNA species occurred during the later stages of auxin treatment, corresponding with the completion of LRP formation. Enhanced expression at this time may reflect an increased need to eliminate proteins which are no longer required, having fulfilled their role in auxin signalling and/or LRP development. The detection of the different sized AP12:3 transcripts may reflect the presence of different polyubiquitin encoding genes, each with a different number of ubiquitin coding repeat units.

5.4.4 Genes Involved in Protein Transport and Secretion

5.4.4.1 DD192.1:2: Synaptobrevin/Vesicle Associated Membrane Protein (*E* value = 0.00034)

Expression characteristics

The expression profile detected by DD192.1:2 in the control samples was typical of the LRP specific pattern. This clone appeared to detect early activated gene expression, with a 32% increase detected above the untreated control at 6 h after auxin stimulation (Fig. 5.20). By 12 h the mRNA transcript had accumulated by a further 46%. At 18 h, expression decreased back to the level observed at 6 h. The amount of mRNA transcript was maintained at this level until expression increased again at 36 h, elevating transcript levels back to that observed at 12 h. At 48 h expression was at its highest before beginning its decline again at 72 h. This expression profile displays characteristics of activated gene expression during the early to middle stages (6-12 h) of LRP induction (Fig. 5.20). Expression was down-regulated during the following 12 h of growth, before a maximum level was reached when LRP numbers were highest (48 h).

Sequence analysis

Translation of the DD192.1:2 nucleotide sequence in all six possible reading frames revealed that this 227 bp clone was not comprised entirely of coding sequence. In order to decipher the translated ORF from the untranslated sequence, two FASTA searches were performed. The first search was conducted against the NR nucleic database, using the DD192.1:2 nucleotide sequence as the query, whilst the second was performed against the

NR protein database, using stretches of the translated sequence which consisted of ten or more amino acid residues.

The nucleic search resulted in two hits to related sequences from *Arabidopsis*, both with *E* values of <0.01. The higher scoring match (72.2% identity over 79 nucleotides, *E*=0.00015) was assigned to an uncharacterised genomic BAC clone T1G11, while the other corresponded to an mRNA sequence for a *vesicle-associated membrane protein 7B* gene (*AtVAMP7B*; 67% identity over 100 nucleotides, *E*=0.00034). In both cases, the region of overlapping nucleotide homology was located at the 5' end of the DD192.1:2 clone.

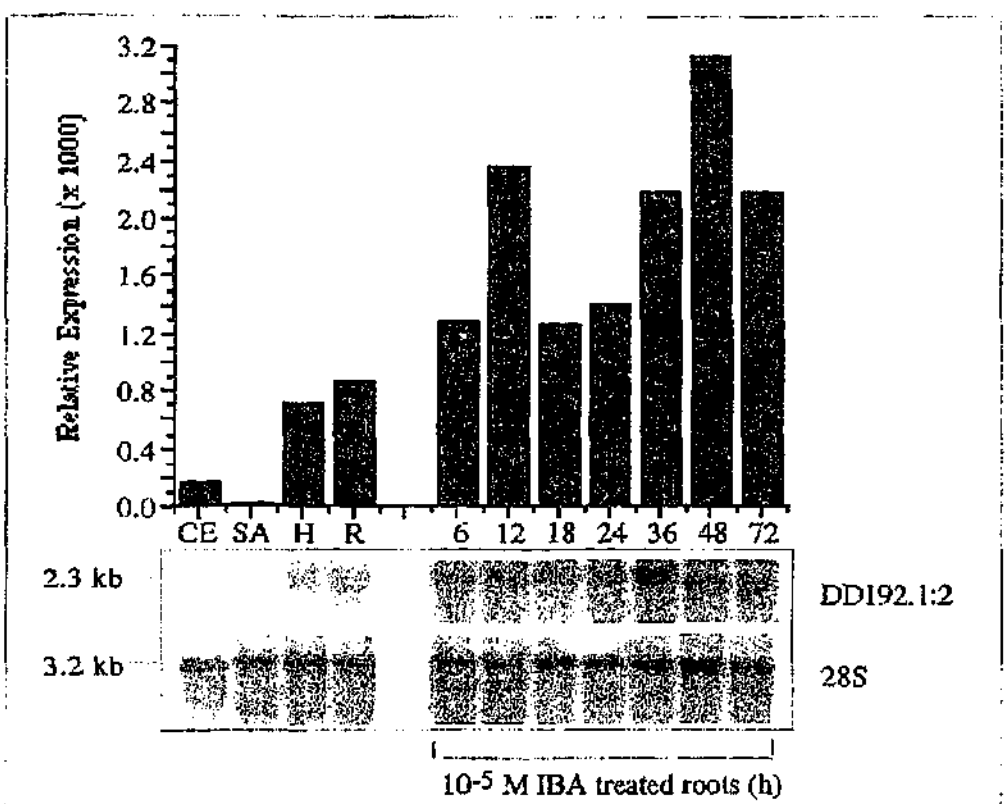


Figure 5.20: Expression profile detected by DD192.1:2 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Results obtained for the deduced amino acid sequence searches, identified a stretch of 26 amino acids, encoded by 78 bp at the 5' end of the DD192.1:2 clone, with significant homology to the same *Arabidopsis* BAC T1G11 (65.4% identity in a 26 amino acid overlap; $E = 7.5e\text{-}06$), and AtVAMP7B (65% identity in a 26 amino acid overlap; $E = 9e\text{-}06$) identified in the earlier nucleotide search. In addition, a significant match was also made to a synaptobrevin-like protein from *Arabidopsis* (SAR1 or HAT24; 65% identity over a 26 amino acid overlap; $E = 9e\text{-}06$), human (SYBL1) and mouse (both exhibiting 56% identity over 25 amino acids, $E = 0.0004$).

SAR1 is itself a vesicle-associated membrane protein (Schena and Davis, 1992). A sequence comparison between the AtVAMP7B and SAR1 polypeptides (Fig. 5.21A) revealed a shared amino acid identity of 95.9% (97.7% similarity), indicating that the two proteins are highly conserved. Furthermore, an alignment of the deduced T1G11 polypeptide, against the AtVAMP7B and SAR1 amino acid sequences, showed that essentially they all represented the same polypeptide, with T1G11 sharing 91.4% identity (95.4% similarity) with AtVAMP7B and 86.9% (92.6% similarity) identity with SAR1 (Fig. 5.21B).

SAR1 was originally isolated as *HAT24* (for homeobox from *Arabidopsis thaliana*) by Schena and Davis (1992) during a screen for homeobox genes from *Arabidopsis*. In animals, homeobox genes control many diverse developmental processes, seemingly acting as a molecular switch to control cellular fate by regulating gene expression (Harrison, 1991). A possible developmental role for homeobox genes in plants was supported when mutations in the maize homeobox gene *Knotted-1*, resulted in altered leaf development (Vollbrecht *et al.*, 1991).

The putative HAT24 homeodomain was reported to exhibit sequence homology to α-helix three of the Antennapedia (Antp) homeodomain of *Drosophila* (Schena and Davis, 1992). However, the authors were led to question the validity of HAT24 as a true homeodomain because it showed little amino acid sequence identity elsewhere within the homeodomain. Nevertheless, despite obvious differences between plant and animal development, there is evidence for some conservation of basic mechanisms of transcriptional regulation (Schena *et al.*, 1991). Consequently, it may be reasonable to suggest that genes used by animals to control development may have equivalents in higher plants.

A.

```
SAR1          1 MAQQSLIYSFVARGTVILVEFTDFKGNFTSIAAQCLQKLPSSNNKFTYNC 50
                |||||||||||||||||||||||||||||||||||||||||||||||||||
AtVAMP7B      1 MAQQSLIYSFVARGTVILVEFTDFKGNFTSIAAQCLQKLPSSNNKFTYNC 50

SAR1         51 DGHTFNYLVEDGFTYCVVAVDSAGRQIPMSFLERVKEDFNKRYGGGKAAT 100
                ||||||||||:|||||||||||||||||||.|||||||||||||||||||
AtVAMP7B     51 DGHTFNYLVENGFTYCVVAVDSAGRQIPMAFLERVKEDFNKRYGGGKAAT 100

SAR1        101 AQANSLNKEFGSKLKEHMQYCMDHPDEISKLAKVKAQVSEVKGVMMENIE 150
                ||||||||||||||||||||||.|||||||||||||||||||||||||||
AtVAMP7B    101 AQANSLNKEFGSKLKEHMQYCMAHPDEISKLAKVKAQVSEVKGVMMENIE 150

SAR1        151 KVLDRGEKIELLVDKTENLRSQAQDFRTTGTQMRRKMWLQNMKIKLIVLA 200
                |||||||||||||||||||||||||| |||||||||:|||||||||||
AtVAMP7B    151 KVLDRGEKIELLVDKTENLRSQAQDFRTQGTQMRRKMWFQNMKIKLIVLA 200

SAR1        201 IIIALILIIVLSVCHGFKC.. 219
                |||||||||:||:| ||.|
AtVAMP7B    201 IIIALILIIILSICGGFNCGK 221
```

B.

```
                    10        20        30        40        50        60
SAR1        MAQQSLIYSFVARGTVILVEFTDFKGNFTSIAAQCLQKLPSSNNKFTYNCDGHTFNYLVE
AtVAMP7B    MAQQSLIYSFVARGTVILVEFTDFKGNFTSIAAQCLQKLPSSNNKFTYNCDGHTFNYLVE
T1G11       MGFGWLHFSMKIWGLIVS------------------------------------------

SAR1        NGFTYCVVAVDSAGRQIPMAFLERVKEDFNKRYGGGKAATAQANSLNKEFGSKLKEHMQY
AtVAMP7B    DGFTYCVVAVDSAGRQIPMSFLERVKEDFNKRYGGGKAATAQANSLNKEFGSKLKEHMQY
T1G11       --FSYCVVAVDSAGRQIPMSFLERVKEDFNKRYGGGKAATAQANSLNKEFGSKLKEHMQY

SAR1        CMAHPDEISKLAKVKAQVSEVKGVMMENIEKVLDRGEKIELLVDKTENLRSQAQDFRTQG
AtVAMP7B    CMDHPDEISKLAKVKAQVSEVKGVMMENIEKVLDRGEKIELLVDKTENLRSQAQDFRTTG
T1G11       CMDHPDEISKLAKVKAQVSEVKGVMMENIEKVLDRGEKIELLVDKTENLRSQAQDFRTTG

SAR1        TQMRRKMWFQNMKIKLIVLAIIIALILIIIILSICGGFNCGK
AtVAMP7B    TQMRRKMWLQNMKIKLIVLAIIIALILIIVLSVCHGFKC--
T1G11       TQMRRKMWLQNMKIKLIVLAIIIALILIIVLSVCHGFKC--
```

Figure 5.21: (A) A GAP amino acid sequence comparison of the AtVAMP7B (GenPept accession number AAC98905) and SAR1 (SWISS-PROT accession number SYBR_ARATH) polypeptides. (B) An alignment of the highly conserved SAR1, AtVAMP7B and T1G11 (GenPept accession number AAB80624) polypeptides. Sequence identity is denoted in red and similarity in blue.

It is evident from the combined nucleotide and amino acid sequence data analysis, that the DD192.1:2 clone comprises of a 5' translated region, representing a partial ORF of 26 amino acids, located upstream of a 3' untranslated region (3' UTR) of 149 bp (Fig. 5.22A). This was confirmed upon comparison to the genomic sequence provided by the BAC T1G11 clone (Fig. 5.22B). In BAC T1G11, the ORF corresponding to the 5' translated region of DD192.1:2, is comprised of 41 amino acid residues in its entirety. The two clones share 65.4% amino acid identity and 71.3% nucleotide identity within the ORF. This level of homology does not extend into the 3' UTR where the two sequences diverge, sharing only 41% identity at the nucleotide level. The *AtVAMP7B* and *SAR1* sequences were derived from mRNA and did not contain any untranslated regions.

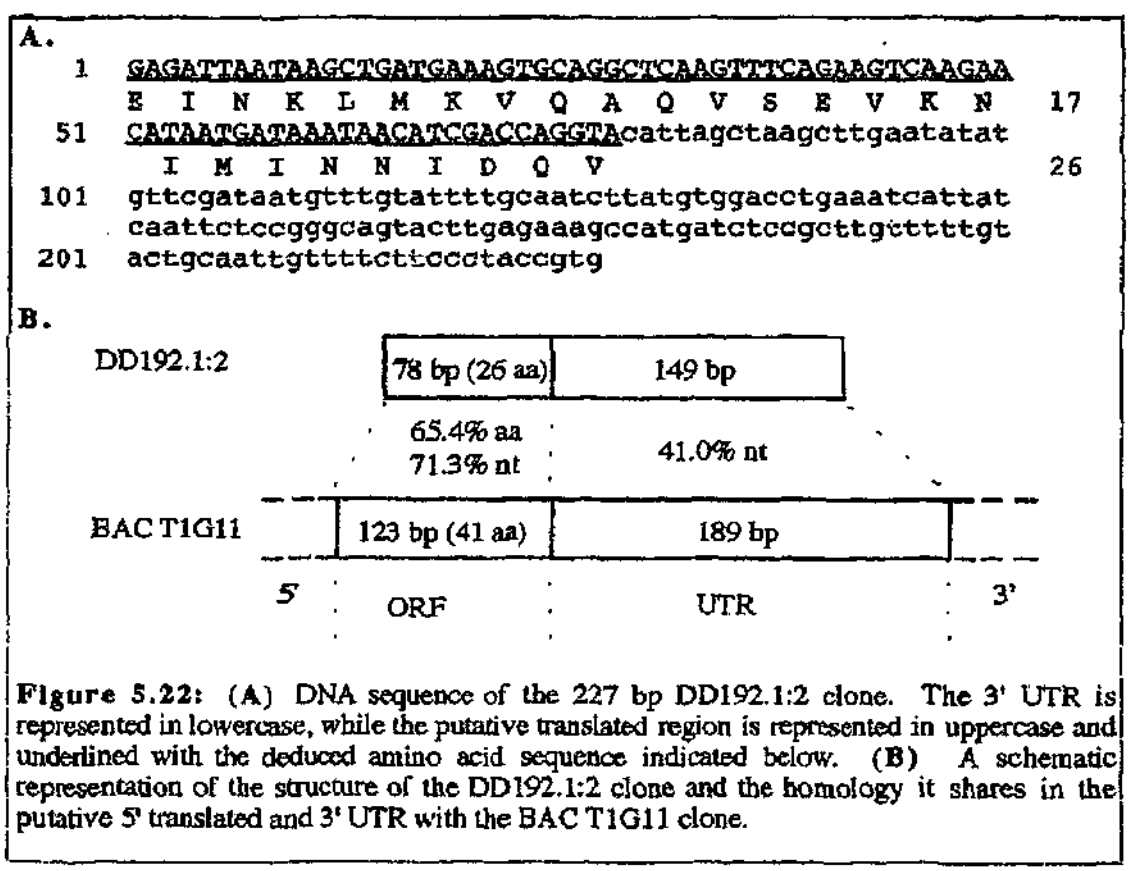


Figure 5.22: (**A**) DNA sequence of the 227 bp DD192.1:2 clone. The 3' UTR is represented in lowercase, while the putative translated region is represented in uppercase and underlined with the deduced amino acid sequence indicated below. (**B**) A schematic representation of the structure of the DD192.1:2 clone and the homology it shares in the putative 5' translated and 3' UTR with the BAC T1G11 clone.

In *Arabidopsis* the *SAR1* (Schena and Davis, 1992) and *AtVAMP7B* (Nikoloff and Somerville, unpublished GenPept accession number AAC98905) messages encode proteins of 221 and 219 amino acids, with calculated weights of 24,884 and 24,764.69 respectively. Both are comparable to the mouse and human synaptobrevin proteins, comprising of 220 and 221 amino acids with calculated molecular weights of 24,966.85 and 24,935 respectively.

Synaptobrevin (Südhof *et al.*, 1989) is an intrinsic vesicle associated membrane protein. It has an unknown function but is highly conserved in mammals, electric ray (where its is known as *VAMP-1*), *Drosophila* and yeast (Gerst *et al.*, 1992). Although, a synaptobrevin-like vesicle associated membrane protein (synaptobrevin/VAMP) has been identified previously in *Arabidopsis* (Schena and Davis, 1992; Nikoloff and Somerville, unpublished GenPept accession number AAC98905), its structural and functional properties have not been characterised in plants. Instead, published reports are generally limited to mammals, yeast and *Drosophila*.

The synaptobrevin/VAMP protein is made up of three (Schena and Davis, 1992; D'Esposito *et al.*, 1996) or four putative domains (Südhof *et al.*, 1989). Although the polypeptides encoded by the *Arabidopsis SAR1* and human *SYBL1* genes exhibit a low level of amino acid identity (36.6%), the similarity (59.3%) is sufficiently high enough to preserve the overall structural features of the protein (Fig. 5.23A and B). The N-terminal cytoplasmic domain is hydrophilic and variable in size, 196 residues in *Arabidopsis* and 188 residues in human. It was in this domain that homology to the predicted 26 amino acid sequence of DD192.1:2 was identified. This is followed by an invariant stretch of 21 hydrophobic amino acid residues which constitute a potential transmembrane region. In human, this region is proposed to function as a signal anchor (D'Esposito *et al.*, 1996). The length of the C-terminal intravesicular domain varies in length between the two species. In *Arabidopsis*, the sequence is shortest comprising of only 4 amino acids, while comparatively longer in human with 11 amino acids. It has been suggested, that while the conserved transmembrane region anchors the protein in the membrane, the rest of the protein is exposed to the cytoplasm, with the variable vesicular domain possessing extracellular potential that may be required to ensure that the transmembrane region crosses the bilayer (Südhof *et al.*, 1989).

A.

```
Human          1 ...MAILPAVVARGTTILAKHAWCGGNFLEVTEQILAKIPSENNKLTYSH 47
                   .:::..|||||.||.. .   ||| .:..|.|.|:||.|||:||.
Arabidopsis    1 MAQQSLIYSFVARGTVILVEFTDFRGNFTSIAAQCLQKLPSSNNKFTYNC 50

Human         48 GNYLFHYICQDRIVYLCITDDDFERSRAFNFLNEIKKRFQTTY.GSRAQT 96
                 :.. |:|:.:: :.| .:. |. :|  ::.||: :|. |.. | |::|.|
Arabidopsis   51 DGHTFNYLVENGFTYCVVAVDSAGRQIPMAFLERVKEDFNKRYGGGKAAT 100

Human         97 ALPYAMNSEFSSVLAAQLKHH.SENKGLDKVMETQAQVDELKGIMVRNID 145
                 | : .:|.||:| | .::.. .. .:::.|: ...|||.|:||:|: ||:
Arabidopsis  101 AQANSLNKEFGSKLKEHMQYCMAHPDEISKLAKVKAQVSEVKGVMMENIE 150

Human        146 LVAQRGERLELLIDKTENLVDSSVTFKTTSRNLARAMCMKNLKLTIIIII 195
                   | :|||::|||:|||||| . . .|:| : .: | | :.|:|:.:|::
Arabidopsis  151 KVLDRGERIELLVDKTENLRSQAQDFRTQGTQMRRKMWFQNMKIKLIVLA 200

Human        196 VSIVFIYIIVSPLCGGFTWPSCVKK 220
                 : |.:|.||: .:||||   .|.|
Arabidopsis  201 IIIALILIIILSICGGF...NCGK. 221
```

B.

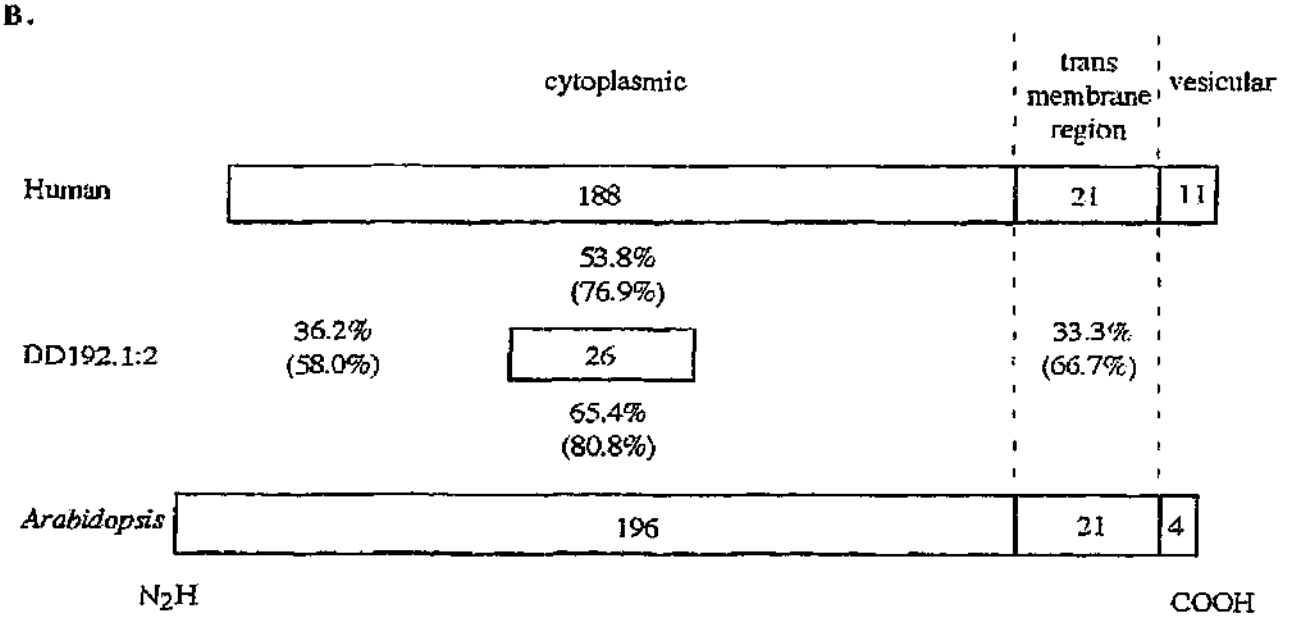


Figure 5.23: (A) A GAP comparison of the amino acid sequences of human (SWISS_PROT accession number SYBL_HUMAN) and *Arabidopsis* (SWISS-PROT accession number SYBR_ARATH) synaptobrevins. Identical residues are denoted by a line, similar residues by dots. The cytoplasmic domain is in black, the transmembrane domain in red and the vesicular domain in blue. (B) A diagrammatic representation of the conservation of synaptobrevin protein structure as a function of its domains. The DD192.1:2 clone has sequence similarity in the cytoplasmic domain of the human and *Arabidopsis* proteins. Inside the boxes, the length of each domain is given in amino acids. The % identity between the domains is indicated between in boxes, with the % similarity in brackets.

Discussion

At present a possible role for synaptobrevin/VAMP in LR development can only be tentatively inferred from its protein structure. In plants, this protein may be important in cell communication by mediating the interactions between the cell and the cytoplasm The

identification of a transmembrane region may imply a signalling function which may be important during processes such as exocytosis where an interaction between cytoplasmic and cellular environments is required. Similarity in the cytoplasmic domain of SAR1 to helix three of the Antp homeodomain may also suggest a putative involvement in transcriptional regulation. Upon isolation of a full length clone corresponding to DD192.1:2, studies aimed at suppression or loss of gene function may help elucidate a precise function for synaptobrevin/VAMP in LR development.

5.4.4.2 DD101.2:4: Rab GTPase Activator Protein (*E* value = 3.1e-62)

Expression characteristics

In the auxin treated root samples, maximum transcript accumulated at 24 h and was maintained at this level to the 48 h time point, after which expression declined at 72 h to levels below the untreated root control (Fig. 5.24). Activated gene expression was associated with the period of LRP growth to maturity, where upon LRP are at maximum numbers in the primary root. Expression in the cell suspension control was also at maximum levels implying regulation by auxin.

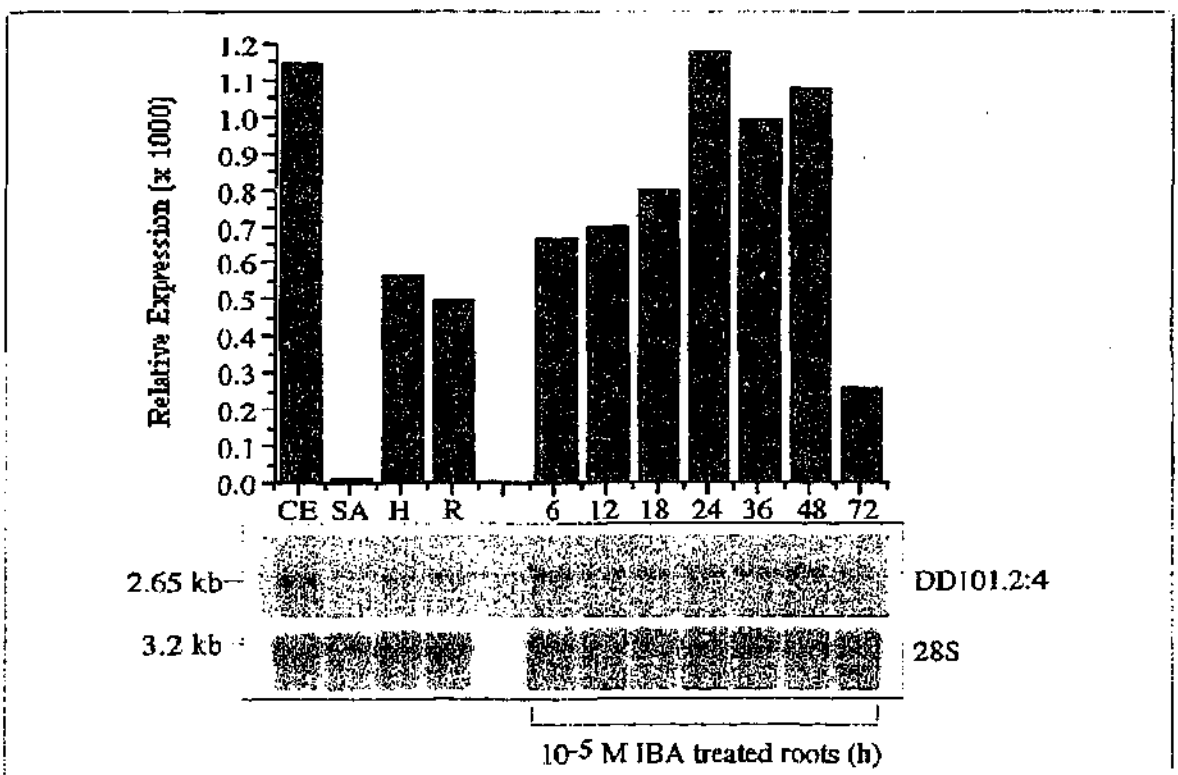


Figure 5.24: Expression profile detected by DD101.2:4 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

A FASTA search using a deduced polypeptide of 161 amino acids encoded by DD101.2:4 (Fig. 5.25) as the query against the NR protein database, identified significant scoring hits to four clones isolated from *Arabidopsis* and one from rice. These clones shared between 78.8% (E = 7.6e-56) and 86.8% (E = 3.1e-62) amino acid homology with the DD101.2:4 sequence, and corresponded to a putative GTPase activator protein, specific for a Rab-like small GTPase-like protein (rabGAP). Lower scoring matches were also made to the homologue from yeast, *Yarrowia lipolytica* (40.5%; E = 2.1e-18), *S. pombe* (36.8%; E = 7e-15) and *Saccharomyces cerevisiae* (37.3%; E = 2.2e-15). The DD1012:4 deduced amino acid sequence has a putative consensus sequence for lipid myristylation which may confer an affinity for membranes (Ferro-Novick and Novick, 1993).

```
  1  CTCGACTAGTTGCAGTTCTCGAAGCCTATGCCCTTTACGACCCTGAAATT
       R  L  V  A  V  L  E  A  Y  A  L  Y  D  P  E  I     6
 51  GGCTATTGCCAGGGTATGAGTGATCTTCTTTCTCCAATAATTACTGTGAT
     G  Y  C  Q  G  M  S  D  L  L  S  P  I  I  T  V  I    33
101  CACTGAAGATCATGAGGCTTTCTGGTGTTTTGTTAGTTTCATGAGGAAGG
       T  E  D  H  E  A  F  W  C  F  V  S  F  M  R  K  A  50
151  CTCGGCATAATTTTAGGCTCGATGAGGTGGGGATAAGAAAGCAGCTCAAT
       R  H  N  F  R  L  D  E  V  G  I  R  K  Q  L  N     66
201  ATTGTCTCCAAAATTATCAAATCCAAGGACTCTCACCTTTACAGGCACTT
     I  V  S  K  I  I  K  S  K  D  S  H  L  Y  R  H  L    83
251  GGAGAAGCTCCAGGCAGAAGATTGTTTCTTCGTTTATAGAATGGTGGTTG
       E  K  L  Q  A  E  D  C  F  F  V  Y  R  M  V  V  V  100
301  TACTATTTAGAAGGGAATTAACCTTTGAACAGACAAGTTGCCTTTGGGAG
       L  F  R  R  E  L  T  F  E  Q  T  S  C  L  W  E    116
351  GTGATGTGGGCTGACCAGGCCGCTATAAGGGCTGGGGTCGGGAAATCGGC
     V  M  W  A  D  Q  A  A  I  R  A  G  V  G  K  S  A   133
401  ATGGAGCCGCATAAGGCAGCGGGCCCCACCGACAGATGATTTGTTGCTGT
       W  S  R  I  R  Q  R  A  P  P  T  D  D  L  L  L  Y  150
451  ACGCAATAGCCGTTCGGTTTTGCAGAAGAGGAAACtgatcatagaggggt
       A  I  A  V  R  F  C  R  R  G  N                    161
501  acaa
```

Figure 5.25: The nucleotide sequence of the DD101.2:4 clone. The putative translated portion of the sequence is represented in uppercase and underlined with the deduced amino acids indicated below. A putative N-myristylation site is highlighted in red.

Discussion

GTPase-activating proteins mediate the hydrolysis step during the cycle of GTP binding and hydrolysis. This cycle, referred to as the GTP/GDP cycle, regulates the active and inactive state of GTPases (Fig. 5.26). The GTPases are GTP-binding proteins and act as a

molecular switch triggered by the transition between their active GTP-bound, inactive GDP-bound and transient unbound forms. This conformational conversion is modulated by the action of specific regulatory proteins including the GTPase-activating proteins (GAPs) and the guanine nucleotide release proteins (GNRPs). The cycle begins with GTPase binding of GTP, enabling it to interact with an effector protein. GAP acts on this active state to catalyse GTP hydrolysis, resulting in the transition of the GTPase to its inactive GDP-bound form, terminating any association with the effector. This step is irreversible, making the cycle unidirectional. The full cycle is completed when GNRP catalyses the release of bound GDP to revert GTPase to the transient unbound state (Bourne *et al.*, 1991; Pryer *et al.*, 1992). The rate of GTP hydrolysis and GDP disassociation is subject to stringent control by GAPs and GNRPs in order to regulate the proportion of active GTP-bound protein for normal cell operation.

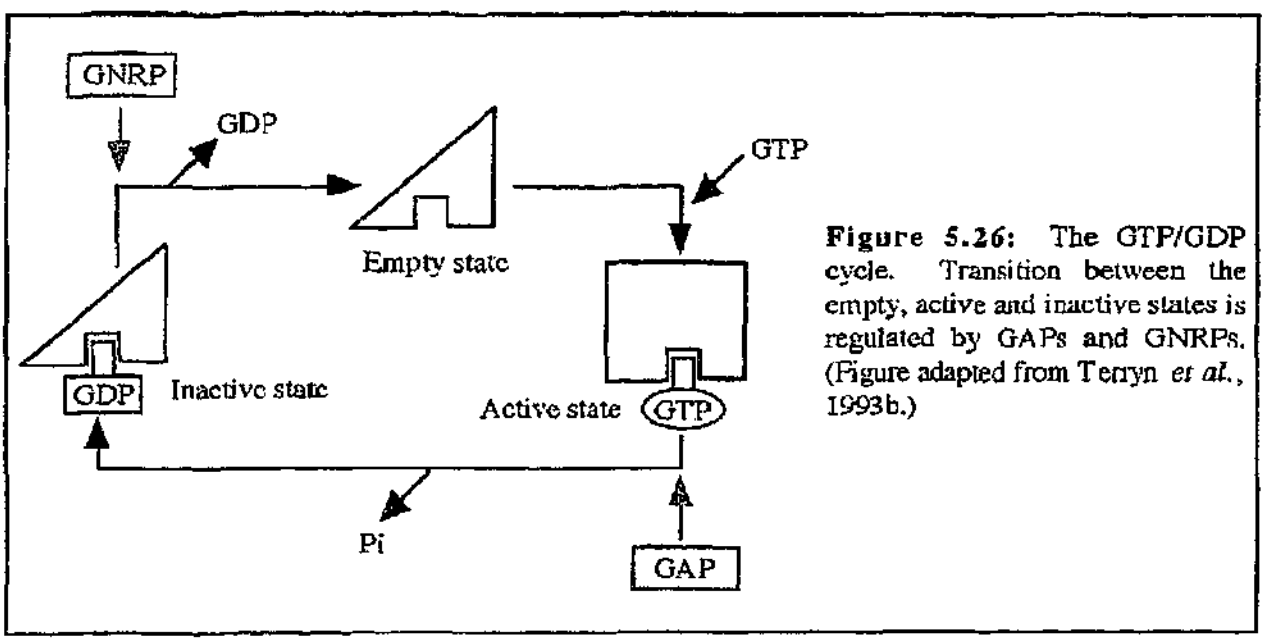


Figure 5.26: The GTP/GDP cycle. Transition between the empty, active and inactive states is regulated by GAPs and GNRPs. (Figure adapted from Terryn *et al.*, 1993b.)

This basic cycle is involved in the synthesis of ribosomal proteins, signal transduction, translocation of proteins into the ER, cell differentiation, proliferation and growth and guiding intracellular vesicular traffic (Bourne *et al.*, 1991; Brunger, 2001; Lin *et al.*, 2001).

GTPases collectively represent the small GTP-binding proteins and the G proteins. Although best characterised in yeast, genes encoding GTPases have been identified in organisms from all kingdoms (Terryn *et al.*, 1993b). The superfamily of small GTPases are sorted into three groups according to their suggested functions. The first group comprises the Rab/Ypt-like proteins which are membrane bound and play a role in vesicular trafficking (Terryn *et al.*, 1993b). The second group contains the Rho/Rac-like

proteins which are also membrane bound but have a postulated function in organisation of the cytoskeleton (Yang and Watson, 1993). The final group are the soluble Ran/Ras-like proteins which are implicated in mitosis and the transport of proteins into the nucleus (Ach and Gruissem, 1994).

The Rab proteins identified in animals and plants, and their Ypt counterparts from yeast, constitute the largest subfamily of small GTPases, some of which are thought to tightly regulate protein transport through the secretory pathway (Moore *et al.*, 1997). Different members of the Rab protein family have been localised to distinct organelles (Pryer *et al.*, 1992) and exhibit strict tissue specificity and stringent regulation of gene expression. In *Arabidopsis* for example, *rha1* is expressed in the guard cells of stomata, stipules, and the root tip of young plants (Terryn *et al.*, 1993a), while *Srab1* and *Srab2* from soybean is expressed in the root nodules and plumule respectively (Kim *et al.*, 1996). Such observations show that different GTPases regulate different cellular processes, or are themselves regulated by different controls. Whichever the case, the differential expression patterns may reflect distinct roles in plant development and morphogenesis for specific Rab-like proteins.

By means of GTP binding and hydrolysis, the small Rab GTPases are thought to regulate the trafficking of secretory vesicles that bud from the donor membrane for targeting to a specific acceptor membrane where they fuse (Ferro-Novick and Novick, 1993; Brunger, 2001). This enables the vesicles to be transported from various subcellular compartments, such as from the ER to the Golgi apparatus, or from the Golgi to the plasma membrane, carrying within them newly synthesised proteins destined for secretion (Pryer *et al.*, 1992). Fusion of the vesicle to the plasma membrane or the vacuole, facilitates the supply of secretory materials required for cell growth and differentiation, including cell wall precursors and membrane associated proteins. This event, known as exocytosis, plays an important role in plant processes including, cell growth and differentiation, accumulation of storage proteins within vacuoles, protein and polysaccharide secretion for cell wall and cell plate formation, and morphogenesis by cell expansion.

Using the bacterial elongation factor EF-Tu as an example, a model has been put forward to link the transport activity of GTPases with exocytosis (Bourne, 1988). This model proposes that a GTPase, recognises a specific protein (X) which is located on post-Golgi secretory vesicles. GTPase targets the vesicle forming a GTPase-protein complex, and transports it for docking to a second protein (Y) located on the plasma membrane. Following GAP mediated GTP hydrolysis, the GTPase returns to target and transport

another secretory vesicle, leaving the two proteins (XY) to complete membrane fusion and exocytosis.

Studies in plant systems have provided evidence to support this model. Vesicle targeting to precise regions of the plasma membrane is demonstrated in maize by the presence of hypersecretory cells in the root cap, which secrete a complex polysaccharide often referred to as slime (Rougier, 1981), while specific putative docking sites have been observed at the plasma membrane of *Micrasterias* (Meindl *et al.*, 1992).

GTPase mediated transport of proteins through the secretory pathway is likely to be important during cell wall differentiation and growth, two processes of paramount importance in the development of LRs. Studies in *Trifolium pratense* L. showed that two cell wall matrix polymers, rhamnogalacturonan I (RG-I) the major pectic polysaccharide, and xyloglucan (XG) a hemicellulose, are present within the Golgi cisternae and vesicles during cell expansion and cell plate formation (Moore and Staehelin, 1988). It was proposed that while these cell wall polysaccharides are synthesised in the Golgi apparatus, they are transported via Golgi derived secretory vesicles. Fusion of the vesicles with the plasma membrane would then allow the release of the polysaccharides into the cell wall, where they become incorporated.

Furthermore, during anaphase of mitotic cell division, the cell wall plate develops within the phagmoplast. This involves the targeted delivery, aggregation and fusion of Golgi vesicles (Battey and Blackbourn, 1993). The cell wall separating the two daughter cells, originates from the cell plate following the secretion of polysaccharide cell wall precursors, while the vesicle membranes form the new plasma membrane (Battey and Blackbourn, 1993).

Exocytosis may also play a role in the auxin induced acid growth phenomenon. Acidification of cell wall components upon auxin stimulation is thought to facilitate cell wall elongation (Hager *et al.*, 1991; Cosgrove, 1997), perhaps as a result of an increase in H^+ secretion at the plasma membrane (Hager *et al.*, 1991). Alternatively, auxin may stimulate the transcription of genes that encode polypeptides that regulate exocytosis, thereby influencing the secretion of cell wall precursors (Theologis, 1986).

Although the DD101.2:4 transcript is detected at relatively high levels in the cell suspension, it is absent from the shoot apex sample. This indicates that DD101.2:4 expression is associated with auxin induction rather than cell division. The elevated transcript levels observed in the cell suspension are also detected during growth of the LRP, 24 to 48 h after treatment of root explants with auxin. This expression pattern seems

to suggest that the rabGAP represented by the DD101.2:4 clone may hydrolyse a specific Rab-like GTPase, to regulate the transport of cell wall proteins and polysaccharides for secretion into the cell wall during growth of the LRP. Elevated DD101.2:4 expression does not persist after growth has ceased at 72 h. This may reflect a decrease in the rate of GTP hydrolysis by rabGAP, as the cells of the LRP cease growth and no longer require secretion of new cell wall components.

5.4.4.3 DD101.2:6: Ornithine Decarboxylase Inducible-Like (*E* value = 3.5e-39)

Expression characteristics

The relative high abundance of the 1.6 kb DD101.2:6 transcript in the cell suspension sample compared to the untreated tissue controls suggest that expression of the associated gene is induced by auxin. The transcript accumulated above the untreated root control after 6 h treatment with 10^{-5} M IBA (Fig 5.27). Levels continued to increase until the transcript was not abundant at 48 h, before declining. This expression profile indicates that the corresponding gene is active during early LRP induction. Activity increases throughout development of the LRP until maturity is reached and LRP numbers are at maximum density 48 h after initial IBA exposure.

Sequence analysis

The DNA sequence of DD101.2:6 is 402 bp long and encodes a deduced amino acid sequence of 133 residues (Fig. 5.28). When searched against the NR protein database, this amino acid sequence found a significant degree of homology to biodegradative ornithine decarboxylase (ODC) from a number of bacterial species, including *Lactobacillus* sp. (74.6% identity; *E* = 3.5e-39) and *Escherichia coli* (65.4%; 1.8e-34). In *Lactobacillus* strain 30a, this protein is encoded by the *ornithine decarboxylase inducible* (*odci*) gene (Hackert *et al.*, 1994). Lower, but still significant scores were assigned to prokaryotic lysine decarboxylase (LDC) and biodegradative arginine decarboxylase (ADC). Homology was not detected to any plant proteins. Similarly, a nucleotide search did not produce a match to any plant or eukaryotic gene sequence within the current NR nucleic database.

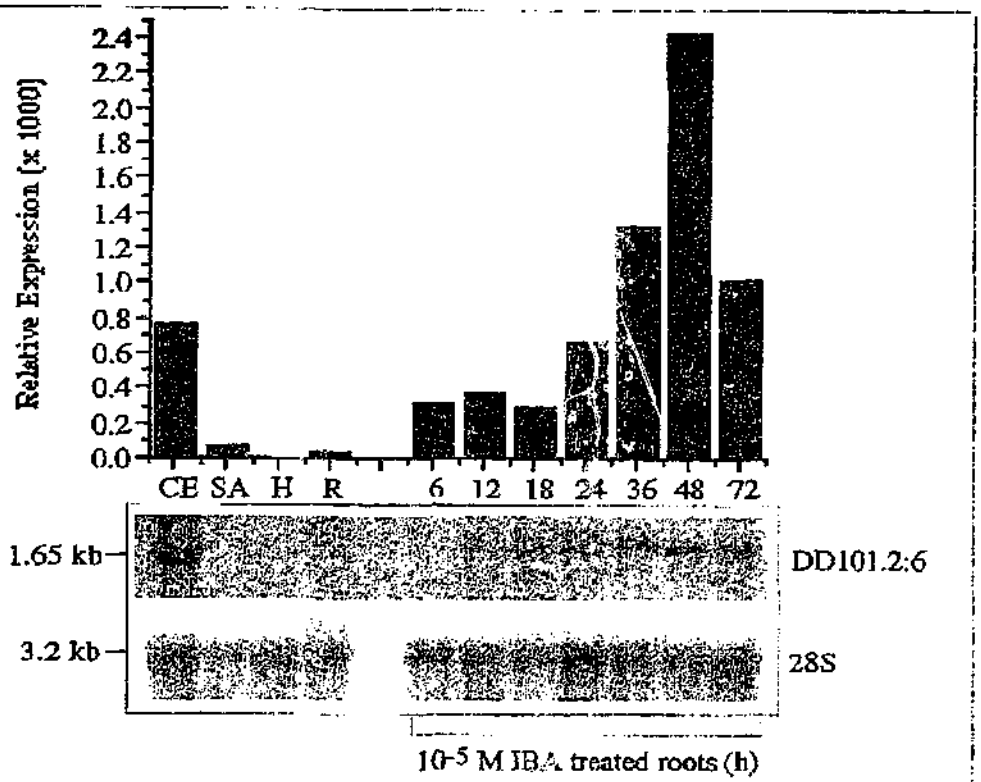


Figure 5.27: Expression profile detected by DD101.2:6 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

```
  1  CTAAAGTATACAATGCAGACAAAACATATTTTGTTATGAATGGTTCTACA
       K  V  Y  N  A  D  K  T  Y  F  V  M  N  G  S  T      16
 51  ACATCTAATAATATCGCTATAACTTCTGCAATAATGCCAGATGACTTAGT
     T  S  N  N  I  A  I  T  S  A  I  M  P  D  D  L  V     33
101  ATTATTTGATACAAATAATCATAAATCTGCTTATAATTCAGCACTTGTTA
      L  F  D  R  N  N  H  K  S  A  Y  N  S  A  L  V  K    50
151  AAGATGGCGGTCGTCCAGTTTATATGCAAACTTCAAGAGATTCTTATGGT
       D  G  G  R  P  V  Y  M  Q  T  S  R  D  S  Y  G     66
201  TTTATAGGTGGTATCTATGAAGAAGATTTTGATGAAAAATATTTAAGAGA
     F  I  G  G  I  Y  E  E  D  F  D  E  K  Y  L  R  E     83
251  ACAAGCTGCTAAAGTAGATCCAGAGAGAGCTAAATGGGAAAGACCATTTA
      Q  A  A  K  V  D  P  E  R  A  K  W  E  R  P  F  R   100
301  GATTAGCAATTATTCAATTAGGAACTTATGATGGAACAATTTATAATGCC
       L  A  I  I  Q  L  G  T  Y  D  G  T  I  Y  N  A     116
351  AAAAAAGTAGTAGAAAAAATTGGTCATTTATGTGATTACATTTTATTTGA
     K  K  V  V  E  K  I  G  H  L  C  D  Y  I  L  F  D    133
401  TT
```

Figure 5.28: The nucleotide and deduced amino acid sequence of the DD101.2:6 clone. The translated DNA sequence is underlined with the deduced amino acids indicated below. The residues denoted in red are involved in PLP binding.

ODC, LDC and ADC are large, pyridoxal-5'-phosphate (PLP) dependent decarboxylases that act on ornithine, lysine or arginine respectively (Momany *et al.*, 1995). Collectively, these enzymes are known as group III decarboxylases (Sandmeier *et al.*, 1994). The deduced DD101.2:6 amino acid sequence falls within the PLP-binding scaffold described for these enzymes (Hackert *et al.*, 1994; Momany *et al.*, 1995). The Gly-111 and Asp-133 of the DD101.2:6 amino acid sequence (Fig. 5.28) have been reported to be conserved amongst the bacterial aminotransferases, and represent two of four residues involved in PLP binding (Hackert *et al.*, 1994). Moreover, the structural similarities of the bacterial group III decarboxylases including the biodegradative forms of ODC, LDC and ADC, in addition to biosynthetic ODC, seem to suggest a common evolutionary ancestor (Sandmeier *et al.*, 1994).

The bacterial form of ODC does not share a high degree of homology to the plant, yeast or human form of the protein (Table 5.10). Pairwise GAP comparisons of the ODCI polypeptides from *L*30a with that from *Datura stramonium* and *N. tabacum* resulted in only 17.3% and 16.9% amino acid identity (44.5% and 42.6% similarity) respectively. Similarly, the amino acid sequence predicted by DD101.2:6 also shares little identity with the plant ODC enzyme. It is possible that the DD101.2:6 clone does not represent a nuclear gene in *E. globulus* but instead may be encoded by a mitochondrial or chloroplast gene that encodes a product with ODCI-like activity.

Species	*Lactobacillus* L30a		DD101.2:6	
	% aa identity	% aa similarity	% aa identity	% aa similarity
D. stramonium	17.3	44.5	47.4	20.3
N. tabacum	16.9	42.6	41.2	20.6
S. cerevisiae	22.4	46.6	49.2	20.5
H. sapiens	20.1	48.6	18.8	47.4

Table 5.10: Shared amino acid homology of *Lactobacillus* L30a ODCI (GenPept AAA64830) and DD101.2:6 to ODC enzymes from *D. stramonium* (SWISS-PROT accession number P50134), *N. tabacum* (GenPept accession number AAK13622), *S. cerevisiae* (SWISS-PROT accession number P08432) and *H. sapiens* (PIR accession number DCHUO).

Discussion

ODC is an enzyme that catalyses ornithine to produce putrescine. This represents the first and rate limiting step in the pathway for polyamine synthesis in cells from a wide range of organisms, including bacteria, humans and plants. In prokaryotes, the biodegradative forms of LDC and ADC catalyse the transformation of lysine and arginine to cadaverine and agmatine respectively. In addition to decarboxylation, these three PLP dependent enzymes also have a role in transamination and elimination. The bacterial group III decarboxylases exist in two forms, biosynthetic and biodegradative (Tabor and Tabor, 1985). While the biosynthetic forms are constitutive, the activity of the biodegradative form of the enzyme is inducible at low pH, suggesting a possible involvement in pH regulation by maintaining pH homeostasis, or by detoxifying the extracellular environment to facilitate extended periods of growth (Tabor and Tabor, 1985; Meng and Bennett, 1992). Consequently, biodegradative ODC is often also referred to as inducible ODC, or ODCI.

Acidification caused by auxin, has been implicated in a number of plant cell wall developmental processes, including exocytosis and pH dependent cell wall extension. Reports that attest to the induction of group III decarboxylases by an acidic pH environment, together with the observed auxin induced expression of DD101.2:6 during LRP formation, suggest that the ODCI-like gene product represented by this clone may play a role in preventing overacidification. Hackert *et al.* (1994) identified a GTP effector site in the *odci* sequence. This suggests that during protein transport via the GTP/GDP binding cycle, the active GTPase-ODCI complex may interact with an effector on the cytoplasmic face of the plasma membrane. This interaction would initiate events that lead to exocytosis (Ferro-Novick and Novick, 1993), allowing proteins to be transported across the membrane and enter the cell, before the bound GTP is hydrolysed by GAPs (possibly encoded by DD101.2:4).

Homeostasis of the cellular pH environment is likely to be achieved by a mechanism similar to that of H^+ removal during decarboxylation (Meng and Bennett, 1992). This is likely to be particularly important during auxin induced LR development in *E. globulus*, more specifically during auxin treatment, exocytosis and cell wall expansion, all of which effect or are affected by an acidic environment.

5.4.5 Clones Representing Genes Involved in Plant Nutrition

5.4.5.1 DD150.2:4: Glutamine Synthetase (*E* value = 0.0056)

Expression characteristics

The pattern of transcript accumulation detected by DD150.2:4 in the control samples indicated that auxin and cell division do not induce expression of this gene (Fig. 5.29). In the treated root samples, expression began to increase above the basal level, indicated by the untreated root, at 12 h after the initial exposure to auxin. The transcript continued to accumulate, so that by 18 h a level double that observed in the untreated root control was reached. This was proceeded by a slight drop in expression at 24-36 h, in the order of about 25%. To this point, the expression pattern coincides with an up-regulation in gene activity during the later stages of LRP induction. When induction is complete, gene activity decreases slightly as the induced primordia continue growth to maturity. The transcript is at its highest abundance at 48 h, corresponding to the time where LRP numbers are at their maximum. Transcript levels dropped by 33% at 72 h, by which time LRP formation is completed and emergence imminent.

Sequence analysis

DD150.2:4 is a 229 bp clone which represents a partial ORF (Fig. 5.30). A FASTA search of the NR nucleic database showed two matches with borderline significance scores, corresponding to a gene encoding the GS1 isoform of glutamine synthetase (GS) from *G. max* (*E*=0.0056) and *Lupinus luteus* (*E*=0.0093). In both cases, the region of significant DNA identity (75% and 74% respectively) did not extend over the entire length of the DD150.2:4 clone. Instead, the homology was confined to a sequence overlap of 72 and 73 nucleotides respectively, at the 3' end of the clone.

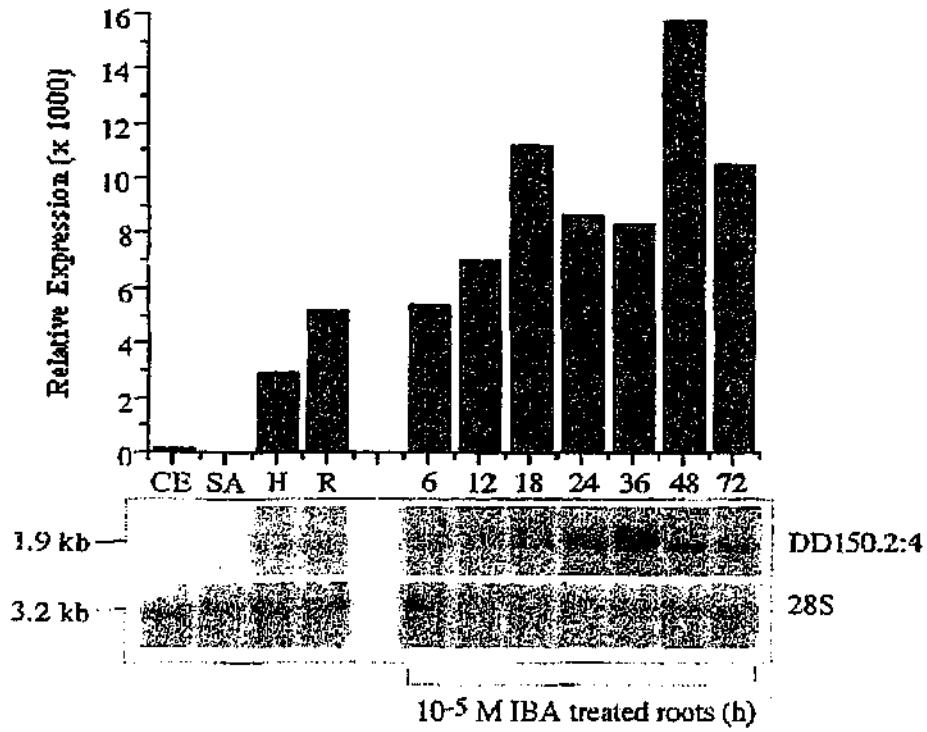


Figure 5.29: Expression profile detected by DD150.2:4 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

```
  1  cagggaagacagtgaagcgatcctatagtaagtgtttgtattatttcttc
 51  atatggatttctgtttgtagtcataagtctcggctcatttgatagaaatc
101  tagtgagatcagtgatttttatcattagagcttgtaggtatcttgagtac
151  tgatgacttcttacccctctttggtatcgactcaatgacacgtacagtCC
                                                      P     1
201  CCAAGCAATTTTCAAGGATCCGTTTAGGT
       Q   A   I   F   K   D   P   F   R                   10
```

Figure 5.30: DNA sequence of the 229 bp clone DD150.2:4. The putative untranslated sequence is represented in lowercase, while the proposed translated region representing an incomplete ORF is represented in uppercase and underlined with the deduced amino acid sequence indicated below.

A comparison of the polypeptide sequence deduced from this homologous region, showed that the 3' end of the DD150.2:4 clone encoded the same ten amino acid residues found at the 5' end of exon 4 of the *GS1* gene from *G. max* (Morey and Sengupta-Gopalan, 1998), and the corresponding *LlNGS1* gene of *L. luteus* (Boron and Legocki, 1993) (Fig. 5.31). It is possible that the remaining 5' portion of the DD150.2:4 clone is untranslated, representing the 3' end of the intron (intron 3) that precedes exon 4 of the published GS sequences.

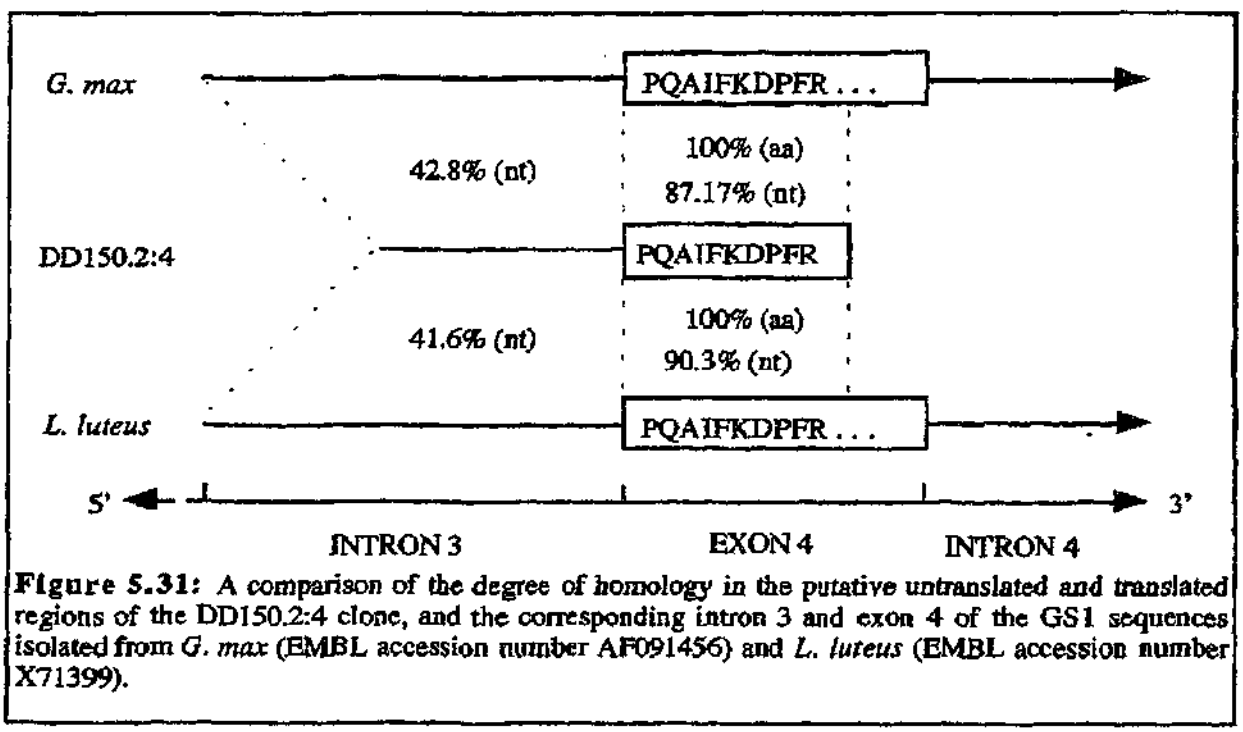


Figure 5.31: A comparison of the degree of homology in the putative untranslated and translated regions of the DD150.2:4 clone, and the corresponding intron 3 and exon 4 of the GS1 sequences isolated from *G. max* (EMBL accession number AF091456) and *L. luteus* (EMBL accession number X71399).

The complete DNA sequence from the genomic clones of *GS1* (Morey and Sengupta-Gopalan, 1998) and *LlNGS1* (Boron and Legocki, 1993), showed that in both species the gene is composed of 12 exons and 11 introns, spanning a region of approximately 4.1 and 3.5 kb respectively. In each species, the respective genes encode polypeptides of 356 amino acids in *G. max* and 353 amino acids in *L. luteus*.

In addition to *G. max* and *L. luteus*, cDNA clones corresponding to *GS1* have been isolated from a number of other plant species including, *Arabidopsis*, *Lupinus angustifolius*, *Pinus sylvestris*, *N. plumbaginifoila*, *N. tabacum*, and *N. sylvestris*. A comparison of the deduced amino acid sequences encoded by these clones shows that they are all structurally similar (Fig 5.32), sharing between 70.5 to 96.6% amino acid identity over the entire deduced polypeptide sequences. The lowest degree of amino acid homology was between *P. sylvestris* and *N. sylvestris*, while the highest was shared between the *L. luteus* and *L. angustifolius* polypeptides. This is indicative of the existence of a conserved gene structure across the angiosperm and gymnosperm plant species.

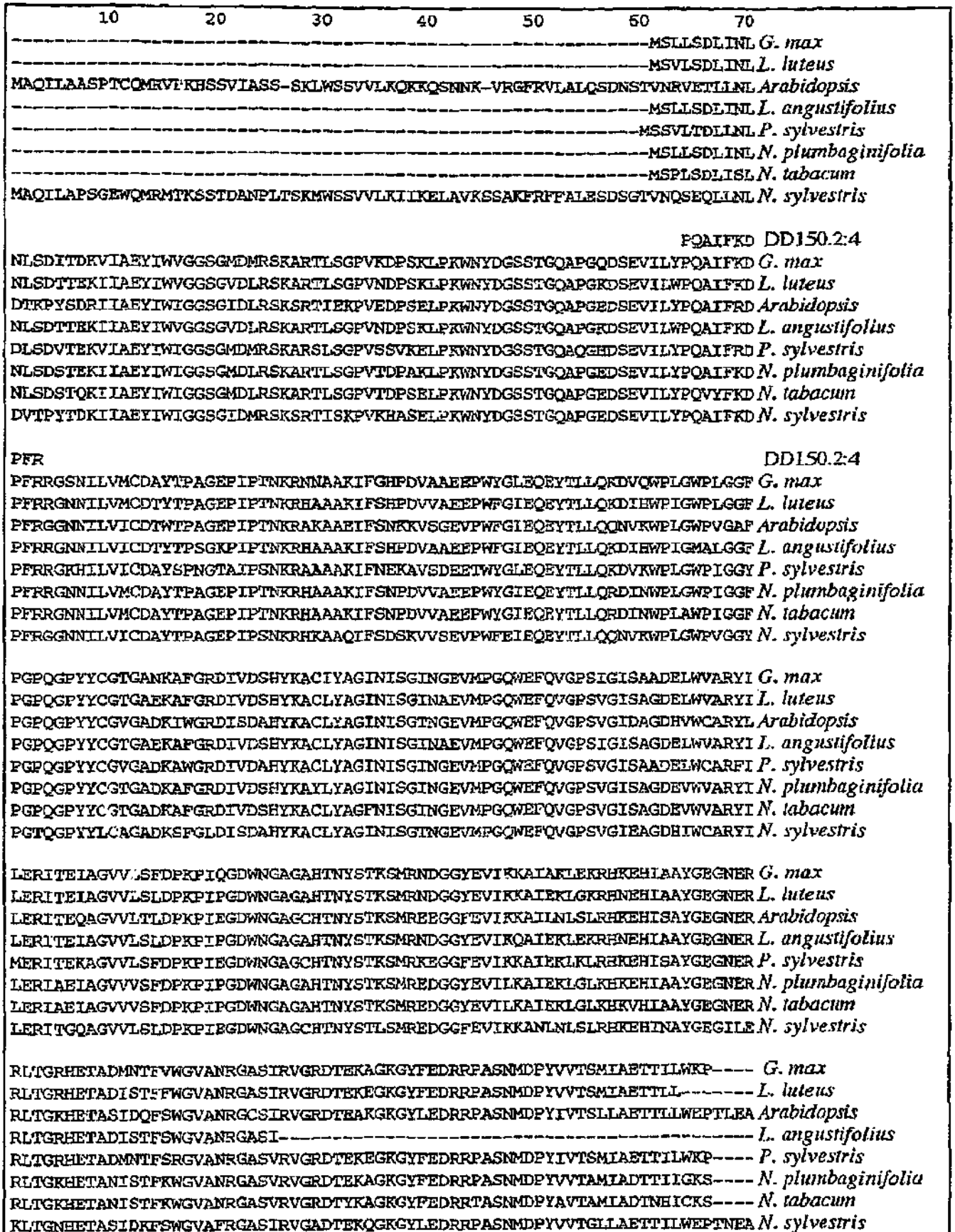


Figure 5.32: Amino acid sequence alignment for DD150.2:4 and the encoded GS proteins from *G. max* (EMBL accession number AF091456, *L. luteus* (EMBL accession number X71399), *Arabidopsis* (GenPept accession number AB015045), *L. angustifolius* (EMBL accession number X15578), *P. sylvestris* (EMBL accession number X66940), *N. plumbaginifolia* (GenPept accession number M19055), *N. tabacum* (EMBL accession number X95933) and *N. sylvestris* (EMBL accession number X66940). The conserved regions of the polypeptide are highlighted in red representing identical amino acid residues, while the blue regions represent similar residues.

Discussion

GS is a key enzyme involved in the assimilation of ammonia in higher plants, and its activity is regulated by the nitrogen status of cells (Temple *et al.*, 1998). Ammonia is available to the plant, from primary nitrogen sources contained in the soil, in the form of ammonium ions or as a product of the reduction of nitrate ions by the plant enzymes nitrate reductase and nitrite reductase (Temple *et al.*, 1998). Plant metabolic pathways provide a secondary source of ammonia from processes including photorespiration, phenylpropanoid metabolism and amino acid catabolism. Furthermore, legumes derive an additional source of ammonia from a symbiotic association with the nitrogen fixing bacterial species of *Rhizobium* and *Bradyrhizobium*, which are capable of reducing nitrogen to ammonia, before it is excreted into the plant cytoplasm (Temple *et al.*, 1998).

The assimilation of ammonia into organic form occurs primarily through the combined action of the two enzymes, glutamine synthetase and glutamate synthase, also known as glutamate 2-oxoglutarate aminotransferase (GOGAT) (Temple *et al.*, 1998). The action of these enzymes via two separate reactions, collectively referred to as the GS/GOGAT cycle, results in the net conversion of ammonium and 2-oxoglutarate to glutamate.

In the first of the two reactions which define the GS/GOGAT cycle, GS catalyses the formation of glutamine from ammonium and glutamate. During the second reaction, GOGAT catalyses the reductive transfer of the amide-nitrogen group of glutamine to 2-oxoglutarate. As a result, the two glutamate molecules formed can be used to top up the pool required for subsequent GS catalysis in a another GS/GOGAT cycle. Alternatively, the glutamate (and the glutamine formed after the first reaction) may be utilised as a substrate by aminotransferases for the biosynthesis of other compounds such as nucleotides, amino acids, chloropylls, polyamines and alkaloids (Temple *et al.*, 1998; Migge *et al.*, 2000). This cycle also constitutes the only possible means by which a plant can biologically synthesise organic nitrogen containing substances from an inorganic form (Temple *et al.*, 1998).

Two classes of GS isoenzymes have been identified in angiosperms, each one having a different cellular location in different plant organs (McNally *et al.*, 1983). DD150.2:4 displays homology to the gene that codes for the cytosolic isoenzyme, GS1. Different forms of GS1 have been found in roots, root nodules, leaves and flowers. The GS2 isoenzyme is restricted to the chloroplasts of photosynthetic tissues and root plastids.

Studies in various plant species have found GS1 to be encoded by a small multigene family (Bennet *et al.*, 1989). The pattern of gene expression amongst the individual *GS1* gene family members was reported to be differentially regulated during development and in response to stimuli such as, nitrogen fixation in legumes, pathogen attack, herbicide treatment, and water stress (Boron and Legocki, 1993; Bauer *et al.*, 1997; Morey and Sengupta-Gopalan, 1998; Pérez-García *et al.*, 1998).

GS1 gene expression in some species of legume is either not detectable in the root, or is constitutively expressed at a very low level in the absence of nitrogen fixation. In roots of *L. luteus* for example, mRNA slot blot hybridisation analyses showed that expression of the *LINGS1* gene was strongly and specifically induced in mature root nodules at the onset of nitrogen fixation (Boron and Legocki, 1993). Elsewhere, the *LINGS1* transcript was also observed in the leaves, but at a barely detectable level. Histochemical analysis of transgenic plants carrying a GUS gene driven by the *LINGS1* promoter supported these findings with very weak staining localised to the vascular tissue of leaves and stems, following a lengthy incubation period. The expression pattern observed by Boron and Legocki (1993), supported the notion that the *LINGS1* transcript was encoded by a nodule enhanced gene for GS.

The specific physiological function of each of the single GS1 gene products remains unclear. In roots, reports have implicated a central role in the primary assimilation of ammonia from soil. GS1 has also been suggested to function in the reassimilation of the ammonia released during processes including senescence (Sakurai *et al.*, 1996), nitrogen fixing in legumes and wound response (Pérez-García *et al.*, 1998). In the shoot, the GS1 protein has been localised to the phloem companion cells of the vascular tissues, where it may have a role in the synthesis of glutamine used in nitrogen transport (Sakurai *et al.*, 1996).

LRP formation and nodulation are two independent processes which share some common developmental characteristics. LRP for instance, are the product of cell division within the pericycle of the primary root. Similarly, root nodules arise from dividing cortical and pericycle cells. It would not be surprising to find that the two processes also share a number of common genes. It is proposed here that GS1 is an example of one such gene.

A cDNA clone corresponding to a GS gene was also isolated in an earlier screen for genes involved in LR formation in radish conducted Sussex *et al.* (1995a). In this study, the clone was isolated following a subtractive hybridisation of a radish cDNA library,

enriched for genes expressed at specific stages in LR development. Expression of the radish GS gene differed considerably from that observed in *E. globulus*, lending support to the notion that the differential expression of individual members of the GS gene family may be regulated in a species specific manner. In radish seedlings, GS expression was highest in both untreated roots, and roots treated with auxin for up to 8 h, after which expression ceased. In *E. globulus* roots, the onset of enhanced expression was observed to coincide with LRP induction, 12 h after the initial exposure to auxin. Unlike the radish roots however, GS expression above the level detected in the untreated root control, persisted until the highest level was observed after 48 h IBA treatment.

The availability and absorption of nutrients is known to greatly influence plant size, root:shoot partitioning of dry matter, and root morphology (Russell, 1977). Early studies conducted in barley, and later in wheat, showed that ammonium or nitrate supplied to a portion of the root system resulted in the localised proliferation of LRs (Drew and Saker, 1975; Bingham *et al.*, 1997). It is possible that the morphological change and the increase in cellular activity that is accompanied by the onset of a newly forming LR places greater demands for nutrients. During the LR forming process, the DD150.2:4 expression data suggests that the demand for nitrogen containing compounds may occur during growth of the LRP. As a consequence, the GS mediated assimilation of ammonia produced by the reduction of nitrate and nitrite may be triggered, reflecting an increase in the local GS expression.

5.4.5.2 DD192.1:6: Pyrophosphate Dependent Phosphofructokinase (*E* value = 4.2e18)

Expression characteristics

The DD192.1:6 probe detected an expression pattern in the control tissues, similar to that observed for DD150.2:4. Transcript levels were extremely low or absent in the cell suspension and shoot apex controls, indicating that expression was not associated with auxin treatment or cell division (Fig. 5.33). The 1.65 kb DD192.1:6 transcript began to accumulate above the untreated root control 12 h after initial exposure to 10^{-5} M IBA. By 18 h the transcript level had accumulated by an additional 48%. This was followed by a decrease, which in this case was only maintained at the 24 h time point. At 36 h, expression began to increase once again until the highest level was detected at 48 h, before transcript levels declined at 72 h. This expression profile correlates with up-regulated gene expression during late LRP induction, at maturity, and at maximum LRP saturation. The

period of LRP growth between induction and maturity appears to reflect a down-regulation in gene expression.

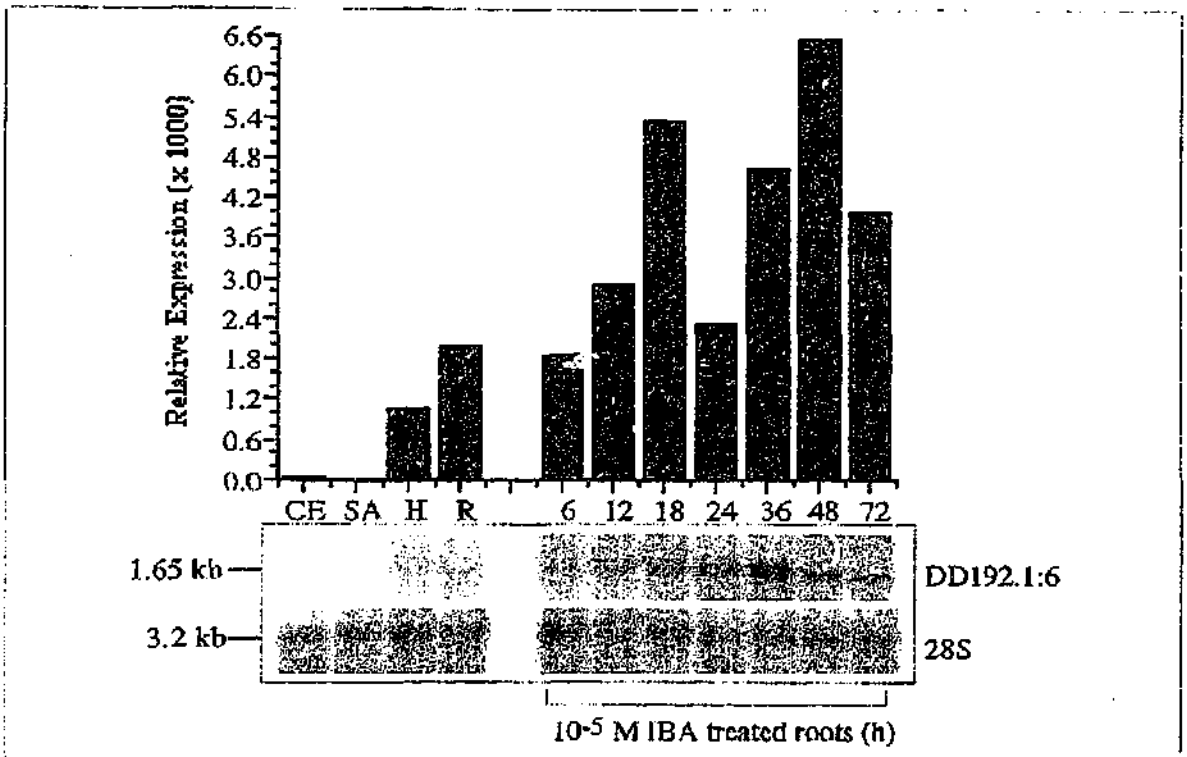


Figure 5.33: Expression profile detected by DD192.1:6 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

The nucleotide and deduced amino acid sequence of the DD192.1:6 clone corresponds to a gene encoding pyrophosphate:fructose-6-phosphate 1-phosphotransferase (also referred to as pyrophosphate-dependent phosphofructokinase; PFP) from *Solanum tuberosum* (potato), *Citrus* x *paradisi* (citrus fruit), and *Ricinus communis* (castor bean) (Table 5.11).

	Nucleotide Search			Amino Acid Search			
Plant	% identity	Length of nt overlap	*E* value	% identity	Length of aa overlap	*E* value	Reference
Potato	86.4	110	4.2e-18	95.8	48	8.6e-18	Carlisle *et al.* (1990)
Citrus fruit	80.2	111	1.5e-14	89.6	48	5.7e-16	Unpublished
Castor bean	75.8	120	1.4e-13	91.7	48	1.3e-16	Todd *et al.* (1995)

Table 5.11: Highest scoring results of FASTA searches. The DD192.1:6 nucleotide and deduced amino acid sequences were used as queries against the NR nucleic and NR protein databases respectively. EMBL accession numbers are as follows STPFB (potato), AF095520 (citrus fruit) and RCPDPBS (castor bean).

The 333 bp sequence of the DD192.1:6 clone is comprised of two putative translated regions separated by a region of non coding sequence (Fig. 5.34). Located at the 5' end of the clone, the first translated region is incomplete and encompasses 33 bp. This is followed by a 111 bp region of untranslated sequence, before a second translated region is found spanning 111 bp in its entirety. The last 78 bp at the 3' end of the clone is also probably untranslated.

PFP is a regulatory enzyme involved in primary carbohydrate metabolism in eukaryotes. Using inorganic pyrophosphate (PP_i) as the phosphoryl donor, PFP catalyses the reversible conversion of fructose 6-phosphate (Fru-6-P) to fructose 1,6-bisphosphate (Fru-1,6-P_2) and inorganic phosphate (P_i) (Xu *et al.*, 1989):

$$\text{Fru-6-P-PP}_i \overset{\text{PFP}}{\leftrightarrow} \text{Fru-1,6-P}_2 + \text{P}_i$$

PFP has also been implicated in having a role in the synthesis of PP_i, which is required by the sucrose synthase pathway for the breakdown of sucrose (Xu *et al.*, 1989).

```
   1  TGTAGTGGTAGAGACAAGATTGAGACTCCAGAGcaggtttttttacgcta
      C  S  G  R  D  K  I  E  T  P  E                    11
  51  atccgctcatatgaaagttgaagtgatcatagattgcagacttctttcag
 101  acctaaagcggttaaccggcaactgcattggacttttgacttttCAGTTT
                                              Q  F    13
 151  AAGCAAGCTGAAGAAACGACAAAGAAGCTCGATTTGGATGGACTTTTGGT
      K  Q  A  E  E  T  T  K  K  L  D  L  D  G  L  L  V  30
 201  CATTGGAGGGGACGATTCCAATACAAATGCGTGCCTTCTTGCCGAGAACT
       I  G  G  D  D  S  N  T  N  A  C  L  L  A  E  N  F  47
 251  TCAGGtaggactaggattctgattaatctgtatgcagtggctattgggtc
        R                                                 48
 301  cgtaacctttggattgggaacgtgatcattctg
```

Figure 5.34: DNA sequence of the 333 bp clone DD192.1:6. The untranslated sequence is represented in lowercase, while the putative translated regions representing a possible partial and a complete ORF are in uppercase and underlined with the deduced amino acid sequence indicated below.

Enzyme purification studies from a number of plant species indicate that PFP is generally made up of two different subunits, designated as α and ß (Yan and Tao, 1984). The role played by each of the subunits is largely unknown. Yan and Tao (1984) proposed that the active site for PFP may be located on the ß-subunit (PFP_β), suggesting its role may be a catalytic one. This idea was supported by Carlisle *et al.* (1990) who observed the presence of four conserved amino acid residues (Gly-51, Arg-115, Asp-145, Ser-147) between the potato PFP_β polypeptide and the *E. coli* ATP-phosphofructokinase I (PFK), located at identical relative positions. These four residues are known to interact with the phosphate region of ATP and not the adenosine, suggesting that this site may be involved in pyrophosphate binding (Carlisle *et al.*, 1990). On the other hand, the α-subunit may

have a function in PFP activation by Fru-2,6-P_2 but may not necessarily be essential to PFP activity (Cheng and Tao, 1990).

Closer comparison of the nucleotide and amino acid sequences of the published potato PFP gene (Carlisle *et al.*, 1990) and the DD192.1:6 clone, revealed that the high degree of sequence similarity was confined to PFP$_\beta$ (Table 5.12). Moreover, the majority of nucleotide divergence between the two sequences can be attributed to wobble, as most nucleotide differences occurred at the third base position of codons. The amino acid comparison between the potato PFP$_\beta$ and DD192.1:6 (Fig. 5.35) also revealed the presence of the conserved Arg-115, Asp-145 and Ser-147 residues which are thought to confer pyrophosphate binding (Carlisle *et al.*, 1990).

PFP subunit	% nt identity	% aa identity
α	52.1	47.9
β	87.5	95.8

Table 5.12: The degree of nucleotide and amino acid identity between DD192.1:6 and the potato PFP α- and β-subunits.

```
                10        20        30        40        50        60
192.1:6   ------------------------------------------------------------
Potato    MALLHLPPVTQRRLQSFFLPYTDNHVSLVPDDSGDVAMNQILKIGVVLSGGQAPGGHNVI

192.1:6   ----------------------------------------------------CSGRDKIET
Potato    SGIFDYLQTHCKGSTMYGFRGGPAGVMKGKYVVLTPEFIYPYRNQGGFDMICSGRDKIET
                                                             *********
192.1:6   PEQFKQAEETTKKLDLDGLLVIGGDDSNTNACLLAENFR--------------------
Potato    PEQFKQAEETAKKLDLDGLVVIGGDDSNTNACLLAENFRSKNLKTRVIGCPKTIDGDLKS
          **********.********.********************
192.1:6   ------------------------------------------------------------
Potato    KEVPTSFGFDTACKIYAEMIGNVMIDARSTGKYYHFVRLMGRAASHITLECALQTHPNVT

192.1:6   ------------------------------------------------------------
Potato    LIGEEVFAKKLTLKNVTDYIADVVCKRAESGYNYGVILIPEGLIDFIPEVQQLIAELNEI

192.1:6   ------------------------------------------------------------
Potato    LAHDVVDEAGVWKKKLTPQCLELFELLPLAIQEQLLLERDPHGNVQVAKIETEKMLIQMV

192.1:6   ------------------------------------------------------------
Potato    ETELDQRKQKGAYNAQFKGQFHFFGYEGRCGLPSNFDSTYCYALGYGAGSLLQSGKTGLI

192.1:6   ------------------------------------------------------------
Potato    SSVGNLAAPVVELTVGGTALTALMDVERRHGKFKPVIKKAMVELEGAPFKKFASKREEWA

192.1:6   ----------------------------------
Potato    LNNRYINPGPIQFVGPVANKVNHTLLLELGVDA
```

Figure 5.35: An alignment of the potato PFP$_\beta$ subunit (GenPept accession number AAA63452) and the deduced amino acid sequence of the DD192.1:6 clone. Amino acid residues which are identical in both sequences are denoted by an asterix (*) and similar residues by a dot (.). The conserved Arg-115, Asp-145 and Ser-147 residues suggested to participate in phosphate binding are highlighted in red.

Discussion

In higher plants the PFP protein is located almost ubiquitously in all tissues. However, enzymatic activity of PFP appears to be highest in young developing and starch storing tissues which are undergoing increased rates of assimilate usage (Xu *et al.*, 1989). Furthermore, PFP activity in crude extracts has been found to vary depending on a number of factors including the plant species, the tissue type, age and development (Nielsen and Wischmann, 1995), growth conditions and nutrition status (Theodorou *et al.*, 1992).

The exact role for PFP in plant metabolism is yet to be determined, although it has a suggested involvement in gluconeogenesis, regulation of the cytosolic PPi concentration, equilibration of the hexose- and triose-phosphate pools, adaptability to stresses such as wounding and anaerobiosis (Sonnewald *et al.*, 1994). Additional roles proposed for PFP in glycolysis, adaptation to suboptimal temperature and limited phosphate and nitrogen availability are greatly debated with conflicting results having been reported (Paul *et al.*, 1995). It is likely that PFP function is dependent upon the plant species and the plant tissue under investigation.

Expression characteristics, as determined by Northern blot analysis, differed between the two species. In potato, PFP_{β} expression was ubiquitous, with a single 2.1 kb transcript detected at similar levels in total RNA samples isolated from mature tubers, rapidly elongating sprouts and leaves. In *E. globulus* however, the DD192.1:6 probe hybridised a slightly larger transcript of 2.3 kb in root and hypocotyl tissues, but was undetected in the shoot apex (Fig 5.33).

During LR differentiation, the *E. globulus* root is undergoing rapid changes in cellular development, to initiate and sustain growth of the LRP. In addition to phytohormones, LR initiation also requires the correct balance of nutrients such as carbohydrates and nitrogenous compounds (Friend *et al.*, 1994). The carbohydrates in particular, are considered to provide the major source of energy during root formation (Haissig *et al.*, 1992). Starch, fructosans and sugars, including sucrose, glucose and fructose, are the predominant forms of carbohydrates available to the plant (Ho, 1988). In 1975, Altman and Wareing were able to show that when hypocotyl cuttings from bean were treated with IAA, sugars accumulated to the site of root formation. This observation suggested to the researchers that auxin may act to stimulate adventitious rooting by increasing the availability of sugar at the site of root initiation. In more recent studies, sucrose was found to be essential to maintain growth, elongation and viability of the apex in cultured roots of tomato (Barlow and Adam, 1988). In olive a supply of sucrose was found to enhance the stimulatory affects of IBA in AR formation and improve root length

(Wiesman and Lavee, 1995). In this study, rooting of IBA treated olive cuttings was improved from 45% to 52% when sucrose was also supplied, and stimulated further (61%) when additional sucrose was also applied every ten days after IBA application. Additional evidence illustrating the important role carbohydrates have in rooting comes from glucose feeding experiments conducted in wheat. Here LRP initiation was increased with 50 mM glucose for 15 h in the absence of exogenous auxin application (Bingham *et al.*, 1997).

PFP may play a role in carbohydrate metabolism by regulating glycolysis and sucrose biosynthesis in the cytosol (Sonnewald *et al.*, 1994). It is likely that LR development involves changes in carbohydrate requirements and utilisation within the root, leading to the observed increase in PFP transcript accumulation, identified by the DD192.1:6 clone during the later stages of LRP initiation and growth.

5.4.6 Clones Representing Previously Uncharacterised Novel Genes

5.4.6.1 DD150.1A:1: Unknown

Expression Characteristics

The pattern of transcript accumulation in the treated root samples (Fig 5.36) was representative of differential expression during LRP formation. Expression at 6 h after initial auxin application, was only marginally above the untreated root control. Transcript abundance dramatically increased at 12 h before dropping back down to control levels at 18 h. At 24 h, the detected expression began to increase again, so that by 36-72 h, expression reached peak levels that were within the range detected at the shorter 12 h treatment. This profile illustrates up-regulated gene expression during the middle stages of LRP induction and again at maturity. During the later stages of induction, the gene represented by this clone appears to be down-regulated until formation of the primordia is complete, and expression once again reaches maximum levels. The comparatively low transcript levels detected in the cell suspension and untreated shoot apex suggest that the observed expression profile was not enhanced by the auxin treatment or cell division.

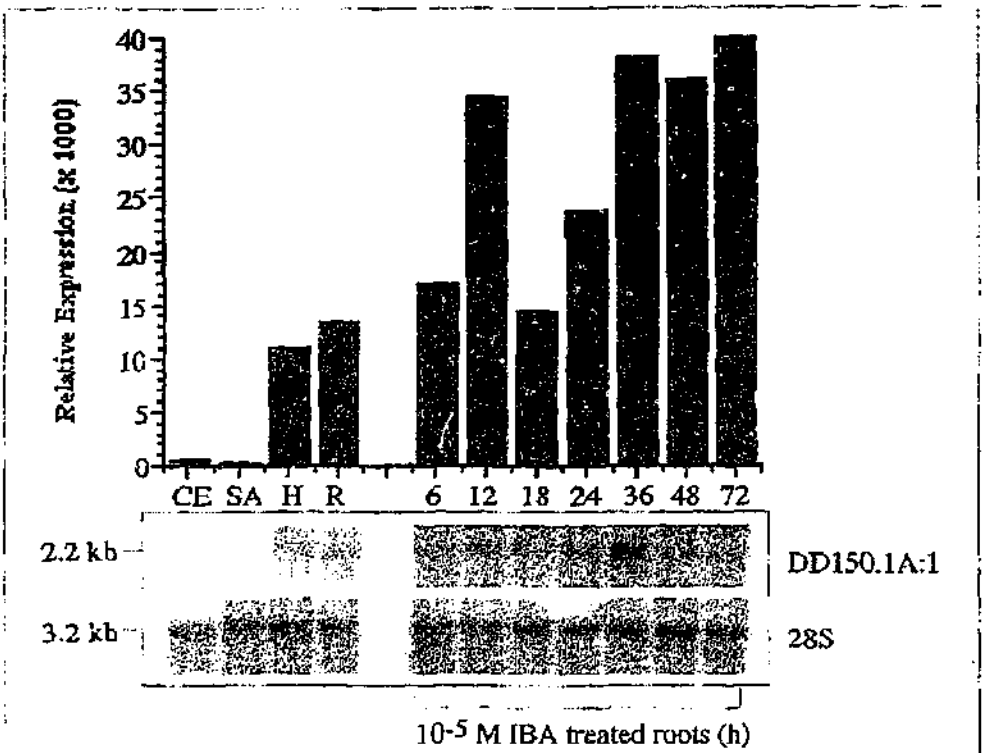


Figure 5.36: Expression profile detected by DD150.1A:1 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

The cDNA sequence of the DD150.1A:1 clone is 501 bp long (Fig. 5.37). FASTA searches of the NR nucleic and protein databases were conducted using the DD150.1A:1 nucleotide and deduced amino acid sequences as queries. The best, and only significant match identified, was to the uncharacterised sequence of *Arabidopsis* chromosome 3 BAC clone F14P13, sharing 60% identity over 322 nucleotides (E = 3e-13), and 50% identity over 106 amino acids (E = 5.3e-12).

```
  1  CCATTCATGTCGAGAGAGGATTGGAGGGTTGCCGAGAGGTTTCAAGCCGG
     P  F  M  S  R  E  D  W  R  V  A  E  R  F  Q  A  G    17
 51  GTTGTCATCTTTTGTAGGCATTGATGATTTGACAAATAATACTCTTGCTG
      L  S  S  F  V  G  I  D  D  L  T  N  N  T  L  A  E   34
101  AAGGTGGGAATGATTCGTCGTTGTTTTCTTTCCAACCCAAAGGCTGCAGC
       G  G  N  D  S  S  L  F  S  F  Q  P  K  G  C  S     50
151  TTTTCTGTACAGGATGCAGAAAAGGATTTGATGGAACTAAGAAAAATGAA
     F  S  V  Q  D  A  E  K  D  L  M  E  L  R  K  M  N    67
201  TAGACATGCTTCTGCTGAATGGCTCGACAAAGGATCAAACGACGTCATTG
      R  H  A  S  A  E  W  L  D  K  G  S  N  D  V  I  G   84
251  GATCGTCAACTTCTAGGTTGGGCACACGGAGGAAGAGTTTTGCTGACATT
       S  S  T  S  R  L  G  T  R  R  K  S  F  A  D  I    100
301  CTTCAGGTAATAAGTACAACTACGAGTAATTTGCTAGGGGGAAAAGTAGA
     L  Q  V  I  S  T  T  T  S  N  L  L  G  G  K  V  D   117
351  TAAAAAGAAATTGACCACAACTTTACTTGCTTGAtgaggacaaaattgcc
      K  K  K  L  T  T  T  L  L  A  STOP                  127
401  atcaaactttcctggtctatctccacacagtttgattctcttggtgttta
451  tctcttaggcttgcccaaaaaaaagaacgttttgactttgttgcagcttg
501  a
```

Figure 5.37: Nucleotide and deduced amino acid sequence of the DD150.1A:1 clone. The putative translated portion of the 501 bp sequence is in uppercase and underlined, with the deduced amino acid sequence indicated below.

5.4.6.2 AP19A:1 Unknown

Expression characteristics

The AP19A:1 transcript exhibited a pattern of accumulation representative of auxin regulation during LRP formation (Fig. 5.38). This was indicated by expression levels detected in the cell suspension, more than twice that observed in the untreated tissues.

Expression of this 1.5 kb transcripts was down-regulated following a 6 h auxin treatment, with transcript detected at levels below that of the untreated root control. The level began to increase above the control at 12 h. The transcript accumulated most substantially between 36 and 48 h, when the detected level doubled to reach its maximum at the 48 h time point. By 72 h the transcript abundance had declined, back to similar levels detected at 36 h. This expression profile coincides with down-regulated gene expression during early LRP induction (6 h). Half way through this stage (12 h), gene expression was activated to the basal level and up-regulated during LRP growth until 48 h when maximum LRP numbers are reached and formation is completed.

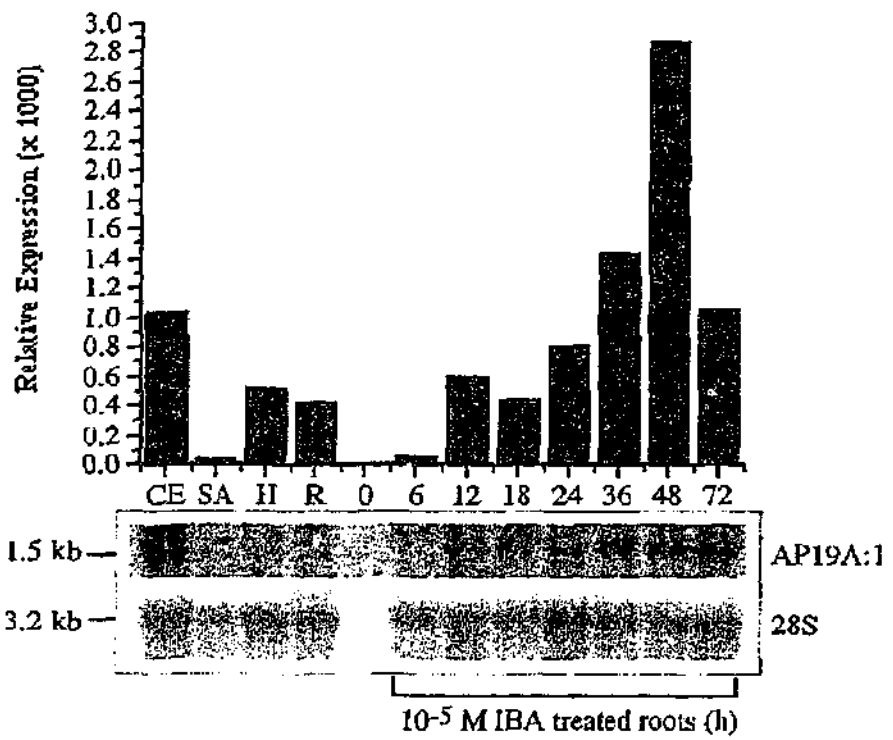


Figure 5.38: Expression profile detected by AP19A:1 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

Sequence analysis

Homology searches using the 96 bp AP19A:1 clone (Fig. 5.39), failed to reveal any significant sequence identity with any gene, clone or protein sequence in the current NR nucleotide and protein databases.

```
  1   GGGAAATCATAGAGAGAGGGACCGTCGCGCCTCCGCAGATCTCCACCGTC
 51   TTCCTCCCTCTCGCCTCCGAAATGGTCACCTTCAGGTTTCACCAGT
```

Figure 5.39: Nucleotide sequence of the AP19A:1 clone.

5.4.6.3 AP21B:4: Unknown

Expression characteristics

AP21B:4 proved to be a difficult clone to categorise because the three transcripts it detected each gave entirely different expression profiles. The smallest 1.2 kb transcript displayed a profile characteristic of an LRP specific clone (Fig. 5.40). This particular transcript was detected at very low levels in the cell suspension indicating that its levels did not accumulate in response to auxin treatment. Levels in the untreated shoot apex and hypocotyl were negligible. In 10^{-5} M IBA treated roots, this transcript accumulated to a maximum level at 24 h during which time the LRP are growing in size, then fell slightly at 36 h. A second increase was detected at 48 h when LRP numbers are highest and formation is completed, before finally declining at 72 h.

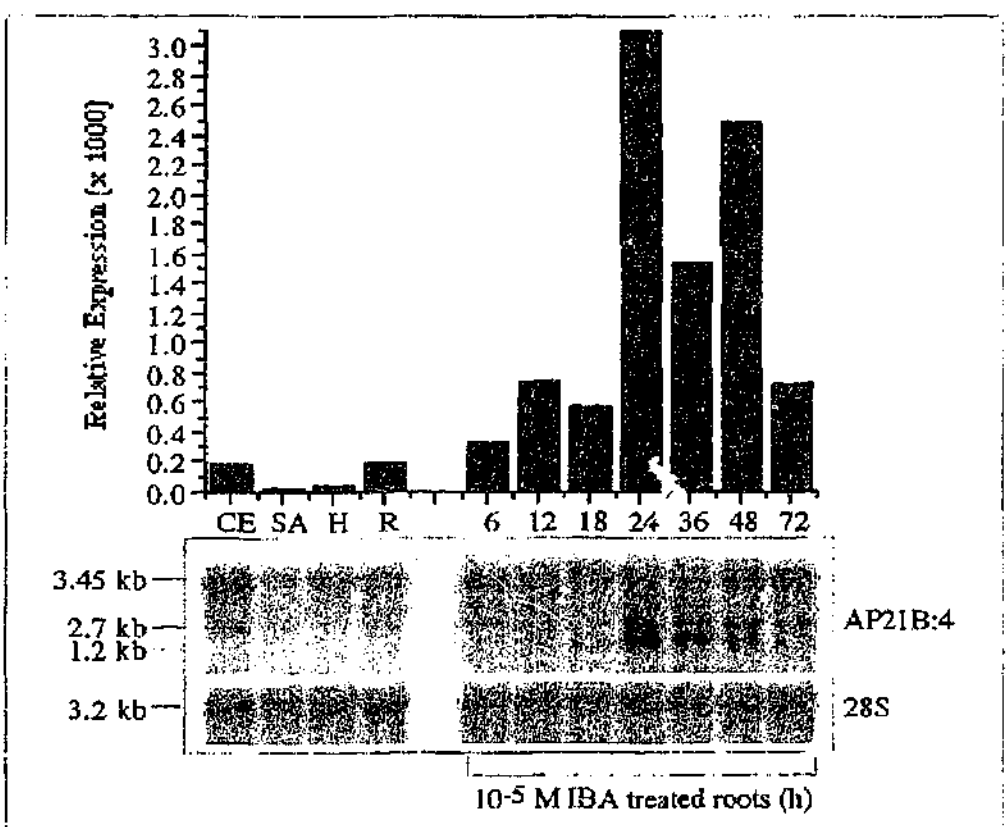


Figure 5.40: Expression profile of the 1.2 kb transcript detected by AP21B:4 on Northern blots containing total RNA from a *E. globulus* cell suspension (CE), untreated explants including, shoot apices (SA), hypocotyls (H), and roots (R); roots treated with 10^{-5} M IBA for 6, 12, 18, 24, 36, 48 and 72 h. The graph shows the calculated relative expression following correction for loading differences against the 28S rRNA transcript detected by the pTA71 probe.

In contrast, the 3.45 kb transcript was constitutively expressed, while the 2.7 kb transcript was induced by auxin, accumulating with increased time on IBA. In the control tissues this transcript was detected in the untreated shoot apex, hypocotyl and root tissues, and was elevated in the cell suspension sample, indicating that expression was stimulated by the presence of auxin rather than differentiation of the LRP.

The presence of three transcripts may suggest that AP21B:4 represents one of a group of related genes. Alternatively, the represented gene may undergo differential splicing, or may possess more than one promoter to regulate expression in response to different conditions.

Sequence analysis

The 327 bp sequence of the AP21B:4 clone (Fig. 5.41), and its amino acid sequence predicted in all possible frames, did not have any significant degree of homology with sequences in the current NR nucleic and protein databases. This suggests that this clone may represent a novel and previously uncharacterised gene. Isolation of a full length cDNA and genomic clones, will facilitate a better understanding of the involvement this AP21B:4 represented gene has in LR formation. This will provide the necessary tools for a detailed investigation into the spatial expression pattern exhibited by this gene, facilitate promoter studies and help predict the structure and function of the encoded protein.

```
  1   TTCATCATAAAAGCTTTCAAGCTTCAAGTACAATCAATTAGCACAAAAAT
 51   AAAGAACAAAAATACTCAAATCATTCTTAATTTTGAAAGGACAATATCCT
101   TCATTTCACCCTACTACTCTCTTTTCCAAGAGGTAGCTCATAGCTGACTA
151   GACTACTTAGAGTGGAAAAAGCAAAATACTTTCTTTTTCCAACACCGTCC
201   TATATTTTATTTTTGCCAATCACATAAAACACACACGACTAAATTCACGG
251   ACGATCAATCTCCATTTCTTCGTCTCCCAAGCAAACCACGCACAACCCTC
301   AGACAGTAAAGTTGACGACCGACCGGT
```

Figure 5.41: Nucleotide sequence of the AP21B:4 clone.

5.5 DISCUSSION

5.5.1 Overview

The results presented here show distinctive expression profiles that illustrate a preference for the middle to late stages of LRP development. Of the 36 clones analysed in Northern blots, 15 detected specific mRNA accumulation during LR development in IBA treated root explants of *E. globulus*. The pattern of transcript accumulation detected by seven of these clones occurred independently of auxin, with transcripts absent or negligible in cell suspensions cultured in the presence this phytohormone. Conversely, the expression profiles detected by the other eight clones appeared to be regulated by auxin treatment, with cell suspension cultures also accumulating transcripts above the basal level detected in the untreated tissues.

Sequence analysis of the cloned inserts and computer assisted searches against the NR nucleic DNA database was used to correlate the 15 clones to known genes. The deduced amino acid sequences of these clones was used to search the NR protein database in order to elucidate a putative function for the encoded protein. Twelve of the 15 clones shared significant nucleotide or amino acid homology to previously characterised genes. Of the remaining three clones, one matched an entry to an unpublished sequence with unknown function, whilst the other two represented novel sequences with no significant match found at the nucleotide or amino acid level.

5.5.2 Assessment of Differential Display and cDNA Subtraction

The DDRT-PCR and cDNA subtraction approaches were both used successfully to isolate cDNA clones that represent genes involved in LR formation in *E. globulus*. During the characterisation analyses, it became apparent that distinctive differences in the type, size, structure and relative abundance of each clone, was dependent upon the particular method employed for its isolation.

Clones derived from DDRT-PCR were relatively large, ranging in size from 229 to 786 bp (mean = 430 bp). However, the sequence of these cDNA clones is often interrupted by one or two introns or UTRs. These non coding regions may limit the usefulness of the cloned sequences when searching for homology in protein databases, to ascertain a

possible inferred function. The cDNA subtracted clones were smaller, ranging in size from 60 to 513 bp (mean = 180 bp), and did not typically contain UTRs.

The higher incidence of UTRs observed in DDRT-PCR clones can be attributed to the use of total RNA as the starting material in this procedure. As a consequence, the cDNA synthesis reaction may have been primed from the primary transcript before it was processed and converted to mRNA. The primary transcript would still contain the untranslated sequences, or introns, embedded within its coding regions. This problem can be easily overcome by using mRNA instead of total RNA for template in the cDNA synthesis step of the technique.

Since mRNA was used as the starting material for the cDNA subtraction procedure, UTRs were less frequent. RNA splicing of the primary transcript ensures the removal of introns to produce an mRNA molecule comprised entirely of coding sequences. As such, when used as a template in cDNA synthesis, the resulting cDNA molecules were free of UTRs. The occasional appearance of UTRs in the clones derived from the cDNA subtraction may have been a reflection of a small proportion of total RNA carry through during mRNA purification.

The cDNA subtraction procedure appeared to be more specific for identifying genes involved in LR formation, with 64.3% of the analysed clones hybridising to LRP (and ARP) specific transcripts (expression profiles I and II, Table 5.13). This was almost twice the frequency of that observed for the DDRT-PCR clones, where 33.3% of the total number of clones analysed fell into the same two categories. The specificity of the DDRT-PCR procedure may be improved by further enhancing gel resolution to simplify accurate reading of the displayed arrays.

Expression profile	# DDRT-PCR clones	# Subtraction clones
I. LR specific	4 (22.2%)	3 (21.4%)
II. LR specific & auxin induced	2 (11.1%)	6 (42.9%)
III. Cell division	1 (5.6%)	2 (14.3%)
IV. False positive	3 (16.7%)	5 (35.7%)
V. No signal	8 (44.4%)	2 (14.3%)
TOTAL	18	14

Table 5.13: A summary of the number of clones isolated from DDRT-PCR and cDNA subtraction detecting each of the five possible expression profiles observed.

The cDNA subtraction procedure could also be improved upon by making the tester and driver samples more specific, to increase the chances of targeting the sequences of interest. As designed in this study, the experimental procedure allowed for the isolation of both auxin induced and non induced sequences by using untreated tissues for driver and auxin treated tissues for tester. It became necessary to include the cell suspension sample in the Northern analyses to help identify clones which represented auxin induced genes but were not necessarily involved in LR formation. Had a cell suspension sample been available at the time, its inclusion as a component of the driver sample would have undoubtedly enhanced the specificity of the cDNA subtraction procedure.

The increased frequency of LRP associated clones isolated by the cDNA subtraction was accompanied by a relative increase in the number of false positives. The subtraction procedure resulted in about twice as many false positives as that offered by DDRT-PCR under optimal conditions. Again, improving the specificity of the driver and tester samples used for subtraction, and further optimisation of the DDRT-PCR gel resolution can aid towards alleviating this problem.

In contrast, the DDRT-PCR procedure yielded more clones which failed to give a signal when used as probes for Northern blots. In fact, for the DDRT-PCR clones this occurrence was observed at a frequency of 44.4%, a similar level as that observed by Li *et al.* (1994). A number of possible explanations have been proposed to account for this incidence, one of which is that the clones may represent genes which are expressed at a level too low to be detected by Northern blotting. If this is indeed the case, the high proportion of DDRT-PCR clones that fall into this category may reflect an increased sensitivity of DDRT-PCR to the isolation of less abundantly represented sequences, such as transcription factors. This hypothesis could be tested further by the use of more sensitive methods of expression analyses, such as quantitative RT-PCR or RNase Protection.

Application of both techniques using the conditions described, resulted in 46.9% of the analysed clones showing correlated expression with LRP development. The largest proportion of these clones were derived using the cDNA subtraction procedure. However this was slightly offset by the higher frequency of false positives generated by the same technique. A number of clones which detected no expression by Northern blot still remain to be studied by other means. If these clones prove to be LRP specific, the potential for isolating sequences corresponding to the lower represented mRNAs may be more successfully provided by DDRT-PCR. A summary of the analysed advantages and disadvantages of these two techniques as used in this study, is represented in Table 5.14,

and should be considered if undertaking a similar molecular search for genes involved in developmental processes.

DDRT-PCR	
Advantages	**Disadvantages**
• Large sized clones (229 - 786 bp)	• Isolated clones may contain UTRs
• Low number of false positives	• Lower specificity
• Possible higher sensitivity to less abundant mRNAs	• Time and labour intensive to optimise conditions and screen an extensive number of primer combinations
cDNA Subtraction	
Advantages	**Disadvantages**
• Infrequent occurrence of UTRs	• More false positives
• Obtain a cDNA library for differential screening if desired	• Less sensitive to low abundant mRNAs
• Relatively quick and easy	

Table 5.14: A summary of the relative advantages and disadvantages determined for the DDRT-PCR and cDNA subtraction methods undertaken in this study.

5.5.3 Gene Preferences Exhibited by Each of the Molecular Cloning Procedures

It appears that the two cloning procedures each exhibited some bias towards the isolation of *E. globulus* cDNA clones representing genes involved in particular plant developmental processes. The majority of characterised clones isolated by cDNA subtraction showed significant sequence homology to genes encoding cell wall proteins (AP9:3/ß-expansin, AP21B:6/AGP and AP1:1/extensin), genes encoding proteins required for cellular functions including transcriptional activation, protein synthesis, protein processing and protein degradation (AP2:2/RNA polymerase, AP3:3/rpL2, AP5:1/CRT and AP12:3/polyubiquitin). In contrast, the clones isolated by DDRT-PCR could be grouped as representing genes with a possible involvement in the pathway for protein transport and secretion (DD192.1:2/synaptobrevin/VAMP, DD101.2:4/rabGAP and DD101.2:6/ODCI-like), and those encoding enzymes of metabolic pathways involved in plant nutrition (DD150.2:4/GS1 and DD192.1:6/PFP_{β}) The proposed induction of ODCI-like activity by an acidic environment to maintain pH homeostasis, also implicates this protein with a

possible involvement during acid growth to mediate cell wall expansion. Three novel clones, DD150.1A:1, AP19A:1 and AP21B:4, represented sequences with no obvious similarity to any entries of the nucleic and protein database searched.

5.5.4 Future Directions

Characterisation of the expression patterns detected by each of the 15 clones described could be expanded to include a detailed study of the spatial location of the corresponding mRNA transcripts. Analysis by *in situ* hybridisation would serve to examine expression at a cellular level to help identify the cell types accumulating the transcript of interest. Moreover, transcripts located specifically in cells of the LRP will be distinguished from those found in the surrounding cells or tissues of the root. The information collected from such studies will help to further decipher and/or confirm a role for each of the represented genes in LR development.

An investigation into the expression characteristics exhibited by each of the clones in response to phytohormones other than auxin, which are known to influence LR development is also merited. Interactions with cytokinin is an obvious starting point with synergistic and antagonistic effects on LR development depending on the concentration applied to *E. globulus* root explants (Pelosi *et al.*, 1995a and 1995b). Expression patterns detected following cytokinin treatment in combination with, or independent of auxin may be compared with the frequency of LRP and emerged LRs in treated root explants of *E. globulus*. The observations made may provide further information regarding possible phytohormone interactions modulating gene expression during development of the LR.

Similarly, treatment with the RNA and protein synthesis inhibitors, actinomycin-D and cycloheximide respectively, was shown to inhibit auxin induced LRP formation in *E. globulus* (Pelosi *et al.*, 1995a and 1995b). This finding suggested that *de novo* transcription and translation of essential genes was required for LR development. It should be feasible to determine whether the isolated clones represent genes regulated at the level of transcription or translation, by examining the expression profiles detected following treatment with either inhibitor.

Isolation of the full length cDNA and genomic clones will provide a valuable tool towards understanding the regulation and structure of the analogous genes. The identification of recognition signals and consensus sequences will provide an insight into

the possible function of the encoded protein. Genomic clones will be of particular interest to search for possible regulatory elements within the promoter regions.

CHAPTER SIX

Auxin Induction of Lateral Root Formation in *Arabidopsis thaliana* and the Isolation of Mutants Showing a Requirement for Indeterminate Growth of the Primary and New Lateral Root Meristem

6.1 INTRODUCTION

The isolation of genetic mutants provides a mechanism by which individual steps in the developmental pathway for LR formation can be identified. As has been noted, LR mutants with altered growth responses to exogenously supplied phytohormones can help identify some of the components of the pathway, facilitating a better understanding of the processes by which plant phytohormones can regulate LR growth.

The extremely long generation time of *Eucalyptus* renders this genus unsuitable for isolating mutant phenotypes and conducting segregation based analyses. Homologues of two *Arabidopsis* floral meristem genes, *LEAFY* and *APETALA1*, have been cloned and characterised in *Eucalyptus globulus* (Kyozuka *et al.*, 1997; Southerton *et al.*, 1998). These studies suggest that basic genetic processes are likely to be conserved between plants. As such, a greater understanding of the pathway that directs LR formation in *Arabidopsis* is likely to be of direct relevance to understanding the process in all higher plants, including eucalypts. This section of the study takes advantage of the considerable potential of *Arabidopsis thaliana* to identify and study genetic components in the pathway for LR development and growth.

6.2 AIM

The aim of this section of the study was to identify putative mutants of *Arabidopsis thaliana* showing altered or aberrant LR formation in response to treatment with auxin and cytokinin. The effect of phytohormone treatment on wildtype seedlings was established to determine the normal wildtype response with respect to LR development in *Arabidopsis* seedling roots. To successfully undertake this study, a time efficient method whereby vast numbers of seedlings could be treated with exogenously supplied phytohormones, and transferred from one set of treatment conditions to the next was devised.

6.3 MATERIALS AND METHODS

6.3.1 Development of an *In Vitro* Screening Method

The procedure used to treat *Arabidopsis* seedlings for phytohormone induction of LRP formation and *in vitro* mutant screens, involved germinating and culturing seedlings for 4 days on the surface of filter discs (Whatman paper No. 1), overlaid on solid MS medium contained within a Petri dish. Seedlings could then be easily transferred simply by lifting the disc from one Petri dish and onto another.

To treat 4 day old seedlings with an exogenous supply of phytohormone, the disc was lifted from the solid medium and placed into a fresh Petri dish. At this point 1 ml of liquid MS medium containing the required level of auxin and/or cytokinin was pipetted onto the surface of the filter disc. The discs together with the seedlings, were rinsed thoroughly between treatments by gently pipetting sterile UHQ water over the surface before application of new medium. At the end of the timed treatment period, seedlings used for phytohormone induction studies were removed and cleared with 1% CrO_3 before assessment.

Due to its toxicity, staining with CrO_3 was not an option for seedlings used in mutant screens. It was essential that these plants remained alive during assessment, so that putative mutants could be isolated and grown to set seed for further analyses. To accomplish this, discs were rinsed with sterile UHQ water, and then lifted and inverted onto the top of hormone free (HF) solid MS medium, so that the seedlings lay between the filter disc and the medium. The disc was then gently peeled away leaving the seedlings behind. Culture of the seedlings on HF medium after auxin induction is essential to enable conversion of LRP into LR (Pelosi *et al.*, 1995a and 1995b). The use of phytagel as the solidifying agent was also found to be important to facilitate examination of seedlings through the transparent medium via use of a stereo microscope.

6.4 RESULTS

6.4.1 Timing of Lateral Root Formation in Untreated Seedlings of *Arabidopsis thaliana*

In order to investigate the onset of LR formation in untreated *Arabidopsis* roots, wildtype seeds were surface sterilised and sown onto ARAB MS (refer to Chapter 2.1.1 for recipe). Plates were kept at 4°C for 48 h to promote even germination, before incubation at 22°C±2°C. Thereafter plates were placed in a vertical position to encourage straight growth of the primary root. From this point (Day 0), seedlings were examined under magnification on a daily basis using a Leica MZ8 stereo microscope. Measurements regarding the number of emerged LRs and the length of the primary root were taken to the nearest mm using graduated paper underlay.

After three days incubation at 22°C, germination of <95% of seedlings had occurred producing seedlings with a primary root ranging in length from 1.5-4 mm. By Day 4 the primary root had grown to between 2 and 9 mm in length, with the majority being 4-5 mm long. LRs were not visible until Day 5, when the primary root averaged 9.7 mm (±0.4 mm). At this point 18% of 126 seedlings sampled, had two LRs appearing at the hypocotyl-root junction. A further 18% displayed a LRP just beginning to emerge from the primary root, while the remaining 64% were yet to form a visible LR. By Day 6 the majority of primary roots had formed at least one visible LR. Elongated LRs were observed to be present on all seedlings at Day 7. On the basis of these observations it was decided to use seedlings for all LRP induction experiments at Day 4, before the visible appearance of LRs.

6.4.2 The Effects of Auxin and Cytokinin Treatment on Lateral Root Development in Roots of *Arabidopsis thaliana*

6.4.2.1 Auxin Induction of Lateral Roots

The parameters required for optimum auxin induction of LRs in *Arabidopsis* seedlings were investigated to determine the most effective auxin type and concentration. From previous studies conducted using *E. globulus* seedlings (Pelosi *et al.*, 1995a, 1995b), and others in the literature (Wightman *et al.*, 1980, MacIsaac *et al.*, 1989), auxin concentrations of 10^{-5} and 10^{-4} M were regarded as being appropriate for maximum stimulation without

toxic effects. Contact with auxin for a period of at least 18 h before transfer to HF, was also previously reported to give rise to maximum numbers of emerged LRs.

Based on these findings, wildtype *Arabidopsis* seedlings of ecotype Landsberg *erecta* were exposed to different auxins at 10^{-5} and 10^{-4} M for 24 h. Treated seedlings were then transferred to HF medium for a further 48 h culture to allow the induced LRP to emerge from primary root as new LRs. Four day old seedlings were treated with either IAA, IBA, IPA, NAA or 2,4-D. Seedlings were rinsed with sterile UHQ water before transfer to HF medium. Control seedlings were manipulated and cultured in the same manner as the treated seedlings, but in the absence of exogenous auxin.

After clearing roots with 1% CrO_3, seedlings were scored for the number of LRP and LRs along the length of the primary root. The LR data was of particular importance because seedlings would not be treated with CrO_3 during a mutant screen, leaving the LR as the only feature visible for scoring. Moreover, it would be necessary to ensure putative mutants showing abnormal LR development be kept alive to produce seeds for subsequent genetic analyses.

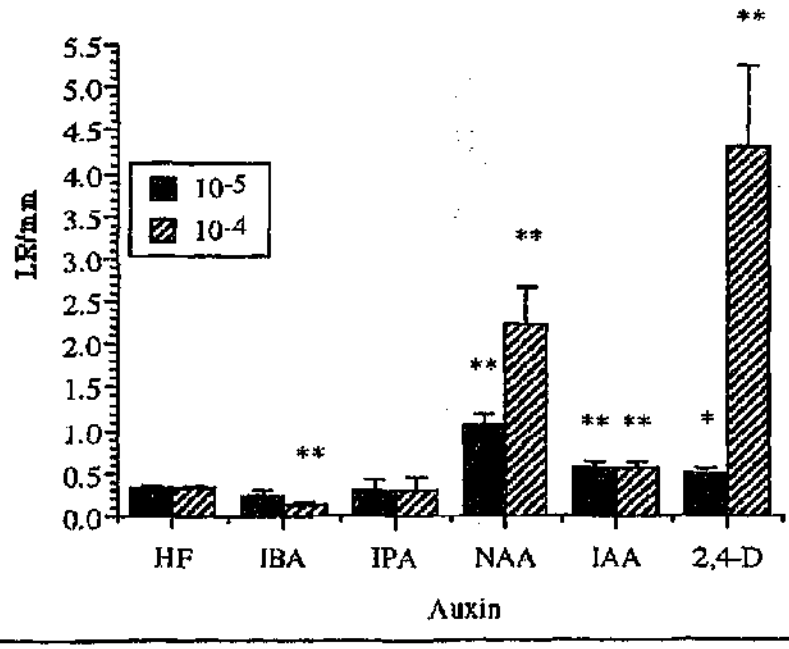


Figure 6.1 : LR induction in *Arabidopsis* seedlings following 24 h treatment with different auxins at 10^{-5} and 10^{-4} M before transfer to HF conditions for a further 48 h culture, and then clearing with CrO_3. Data represent means of 5 to 32 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from the HF control at $p<0.05$ and at $p<0.01$ are indicated by * and ** respectively.

A comparison of the two auxin concentrations, revealed that LR production was greatest when seedlings were treated with NAA (2.2±0.4 LR/mm) or 2,4-D (4.3±1.0 LR/mm) at 10^{-4} M (Fig 6.1). Other treatments producing significantly more LR/mm than the HF control ($p<0.01$) were IAA at both 10^{-5} and 10^{-4} M. IBA at the higher 10^{-4} M concentration was the only exception resulting in fewer LRs than the HF control ($p<0.01$).

Although the 10^{-4} M 2,4-D treatment resulted in slightly more LR/mm than NAA at the same concentration, this difference was not statistically significant ($t_{45} = 1.5$, $p>0.05$, two-tailed test). Analysis of the LRP data revealed that while all the auxins tested at 10^{-4} M, with the exception of IAA, produced significantly more LRP/mm than the HF controls ($p<0.05$), the suboptimal conversion of these LRP to LRs was adversely affected in some instances (Fig. 6.2). This could be attributed either to an inhibitory effect some auxins have on LR emergence, and/or the 48 h HF culture being insufficient time to promote conversion. The rate of LR emergence was particularly inhibited in IBA-treated seedlings which resulted in significantly more LRP than LRs. The effects of IPA and 2,4-D were less severe but did not yield a significant difference between the density of LRP and LRs being formed.

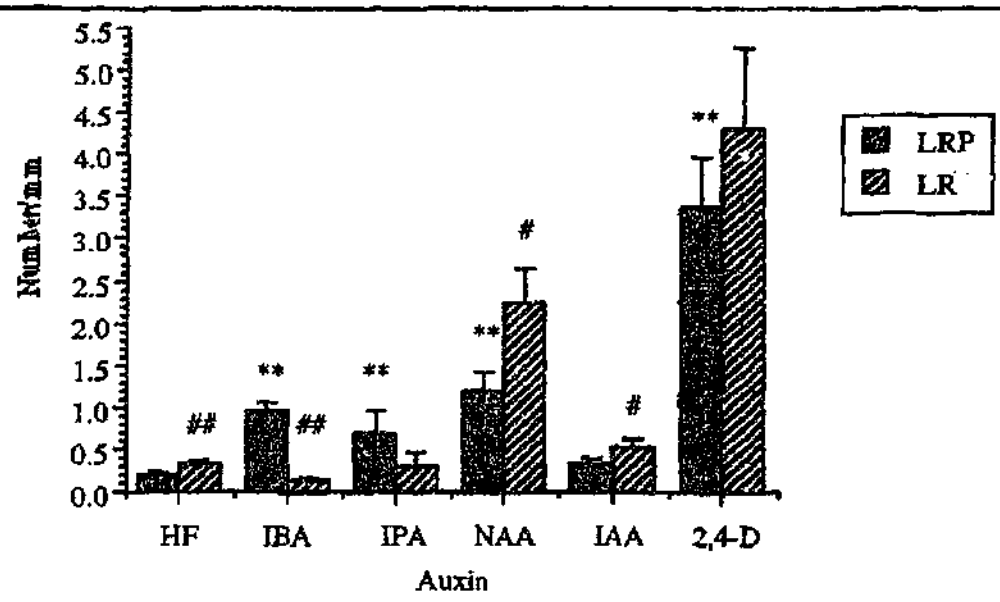


Figure 6.2: LRP and LR formation in *Arabidopsis* seedlings following 24 h treatment with different auxins at 10^{-4} M before transfer to HF conditions for a further 48 h culture, before CrO_3 analysis. Data represent means of 5 to 32 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences in LRP/mm from the HF control at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively. Significant differences between LR and LRP/mm at $p<0.05$ and $p<0.01$ are indicated by # and ## respectively.

Despite the fact 2,4-D is the strongest LRP inducer, it was decided that less than 50% conversion of LRP to LRs would not be optimal during a mutant screen, as it is essential that LRs be easily visible and readily scoreable. A 48 h period of HF culture following auxin treatment was the maximum that could be accommodated as emerged LRs became long, entangled, and started to branch after this point, complicating observations. Based on this criteria, IBA, IPA and 2,4-D were deemed not the auxins of choice.

Therefore, treatment with a high level of NAA (10^{-4} M) for 24 h, followed by transfer to HF for 48 h, was chosen as the preferred treatment to ensure LR formation. Under these conditions maximum induction of LRP was achieved and the subsequent emergence of more than 50% LRs was permitted.

6.4.2.2 The Effects of Cytokinin Prior to, and During Auxin Induced Lateral Root Development

In the presence of an endogenous or exogenous supply of auxin, LRP formation can be inhibited by treatment with cytokinin in the range of 10^{-4}-10^{-6} M (Wightman *et al.*, 1980; MacIsaac *et al.*, 1989; Pelosi *et al.*, 1995a, 1995b). Very low levels on the other hand, can have a slight stimulatory effect in the presence of optimal concentrations of auxin (Wightman *et al.*, 1980; Biddington and Dearman, 1982). Seedlings of *Arabidopsis* were thus exposed to cytokinin before, during and after auxin induction of LRP, to determine which concentrations and cytokinin types are most inhibitory. The possible existence of a concentration window, where cytokinin exhibits a stimulatory effect on LR formation was also investigated.

The initial cytokinin trial involved pretreating 4 day old seedlings with BAP, kinetin, 2*iP* or zeatin for 24 h, at concentrations ranging from 10^{-3} to 10^{-6} M. In the continued presence of cytokinin, seedlings were then supplied with NAA at 10^{-4} M for a further 48 h, making the total treatment period 72 h long. The formation of LRP and LRs in these cytokinin treated seedlings was compared to that of control seedlings which were either subjected to HF conditions, or auxin induction in the absence of cytokinin. The auxin induced controls were exposed to HF conditions for the 24 h pretreatment before transfer to 10^{-4} M NAA for a further 48 h, while the HF controls were cultured in the absence of exogenous phytohormone for the duration of the 72 h period. A summary of the cytokinin and control treatments is represented in Figure 6.3.

Cytokinin treatment:-
cytokinin (24 h) → 10^{-4} M NAA + 10^{-3}-10^{-6} M cytokinin (48 h)

Controls:-
HF → 10^{-4} M NAA (48 h)
HF (72h)

Figure 6.3: An outline of the treatments used to assess the effect cytokinin has on LRP and LR formation when present before and during NAA induction.

When the LRP and LR data was combined (collectively referred to as LR initials), all the cytokinins tested at each of the concentrations, 10^{-3} to 10^{-6} M, clearly inhibited the overall production of NAA-induced LR initials (Fig. 6.4). At 10^{-3} M all the cytokinins tested resulted in a complete absence of LRP and LRs. At 10^{-4} M, with the exception of zeatin, LR initials were either still absent, or significantly fewer than the HF control. In contrast, 10^{-4} M zeatin-treated seedlings behaved as the HF control, giving rise to a similar number of LR initials along the length of the primary root. At 10^{-5} and 10^{-6} M, cytokinin inhibition of LR formation was slightly less severe with the number of LR initials permitted to form along the primary root ranging from 22% (10^{-5} M zeatin) to 63% (10^{-6} M kinetin) of that observed in the 10^{-4} M NAA control.

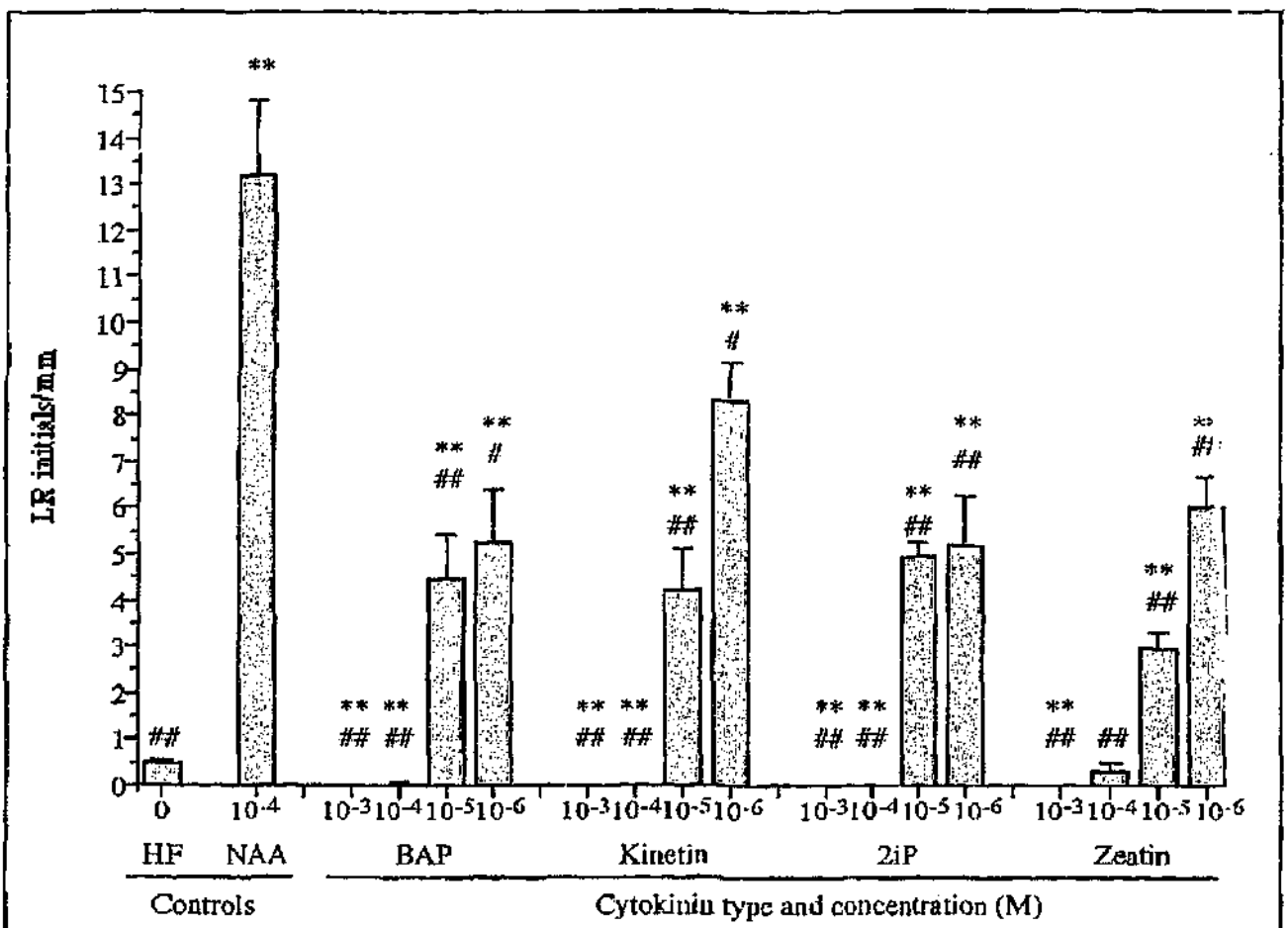


Figure 6.4: The formation of LR initials (combined LRP and LR) in *Arabidopsis* seedlings following 24 h cytokinin pretreatment before the inclusion of 10^{-4} M NAA for a further 48 h culture. Controls include a HF treatment for 72 h, and exposure to 10^{-4} M NAA for 48 h following a 24 h HF pretreatment. Data represent means of 3 to 12 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from the HF controls at $p<0.01$ are indicated by **. Significant differences from the 10^{-4} M NAA controls at $p<0.01$ and $p<0.05$ are indicated by ## and # respectively.

When stimulated to form by 10^{-4} M NAA, LRP as opposed to LRs constituted the majority of the LR initials which were produced in the presence of 10^{-5} and 10^{-6} M cytokinin (Table 6.1). As expected, this was also true for the NAA control. Although auxin at 10^{-4} M has been shown to be an effective inducer of LRP formation in various plant species, prolonged exposure will inhibit the process of LRP conversion to LRs. At 10^{-6} M kinetin, LR emergence although still inhibited, was observed to occur at a significantly higher frequency than in the NAA control. This suggests that in the presence of 10^{-4} M NAA, this level of kinetin can begin to alleviate some of the inhibition exerted by auxin on LR emergence. These observations indicate that the presence of 10^{-5} and 10^{-6} M cytokinin can exert an inhibitory effect on NAA induction of LRP, and that these same levels of cytokinin do not completely overcome the inhibitory effect exerted by auxin on subsequent LR emergence.

Treatment	LRP/mm	LR/mm
Controls		
HF	0.1±0.02	0.3±0.03##
10^{-4} M NAA	13.2±1.65	0
Cytokinin		
10^{-5} M BAP	4.5±0.94	0
10^{-6} M BAP	5.2±1.14	0
10^{-5} M Kinetin	4.1±0.86	0.1±0.08
10^{-6} M Kinetin	7.5±0.92	0.8±0.17#
10^{-5} M 2iP	..9±0.27	0±0.03
10^{-6} M 2iP	5.2±1.04	0.1±0.06
10^{-5} M Zeatin	2.8±0.39	0.1±0.08
10^{-6} M Zeatin	5.7±0.77	0.3±0.17

Table 6.1: LRP and LR formation in *Arabidopsis* seedlings following 24 h cytokinin pretreatment before the inclusion of 10^{-4} M NAA for a further 48 h culture. Controls include a HF treatment for 72 h, and exposure to 10^{-4} M NAA for 48 h following a 24 h HF pretreatment. Data represent means of 3 to 12 replicates ± SEM. Significant differences from the 10^{-4} M NAA control at $p<0.01$ and $p<0.05$ are indicated by ## and # respectively.

The prevention, or marked inhibition of auxin induced LRP formation observed here by cytokinin concentrations ranging from 10^{-3}-10^{-6} M, is in agreement with the previous findings reported for pea, lettuce and *E. globulus* (Wightman *et al.*, 1980; MacIsaac *et al.*, 1989; Pelosi *et al.*, 1995a, 1995b). In these experiments, a marked reduction in LRP initiation was evident when inhibitory concentrations of cytokinin were supplied either before or during auxin induction.

6.4.2.3 The Effect of Kinetin on Lateral Root Induction and Emergence in NAA Treated Roots

In a further trial, the consequences of a kinetin treatment on LR emergence in NAA pre-induced *Arabidopsis* roots was investigated in more detail. Four day old seedlings were pretreated with 10^{-4} M NAA for 24 h to induce the formation of LRP, and then transferred to medium containing kinetin at concentrations varying between 10^{-3} M and 10^{-12} M for 48 h. The effect of these kinetin treatments on LRP induction and subsequent conversion to LRs was assessed and compared to NAA and HF controls. The NAA treated controls were transferred to HF conditions for 48 h following induction, while HF controls were cultured in the absence of exogenous phytohormone for the duration of the full 72 h treatment period (Fig. 6.5).

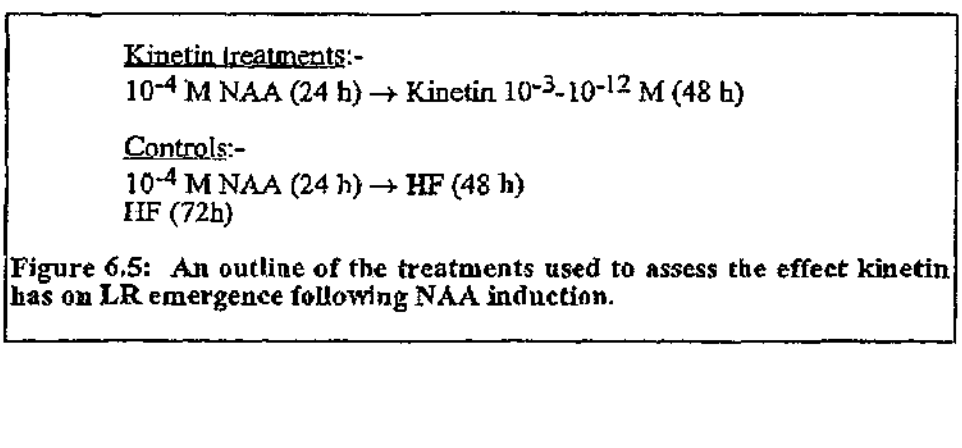


Figure 6.5: An outline of the treatments used to assess the effect kinetin has on LR emergence following NAA induction.

In this experiment, although NAA treatment was performed in the absence of cytokinin, transfer after 24 h to kinetin at concentrations between 10^{-3} and 10^{-5} M, significantly reduced the combined number of formed LRP and LRs along the length of the primary root (Fig. 6.6). In particular, transfer to medium containing 10^{-3} M kinetin effectively blocked NAA induction of LRP, resulting in significantly fewer LR initials than was observed even in the HF controls. The lower concentrations (10^{-6}-10^{-12} M) enabled the formation of these LR initials to a similar number as that produced by the NAA control, but did not enhance production above this level.

A comparative analysis of the LRP and LR data obtained from this experiment, showed that following auxin induction the process whereby LRP are converted to LRs was effectively inhibited upon transfer to 10^{-4} and 10^{-5} M kinetin (Fig. 6.7). At these kinetin concentrations, the number of observed LRP was significantly higher than the number of emerged LRs. The proportion of LRP and LRs along the primary root began to equalise following transfer to moderate kinetin concentrations of between 10^{-6} and 10^{-8} M. Thereafter, the balance shifted at the lower end of the scale such that, at kinetin concentrations of 10^{-9} M and less, LR emergence was promoted significantly (Fig. 6.7).

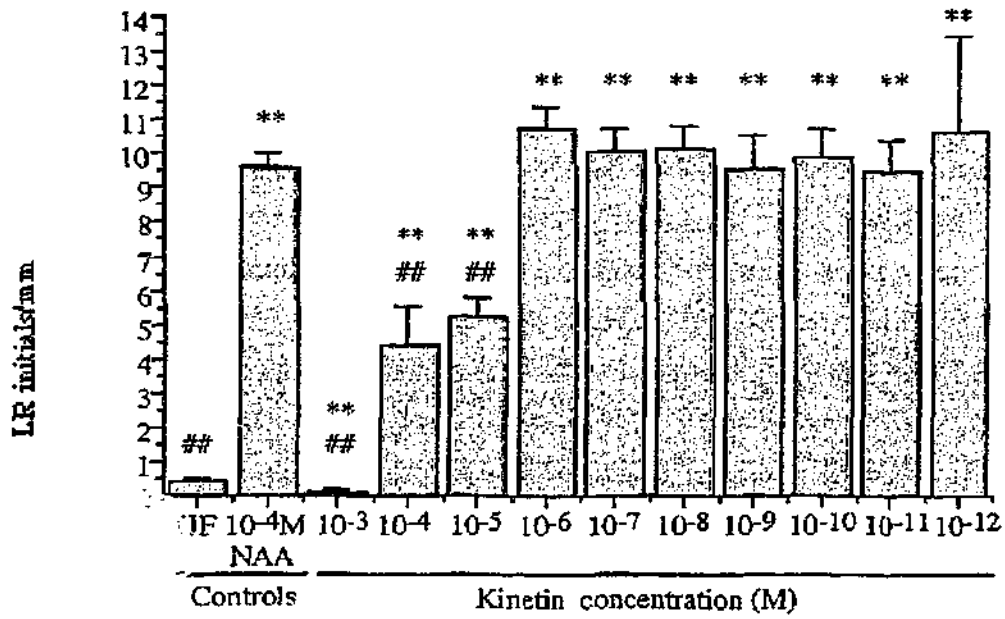


Figure 6.6: The formation of LR initials (LRP and LRs combined) in *Arabidopsis* seedlings following a 24 h induction by 10^{-4} M NAA before transfer to kinetin at a range of concentrations for 48 h. Controls include a HF treatment for 72 h, and transfer to HF for 48 h following a 24 h 10^{-4} M NAA induction. Data represent means of 8 to 20 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from the HF and 10^{-4} M NAA controls at $p<0.01$ are indicated by ** and ## respectively.

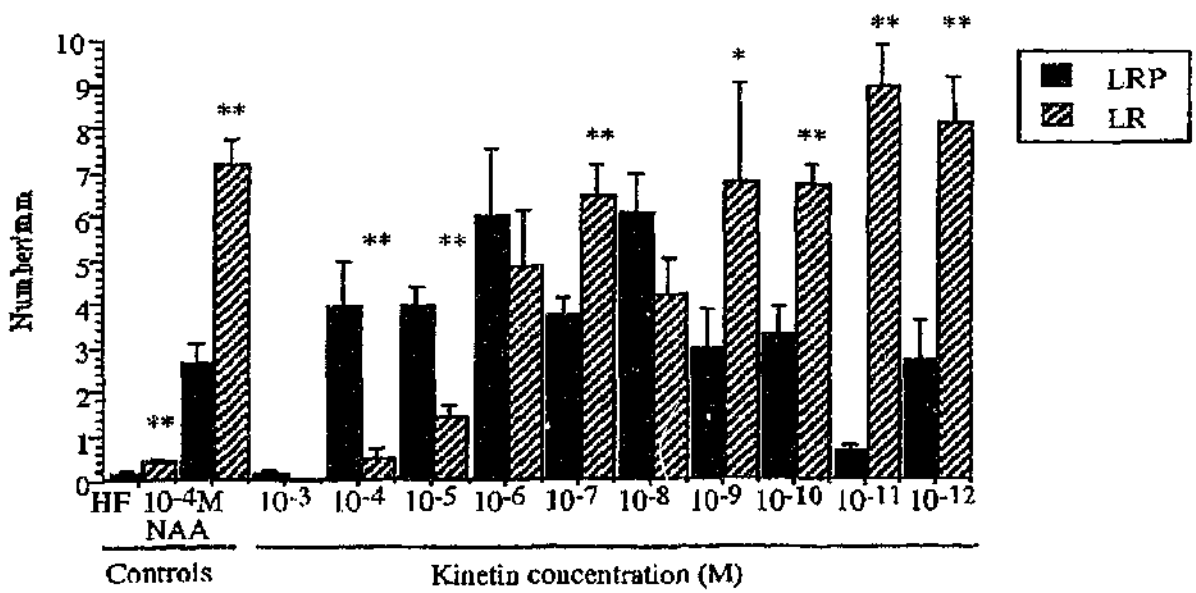


Figure 6.7: The conversion of LRP to LRs in *Arabidopsis* roots after 24 h induction by 10^{-4} M NAA followed by transfer to kinetin at a range of concentrations for a further 48 h culture. Data represent means of 8 to 20 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences between LR and LRP/mm at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively.

These findings show that when kinetin is supplied after NAA treatment, high concentrations (10^{-3}-10^{-5} M) can effectively inhibit LR formation on two levels, induction and subsequent emergence. At low concentrations, these inhibitory effects are reversed. NAA induction of LRP is restored by transfer to kinetin at concentrations of 10^{-6} M or less, while the subsequent conversion of these LRP to LRs is stimulated at levels of 10^{-9} M and below. In particular, 10^{-11} M was effective in stimulating the conversion of LRP to LRs which appears to be in general agreement with the findings reported in lettuce by Biddington and Dearman (1982), who showed a small but reproducible stimulation in rates of LRP to LR conversion in response to treatment with concentrations of zeatin below 10^{-8} M. Similar findings were also reported in *E. globulus* where concentrations of BAP ranging from 10^{-9} to 10^{-12} M were effective stimulators of LR emergence (Pelosi *et al.*, 1995a).

6.4.3 The Screen for Lateral Root Mutants

Following EMS mutagenesis of wildtype *Arabidopsis* (ecotype Landsberg *erecta*) seed, approximately 54,000 of the resulting M1 seeds were planted in soil and allowed to self fertilise. The M2 seed was collected into 27 families from about 9,000 M1 germinated plants. An estimated 3,200 M2 plants were screened *in vitro* for altered LR formation following exposure to various phytohormone treatments (Fig. 6.8). The treatments were applied to 4 day old M2 plants and were designed to target putative mutants with an increased sensitivity to suboptimal LRP induction levels of auxin (10^{-5} M NAA), or conversely a resistance to auxin induction when optimum conditions (10^{-4} M NAA) were employed. In addition auxin-cytokinin combinations were also used in an attempt to identify mutant plants exhibiting altered patterns of LR emergence.

Auxin induction treatments:-

10^{-4} M NAA (24 h) → HF (48 h)

10^{-5} M NAA (24 h) → HF (48 h)

Auxin and cytokinin treatments:-

10^{-5} M NAA (24 h) → 10^{-5} M kinetin (48 h)

10^{-5} M NAA (24 h) → 10^{-5} M NAA +10^{-5} M kinetin (48 h)

Figure 6.8: An outline of the treatments used to screen for LR mutants in *Arabidopsis*.

The density of LRs formed along the length of the primary root was determined for all the seedlings screened. M2 plants which showed an altered root morphology, including LR numbers outside the normal range of wildtype controls were isolated and cultured *in vitro* in HF medium to enable self fertilisation. Because a wide range of variation in LR densities was observed in wildtype plants during the screens, it was decided that secondary screens were essential to determine whether the isolated phenotypes were consistently different from wildtype. To confirm abnormal root phenotypes and to conduct segregation analysis, M3 seed was collected from the isolated putative mutants. An outline of the mutant screening procedure and subsequent analyses is represented in Figure 6.9.

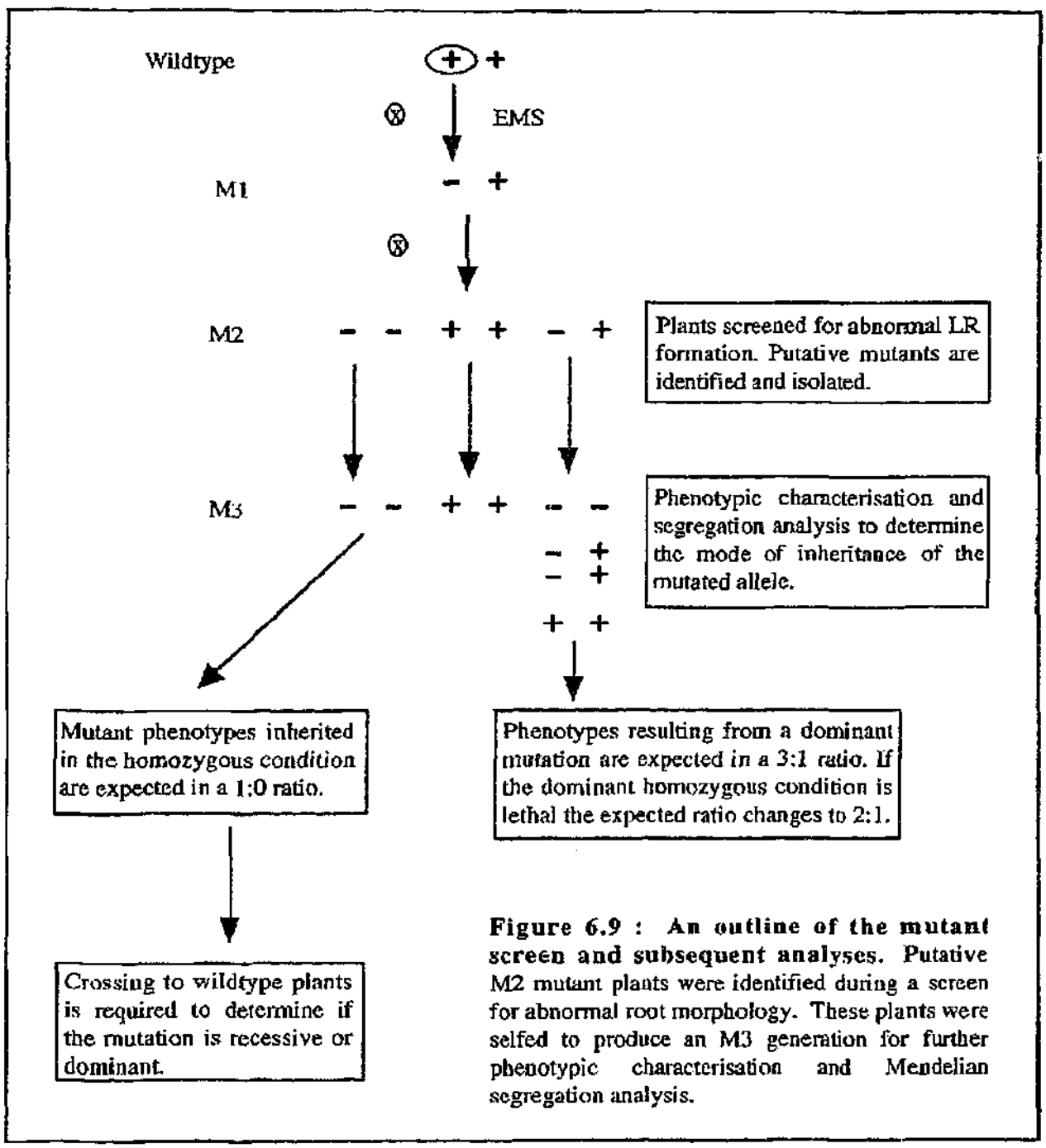


Figure 6.9 : An outline of the mutant screen and subsequent analyses. Putative M2 mutant plants were identified during a screen for abnormal root morphology. These plants were selfed to produce an M3 generation for further phenotypic characterisation and Mendelian segregation analysis.

In total, 193 putative mutant seedlings were isolated from a series of primary screens, and seed harvested for further analyses in secondary screens. Resource availability enabled a limited number of putative mutants to be assessed in detail. Rescreening was undertaken on nine of the putative mutant lines that were isolated. Obtaining sufficient quantities of viable seed did prove difficult from five out of the nine putative mutant lines. In two cases the isolated plants (Le1a:2.4 and Le1a:3.22) produced seed which failed to germinate and therefore could not be analysed further. The reduced fertility of a further three putative mutants (Le2a:5.25, Le3a:2.3 and Le5a:10.5) produced a small seed set, complicating analyses with insufficient sample sizes. Seed quantities were adequate to conduct secondary screens for the other four putative mutant lines isolated (Le1a:2.1, Le1a:2.14, Le2a:26.1 and Le4a:3.2).

6.4.3.1 Le1a:2.1

Le1a:2.1 is a putative mutant line which was selected from a screen of 4 day old M2 seedlings subjected to a 24 h pulse treatment with 10^{-4} M NAA, before transfer back to HF conditions. After a further 48 h culture, the original Le1a:2.1 isolate exhibited an atypical response compared to wildtype plants.

The Le1a:2.1 root morphology consisted of a primary root measuring 6 mm in length with a single LR. Its most distinguishing feature was the 24 'bulging' LRP, visible as small bumps along the length of the transparent primary root. It was unclear at this stage whether these features represented non emerged LRP or stunted LRs. Under identical conditions, the wildtype seedlings produced LRs in the order of 1-4.6 LR/mm (mean = 2.7±0.2). The primary root of wildtype seedlings was 1-11 mm long, but with no apparent bulging. At the 4-leaf stage it became apparent that the bulging along the Le1a:2.1 primary root corresponded to LRs which had arrested during development (Fig. 6.10).

The Le1a:2.1 isolate was cultured *in vitro* on HF medium where it was allowed to self fertilise to produce seed for the M3 generation. M3 plants were used for a preliminary phenotypic study and segregation analysis.

6.4.3.1.1 The Le1a:2.1 Phenotype

M3 plants were germinated and cultivated *in vitro* on HF medium. Wildtype seeds germinated at a frequency of 74%, while germination of Le1a:2.1 seeds was reduced at 35%. At 4 days old, some plants were subjected to a single 24 h 10^{-4} M NAA treatment before transfer back to HF medium. The remaining untreated plants were cultivated in HF conditions throughout growth.

Phenotypic observations were made on 8, 12, 15 and 21 day old Lela:2.1 seedlings and compared to wildtype plants of the same age and grown in identical conditions. At 8 days old, all Lela:2.1 seedlings had a primary root which was significantly shorter than that of wildtype seedlings (HF $t_{13} = 3.2$, $p>0.01$, two-tailed test; NAA treated $t_{24} = 2.8$, $p>0.01$, two-tailed test). This was true regardless of whether or not the seedlings had received NAA treatment (Fig. 6.11).

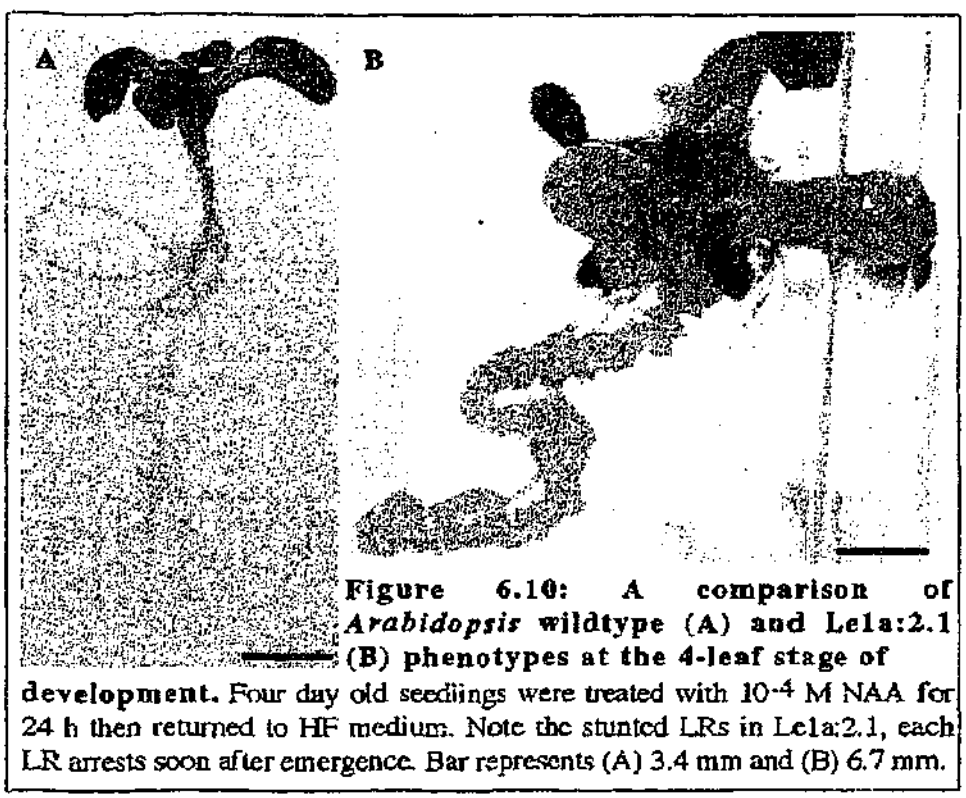


Figure 6.10: A comparison of *Arabidopsis* wildtype (A) and Lela:2.1 (B) phenotypes at the 4-leaf stage of development. Four day old seedlings were treated with 10^{-4} M NAA for 24 h then returned to HF medium. Note the stunted LRs in Lela:2.1, each LR arrests soon after emergence. Bar represents (A) 3.4 mm and (B) 6.7 mm.

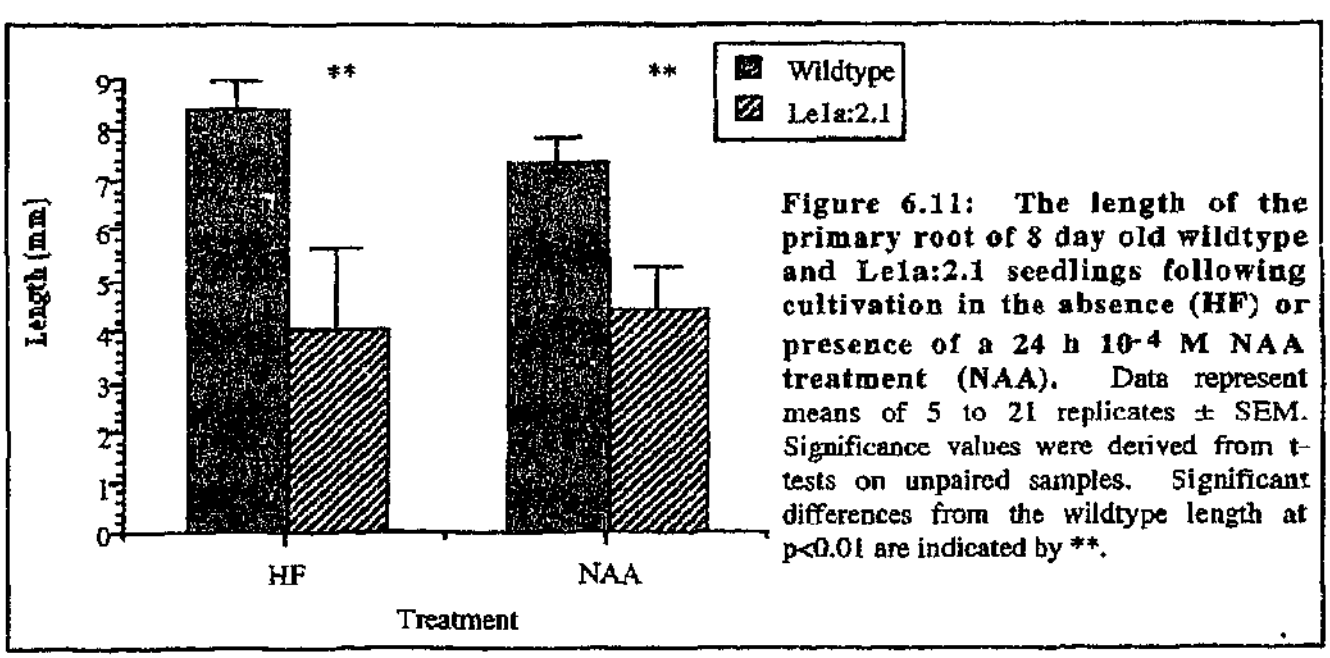


Figure 6.11: The length of the primary root of 8 day old wildtype and Lela:2.1 seedlings following cultivation in the absence (HF) or presence of a 24 h 10^{-4} M NAA treatment (NAA). Data represent means of 5 to 21 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from the wildtype length at $p<0.01$ are indicated by **.

Staining of a number of wildtype and Lela:2.1 plants with CrO_3 revealed that the relative density of formed LRP (t_{13} = 1.4, $p<0.05$, two-tailed test on HF data; t_{24} = 0.5, $p<0.05$, two-tailed test on NAA data) and LRs (t_{13} = 1.3, $p<0.05$, two-tailed test on HF data; t_{24} = 0.1, $p<0.05$, two-tailed test on NAA data) did not differ between the two plant lines (Fig. 6.12). In addition, the Lela:2.1 primary root tissue near the hypocotyl junction, sustained a high degree of breakage, perhaps caused by damage inflicted by the emerging LR (Fig. 6.13). Like the primary root, the emerged LRs of the 12 and 15 d old Lela:2.1 plants were similarly stunted (Fig. 6.14B and C).

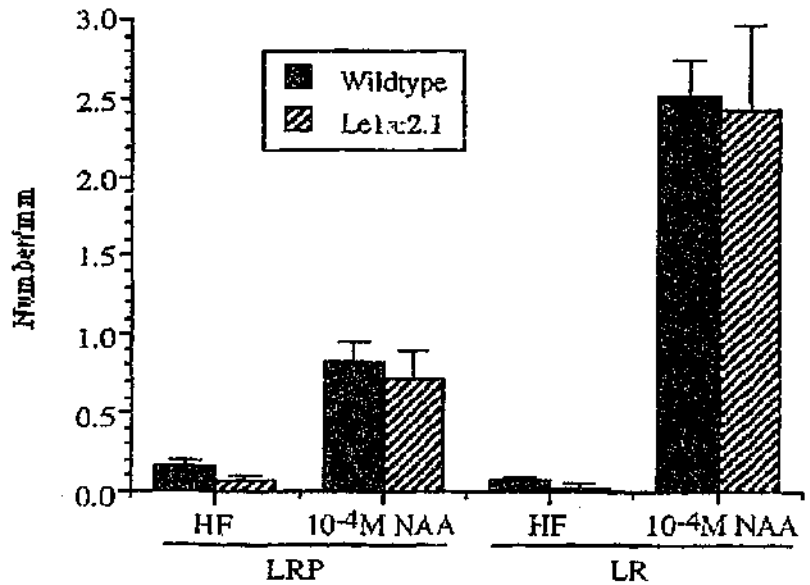


Figure 6.12 : Combined LRP and LR formation in 8 day old wildtype and Lela:2.1 seedlings following cultivation in the absence (HF) or presence of a 24 h 10^{-4} M NAA treatment. Data represent means of 5 to 21 replicates ± SEM.

Figure 6.13: A comparison of the hypocotyl-root junction in the (A) wildtype and (B) Lela:2.1 seedling. At 4 days old, seedlings were treated with 10^{-4} M NAA and then transferred to HF medium for a further 3 days culture. Note damage to the tissue at the hypocotyl root junction in Lela:2.1. Bar represents (A) 7.2 mm and (B) 6.1 mm.

When grown *in vitro*, obvious phenotypic differences were also apparent in the aerial part of the plant. At 8 days old, the Le1a:2.1 shoot was dwarfed with two cotyledons. At the same age, the wildtype shoot had reached the four leaf stage (Fig. 6.14A). The Le1a:2.1 seedlings did not develop their second pair of leaves until they are 12-15 days old (Fig. 6.14B and C). At 21 days wildtype seedlings had reached the 8-leaf stage and had a comparatively dense root system. In comparison the Le1a:2.1 shoot was severely stunted with a very sparse root system (Fig. 6.14D). When Le1a:2.1 seedlings at this stage of development were transferred to soil, their small root system was unable to sustain plant growth.

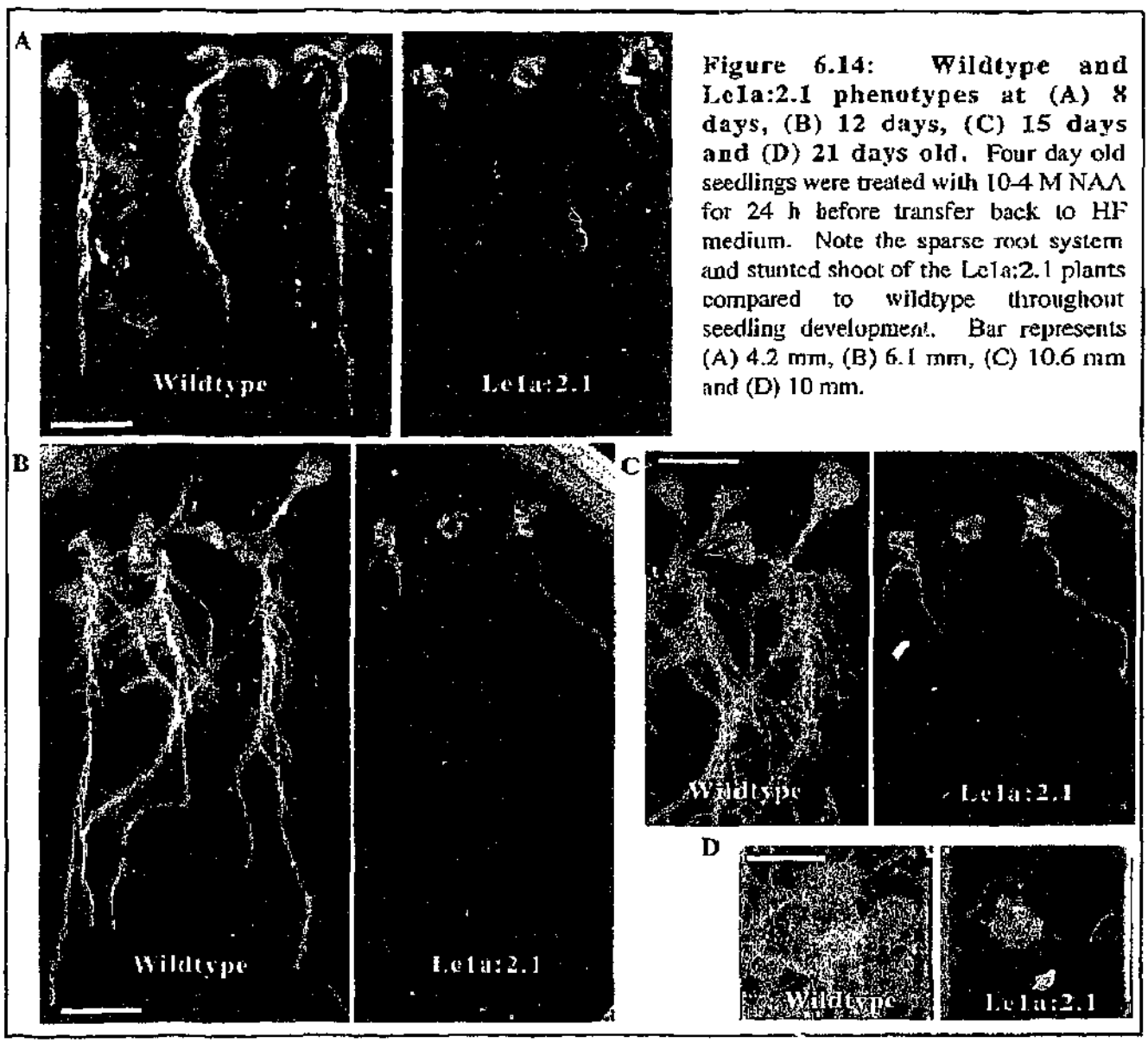


Figure 6.14: Wildtype and Le1a:2.1 phenotypes at (A) 8 days, (B) 12 days, (C) 15 days and (D) 21 days old. Four day old seedlings were treated with 10-4 M NAA for 24 h before transfer back to HF medium. Note the sparse root system and stunted shoot of the Le1a:2.1 plants compared to wildtype throughout seedling development. Bar represents (A) 4.2 mm, (B) 6.1 mm, (C) 10.6 mm and (D) 10 mm.

Although the preliminary data suggests that Le1a:2.1 does not show aberrations in the initiation of LRP or the emergence of LRs, its phenotypic defect with respect to root morphology and growth, appears to be confined to the reduced length of both the primary and secondary roots. This could be attributed to an inability of cells to elongate, a reduction in cell division, or an inability to maintain indeterminate root growth. Whatever the defect, it is not exclusive to the Le1a:2.1 root, but was also noticeable in the shoot, giving plants a dwarfed appearance.

6.4.3.2 Le1a:2.14

Le1a:2.14 was another putative mutant identified in the same screen. The pattern of LR development in this plant initially suggested a possible increased sensitivity to auxin treatment. At 7.7 LR/mm, the density of LRs formed by Le1a:2.14 was higher than the 0-5 LR/mm detected for wildtype controls. The Le1a:2.14 plant was isolated and self fertilised to conduct a secondary screen of the M3 generation.

For the secondary screen, 4 day old Le1a:2.14 plants underwent a 10^{-4} M NAA treatment for 24 h before a further 2 days culture on HF medium. At this stage, the 7 day old plants were cleared with CrO_3 and compared to wildtype plants cultured in identical conditions. The root lengths were measured and the LRP and LR densities calculated. A statistical analysis using the student t-test on unpaired samples was performed to determine whether the phenotypic characteristics exhibited by the roots of the Le1a:2.14 line were significantly different from those of wildtype plants (Table 6.2).

Characteristic	Wildtype	Le1a:2.14
Length (mm)	14.8±0.9	9.0±1.3*
LRP/mm	0.5±0.3	0.3±0.1
LR/mm	0.8±0.1	1.1±0.2

Table 6.2: Root length, LRP and LR formation in wildtype and Le1a:2.14 seedlings following 24 h treatment with 10^{-4} M NAA before transfer to HF culture for a further 48 h. Measurements represent means of 3 to 8 replicates ± SEM. Significant differences ($p<0.05$) from wildtype are indicated by *.

The only root characteristic to differ between Le1a:2.14 plants and wildtype was the root length. In this respect Le1a:2.14 was similar to Le1a:2.1 in that both produced a shorter primary root than wildtype. Unlike Le1a:2.1 however, the hypocotyl-root junction was unaffected in Le1a:2.14 plants and the LRs showed no apparent signs of arrested

development. A comparison of 15 day old wildtype and Le1a:2.14 seedlings showed that although root growth in general did not seem to be severely affected in Le1a:2.14, root length continued to be shorter than wildtype (Fig 6.15). Moreover, the shoot of Le1a:2.14 seedlings was also slightly stunted compared to wildtype. The phenotypic data seems to suggest that Le1a:2.14 has a slower growth rate overall, but LR development appears to be unaffected. As such this mutant could not be classed as a LR mutant.

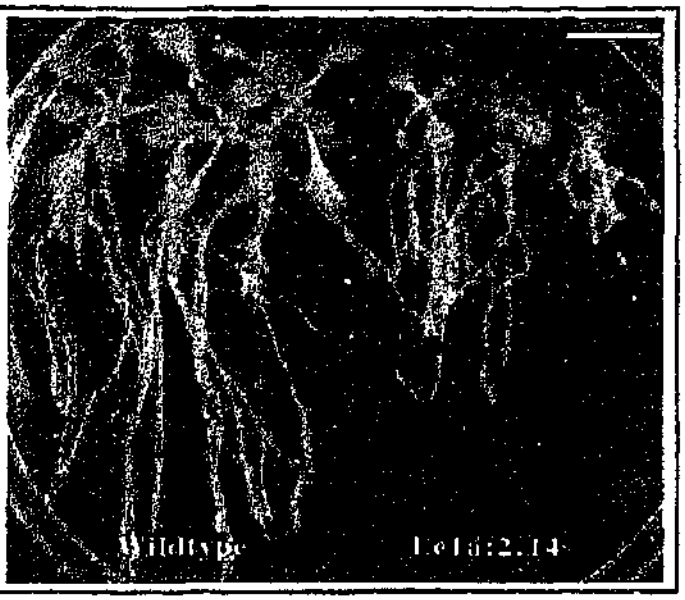


Figure 6.15: A comparison of wildtype and Le1a:2.14 seedlings at 15 days old. Four day old seedlings were treated with 10^{-4} M NAA for 24 h before transfer back to HF medium for a further 10 days culture. Bar represents (A) 6.1 mm and (B) 10.6 mm.

6.4.3.3 Le2a:26.1

Le2a:26.1 was also isolated as a potential auxin-sensitive mutant with an apparent increase in LR density from wildtype following a 24 h 10^{-4} M NAA treatment before culture on HF medium. Upon rescreening, this plant line showed no apparent differences in LR formation or general root morphology from wildtype plants when NAA treatment was given, or when cultured in HF conditions. This plant line was deemed to display a wildtype phenotype and analysis was discontinued.

6.4.3.4 Le4a:3.2

In an attempt to target mutations that may affect LR emergence, screens were also conducted using an inhibitory concentration of cytokinin either during or after auxin induction of LRP. Mutants insensitive to the inhibitory effects were expected to display increased densities of LRs. Amongst the putative mutants identified with increased LR formation was one notable exception. This seedling, designated Le4a:3.2, was

conspicuous for its lack of a primary root. The Le4a:3.2 seedling was isolated, grown *in vitro*, and observed over time to determine whether a primary root would eventually emerge. At 11 days old, the seedling finally produced a short primary root. At this stage it was unclear whether the retarded development of the primary root was related to a general defect in the process of emergence. If so, it was possible that LR emergence was also affected. This plant was allowed to self fertilise for a preliminary phenotypic study of the M3 progeny.

6.4.3.4.1 The Le4a:3.2 Phenotype

Four day old M3 Le4a:3.2 plants were treated with 10^{-5} M NAA for 24 h before transfer to medium containing 10^{-5} M NAA and 10^{-5} M kinetin for 4 days. Following treatment, the 9 day old seedlings were cultured on HF medium and assessed at various ages. Wildtype controls were treated and cultivated in the same manner for comparison. Le4a:3.2 seeds germinated at a frequency of 63%, lower than the 85% observed for wildtype seeds in this experiment.

The primary root of 9 day old wildtype seedlings was on average 4.7±1.1 mm long, compared to Le4a:3.2 seedlings which had still not formed a primary root at this stage (Fig. 6.16A). Le4a:3.2 seedlings did not produce a primary root until they reached 11-15 days old (Fig. 6.16B). Development of the shoot of plants cultured *in vitro* did not appear to be retarded but typically was smaller in stature and leaf size.

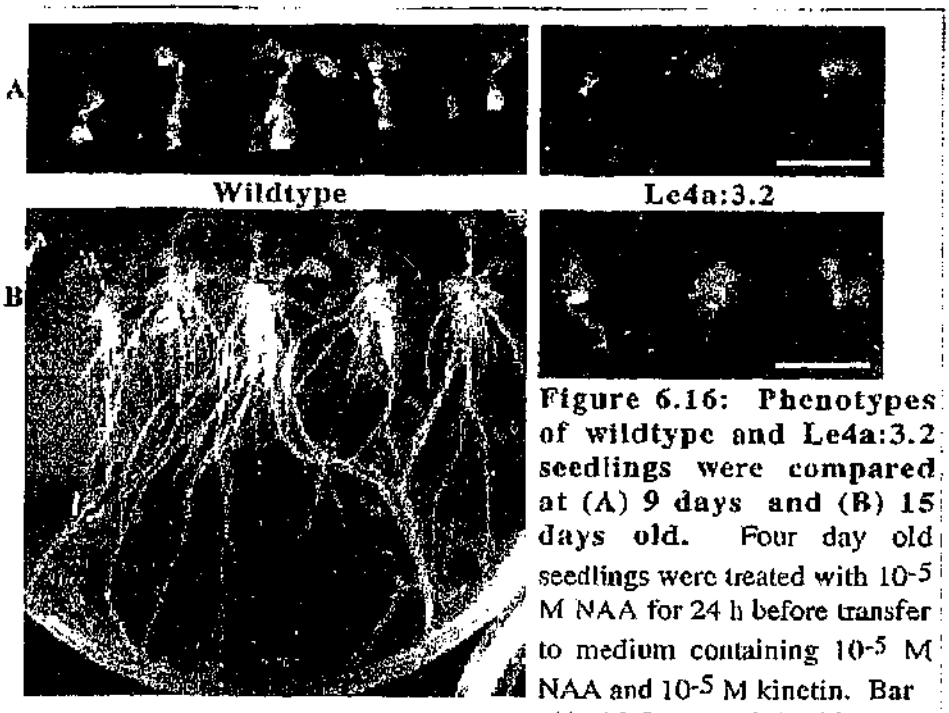


Figure 6.16: Phenotypes of wildtype and Le4a:3.2 seedlings were compared at (A) 9 days and (B) 15 days old. Four day old seedlings were treated with 10^{-5} M NAA for 24 h before transfer to medium containing 10^{-5} M NAA and 10^{-5} M kinetin. Bar represents (A) 38.5 mm and (B) 23.7 mm.

To assess LR formation in the Le4a:3.2 putative mutant line, seedlings were subjected to one of three alternative treatments outlined in Figure 6.17. Due to the delayed formation of the primary root in the Le4a:3.2 line, seedlings were taken for treatment at 11 days old. In contrast, wildtype seedlings were treated when 4 days old. Following treatment, seedlings were cleared in 1% CrO_3, and the length of the primary root was measured and scored for the number of visible LRP and LRs.

Treatment A:-
10^{-5} M NAA (24 h) → 10^{-5} M NAA + 10^{-5} M kinetin (48 h)

Treatment B:-
10^{-5} M NAA (24 h) → HF (48 h)

Treatment C:-
HF (72h)

Figure 6.17: An outline of the treatments used to assess the effect cytokinin has on NAA induced LR formation.

Comparisons conducted between Le4a:3.2 and wildtype plants revealed that Le4a:3.2 produced a significantly shorter primary root in all three treatments (Fig. 6.18). Interestingly, the length of the Le4a:3.2 primary root remained relatively unaffected by each treatment type. This was in contrast to the results obtained for wildtype plants, where treatment C produced the longest primary root, while treatment B was inhibitory, producing the shortest root. Treatment A gave rise to a primary root of intermediate length which was statistically different ($p<0.05$) from that produced by treatments B or C.

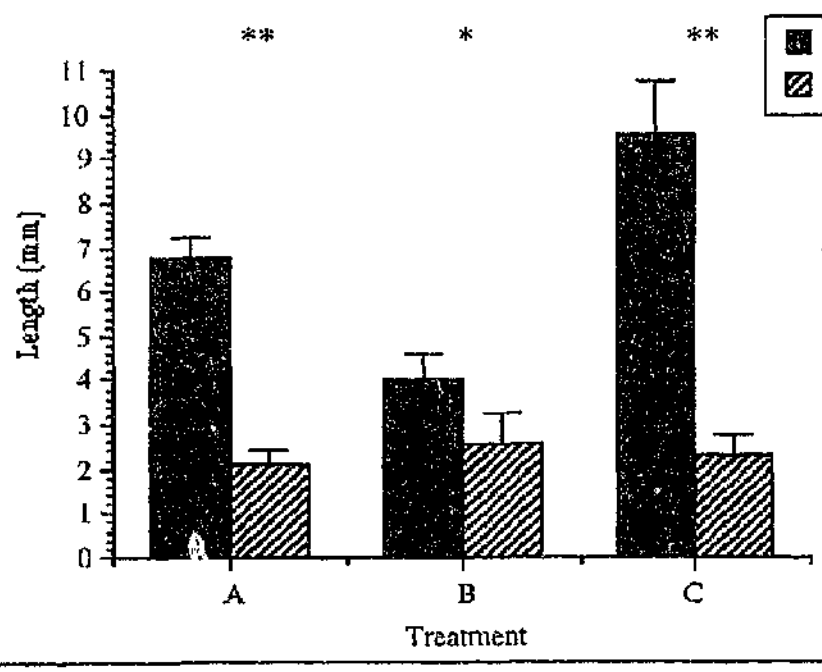


Figure 6.18: The length of the primary root of wildtype and Le4a:3.2 seedlings following treatment A, B or C. Data represent means of 6 to 12 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences from the wildtype length at $p<0.05$ and $p<0.01$ are indicated by * and ** respectively.

Although some distinct variations in LRP and LR density were detected between the three treatments, the Le4a:3.2 response did not alter significantly from that observed for wildtype seedlings following any one of the three treatments. However, there was significant inhibition of LR emergence exhibited by Le4a:3.2 following treatments A and B (Fig. 6.19). As in the case of the primary root, the LRs that did emerge were noticeably shorter than wildtype. It is likely that reduced rate of LR emergence observed in Le4a:3.2 is a result of arrested growth of the LRP at or near the time of emergence.

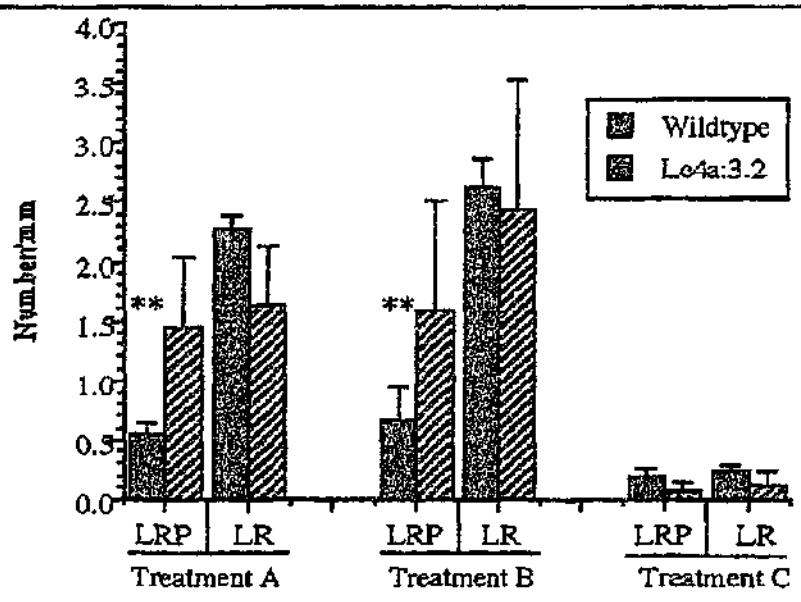


Figure 6.19: The conversion of LRP to LRs in wildtype and Le4a:3.2 roots following treatment A, B or C. Data represent means of 6 to 21 replicates ± SEM. Significance values were derived from t-tests on unpaired samples. Significant differences between LRP and LR/mm at $p<0.01$ are denoted by **. LR emergence was inhibited when the difference between LRP and LR/mm was not significant.

These observations indicate that Le4a:3.2 displays increased auxin sensitivity with respect to root elongation and LR emergence, but was not altered in its sensitivity to LRP induction by this phytohormone. This suggests that auxin acts in separate pathways to affect the processes of root elongation and LRP induction.

It was particularly interesting that LR emergence was inhibited in Le4a:3.2 seedlings treated with NAA. Neither the addition of kinetin 24 h after initial NAA exposure (treatment A), nor the transfer to HF medium (treatment B) was able to promote the conversion of LRP to LRs as it did in wildtype seedlings. This suggests that the auxin sensitivity of Le4a:3.2 seedlings is too strong for the kinetin or HF culture to overcome or reverse the inhibition towards LR emergence. It is not clear at this stage whether the inhibited LR emergence is related to a general defect in root elongation, in which case the LR fails to emerge due to its inability to elongate. The less probable alternative is that the Le4a:3.2 defect affects the two events independently.

Unlike the Le1a:2.1 seedlings, the Lc4a:3.2 plants survived transfer to soil. The mature Le4a:3.2 plant was of similar size to wildtype, suggesting that the defective elongation is specific to the root. Although the Le4a:3.2 flower appeared bigger than wildtype with comparatively larger petals and sepals, the siliques were noticeably smaller (Fig. 6.20).

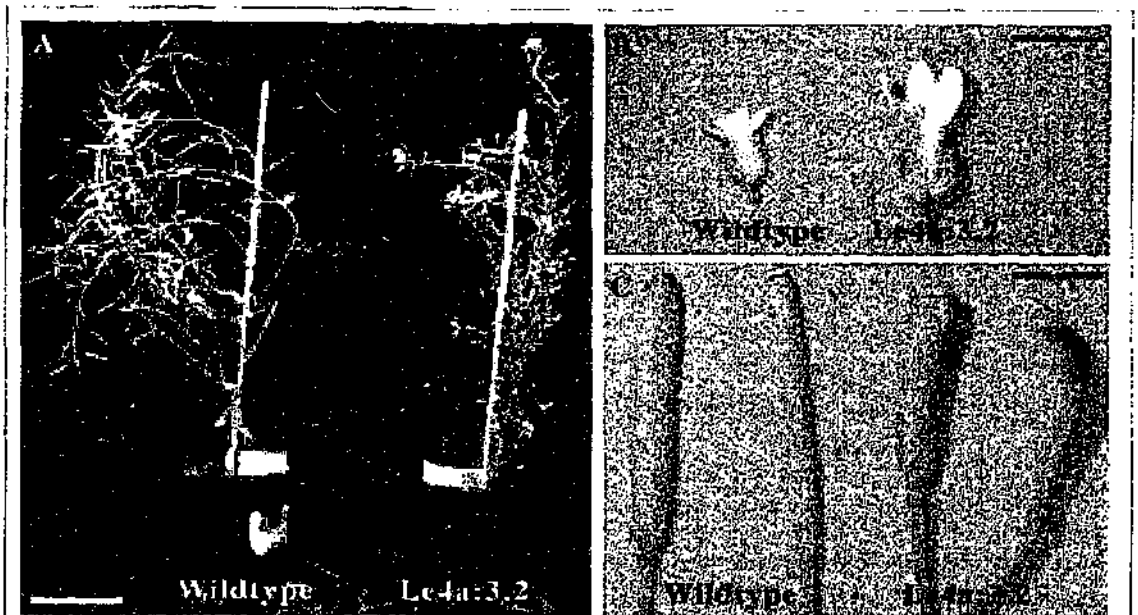


Figure 6.20: A phenotypic comparison of (A) the shoot, (B) the flower and (C) the siliques of mature soil grown wildtype and Le4a:3.2 plants. Bar represents (A) 46.7 mm (B) 3 mm and (C) 3.6 mm.

6.4.3.5 Mendelian Segregation Analysis

To ascertain the nature of inheritance of the Le1a:2.1, Le1a:2.14 and Le4a:3.2 phenotypes, a preliminary Mendelian segregation analysis was performed. Data was collected from the progeny of the three selfed original isolated plants, to analyse heritability and segregation of the respective putative mutant phenotypes amongst the M3 generation.

6.4.3.5.1 Le1a:2.1

Plants were scored at 8, 9, 11, 14 and 16 days old to ensure that the phenotypic classification was consistent. Segregation of the Le1a:2.1 phenotype, consisting of a dwarfed shoot with a short root, was scored by comparing the length of the primary root and the size of the shoot to known wildtype plants. The Le1a:2.1 phenotype, segregated in a ratio that was statistically indistinguishable from a 2:1 ratio (Table 6.3), suggesting that the Le1a:2.1 phenotype results from a dominant mutation, which is lethal when present in the homozygous condition. Le1a:2.1 plants are clearly heterozygotes carrying one

dominant allele. Attempts to cross Le1a:2.1 plants to wildtype failed, suggesting that these putative mutants may have reduced fertility.

Phenotype	*N*	Mutant		Wildtype		Ratio tested	χ^2
		Observed	Expected	Observed	Expected		
Le1a:2.1	64	40	42.7	24	21.3	2:1	0.5
Le1a:2.14	44	39	44	5	0	1:0	0.6
Le4a:3.2	38	32	38	2	0	1:0	1.0

Table 6.3: Segregation analysis in the M3 generation of the selfed Le1a:2.1, Le1a:2.14 and Le4a:3.2 putative mutants. *N* = total number of seedlings observed. The expected number was calculated given a 2:1 ratio of segregation for Le1a:2.1, and 1:0 for Le1a:2.14 and Le4a:3.2.

6.4.3.5.2 Le1a:2.14

Phenotypes of 9, 10, 13 and 16 day old plants were classified to ensure that the Le1a:2.14 phenotype was reliably scored. The dwarfed Le1a:2.14 phenotype segregated in a ratio that was statistically indistinguishable from 1:0, suggesting that the mutant phenotype is the product of the homozygous condition (Table 6.3). Whilst likely a recessive trait, to determine whether the mutation is a dominant or recessive Le1a:2.14 plants will need to be crossed to wildtype.

6.4.3.5.3 Le4a:3.2

At 15 days old, segregation of the Le4a:3.2 phenotype was scored by comparing the length of the primary root and the size of the leaves to known wildtype plants. The short root and small leaf characteristics of the Le4a:3.2 phenotype segregated in a ratio that was statistically insignificant from 1:0 (Table 6.3), suggesting that the Le4a:3.2 mutant phenotype is a homozygote, carrying two copies of the mutation. Although this mutation is likely to be recessive, further work needs to be undertaken to cross Le4a:3.2 to wildtype for confirmation.

6.5 DISCUSSION

6.5.1 The Interacting Effects of Auxin and Cytokinin on Lateral Root Formation in *Arabidopsis thaliana*

In *Arabidopsis*, as in *E. globulus*, LR formation is influenced by the synergistic and antagonistic interactions of auxin and cytokinin. Auxin induction of LRP in *Arabidopsis* was optimally achieved by treatment with the synthetic auxin NAA at a concentration of 10^{-4} M. NAA-stimulated LRP formation could be prevented or severely inhibited by the simultaneous presence of kinetin at levels between 10^{-3} and 10^{-6} M within the first 24 h of induction.

The antagonised induction of LRP imposed by cytokinin when present in combination with NAA may be mediated through the cell cycle. Roles for auxin and cytokinin have been identified at specific control points within the cell cycle, at late G1 and at the G2/M transition (Zhang *et al.*, 1996). Induction of LRP by auxin is correlated with an induction in the levels of the $p34^{cdc2}$ protein kinase (John *et al.*, 1993). Progression through the cell cycle in plants is dependent upon the phosphorylation state of this protein and its catalytic activity. When $p34^{cdc2}$ is active, cells are permitted entry into mitosis.

While auxin acts to induce $p34^{cdc2}$ (Zhang *et al.*, 1996), its levels are regulated by the auxin to cytokinin ratio (John *et al.*, 1993; John and Zhang 2001). Studies in pea have shown that when cytokinin levels are elevated, an antagonistic effect is produced (John *et al.*, 1993). The auxin to cytokinin ratio decreases resulting in inhibited LRP initiation and a concomitant reduction in $p34^{cdc2}$ protein kinase. When the higher auxin to cytokinin ratio was restored by exogenous auxin treatment, so too were the levels of $p34^{cdc2}$.

Further investigation using cell suspension cultures of *Nicotiana plumbaginifolia* revealed that auxin or cytokinin alone could not stimulate cell cycle activity (Zhang *et al.*, 1996). In fact, active cell proliferation was dependent on the simultaneous presence of both phytohormones. Auxin was required for cell cycle progression through G1 to S phase, and from G2 to M. At the G2 phase control point, auxin stimulates the accumulation of the $p34^{cdc2}$ protein kinase, while cytokinin is essential for its tyrosine dephosphorylation and activation, permitting progression through mitosis and resulting in cell doubling. It was also suggested that low levels of cytokinin can lead to arrest in G2 resulting in the cessation of cell proliferation.

In experiments devised here, two concepts of root development were examined, (i) LRP initiation, and (ii) LRP to LR conversion. The cytokinin inhibition of LRP initiation observed during NAA induction in wildtype *Arabidopsis* roots (Fig. 6.4) may be explained in terms of the auxin to cytokinin ratio. The high concentrations of cytokinins used in this experiment presumably decreased the auxin to cytokinin ratio, such that the accumulation of $p34^{cdc2}$-like protein kinase was inhibited, preventing cells from entering mitosis and thus inhibiting the formation of LRP. At 10^{-3} and 10^{-4} M cytokinin, induction of $p34^{cdc2}$ may have been entirely prevented by an extremely low auxin to cytokinin ratio. However, 10^{-5} and 10^{-6} M cytokinin may have resulted in altering the relative level of auxin to cytokinin, such that the ratio enabled $p34^{cdc2}$ levels to increase slightly and become activated, permitting a slight degree of cell proliferation.

When *Arabidopsis* seedlings were cultured on medium containing levels of kinetin at or below 10^{-9} M following NAA induction, LRP initiation was restored and LR emergence was promoted particularly at 10^{-11} M kinetin (Fig. 6.6 and 6.7). This finding supports earlier studies conducted in lettuce and also *E. globulus* where similar levels of exogenously supplied cytokinin enhanced emergence of the LR (Biddington and Dearman, 1982; Pelosi *et al.*, 1995a).

The increased rates of conversion of LRP to LRs following culture on low concentrations of cytokinin, after auxin treatment (Fig. 6.6 and 6.7), may also be explained in terms of our current understanding of control points in the cell cycle. When roots were transferred to 10^{-3}-10^{-5} M kinetin for example, the lowered auxin to cytokinin ratio effectively inhibited LRP formation possibly by reducing $p34^{cdc2}$ accumulation. Restored rates of LRP formation upon transfer to kinetin at or below 10^{-6} M, may have resulted from an auxin to cytokinin ratio that was conducive to the activation of the induced $p34^{cdc2}$ protein kinase, and thus enabling progression from G2 to mitosis. As the LRP makes the developmental transition to a LR, mitotic activity decreases, and the cells elongate and expand to emerge from the primary root (MacLeod, 1972; Malamy and Benfey, 1997b). The cessation of cell division in the elongating cells of the LRP may also be achieved through the modification of $p34^{cdc2}$ levels. High levels of cytokinin inhibit cell division in the elongation zone of the root (John *et al.*, 1993) and it is believed that cytokinin acts in this region to prevent $p34^{cdc2}$ induction and promotes breakdown of any remaining $p34^{cdc2}$ protein (John *et al.*, 1993). Paradoxically, low levels of cytokinin promote cell division. It is possible that transfer to 10^{-11} M kinetin produced an optimal auxin to cytokinin ratio in *Arabidopsis* root tissue, resulting in elevated rates of conversion of LRP to emerged LRs.

6.5.2 The Isolation of Two Mutants With Altered Lateral Root Development

Altogether, secondary screens were possible for four of the putative mutant lines isolated. Two of these, Le1a:2.1 and Le4a:3.2 showed consistent defects in LR development. Le1a:2.1 plants produced a wildtype-like response with respect to LRP induction and LR emergence when cultured on HF medium both with and without a 24 h NAA induction treatment. Once the LRs have emerged in Le1a:2.1, they arrest growth resulting in shorter roots than wildtype. This observation seems to suggest that the Le1a:2.1 defect is not in auxin recognition or signalling. Rather, it seems likely that the putative mutation may be in a gene involved in cell expansion and operating in a pathway distinct from LRP induction. A detailed phenotypic comparison of Le1a:2.1 and wildtype plants at the cellular level is required to test this hypothesis.

The retarded growth of the primary root and LRs of both Le1a:2.1 and Le4a:3.2, and the reduced rates conversion of LRP to LRs in Le4a:3.2, appear to suggest that the respective mutations may be explained if the defects lie in the inefficient transport of auxin to the root tip. The consequences of such an aberration may be that cells of the primary and LR meristems fail to divide or elongate after emergence. If this were to occur, the root meristems would become non viable and arrest growth. This scenario would have no apparent effects on rates of LR initiation which takes place before the apical meristem is established (see Chapter 1).

The arrested growth of the LRs at or near the time of emergence is a phenotypic trait that Le4a:3.2 has in common with the *alf3-1* mutant characterised by Celenza *et al.* (1995). Examination of the arrested *alf3-1* LRP revealed that they lacked the cellular organisation that was typical of wildtype LRP. The *alf3-1* LRP resembled an undifferentiated mass of cells that did not elongate, and rarely developed vascular tissue. An examination of Le4a:3.2 needs to be conducted at the cellular level to determine its organisational and patterning status. Aberrant cellular organisation may support the hypothesis for a defect in the transport of auxin and/or nutrients contributing to a loss of indeterminate growth of the primary and LRs.

The similarity in phenotypes between *alf3-1* and Le4a:3.2 however appear to end here. The *alf3-1* mutation also causes an increased frequency of LR initiation which is not the case for Le4a:3.2. Moreover, growth on medium supplemented with IAA or indole, rescued the *alf3-1* phenotype. In the present study, a 24 h treatment with 10^{-5} M NAA (Figs. 6.18 and 6.19, treatment B) was not sufficient to rescue the Le4a:3.2 phenotype,

however extended growth periods on auxin containing medium needs to be examined to explore the possibility further.

6.5.3 The Identification of Three Stages in Lateral Root Development.

Interpretation of the data obtained from the phytohormone studies, combined with the preliminary phenotypic analysis of the two putative mutant lines, supports current views on LR formation which involve three discrete stages for LR formation (see Chapter 1). The first stage is the induction of LRP within 24 h of a high dose auxin treatment. In this respect the Le1a:2.1 and Le4a:3.2 mutant response was indistinguishable from wildtype, each producing LRP along the length of the primary root at a density similar to wildtype.

The second stage involves the conversion of LRP to LRs within a further 48 h. The phytohormone application studies showed that in wildtype plants, LR emergence is inhibited if roots are cultured in the presence of high levels of NAA and/or kinetin. Conversely, the process can be promoted by transfer to very low kinetin or HF conditions. The Le4a:3.2 line differs from wildtype at this stage of LR development. Culture in HF conditions following LRP induction by 10^{-5} M NAA, was unable to significantly promote LR emergence in Le4a:3.2 plants. The same inhibited response was also observed after transfer to 10^{-5} M NAA with 10^{-5} M kinetin. In contrast, both treatments permitted the conversion of LRP to LRs in wildtype seedlings.

The third and final stage requires that the newly emerged LR continues to grow, and is itself capable of further LRP formation. Here both putative mutant lines appear to be different from wildtype. In both cases, Le1a:2.1 and Le4a:3.2 produce significantly shorter primary and LRs. The conversion of LRP to LRs is unaffected by the Le1a:2.1 mutation, suggesting that arrested growth of the LRs occurs after they emerge. Reduced rates of LR emergence in Le4a:3.2 suggests that this mutation acts to arrest LR growth before Le1a:2.1. Presumably LRs of Le4a:3.2 cease development at or just prior to emergence, resulting in the decreased rates of LRP to LR conversion observed for this mutant. At this stage, LR emergence and subsequent growth of the new organ may require the coordinated activity of genes involved in cell elongation, cell division and the establishment and maintenance of an active root apical meristem.

6.5.4 Future Directions

As was noted in Chapter 1, short root systems have been previously described for several root morphology mutants with altered radial organisation and patterning during root development. In *Arabidopsis*, *shortroot* (*shr*), *scarecrow* (*scr*), *gollum* (*glm*) *rootmeristemless* (*rml*) and *wooden leg* (*wol*) are some characterised examples of mutations that give rise to a shortened root system. In each case the mutations affected the patterning of different tissue types within the root as a result of altered cell division or tissue specification (Scheres *et al.*, 1995). While the *scr* (Scheres *et al.*, 1995) mutants were found to lack a ground tissue layer (cortex or endodermis), the *shr* mutation results in the loss of the endodermis and part of the stele, as well as a loss in the indeterminate growth of the primary and LR (Benfey *et al.*, 1993). The *glm* mutation disrupts the organisation of the vascular tissue and pericycle and *wol* affects the vascular tissue alone (Scheres *et al.*, 1995). As some of these mutations also affected the aerial phenotype, it has been proposed that the wildtype gene products act in pathway(s) that are not exclusive to the root. Benfey *et al.* (1993) suggested that the absence of, or disruption to a particular cell layer may cause problems in the nutrient balance and/or phytohormone transport to the root tip, resulting in arrested growth of the root.

Cloning of the *SCR* and *SHR* genes revealed that both encode members of the GRAS family of transcription factors with putative roles in regulating the radial organisation of the root (Di Laurenzio *et al.*, 1996; Pysh *et al.*, 1999; Helariutta *et al.*, 2000). Gene expression studies suggest that *SHR* functions upstream of *SCR* to direct the asymmetric cell division that gives rise to the ground tissue (endodermis and cortex) (Helariutta *et al.*, 2000). SHR has been shown to move from the stele to the ground tissue where it positively regulates the transcription of *SCR* (Di Laurenzio *et al.*, 1996; Nakajima *et al.*, 2001). The asymmetric cell divisions that generate the phloem and procambium require the *WOL* gene. The deduced amino acid sequence of WOL suggested that it represents a novel two-component histidine kinase with receptor domains (Mähönen *et al.*, 2001). Taken together with expression studies, a function for WOL as a signal transducer during vascular morphogenesis was postulated.

Small roots have also been described for the *rml1* and *rml2* mutants (Cheng *et al.*, 1995). It appears that these mutants lack a functional root meristem following the failure to activate cell division in the apical cells of the primary root during early development, resulting in arrested growth. LRs and roots generated from *rml1* callus show the same defect. The LRP of *rml1* mutants emerge from the primary root but abort further development, presumably through a failure to form an active meristem. This arrested

development of LRs is also observed here in Le4a:3.2, and was reported for the *alf3-1* mutant isolated by Celenza *et al.*, 1995. Further work is required to assess if Le1a:2.1 and Le4a:3.2 are allelic to any of these genes or represent novel mutations.

If terminal growth of the Le1a:2.1 and Le4a:3.2 LRs is caused by an inactive LR meristem, then cytokinin treatment may rescue the defect by promoting progression through the cell cycle. If a population of rapidly dividing cells can be maintained within the LR meristem by the presence of cytokinin the meristem should remain active and indeterminate growth restored to the LRs of Le1a:2.1 and Le4a:3.2 plants. This hypothesis is worthy of further investigation, particularly in light of the fact that mutants with altered responses to cytokinin have not yet been described with respect to LR development. Rescue of either or both mutations by cytokinin would suggest that Le1a:2.1 and Le4a:3.2 represent novel genes that identify a role for cytokinin in LR development.

Morphological and molecular based mapping of Le1a:2.1 and Le4a:3.2 may also be considered for future work. Since the sequence of the *Arabidopsis* genome was completed, the task of map-based cloning of genes identified by mutant phenotypes should be a relatively accelerated and straight forward process. Once cloned expression studies and sequence analysis will help elucidate a role for the encoded Le1a:2.1 and Le4a:3.2 proteins in the LR developmental program.

A further 187 putative mutant lines isolated from the screens described here are available for secondary screening when adequate resources become available. Extrapolating from current analyses, at most 20% of these isolated lines will show reproducible and consistent aberrations in LR development. However, finding mutations that specifically effect LR formation is expected to be rare, given that many mutants isolated to date also display defects in primary root development and shoot phenotype. It should also be noted that less severe mutations may be difficult to find amongst the natural variation that exists in the number of LRs that form along the length of the primary root.

CHAPTER SEVEN

Final Discussion

7.1 SYNOPSIS

A healthy and prolific root system is important for normal plant growth and development and also vital for the establishment of clonal forestry programs by micropropagation. In eucalypts, as in most plants, a substantial proportion of the root system is contributed by LRs, yet our knowledge of the mechanisms that govern LR formation is unclear. To elucidate a better understanding of LR development, this study was principally aimed towards the isolation and characterisation of genes involved in LR formation in *E. globulus*. In achieving this objective, some of the regulatory processes involved at different stages of LR development have been identified.

To attain these goals, the PCR-based differential display technique was modified and optimised in this study to identify sequences in *E. globulus* that participate in the development and growth of LRs. A cDNA subtraction procedure was also conducted to complement and possibly increase the array of mRNAs represented. Isolated sequences were cloned, sequenced and assigned to a homologous family before further processing was conducted to confirm the differential expression profiles.

As described previously, auxin induces a primary response within minutes of treatment, including increased Ca^{+} levels, acidification and the transcription of early response genes (Barbier-Brygoo, 1995; Abel and Theologis, 1996). Maximal transcriptional activation detected by the clones identified in this study occurs 12-72 h after auxin suggesting that they are not involved in the primary auxin response, but rather act downstream. Whether or not expression of these identified genes are regulated by the upstream events involved in the primary auxin response is a question for further research.

Northern blot analysis facilitated the classification of LR-related clones, based on the pattern of transcript accumulation during LR formation in auxin-treated root explants of *E. globulus*. The molecular data, summarised in Figure 7.1, clearly demonstrated that a number of genes are specifically regulated during the first 72 h of LR formation. This time frame suggests that the genes represented by 15 clones characterised in this study are important at various stages in the differentiation, development and growth of the LR in *E. globulus*.

Sequence data, obtained from the clones of interest, was searched against the current nucleotide and protein databases to identify any significant homology to previously characterised clones. A specific, putative function in LR development was inferred when

significant matches were found, although of course information of this nature is only predictive at best. In particular, clones representing novel genes will require further characterisation to elucidate a possible role in this process.

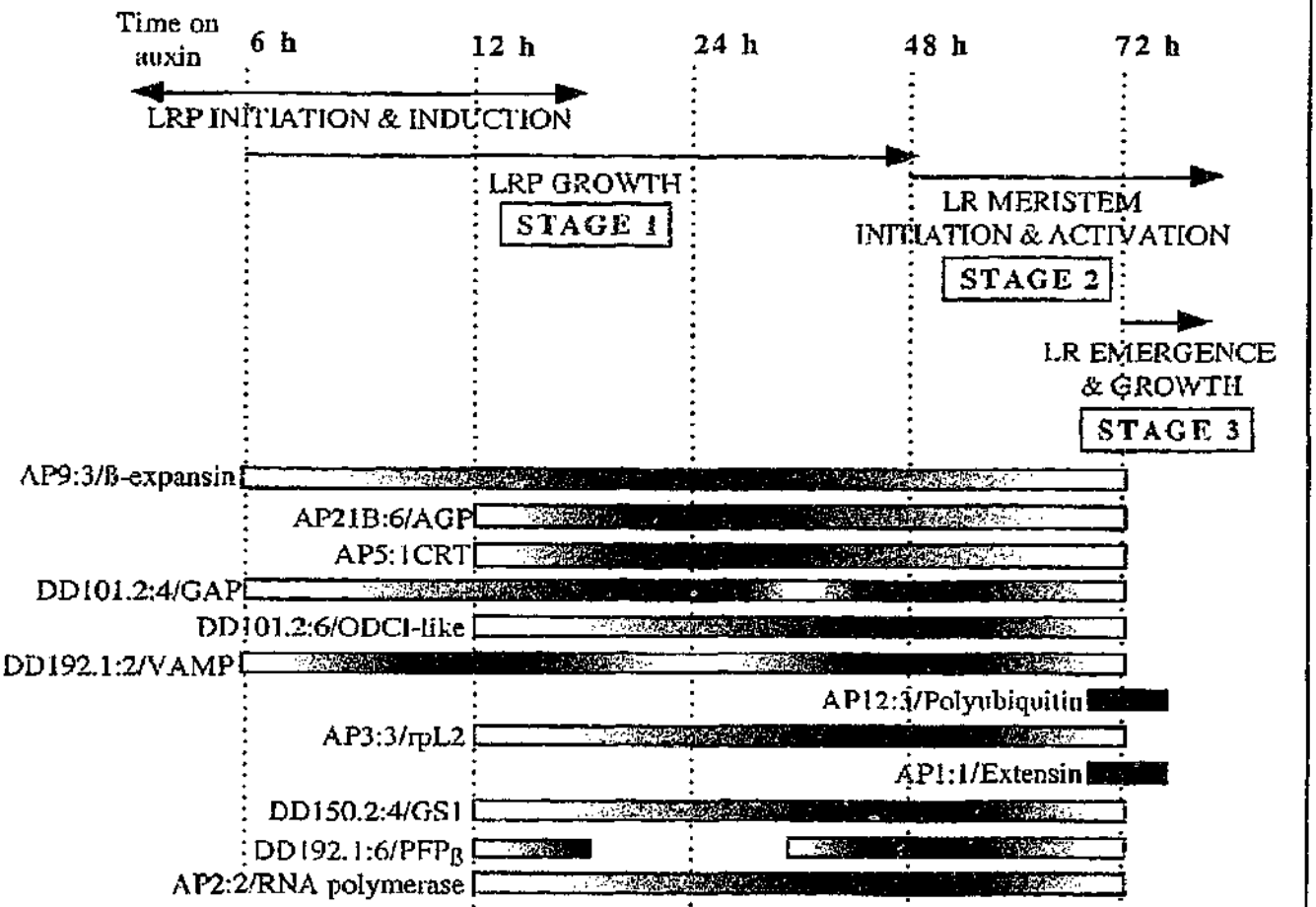


Figure 7.1: A summary of the temporal expression profiles exhibited in IBA treated roots of *E. globulus* by the clones showing significant homology to characterised genes and proteins. The boxes indicate transcript accumulation above the level detected for the untreated root control. The shading in the boxes represents the relative expression level over the treatment period, with darker shading correlating with higher expression. Maximum expression can be correlated with physiological events occurring during various stages in development of auxin induced roots. Stage 1 encompasses LRP initiation, induction and growth; Stage 2 includes initiation and activation of the new LR meristem; and Stage 3 involves LR emergence and growth.

A complementary approach was to screen for mutants affecting LR growth. A specialised screening method was devised to target mutations in *Arabidopsis* that alter LR emergence and subsequent growth of the new organ. Mutant screens conducted on medium containing various combinations of NAA and kinetin resulted in the identification of two putative mutants, Le1a:2.1 and Le4a:3.2 with possible defects in the process of LR emergence and subsequent growth.

Auxin stimulated LRP initiation was unaffected in both putative mutants isolated. However, the primary and LRs of both Le1a:2.1 and Le4a:3.2 were significantly shorter than wildtype when grown in the presence or absence of NAA. This arrested growth occurs earliest in Le4a:3.2, at or near the time of LR emergence. Consequently, the observed rate of LRP conversion to LRs in this mutant was significantly reduced compared to wildtype. In contrast, LRs of Le1a:2.1 plants do emerge but arrest growth soon after. These observations indicate that the pathway that regulates indeterminate root growth is distinct from that involved in the induction and initiation of LRP. The literature on root mutants in *Arabidopsis*, including *shortroot*, *scarecrow*, *gollum*, *rootmeristemless*, *wooden leg* and *alf3-1* (Benfey *et al.*, 1993; Celenza *et al.*, 1995, Cheng *et al.*, 1995; Scheres *et al.*, 1995), suggests that the defects responsible for short root phenotypes similar to that seen in Le1a:2.1 and Le4a:3.2, may lie in meristem activation, cell expansion, cell division, or cell patterning. Both mutants also exhibited reduced aerial stature suggesting that the affected mechanism regulating indeterminate root growth also operates in the shoot.

7.2 THREE DEVELOPMENTAL STAGES FOR LATERAL ROOT DEVELOPMENT

The molecular and genetic data collected in this study is consistent with the consensus in the literature that auxin induced LRs in *E. globulus*, as in other species, progresses through a number of discrete developmental stages following initiation (Blakely *et al.*, 1982; MacIsaac *et al.*, 1989; Celenza *et al.*, 1995; Laskowski *et al.*, 1995; Pelosi *et al.*, 1995a and 1995b; Malamy and Benfey, 1997a and 1997b). The current study helps to define three of these stages in *E. globulus*, (i) LRP initiation and LRP growth (~6-48 h); (ii) LR meristem initiation and activation (~48-72 h); and (iii) LR emergence and subsequent growth (~72^{+} h), as shown in Figure 7.1.

7.2.1 Proposed Function of Cell Signalling and Cell Wall Modification During Stage 1: Initiation and Growth of the Lateral Root Primordium

LRP development requires the synthesis of new cell walls and subsequent cell wall modification to accommodate cell expansion within the growing LRP (Cosgrove, 1997). As the cells of the LRP expand, new structural components are deposited along the length

of the cell wall (Cho and Kende, 1998). This is accomplished by an increase in the synthesis of new structural proteins and their targeted transport to the apoplast for incorporation into the new cell wall (Cosgrove, 1997; Cosgrove, 2000). The cell wall is able to yield to the increasing turgor of the expanding cell by the breaking and reformation of bonds in its polymer network, to accommodate deposition of these new structural materials (Shieh and Cosgrove, 1998). It then undergoes rigidification, preventing the breaking and deposition of additional cell wall components (Cosgrove and Li, 1993; Cosgrove, 1997). At this point the cell has ceased its growth and is able to withstand and resist turgor pressure exerted by the protoplast contained within. Thus at the very least, it is likely that growth of LRP to form new LRs requires the up-regulated activity of genes that encode cell wall components and regulate rigidification. This requires that genes involved in upstream events including cell signalling, transcription and translation, and the processing, trafficking and secretion of the encoded cell wall component, are also activated during the process.

The temporal expression profiles detected by two of the putative cell wall protein encoding clones identified in this study (AP9:3/β-expansin and AP21B:6/AGP), suggest that they may be correlated with physiological events that occur during the growth phase of LRP development. As the LRP increases in size, the cells contained within grow largely through expansion (Malamy and Benfey, 1997b). The timing of this process is consistent with up-regulated activity of the corresponding AP9:3 and AP21B:6 genes, with related transcripts accumulating to their most abundant level after 24 h auxin treatment (Fig 7.1).

AP21B:6/AGP may have multiple roles during development of the LRP. Early AP21B:6 expression at induction, may be indicative of the speculated function AGP has in cell signalling and cell-cell interaction, as cells of the incipient LRP are recruited to proliferate upon perception of the auxin stimulus (Cassab, 1998). As the LRP grows, some neighbouring cells may be forced to collapse under the pressure exerted by the expanding cells of the primordium (Peterson and Peterson, 1986). High levels of AP21B:6 expression at this stage may be reflective of the proposed role for AGPs in programmed cell death (Gao and Showalter, 2000). The decline in AP9:3 and AP21B:6 expression correlating as it does with decelerated growth of LRP, is consistent with a decrease in the rate of cell division within the mature primordium just prior to its emergence from the primary root (MacLeod, 1972; Laskowski *et al.*, 1995; Malamy and Benfey, 1997b)

The previously proposed role for AP5:1/CRT in protein folding (Vassilakos *et al.*, 1998) suggests that it may function to prepare newly synthesised cell wall glycoproteins such as AP21B:6/AGP, for deposition into expanding cell walls within the growing LRP.

Consistent with this idea is the observation that activity of the CRT-encoding gene, represented by AP5:1, is elevated from as early as 12 h auxin treatment, reaching maximum abundance at 24 h (Fig. 7.1). This observation is also indicative of an increased requirement for CRT during growth of the LRP, perhaps to help meet an increased demand for correctly folded glycoproteins to be incorporated into the cell wall matrix of growing cells within the LRP. CRT function in protein folding is believed to be regulated by the level of free Ca^{2+} within the cell. In light of the fact that an increase in free Ca^{2+} has been established as an early-auxin response (Barbier-Brygoo, 1995; Abel and Theologis, 1996), it is not surprising that AP5:1/CRT activity is up-regulated during auxin-induced LR formation in *E. globulus*.

Once newly synthesised proteins are correctly folded, they are packaged into vesicles to facilitate their transport through the secretory pathway to target membranes and various cellular compartments (Pryer *et al.*, 1992; Moore *et al.*, 1997; Ferro-Novick and Novick, 1993; Brunger, 2001). Proteins encoded by genes corresponding to DD101.2:4, DD101.2:6 and DD192.1:2, may play a role in these transport mechanisms. DD101.2:4/rabGAP for instance, is likely to be involved in regulating vesicular traffic through the secretory pathway (Terryn *et al.*, 1993b; Brunger, 2001). Upon arrival to the appropriate target membrane, DD192.1:2/VAMP may function in mediating the signalling between the cell and its extracellular environment to aid exocytosis, perhaps by facilitating the fusion between the vesicle and the acceptor membranes (Brunger, 2001). DD101.2:6/ODCI-like may also play a role in exocytosis, as the formation of a GTPase-ODCI complex and its interaction at the cytoplasmic face of the cell membrane is believed to facilitate protein transport across the membrane and entry into the bacterial cell (Hackert *et al.*, 1994). At this point, CRT may have a second function to mediate Ca^{2+} signalling to facilitate exocytosis (Corbett and Michalak, 2000). Collectively the activity of these encoded proteins would ensure that a supply of cell wall precursors and membrane proteins is maintained during growth of the LRP.

The expression data suggests that these mechanisms operate during growth of the LRP (Fig. 7.1). DD101.2:4, DD101.2:6 and DD192.1:2 clones were largely expressed throughout development of the LRP, from early induction to maturity. The respective transcripts accumulated to maximum abundance around 24 to 48 h, with only DD101.2:6 having a well defined peak at 48 h.

7.2.2 Proposed Function of Cell Division, Nutrition and Indeterminate Growth During Stage 2: Lateral Root Meristem Initiation and Activation

The development of the new LR organ and its indeterminate growth requires active cell division and a sufficient supply of phytohormones and nutrients (Barlow and Adam, 1988; Celenza *et al.*, 1995; Cheng *et al.*, 1995). Results from auxin treated *E. globulus* roots (Pelosi *et al.*, 1995a and 1995b), together with the molecular data presented in this study, indicate that the meristem of the new LR is initiated 48 h after initial exposure to auxin in *E. globulus*. This is in agreement with earlier findings that suggest formation of the LR meristem requires 24-48 h in most plants studied (Laskowski *et al.*, 1995; Sussex *et al.*, 1995a).

The expression data suggests that signalling and exocytosis may be just as important during this phase of LR development as it is during earlier events including LRP initiation and growth. This is illustrated by the relatively high level of transcripts corresponding to DD101.2:4/GAP, DD101.2:6/ODCI-like and DD192.1:2/VAMP after 48 h auxin treatment (Fig. 7.1). Elevated rates of cell differentiation and cell proliferation during LR meristem initiation (Laskowski *et al.*, 1995; Sussex *et al.*, 1995a) may place increased demands on mechanisms that involve GAP and ODCI-like activity to regulate protein delivery and secretion (Battey and Blackbourn, 1993; Brunger, 2001). As a consequence signalling proteins such as VAMP are expected to play an important role in mediating interactions between the intra- and extracellular environments (Schena and Davis, 1992; D'Esposito *et al.*, 1996).

Evidence collated in the present study indicates that the rpL2 protein encoded by AP3:3 is likely to function in the synthesis of new proteins required during development of the LR meristem (Fig. 7.1). The relevant literature suggests that expression of the *rpL2* gene may be associated with active and rapid cell division and cell proliferation in the root apical meristem (Köhler *et al.*, 1992; Marty and Meyer, 1992). When the AP3:3 expression data is viewed in light of these studies, a possible correlation may be drawn between maximum transcript abundance after 48 h auxin treatment (Fig. 7.1), and an increase in the rate of cell division during the initiation of the LR meristem.

The availability of nutrients including nitrogenous compounds and carbohydrates is essential to the development of LRs (Drew and Saker, 1975; Friend *et al.*, 1994; Bingham *et al.*, 1997). Whilst nitrogenous compounds appear to promote LR proliferation in wheat (Drew and Saker, 1975; Bingham *et al.*, 1997), carbohydrates are considered to be

particularly important to maintain an active root meristem in tomato (Barlow and Adam, 1988). Consistent with these observations, high activity of genes encoding GS1 and PFP_{β}, represented in this study by DD150.2:4 and DD192.1:6 respectively, appears to coincide with initiation and activation of the new LR meristem after 48 h auxin treatment (Fig. 7.1). This may be indicative of a requirement for increased rates of ammonium assimilation and carbohydrate metabolism, presumably to sustain a viable LR meristem. Moreover, the expression data also suggests that DD192.1:6/PFP_{β} is seemingly active 12-18 h after auxin treatment and then inactivated for a period of 24 h during LRP growth, before reactivation just prior to initiation of the LR meristem. Perhaps this is suggestive of an increased rate of assimilate usage in periods of relatively high mitotic activity, firstly during initiation of the LRP and then again during initiation of the LR meristem (MacLeod, 1972; Laskowski *et al.*, 1995; Malamy and Benfey, 1997b). Therefore, it is likely that DD192.1:6/PFP_{β} functions in a metabolic pathway that ensures specific nutritional requirements are met during initiation of LRP and the LR meristem.

The inhibited LR emergence exhibited by the Le4a:3.2 putative mutant of *Arabidopsis*, together with the retarded primary and LR growth displayed by both the Le4a:3.2 and Le1a:2.1 mutants are suggestive of a partial loss of capacity for indeterminate root growth post germination. During LR formation, the putative Le4a:3.2 defect acts just prior to, or at around the time of LR emergence. In normal development, it is at this time that the LR meristem is organised from a subset of actively dividing cells within the LRP (Peterson and Peterson, 1986; Dolan *et al.*, 1993; Laskowski *et al.*, 1995; Malamy and Benfey, 1997b). Whether a Le4a:3.2 causes a defect in the development of the meristem of the LR warrants further investigation.

7.2.3 Proposed Function of Rigidification and Maintenance of the Lateral Root Meristem During Stage 3: Lateral Root Emergence and Subsequent Growth

When the mature LRP is ready to emerge, AP1:1/extensin activity is at its peak (Fig. 7.1), quite possibly to strengthen and reinforce the cell walls of the primary root surrounding the LRP as it emerges, as suggested for sbHRGP3 by Ahn *et al.* (1996 and 1998). Alternatively, AP1:1/extensin may function in a capacity that is similar to that speculated for HRGPnt3 by Keller and Lamb (1989). In this scenario, AP1:1/extensin may promote rigidification of the cells of the LRP itself, either to stop cell expansion and/or to protect the root tip against the imminent mechanical force exerted as it pushes its way through the cortex and epidermis. However, unlike HRGPnt3, expression of the gene represented by

AP1:1 was not associated with LR initiation. The low levels of AP1:1 transcript detected during LRP growth (from 24 h auxin treatment) may represent increasing mechanical pressure exerted on cells adjacent by the expanding LRP. The comparatively delayed onset of peak AP1:1 expression (72 h after auxin treatment), suggests that the encoded extensin has a role in the latter stages of LR development. As such, AP1:1/extensin activity is more likely to be correlated with LR emergence rather than LR initiation, and conceivably has a sbHRGP3-like function (Ahn *et al.*, 1998) as opposed to HRGPnt3-related activity (Keller and Lamb 1989; Vera *et al.*, 1994). This hypothesis is supported in this study by sequence data which shows significant homology between AP1:1 and sbHRGP3 and not between AP1:1 and HRGPnt3.

Once the LR has emerged, its activated meristem must be maintained to ensure indeterminate growth of the new LR (Laskowski *et al.*, 1995; Sussex *et al.*, 1995a). Vascular differentiation, cell patterning, active cell division and nutrient acquisition are some of the processes operating that ensure growth of the root tip is maintained (Benfey *et al.*, 1993; Di Laurenzio *et al.*, 1996; Cheng *et al.*, 1995; Pysh *et al.*, 1999; Scheres *et al.*, 1995; Helariutta *et al.*, 2000). Le1a:2.1 mutants may be defective in any one or more of these processes resulting in arrested growth of the LR meristem to produce the stunted root system which is characteristic of the mutant phenotype. As the Le1a:2.1 mutation does not appear to affect LR emergence it is likely to act later than Le4a:3.2 during outgrowth of the LR (Fig. 7.1).

7.2.4 Proposed Function of RNA Polymerase and Protein Degradation During Lateral Root Formation

It has been well documented that LR formation requires the regulated transcription of a vast array of genes, and thus it is possible that the RNA polymerase encoded by the corresponding AP2:2 gene functions in regulating general transcriptional activation. AP2:2 expression level was maintained at elevated levels from 12 h onwards reaching its highest peak at 48 h auxin treatment (Fig. 7.1).

The comparatively late onset of AP12:3 expression at 72 h after auxin treatment (Fig 7.1), may be explained by a postulated role for polyubiquitin in the degradation of proteins that were incorrectly folded or are no longer required for LR formation (Klausner and Sitia, 1990; von Kampen *et al.*, 1996). By 72 h, formation of the LRP is complete and ready to emerge as a new LR. It is expected that once the proteins required for LRP

development have fulfilled their purpose, they are ubiquitinated and targeted for degradation by the proteasome. While protein degradation is an ongoing process, completion of LR formation at 72 h coincides with a peak in polyubiquitin, probably to get rid of proteins that are no longer needed for LR formation.

7.2.5 A Proposed Model For Lateral Root Development Based on Data From *Eucalyptus globulus*

The genetic and molecular evidence obtained in the present study was considered in light of the relevant literature to propose a model that explains the possible function and interactions genes identified in this study may possess during of LR development (Fig. 7.2). Signalling proteins (e.g. DD192.1:2/VAMP, AP5:1/CRT) probably act throughout LR development to mediate cell communication by facilitating interactions between cell and cytoplasm. An increased requirement for proteins involved in regulating the trafficking and delivery of molecules to their site of action (e.g. DD101.2:4/rabGAP, DD192.1:2/VAMP, DD101.2:6/ODCI-like) is probably brought about to cope with cellular changes occurring within the growing LRP. Cell wall proteins appear to be particularly important to facilitate cell expansion during periods of LRP growth (e.g. AP9:3 and AP21B:6) and to provide mechanical strength (e.g. AP1:1/extensin) as the LRP is converted to a LR. Development of the LR meristem is likely to involve the expression of genes required for its initiation and activation (Le4a:3.2), as well as genes and associated with rapid cell division (AP3:3/rpL2). Once established the active LR meristem must remain viable in order to sustain indeterminate growth of the new LR organ (Le1a:2.1). This can be in part achieved by and adequate supply of nutrients to the root apex (DD150.1A:1/GS1 and DD192.1:6/PFP$_{\beta}$). Genes involved in general transcription and protein degradation are also important in LR development to ensure genes are activated when needed and their encoded products broken down when no longer required.

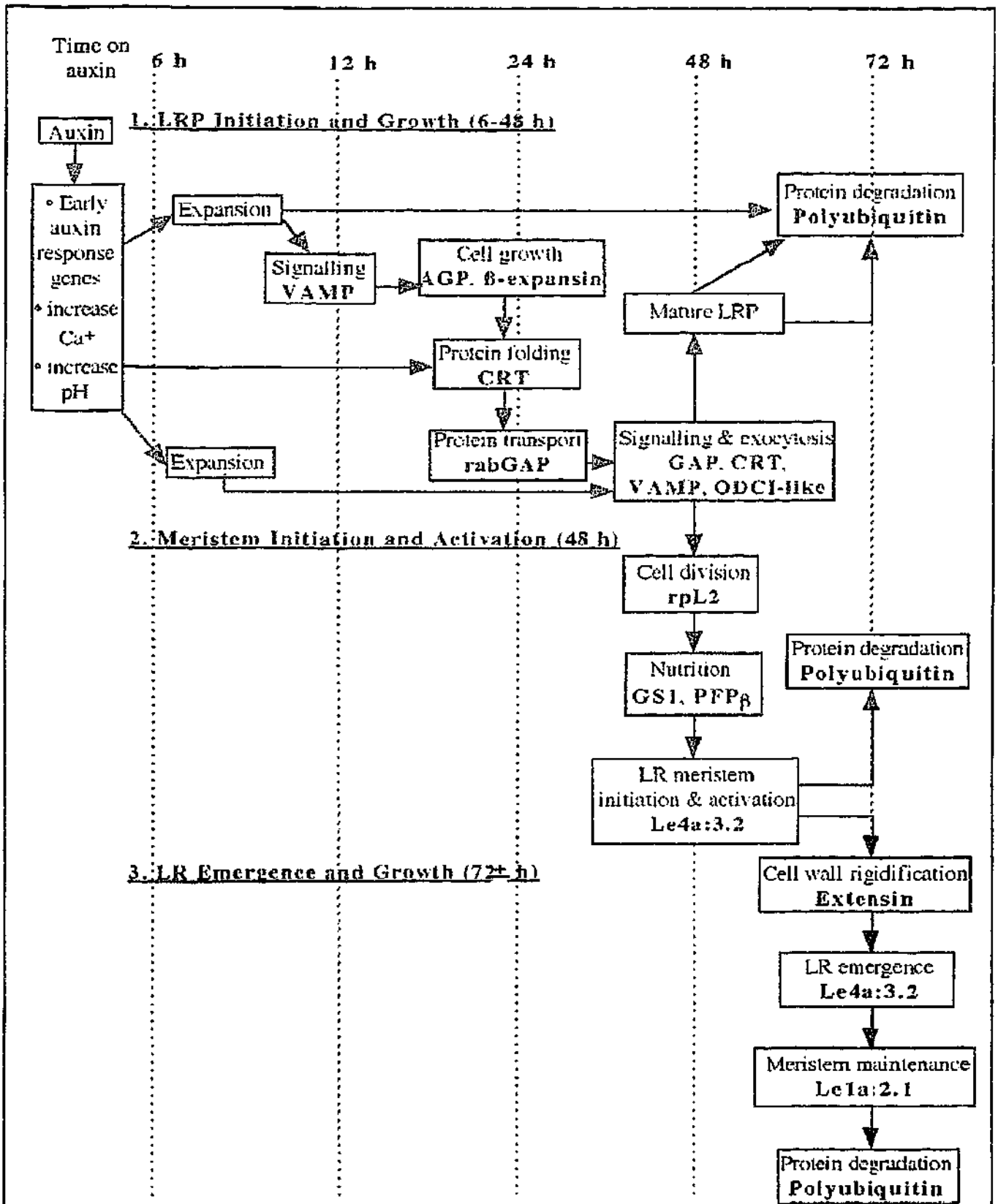


Figure 7.2: A model deduced from the molecular and genetic evidence collected in this study and the relevant literature available. The model proposes that the genes identified operate at one or more stages in LR development. The suggested function and interactions of these genes are outlined for each stage in the text.

7.3 FUTURE DIRECTIONS

It is clear that LR formation is a complex process of sequential metabolic events involving the action of plant phytohormones and the coordinated regulation of a vast array of genes operating in many diverse biochemical and biological pathways. To date relatively few of the characterised genes reported in the literature have been found to function exclusively in the induction, initiation, development and/or growth of the new LR organ. Many of the genes identified in this current study show up-regulated transcriptional activity during both LR and AR formation. In ensuing studies, isolation of the respective genomic clones will facilitate promoter analyses to identify possible regulatory sequences of selected genes. Furthermore, the additional 38 DNA clones from *E. globulus* and 187 putative mutant lines of *Arabidopsis* isolated here will provide a useful supplementary resource for future analyses.

In order to further examine the function of these genes in LR development, gain of function experiments may be preferred. Full length sequences may be introduced under the control of either endogenous promoters or constitutive promoters such as CaMV35S into *E. globulus* and other plant species, including *N. tabacum* or *Arabidopsis*. Antisense and/or deliberate cosuppression (Waterhouse *et al.*, 2001) experiments may also provide information on effects of down-regulating gene activity with regard to LR development and morphology.

Rooting performance in *Eucalyptus* in response to treatment with auxins, is often inconsistent (Hartney, 1980; Zobel, 1993; Bennett *et al.*, 1994; Sasse and Sands, 1995). To address the problem of recalcitrant rooting in *Eucalyptus*, the expression of these LR- (and AR-) related genes should be compared amongst the micropropagated clones designated as hard- and easy-to-root following a root-inducing treatment with phytohormones. Recalcitrant rooting is of course a common problem in woody species. Hutchison *et al.* (1999) hypothesised that decreased rootabilty in conifers is brought about by the loss of cells capable of responding to auxin with respect to induction of AR formation. Whether this could be as a result of a loss of specific cell types, an inability of specific cells to perceive the auxin signal, or the transcriptional suppression of root forming genes is unknown. This question is also applicable to *Eucalyptus* and warrants further investigation.

If differences in specific root gene expression can be detected between hard- and easy-to-root clones, the associated gene may serve as a genetic marker for rootabilty. Of

the genes identified in this study, AP1:1/extensin is the most likely potential candidate for such a rooting marker, as expression studies in *E. globulus* (this study) and in soybean (Ahn *et al.*, 1996; Ahn *et al.*, 1998) show that activity of this gene is specifically associated with LR and AR formation. Such a marker may provide a reliable means by which large scale screening of elite *Eucalyptus* clones can be conducted to determine whether expression of levels of AP1:1/extensin can be correlated with rooting propensity. A root marker could also be used to investigate how certain variables, such as altered culture conditions, the age of cuttings used for vegetative propagation, or the length of time the micropropagated material is in culture may affect rooting competence. If recalcitrance can be detected at the molecular level, means to overcoming the problem could potentially be solved by genetically engineering elite trees to overexpress the relevant gene.

7.3.1 Implications of the Present Research in Breeding Strategies for Micropropagated Eucalypts

The use of genetic linkage maps has become an increasingly powerful tool in genetic studies and modern breeding programs to resolve genotypes, to determine genetic variation, and to facilitate marker-based selection of desirable genetic traits for breeding. This can be achieved by associating genetic traits of interest with DNA markers such as random fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNA (RAPDs) and amplified fragment length polymorphisms (AFLPs), to identify quantitative trait loci (QTLs).

Genetic linkage maps have been constructed for several species of *Eucalyptus* including *E. grandis* and *E. urophylla* (Grattapaglia and Sederoff, 1994), *E. nitens* (Byrne *et al.*, 1995), *E. tereticornis* and *E. globulus* (Marques *et al.*, 1998; Thamarus *et al.*, 2002), using one or a combination of RFLP, RAPD, AFLP, microsatellite and isozyme markers. These maps have been particularly useful in identifying QTLs for frost tolerance, seedling height and leaf area in *E. nitens* (Byrne *et al.*, 1997a and 1997b); and floral development and wood and fibre properties in *E. globulus* (Thamarus *et al.*, 2002).

Major advances in dealing with the problem of rooting recalcitrance in micropropagated eucalypts could potentially be provided through the identification of QTLs that influence root formation. Importantly, if any of the LR genes identified in the present study were found to map in or around the DNA markers located on existing linkage

maps, the associated QTLs may be useful as molecular markers for breeding programs to improve rooting propensity in elite trees.

In addition, cloning and molecular genomic characterisation analyses of the genes represented by the Le1a:2.1 and Le4a:3.2 mutations may be facilitated through the identification of their respective chromosomal locations on both the morphological and DNA marker maps of *Arabidopsis*. Once cloned, the *E. globulus* homologues could be recovered and molecular characterisation studies performed.

7.3.2 Applicability of Differential Display, cDNA Subtraction and Eucalypts for Gene Discovery in the Future

The recent advent of the completed *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative, 2000) has paved the way for functional genomics, a holistic approach to studying genes and their role in plant growth and development. Functional genomics encompasses the use of high throughput technologies such as DNA microarrays to analyse thousands of genes, transcripts, proteins or metabolites simultaneously (Colebatch *et al.*, 2002). Basically, microarrays consist of DNA spots representing known ESTs, immobilised on a glass slide, and then hybridised to fluorescently labelled cDNA probes. Typically, cDNA derived from control and experimental samples are each labelled with a different dye and together are hybridised to a single microarray. In this way differentially expressed genes can be detected by comparing the relative signal intensities of the two probes (Colebatch *et al.*, 2002).

This promising and exciting new technology enables almost the entire *Arabidopsis* genome to be scanned in a single experiment. Accordingly it may be tempting, and certainly feasible, to concentrate future gene discovery research in *Arabidopsis*, when searching for genes involved in fundamental plant processes. However, DNA microarray technology is not applicable to non-model species such as *E. globulus*, nor is *Arabidopsis* practical if searching for genes with specialised functions, such as nitrogen fixing in legumes or lignin biosynthesis and fibre length in woody species.

When searching for genes that are likely to be specific to species other than *Arabidopsis*, the DDRT-PCR and cDNA subtraction procedures are worthy of consideration. Like DNA microarrays, these techniques also facilitate a functional genomic approach to identifying differentially expressed genes, albeit at a smaller scale.

As applied in this study, DDRT-PCR and cDNA subtraction provide efficient means for rapidly screening a population of mRNAs for transcriptionally active genes in species such as *E. globulus*, where the complete genome is not available. Employment of these procedures in this study, led to the recovery of three sequences (AP19A:1/unknown, AP21B:6/unknown and DD101.2:6/ODCI-like) from a total of 15 that showed no significant homology to *Arabidopsis* genes, proteins or ESTs. This suggests that although the developmental root program is likely to be largely conserved among higher plants, it may be plausible to expect that a subset of genes required for LR development in *E. globulus* may be unique to woody species.

7.4 CONCLUDING REMARKS

The preliminary characterisation of the genes and mutants identified in this experimental program has helped to define three developmental stages in the pathway for LR formation in *Eucalyptus*, possibly common to higher plants. In addition, the information obtained has served to increase our understanding of some of the molecular and genetic mechanisms involved in the rooting process. This knowledge is of fundamental importance and may also be of practical value, in the longer term, to improve the feasibility of clonal forestry and represents valuable resources that may facilitate the selection of desired genotypes from preferred varieties of *Eucalyptus* and other woody species for plantation establishment programs.

APPENDIX

A-1: Adapters and Primers Used in the cDNA Subtraction Procedure

Adapter 1

```
5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
                                        3'-GGCCCGTCCA-5'
```

5'-TCAAGCGGCCGCCCGGGCAGGT-3' **Nested PCR primer 1**

5'-CTAATACGACTCACTATAGGGC-3' **PCR primer 1**

Adapter 2

```
5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3'
                                        3'-CGGCTCCA-5'
```

5'-AGCGTGGTCGCGGCCGAGGT-3' **Nested PCR primer 2**

A-2: Primers Used for Differential Display

$T_{11}AA$	5'-TTTTTTTTTTAA-3'
$T_{11}AC$	5'-TTTTTTTTTTAC-3'
$T_{11}AG$	5'-TTTTTTTTTTAG-3'
OPAP-01	5'-AACTGGCCCC-3'
OPAP-02	5'-TGGTCATCCC-3'
OPAP-14	5'-TGCCATGCTG-3'

REFERENCES

Abel, S., Nguyen, M.D. and Theologis, A. (1995). The *PS-IAA/5*-like family of early auxin-inducible mRNAs in *Arabidopss thaliana*. *Journal of Molecular Biology*, **251**(4): 533-549.

Abel, S., Oeller, P.W. and Theologis, A. (1994). Early auxin-induced genes encode short-lived proteins. *Proceedings of the National Academy of Sciences of the United States of America*, **91**: 326-330.

Abel, S. and Theologis, A. (1996). Early genes and auxin action. *Plant Physiology*, **111**: 9-17.

Ach, R.A. and Gruissem, W. (1994). A small nuclear GTP-binding protein from tomato suppresses a *Schizosaccharomyces pombe* cell-cycle mutant. *Proceedings of the National Academy of Sciences of the United States of America*, **91**: 5863-5867.

Agarwal, M.L. and Cullis, C.A. (1991). The ubiquitin-encoding multigene family of flax, *Linum usitatissimum*. *Gene*, **99**: 69-75.

Aguilar, M., Osuna, D., Caballero, J.L. and Munoz, J. (1997). Isolation and characterization of a cDNA encoding a polyubiquitin protein (Accession No. U82086) from strawberry fruit (PGR97-030). *Plant Physiology*, **113**: 665.

Ahn, J.H., Choi, Y., Kim, S.G., Kwon, Y.M., Choi, Y.D. and Lee, J.S. (1998). Expression of a soybean hydroxyproline-rich glycoprotein gene is correlated with maturation of roots. *Plant Physiology*, **116**: 671-679.

Ahn, J.H., Choi, Y., Kwon, Y.M., Kim, S.G., Choi, Y.D. and Lee, J.S. (1996). A novel extensin gene encoding a hydroxyproline-rich glycoprotein requires sucrose for its wound-inducible expression in transgenic plants. *Plant Cell*, **8(9)**: 1477-1490.

Altman, A. and Wareing, P.F. (1975). The effect of IAA on sugar accumulation and basipetal transport of ^{14}C-labelled assimilates in relation to root formation in *Phaseouls vulgaris* cuttings. *Plant Physiology*, **33**: 32-38.

An, G., Luo, G., Veltri, R.W. and O'Hara, S.M. (1996). Sensitive, nonradioactive differential display method using chemiluminescent detection. *BioTechniques*, **20(3)**: 342-346.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M. (1999). Differential display of eukaryotic mRNA: Meeting the demands of the new millennium? *Journal of Plant Physiology*, **154(5-6)**: 561-570.

Arabidopsis Genome Initiative, The (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**: 796-815.

Averboukh, L., Douglas, S.A., Zhao, S., Lowe, K., Maher, J. and Pardee, A.B. (1996). Better gel resolution and longer cDNAs increase the precision of differential display. *BioTechniques*, **20(5)**: 918-921.

Avila, C., Suárez, M.F., Gómez-Maldonado, J. and Cánovas, F.M. (2001). Spatial and temporal expression of two cytosolic glutamine synthetase genes in Scots pine:

functional implications on nitrogen metabolism during early stages of conifer development. *The Plant Journal*, 25(1): 93-102.

Baksh, S. and Michalak, M. (1991). Expression of calreticulin in *Escherichia coli* and identification of its Ca^{2+} binding domains. *Journal of Biological Chemistry*, **266(32)**: 21458-21465.

Ballas, N., Wong, L., Ke, M. and Theologis, A. (1995). Two auxin-responsive domains interact positively to induce expression of the early indoleacetic acid-inducible gene *PS-IAA4/5*. *Proceedings of the National Academy of Sciences of the United States of America*, **92(8)**: 3483-3487.

Barbier-Brygoo, H. (1995). Tracking auxin receptors using functional approaches. *Critical Reviews in Plant Sciences of the United States of America*, **14(1)**: 1-25.

Barlow, P.W. and Adam, J.S. (1988). The position and growth of lateral roots on cultured root axes of tomato, *Lycopersicon esculentum* (*Solanaceae*). *Plant Systematics and Evolution*, **158**: 141-154.

Battey, N.H. and Blackbourn, H.D. (1993). Tansley review no. 57. The control of exocytosis in plant cells. *New Phytologist*, **125**: 307-338.

Baskin, T.I., Betzner, A.S., Hoggart, R., Cork, A. and Williamson, R.E. (1992). Root morphology mutants in *Arabidopsis thaliana*. *Australian Journal of Plant Physiology*, **19**: 427-437.

Bauer, D., Biehler, K., Fock, H., Carrayol, E., Hirel, B., Migge, A. and Becker, T.W. (1997). A role for cytosolic glutamine synthetase in the remobilization of leaf nitrogen during water stress in tomato. *Physiologia Plantarum*, **99**: 241-248.

Bauer, D. Müller, H., reich, J., Riedei, H., Ahrenkiel, V., Warthoe, P. and Strauss, M. (1993). Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research*, **21(18)**:4272-4280.

Benedetti, C.E. and Turner, J.G. (1995) Nucleotide Sequence of an *Arabidopsis thaliana* cDNA (Accession U27698) Encoding a Protein Homologous to Plant and Animal Calreticulins (PGR95-047). *Plant Physiology*, **109**: 338.

Benfey, P.N., Linstead, P.J., Roberts, K., Schiefelbein, J.W., Hauser, M. and Aeschbacher, R.A. (1993). Root development in *Arabidopsis*: four mutants with dramatically altered root morphogenesis. *Development*, **119**: 57-70.

Benito, E.P., Prins, T. and van Kan, J.A.L. (1996). Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Molecular Biology*, **32**: 947-957.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offringa, R. (2001). The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development*, **128**: 4057-4067.

Bennett, S.R.M., Alvarez, J., Bossinger, G. and Smyth, D.R. (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. *The Plant Journal*, **8**: 505-520.

Bennett, I.J., McComb, J.A., Tonkin, C.M. and McDavid, D.A.J. (1994). Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Annals of Botany*, **74**: 53-58.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B. and Feldmann, K.A. (1996). *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. *Science*, **273**: 948-950.

Bennet, M.J., Lightfoot, D.A. and Cullimore, J.V. (1989). cDNA sequence and differentiatial expression of the gene encoding the glutamine synthetase γ polypeptide of *Phaseolus vulgaris* L. *Plant Molecular Biology*, **12**: 553-565.

Bertioli, D.J., Schlichter, U.H.A., Adams, M.J., Burrows, P.R., Steinbiß and Antoniw, J.F. (1995). An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acids Research*, **23(21)**: 4520-4523.

Biddington, N.L and Dearman, A.S. (1982). The involvement of the root apex and cytokinins in the control of lateral root emergence in lettuce seedlings. *Plant Growth Regulation*, **1**: 183-193.

Binet, M.-N., Weil, J.-H. and Tessier, L.-H. (1991). Structure and expression of sunflower ubiquitin genes. *Plant Molecular Biology*, **17(3)**: 395-407.

Bingham, I.J., Blackwood, J.M. and Stevenson, E.A. (1997). Site, scale and time-course for adjustments in lateral root initiation in wheat following changes in C and N supply. *Annals of Botany*, **80**: 97-106.

Blakely, L.M. and Evans, T.A. (1979). Cell dynamics studies on the pericycle of radish seedling roots. *Plant Science Letters*, **14**: 79-83.

Blakely, L.M., Rodaway, S.J., Hollen, L.B. and Croker, S.G. (1972). Control and kinetics of branch root formation in cultured root segments of *Haplopappus ravenii*. *Plant Physiology*, **50**: 35-42.

Blakely, L.M., Durham, M., Evans, T.A. and Blakely, R.M. (1982). Experimental studies on lateral root formation in radish seedling roots. I. General methods, developmental stages, and spontaneous formation of laterals. *Botanical Gazette*, **143(3)**: 341-352.

Boerjan, W., Cervera, M., Delarue, M., Beeckman, T., Dewitte, W., Bellinin, C., Caboche, M.m Van Onckelen, H., Van Montagu, M. and Inzé, D. (1995). *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. *The Plant Cell*, **7**: 1405-1419.

Borisjuk, N., Sitailo, L., Adler, K., Malysheva, L., Tewes, A., Borisjul, L. and Manteuffel, R. (1998). Calreticulin expression in plant cells: developmental regulation, tissue specificity and intracellular distribution. *Planta*, **206(4)**: 504-514.

Boron L.J. and Legocki, A.B. (1993). Cloning and characterisation of a nodule-enhanced glutamine synthetase-encoding gene from *Lupinus luteus*. *Gene*, **136**: 95-102.

Bourne, H.R. (1988). Do GTPases direct membrane traffic in secretion? Cell, **53**: 669-671.

Bourne, H.R., Sanders, D.A. and McCormick, F. (1991). The GTPase superfamily: conserved structure and molecular mechanism. *Nature*, **349**: 117-127.

Brenner, S.E. (1998). Practical database searching. *Trends Guide to Bioinformatics*, **Supplement:** 9-12.

Brunger, A.T. (2001). Structural insights into the molecular mechanism of calcium-dependent vesicle-membrane fusion. *Current Opinion in Structural Biology*, **11(2)**: 163-173.

Byrne, M., Murrell, J.C., Allen, B. and Moran, G.F. (1995). An integrated genetic linkage map of eucalypts using RFLP, RAPD and isozyme markers. *Theoretical and Applied Genetics*, **91**: 869-875.

Byrne, M., Murrell, J.C., Owen, J.V., Kriedemann, P., Williams, E.R. and Moran, G.F. (1997a). Identification and mode of action of quantitative trait loci affecting seedling height and leaf area in *Eucalyptus nitens*. *Theoretical and Applied Genetics*, **94**:674-681.

Byrne, M., Murrell, J.C., Owen, J.V., Williams, E.R. and Moran, G.F. (1997b). Mapping of quantitative trait loci influencing frost tolerance in *Eucalyptys nitens*. *Theoretical and Applied Genetics*, **95**: 975-979.

Callard, D., Lescure, B. and Mazzolini, L. (1994). A method for the elimination of false positives generated by the mRNA differential display technique. *BioTechniques*, **16(6)**:1096-1103.

Callis, J. Carpenter, T., Sun, C.-W. and Viestra, R.D. (1995). Structure and evolution of genes encoding polyubiquitin-like proteins in *Arabidopsis thalian* ecotype Columbia. *Genetics*, **139**: 921-939.

Carlisle, S.M., Blakeley, S.D., Hemmingsen, S.M., Trevanion, S.J., Hiyoshi, T., Kruger, N.J. and Dennis, D.T. (1990). Pyrophosphate-dependent phosphofructokinase. Conservation of protien sequence between the α- and β-subunits and with the ATP-dependent phosphofructokinase. *The Journal of Biological Chemistry*, **265(30)**: 18366-18371.

Carpita, N.C. and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, **3(1)**: 1-30.

Casero, P.J., Casimiro, I. and Lloret, P.G. (1995). Lateral root initiation by asymmetrical transverse divisions of pericycle cells in four plant species: *Raphanus sativus*, *Helianthus annuus*, *Zea mays*, and *Daucus carota*. *Protoplasma*, **188**: 49-58.

Cassab, G.I. (1986). Arabinogalactan proteins during the development of soybean root nodules. *Planta*, **168**: 441-446.

Cassab, G.I. (1998). Plant cell wall proteins. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**: 281-309.

Catalá, C., Rose, J.K.C. and Bennett, A.B. (2000). Auxin-regulated genes encoding cell wall-modifying proteins are expressed during early tomato fruit growth. *Plant Physiology*, **122(2)**: 527-534.

Celenza, J.L., Grisafi, P.L., and Fink, G.R. (1995). A pathway for lateral root formation in *Arabidopsis thaliana*. *Genes and Development*, **9**: 2131-2142.

Cernac, A., Lincoln, C., Lammer, D. and Estelle, M. (1997). The *SAR1* gene of *Arabidopsis* acts downstream of the *AXR1* gene in auxin response. *Development*, **124**: 1583-1591.

Chen, F., Hayes, P.M., Mulrooney, D.M. and Pan, A. (1994). Identification and characterization of cDNA clones encoding plant calreticulin in barley. *The Plant Cell*, **6**: 835-843.

Chen, J. and Varner, J.E. (1985a). An extracellular matrix protein in plants: characterization of a genomic clone for carrot extensin. *The EMBO Journal*, **4(9)**: 2145-2151.

Chen, J. and Varner, J.E. (1985b). Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proceedings of the National Academy of Sciences of the United States of America*, **82**: 4399-4403.

Cheng, J.C., Seeley, K.A. and Sung, Z.R. (1995). *RML1* and *RML2*, *Arabidopsis* genes required for cell proliferation at the root tip. *Plant Physiology*, **107**:365-376.

Cheng, H.F. and Tao, M. (1990). Differential proteolysis of the subunits of pyrophosphate-dependent 6-phosphofructo-1-phosphotransferase. *Journal of Biological Chemistry*, **265**: 2173-2177.

Cho, H.-T. and Kende, H. (1998). Tissue localization of expansins in deepwater rice. *The Plant Journal*, **15(6)**: 805-812.

Chung, C. and Baek, S. (1999). Deubiquitinating enzymes: their diversity and emerging roles. *Biochemical and Biophysical Research Communications*, **266(3)**: 633-640.

Christensen, S.K., Dagenais, N., Chory, J. and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. *Cell*, **100(4)**: 469-478.

Chung, C.T. and Miller, R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research*, **16**: 3580.

Colebatch, G., Trevaskis, B. and Udvardi, M. (2002). Functional genomics: tools of the trade. *New Phytologist*, **153**: 27-36.

Colonna-Romano, S., Leone, A. and Maresca, B. (1998). Differential Display Reverse Transcription PCR (DDRT-PCR). Springer-Verlag, Heidelberg.

Conkling, M.A., Cheng, C., Yamamoto, Y.T. and Goodman, H.M. (1990). Isolation of transcriptionally regulated root-specific genes from tobacco. *Plant Physiology*, **93**: 1203-1211.

Cooperman, B.S., Wooten, T., Romero, D.P. and Traut, R.R. (1995). Histidine 229 in protein L2 is apparently essential for 50S peptidyl transferase activity. *Biochemistry and Cell Biology*, **73**: 1087-1094.

Corbett, E.F. and Michalak, M. (2000). Calcium, a signaling molecule in the endoplasmic reticulum? *Trends in Biochemical Sciences*, **25(7)**: 307-311.

Corbin, D.R., Sauer, N. and Lamb, C.J. (1987). Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. *Molecular and Cellular Biology*, **7(12)**: 4337-4344.

Cosgrove, D.J. (1993). Wall extensibility: its nature, measurement and relationship to plant cell growth. *New Phytologist*, **124**: 1-23.

Cosgrove, D.J. (1997). Assembly and enlargement of the primary cell wall in plants. *Annual Review of Cell and Developmental Biology*, **13**: 171-201.

Cosgrove, D.J. (2000). Loosening of plant cell walls by expansins. *Nature*, **407(6802)**: 321-326.

Cosgrove, D.J., Bedinger, P. and Durachko, D.M. (1997). Group I allergens of grass pollen as cell wall-loosening agents. *Proceedings of the National Academy of Sciences of the United States of America*, **94(12)**: 6559-6564.

Cosgrove, D.J. and Li, Z.-C. (1993). Role of expansin in cell enlargement of oat coleoptiles. *Plant Physiology*, **103(4)**: 1321-1328.

Coughlan, S.J., Hastings, C. and Winfrey, R. (1997). Cloning and characterization of the calreticulin gene from *Ricinus communis* L. *Plant Molecular Biology*, **34**: 897-911.

Crofts, A.J and Denecke, J. (1998). Caltreticulin and calnexin in plants. *Trends in Plant Science*, **3(10)**: 396-399.

Crofts, A.J., Leborgne-Castel, N., Pesca, M., Vitale, A. and Denecke, J. (1998). BiP and calreticulin form an abundant complex that is independent of endoplasmic reticulum stress. *The Plant Cell*, 10: 813-823.

Crowell, D.N. (1994). Cytokinin regulation of a soybean pollen allergen gene. *Plant Molecular Biology*, **25**: 829-835.

Danilczyk, U.G., Cohen-Doyle, M.F. and Williams, D.B. (2000). Functional relationship between calreticulin, calnexin, and the endoplasmic reticulum luminal domain of calnexin. *The Journal of Biological Chemistry*, **275(17)**: 13089-13097.

Dargeviciute, A., Roux, C., Decreux, A., Sitbon, F. and Perrot-Rechenmann, C. (1998). Molecular cloning and expression of the early auxin-responsive *Aux/IAA* gene family in *Nicotiana tabacum*. *Plant Cell Physiology*, **39(10)**: 993-1002.

Davies, P.J. (1987) The plant hormones: Their nature, occurrence and functions. In Davies, PJ. (ed). *Plant Hormones and their Role in Plant Growth and Development*. Martinus Nijhoff Publishers, Dordrecht, pp. 1-23.

Delbarre, A., Muller, P., Imhoff, V. and Guern, J. (1996). Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. *Planta*, **198**: 532-541.

del Pozo, J.C. and Estelle, M. (1999a). The *Arabidopsis* cullin AtCUL1 s modified by the ubiquitin-related protein RUB1. *Proceedings of the National Academy of Sciences of the United States of America*, **96(26)**: 15342-15347.

del Pozo, J.C. and Estelle, M. (1999b). Function of the ubiquitin-proteosome pathway in auxin response. *Trends in Plant Science*, **4(3)**: 107-112.

del Pozo, J.C., Timpte, C., Tan, S., Callis, J. and Estelle, M. (1998). The ubiquitin-related protein RUB1 and auxin response in *Arabidopsis*. *Science*, **280**: 1760-1763.

Denecke, J., Carlsson, L.E., Vidal, S., Höglund, A-S., Ek, B., van Zeijl, M.J., Sinjoro, K.M.C. and Palva, E.T. (1995). The tobacco homolog of mammalian calreticulin is present in protein complexes *in vivo*. *The Plant Cell*, **7**: 391-406.

D'Esposito, M., Ciccodicola, A., Gianfrancesco, F., Esposito, T., Flagiello, L., Mazzarella, R., Sclessinger, D. and D'Urso, M. (1996). A synaptobrevin-like gene in the Xq28 pseudoautosomal region undergoes X inactivation. *Nature Genetics*, 13: 227-229.

De Tullio, M.C., Paciolla, C., Dalla Vecchia, F., Rascio, N., D'Emerico, S., De Gara, L., Liso, R. and Arrigoni, O. (1999). Changes in onion root development induced by the inhibition of peptidyl-prolyl hydrolase and influence of the ascorbate system on cell division and elongation. *Planta*, **209**: 424-434.

Dhindsa, R.S., Dong, G. and Lalonde, L. (1987). Altered gene expression during auxin-induced root development from excised mung bean seedlings. *Plant Physiology*, 84: 1148-1153.

Diachenko, L.B., Ledesma, J., Chenchik, A.A. and Siebert, P.D. (1996). Combining the technique of RNA fingerprinting and differential display to obtain differentially expressed mRNA. *Biochemical and Biophysical Communications*, **219**: 824-828.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Psch, L., Helariutta, Y., Freshour, G., Hahn, M.G., feldman, K.A. and Benfey, P.N. (1996). The *SCARCROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root. *Cell*, **86(3)**: 423-434.

Din, N., Forsythe, I.J., Burtnick, L.D., Gikes, N.R., Miller, R.C., Warren, R.A.J. and Kilburn, D.G. (1994). The cellular-binding domain of endoglucanase A (CenA) from *Cellulomonas fimi*: evidence for the involvement of tryptophan residues in binding. *Molecular Microbiology*, **11(4)**: 747-755.

Doerner, P., Jørgensen, J., You, R., Steppuhn, J. and Lamb, C. (1996). Control of root growth and development by cyclin expression. *Nature*, 380: 520-523.

Dohmen, R.J., Stappen, R., McGrath, J.P., Forrová, H., Kalarov, J., Goffeau, A. and Varshavsky, A. (1995). An essential yeast gene encoding a homology of ubiquitin-activating enzyme. *Journal of Biological Chemistry*, **270(30)**: 18099-18109.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993). Cellular organisation of the *Arabidopsis thaliana* root. *Development*, **119**: 71-84.

Dresselhaus, T., Hagel, C., Lörz, H. and Kranz, E. (1996). Isolation of a full-length cDNA encoding calreticulin from a PCR library of *in vitro* zygotes of maize. *Plant Molecular Biology*, **31(1)**: 23-34.

Drew, M.C. and Saker, L.R. (1975). Nutrient supply and the growth of the seminal root system in barley. II. Localized compensatory increases in lateral root growth and rates of nitrate uptake when nitrate supply is restricted to only part of the root system. *Journal of Experimantal Botany*, **26**: 79-90.

Dubrovsky, J.G., Doerner, P.W., Colón-Carmona, A. and Rost, T.L. (2000). Pericycle cell proliferation and lateral root initiation in *Arabidopsis*. *Plant Physiology*, **124(4)**: 1648-1657.

Dubrovsky, J.G., Rost, T.L., Colón-Carmona, A. and Doerner, P. (2001). Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. *Planta*, **214(1)**: 30-36.

Eckardt, N.A. (2001). Auxin and the power of the proteasome in plants. *The Plant Cell*, **13**: 2161-2163.

Egertsdotter, U. and Van Arnold, S. (1995). Importance of arabinogalactan proteins for the development of somatic embryos of norway spruce (*Picea abies*). *Physiologia Plantarum*, **93**: 334-345.

Eldridge, K., Davidson, J., Harwood, C. and van Wyk, G. (1994). *Eucalypt Domestication and Breeding*. Clarendon Press, Oxford.

Esau, K. (1977). *Anatomy of Seed Plants*. John Wiley and Sons, USA.

Estelle, M. (2001). Plant hormones - Transporters on the move. *Nature*, **413(6854)**: 374-375.

Estelle, M.A. and Somerville, C. (1987). Auxin-resistant mutants of *Arabidopsis thaliana* with altered morphology. *Molecular and General Genetics*, **206**: 200-206.

Etter, A., Bernard, V., Kenzelmann, M., Tobler, H. and Müller, F. (1994). Ribosomal heterogeneity from chromatin diminution in *ascaris lumbricoides*. *Science*, **265**: 954-956.

Evans, M.L., Ihikawa, H. and Estelle, M.A. (1994). Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: comparison of wild type and auxin-response mutants. *Planta*, **194**: 215-222.

FAO (1979). *Eucalypts for Planting*. FAO Forestry Series No. II. Food and agriculture Organization of the United Nations, Rome.

Ferreira, P.C.G., Hemerly, A.S., de Almeida Engler, J., Van Montagu, M., Engler, G. and Inzé, D. (1994). Developmental expression of the *Arabidopsis* cyclin gene *cycIAt*. *The Plant Cell*, **6**:1763-1774.

Ferreira, P.C.G., Hemerly, A.S., Villarroel, R., Van Montagu, M. and Inzé, D. (1991). The *Arabidopsis* functional homolog of the $p34^{cdc2}$ proein kinase. *The Plant Cell*, **3**: 531-540.

Ferro-Novick, S. and Novick, P. (1993). The role of GTP-binding proteins in transport along the exocytic pathway. *Annual Review of Cell Biology*, **9**: 575-599.

Fincher, G.B. and Stone, B.A. (1983). Arabinogalactan-proteins: structure, biosynthesis, and function. *Annual Review of Plant Physiology and Plant Molecular Biology*, **34**: 47-70.

Fleming, A.J., McQueen-Mason, S., Mandel, T., Kuhlemeir, C. (1997). Induction of leaf primordia by the cell wall protein expansin. *Science*, **276**: 1415-1418.

Fogel, R. (1985). Roots as primary producers in below-ground ecosystems. In Fitter, A.H., Atkinson, D., Read,D.J. and Usher, M.B. (eds). *Ecological Interactions in Soil*. Blackwell, Oxford, pp 23-36.

Fong, C., Kieliszewski, M.J., de Zacks, R., Leykam, J.F. and Lamport, D.T.A. (1992). A gymnosperm extensin contains serine-tetra hydroxyproline motif. *Plant Physiology*, **99**: 548-552.

Franco, A.R., Gee, M.A. and Guilfoyle, T.J. (1990). Induction and superinduction of auxin-responsive mRNAs with auxin and protein synthesis inhibitors. *Journal of Biological Chemistry*, **265(26)**: 15845-15849.

Friend, A.L., Coleman, M.D. and Isebrands, J.G. (1994). Carbon allocation to root and shoot systems of woody plants. In Davis, T.D. and Haissig, B.E. (eds.) *Biology of Adventitious Root Formation*. Plenum Press, New York, pp245-273.

Gao, M., Kieliszewski, M.J., Lamport, D.T.A. and Showalter, A.M. (1999). Isolation, characterisation and immunolocalisation of a novel, modular tomato arabinogalactan-protein corresponding to the *LeAGP-1* gene. *The Plant Journal*, **18(1)**: 43-55.

Gao, M. and Showalter, A.M. (2000). Immunolocalisation of LeAGP-1, a modular arabinogalactan-protein, reveals its developmentally regulated expression in tomato. *Planta*, **210(6)**: 865-874.

Geldner, , N., Friml, J., Stierhof, Y.-D., Jürgens, G. and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature*, **413(6854)**: 425-428.

Genschik, P., Marbach, M., Uze, M., Feuerman, B., Plesse, B. and Fleck, J. (1994). Structure and promoter activity of a stress and developmentally regulated polyubiquitin-encoding gene of *Nicotiana tabacum*. *Gene*, **148(2)**: 195-202.

Genschik, P., Parmentier, Y., Durr, A., Marbach, J., Criqui, M.-C., Jamet, E. and Fleck, J. (1992). Ubiquitin genes are differentially regulated in protoplast-derived cultures of *Nicotiana sylvestris* and in response to various stresses. *Plant Molecular Biology*, **20**: 897-910.

Gerlach and Bedbrook (1979). Cloning and characterization of ribosmal genes from wheat and barley. *Nucleic Acids Research*, **7(7)**: 1869-1885.

Gerst, J.E., Rodgers, L., Riggs, M. and Wigler, M. (1992). *SNC1*, a yeast homolog of the synaptic vesicle-associated membrane protein/synaptobrevin gene family: genetic interactions with the *RAS* and *CAP* genes. *Proceedings of the National Academy of Sciences of the United States of America*, **89**: 4338-4342.

Gil, P. and Green, P.J. (1997). Regulatory activity exerted by the *SAUR-AC1* promoter region in transgenic plants. *Plant Molecular Biology*, **34**: 803-808.

Gil, P., Liu, Y., Orbovic, V., Verkamp, E. Poff, K.L. and Green, P.J. (1994). Characterization of the auxin-inducible *SAUR-AC1* gene for use as a molecular genetic tool in *Arabidopsis*. *Plant Physiology*, **104**: 777-784.

Grattapaglia, D. and Sederoff, R. (1994). Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using pseudo-testcross: mapping strategy and RAPD markers. *Genetics*, **137**: 1121-1137.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H. and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes and Development*, **13**: 1678-1691.

Gray, W.M. and Estelle, M. (2000). Function of the ubiquitin-proteasome pathway in auxin response. *Trends in Biochemical Sciences*, **25(3)**: 133-138.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O. and Estelle, M. (2001). Auxin regulates SCF^{TIR}-dependent degradation of Aux/IAA proteins. *Nature*, **414**: 271-276.

Gray, W.M., Östin, A., Sandberg, G., Romano, C.P. and Estelle, M. (1998). High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*.

Proceedings of the National Academy of Sciences of the United States of America, **95**: 7197-7202.

Guilfoyle, T.J. (1998). Aux/IAA proteins and auxin signal transduction. *Trends in Plant Science*, **3(6)**: 205-207.

Guilfoyle, T.J., Hagen, G., Li, Y., Ulmasov, T., Liu, Z., Strabala, T. and Gee, M. (1993). Auxin-regulated transcription. *Australian Journal of Plant Physiology*, **20**: 489-502.

Haag, E. and Raman, V. (1994). Effects of primer choice and source of *Taq* DNA polymerase on the banding patterns of differential display RT-PCR. *BioTechniques*, **17(2)**: 226-228.

Hackert, M.L., Carroll, D.W., Davidson, L., Kim, S-O., Momany, C., Vaaler, G.L. and Zhang, L. (1994). Sequence of ornithine decarboxylase from *Lactobacillus* sp. strain 30a. *Journal of Bacteriology*, **176(23)**: 7391-7394.

Hagen, G. and Guilfoyle, T.J. (1985). Rapid induction of selective transcription by auxins. *Molecular and Cellular Biology*, **5(6)**: 1197-1203.

Hagen, G., Kleinschmidt, A. and Guilfoyle, T. (1984). Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. *Planta*, **162**: 147-153.

Hager, A., Debus, G., Edel, H.-G., Stransky, H. and Serrano, R. (1991). Auxin induces exocytosis and the rapid synthesis of a high-turnover pool of plasma-membrane H^+-ATPase. *Planta* **185**: 527-537.

Haissig, B.E., Davis, T.D. and Riemenschneider, D.E. (1992). Researching the controls of adventitious rooting. *Physiologia Plantarum*, **84**: 310-317.

Hammerschmidt, R., Lamport, D.T.A. and Muldoon, E.P. (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber to *Cladosporium cucumerinum*. *Physiological Plant Pathology*, **24**: 43-47.

Harrison, S.C. (1991). A structural taxonomy of DNA-binding domains. *Nature*, **353**: 715-719.

Hartney, V.J. (1980). Vegetative propagation of the eucalypts. *Australian Forest Research*, **10**: 191-211.

Hauser , M., Morikami, A. and Benfey, P.N. (1995). Conditional root expansion mutants of *Arabidopsis*. *Development*, **121**: 1237-1252.

Hegde, A.N., Broome, B.M., Qiang, M. and Schwartz, J.H. (2000). Structure and expression of the *Aplysia* polyubiquitin gene. *Molecular Brain Research*, **76**: 424-428.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M.T. and benfey, P.N. (2000). The *SHORT-ROOT* gene controls radial patterning of the *Arabidopsis* root through radial signaling. *Cell*, **101(5)**: 555-567.

Hemerly, A., Bergounioux, C., Van Montagu, M., Inzé, D. and Ferreira, P. (1992). Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, **89**: 3295-3299.

Hemerly, A.S., Ferreira, P., de Almeida Engler, J. Van Montagu, M., Engler, G. and Inzé, D. (1993). *cdc2a* expression in *Arabidopsis* is linked with competence for cell division. *The Plant Cell*, 5: 1711-1723.

Hess, W.R., Hoch, B., Zeltz, P., Hübschmann, T. Kössel, H. and Börner, T. (1994). *Inefficient rpl2* splicing in barley mutants with ribosome-deficient plastids. *The Plant Cell*, **6(10)**: 1455-1465.

High, S., Lecomte, F.J.L., Russell, S.J., Abell, B.M., Oliver, J.D. (2000). Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? *FEBS Letters*, **476(1-2)**: 38-41.

Hirayama, T., Imajuku, Y., Anai, T., Matsui, M., and Oka, A. (1991). Identification of two cell-cycle-controlling *cdc2* gene homologs in *Arabidopsis thaliana*. *Gene*, **105**: 159-165.

Hirsinger, C., Salvà, I., Marbach, J., Durr, A., Fleck, J. and Jamet, E. (1999). The tobacco extensin gene *Ext 1.4* is expressed in cells submitted to mechanical constraints and in cells proliferating under hormone control. *Journal of Experimental Botany*, **50(332)**: 343-355.

Ho, L.C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annual Review of Plant Physiology and Plant Molecular Biology*, **39**: 355-378.

Hobbie, L. and Estelle, M. (1994). Genetic approaches to auxin action. *Plant, Cell and Environment*, **17**: 525-540.

Hobbie, L. and Estelle, M. (1995). The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. *The Plant Journal*, 7(2): 211-220.

Hobbie, L., Timpte, C. and Estelle, M. (1994). Molecular genetics of auxin and cytokinin. *Plant Molecular Biology*, **26**: 1499-1519.

Holaska, J.M., Black, B.E., Love, D.C., Hanover, J.A., Leszyk, J. and Paschal, B.M. (2001). Calreticulin is a receptor for nuclear export. *The Journal of Cell Biology*, **152(1)**: 127-140.

Hooft van Huijsduijen, R.A.M., Cornelissen, B.J.C., van Loon, L.C., van Boom, J.H., Tromp, M. and Bol, J.F. (1985). Virus-induced synthesis of messenger RNAs for precursors of pathogenesis-related proteins in tobacco. *The EMBO Journal*, 4(9): 2167-2171.

Hutchison, K.W., Singer, P.B., McInnis, S., Diaz-Sala, C. and Greenwood, M.S. (1999). Expansins are conserved in conifers and expressed in hypocotyls in response to exogenous auxin. *Plant Physiology*, **120**: 827-831.

Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (1990). *PCR Protocols. A Guide to Methods and Applications*. Academic Press, Inc., San Diego.

Ito, M., Kodama, H., Komamine, A. and Watanabe, A. (1998). Expression of extensin genes is dependent on the stage of the cell cycle and cell proliferation in suspension-cultured *Catharanthus roseus* cells. *Plant Molecular Biology*, 36(3): 343-351.

John, P.C.L. and Zhang, K. (2001). Cytokinin control of cell proliferation in plant development. In Francis, D. (ed). *The Plant Cell Cycle and its Interfaces*. Sheffield Academic Press, England.

John, P.C.L., Zhang, K., Dong, C., Diederich, L. and Wightman, F. (1993). $P34^{cdc2}$-related proteins in control of cell cycle progression, the switch between division and differentiation in tissue development and stimulation of division by auxin and cytokinin. *Australian Journal of Plant Physiology*, **20**:503-526.

Johnson, E.S., Schwienhorst, I., Dohmen, R.J. and Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an AoS1p/Uba2p heterodimer. *The EMBO Journal*, **16(18)**: 5509-5519.

Jones, A. M. (1998). Auxin transport: Down and out and up again. *Science*, **282**: 2201-2202.

Joshi, A.K., Baichwal, V. and Ames, G.F. (1991). Rapid polymerase chain reaction amplification using intact bacterial cells. *BioTechniques*, **10(1)**: 42-44.

Kantharaj, G.R., Mahadevan, S. and Padmanabhan, G. (1985). Tubulin synthesis and auxin-induced root initiation in Phaseolus. *Phytochemistry*, **24(1)**: 23-27.

Kehlenbach, R.H., Dickmanns, A., Guan, T. and Gerace, L. (1999). A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. *The Journal of Cell Biology*, **145(4)**: 645-657.

Keller, B. and Lamb, C.J. (1989). Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes and Development*, **3**: 1639-1646.

Kiegle, E., Gilliham, M., Haseloff, J. and Tester, M. (2000). Hyperpolarisation-activated calcium currents found only in cells from the elongation zone of *Arabidopsis thaliana* roots. *The Plant Journal*, **21(2)**: 225-229.

Kieliszewski, M.J. and Lampot, D.T.A. (1994). Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *The Plant Journal*, **5(2)**: 157-172.

Kim, W.Y., Cheong, N.E., Lee, D.C., Lee, K.O., Je, D.Y., Bahk, J.D., Cho, M.J. and Lee, S.Y. (1996). Isolation of an additional soybean cDNA encoding *Ypt/Rab*-related small GTP-binding protein and its functional comparison to *Sypt* using a yeast *ypt1-1* mutant. *Plant Molecular Biology*, **31**: 783-792.

Kim, J., Harter, K. and Theologis, A. (1997). Protein-protein interactions among the Aux/IAA proteins. *Proceedings of the National Academy of Sciences of the United States of America*, **94(22)**: 11786-11791.

King, J.J., Stimart, D.P., Fisher, R.H. and Bleeker, A. B. (1995). A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. *The Plant Cell*, **7(12)**: 2023-2037.

Klausner, R.D. and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. *Cell*, **62**: 611-614.

Köhler, S.,. Coraggio, I., Becker, D. and Salamini, F. (1992). Pattern of expression of meristem-specific cDNA clones of barley (*Hordeum vulgare* L.). *Planta*, **186**: 227-235.

Komitzer, D. and Ciechanover, A. (2000). Modes of regulation of ubiquitin-mediated protein degradation. *Journal of Cellular Physiology*, **182**: 1-11.

Koshiba, T., Ballas, N., Wong, M. and Theologis, A. (1995). Transcriptional regulation of *PS-IAA4/5* and *PS-IAA6* early gene expression by indoleacetic acid and protein synthesis inhibitors in pea (*Pisum sativum*). *Journal of Molecular Biology*, **253**: 396-413.

Krause, K.-H. and Michalak, M. (1997). Calreticulin. *Cell*, **88**: 439-443.

Kubo, N., Ozawa, K., Hino, T. and Kadowaki, K. (1996). A ribosomal protein L2 gene is transcribed, spliced, and edited at one site in rice mitochondria. *Plant Molecular Biology*, **31(4)**: 853-862.

Kyozuka, J., Harcourt, R., Peacock, W.J. and Dennis, E.S. (1997). *Eucalyptus* has functional equivalents of the *Arabidopsis AP1* gene. *Plant Molecular Biology*, **35**: 573-584.

Labriola, C., Cazzulo, J.J. and Parodi, A.J. (1999). *Trypanosoma cruzi* calreticulin is a lectin that binds monoglucosylated oligosaccharides but not protein moieties of glycoproteins. *Molecular Biology of the Cell*, **10(5)**: 1381-1394.

Langan, K.J. and Nothangel, E.A. (1997). Cell surface arabinogalactan-proteins and their relation to cell proliferation and viability. *Protoplasma*, **196**: 87-98.

Laskowski, M.J., Williams, M.E., Nusbaum, H.C. and Sussex, I.M. (1995). Formation of lateral root meristems is a two stage-process. *Development*, **121**: 3303-3310.

Lester, D. R., Speirs, J., Orr, G., and Brady, C.J. (1994). Peach (*Prunus persica*) endopolygalcturonase cDNA isolation and mRNA analysis in melting and nonmelting peach cultivars. *Plant Physiology*.,**105**: 225-231.

Leyser, O. (1997). Auxin: lessons from a mutant weed. *Physiologia Plantarum*, **100**: 407-414.

Leyser, O. and Berleth, T. (1999). A molecular basis for auxin action. *Seminars in Cell and Developmental Biology*, **10(2)**: 131-137.

Leyser, H.M., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J. and Estelle, M. (1993). *Arabidopsis auxin-resistance gene AXR1* encodes a protein related to ubiquitin-activating enzyme E1. *Nature*, **364**: 161-164.

Leyser, H.M.O., Pickett, F.B., Dharmasiri, S. and Estelle, M. (1996). Mutations in the *AXR3* gene of *Arabidopsis* result in altered auxin response including ectopic expression from the *SAUR-AC1* promoter. *The Plant Journal*, **10(3)**: 403-413.

Li, F., Barnathan, E.S. and Karikó K. (1994). Rapid method for screening and cloning cDNAs generated in differential mRNA display: application of Northern blot for affinity capturing of cDNAs. *Nucleic Acids Research*, **22(9)**: 1764-1765.

Liakopoulos, D., Büsgen, T., Brychzy, A., Jentsch, S. and Dause, A. (1999). Conjugation of the ubiquitin-like protein NEDD8 to cullin-2 is linked to von Hippel-Lindau tumor suppressor function. *Proceedings of the National Academy of Sciences of the United States of America*, **96**: 5510-5515.

Liakopoulos, D., Doenges, G., Matuschewski, K. and Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. *The EMBO Journal*, **17(1)**: 2208-2214.

Liang, P., Averboukh, L., Keyomarsi, K., Sager, R. and Pardee, A.B. (1992). Differential display and cloning of messanger RNAs from human breast cancer versus mammary epithelial cells. *Cancer Research*, 52:6966-6968.

Liang, P., Averboukh, L. and Pardee, A.B. (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Research*, **21(14)**: 3269-3275.

Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**: 967-971.

Liang, P. and Pardee, A.B. (1995). Response to alternatives to ^{35}S as a label for the differential display of eukaryotic messenger RNA (Trentmann *et al.*). *Science*, 267: 1186-1187.

Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connell, P.O., Averboukh, L., Wang, F. and Pardee, A.B. (1994). Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Research*, **22(25)**: 5763-5764.

Lincoln, C., Britton, J.H. and Estelle, M. (1990). Growth and development of the *axr1* mutants of *Arabidopsis*. *The Plant Cell*, **2**: 1071-1080.

Li, S. and Showalter, A.M. (1996). Cloning and developmental/stress-regulated expression of a gene encoding a tomato arabinogalactan protein. *Plant Molecular Biology*, **32**: 641-652.

Lin, Y., Seals, D.F., Randall, S.K. and Yang, Z. (2001). Dynamic localization of Rop GTPase to the tonoplast during vacuole development. *Plant Physiology*, **125**: 241-251.

Liu, L., Maillet, D.S., Frappier, J.R.H., Walden, D.B. and Atkinson, B.G. (1995). Characterization, chromosomal mapping, and expression of different polyubiquitin genes in tissues from control and heat-shocked maize seedlings. *Biochemistry and Cell Biology*, **73(1-2)**: 19-30.

Lohmann, J., Schickle, H. and Bosch, T.C.G. (1995). REN display, a rapid and efficient method for nonradioactive differential display and mRNA isolation. *BioTechneques*, **18(2)**: 200-202.

Ludevid, M.D., Ruiz-Avila, L., Vallés, M.P., Stiefel, V., Torrent, M., Torne, J.M. and Puigdomènech, P. (1990). Expression of genes for cell-wall proteins in dividing and wounded tissues of *Zea mays* L. *Planta*, **180**: 524-529.

McClure, B.A. and Guilfoyle, T. (1987). Characterization of a class of small auxin-inducible soybean polyadenylated RNAs. *Plant Molecular Biology*, **9**: 611-624.

MacIsaac, S.A. and Sawhney, V.K. (1990). Protein changes associated with auxin-induced stimulation and kinetin-induced inhibition of lateral root initiation in lettuce (*Lactuca sativa*) roots. *Journal of Experimental Botany*, **41(299)**: 1039-1044.

MacIsaac, S.A., Sawhney, V.K. and Pohorecky, Y. (1989). Regulation of lateral root formation in lettuce (*Lactuca sativa*) seedling roots: interacting effects of a-naphthaleneacetic acid and kinetin. *Physiologia Plantarum*, 77: 287-293.

McKendree, W.L., Nairn, C.J. and Bausher, M.G. (1995). Differential display from plant leaves using oligo(dT) magnetic bead mRNA isolation and hot air PCR. *BioTechniques*, **19(5)**: 715-719.

MacLeod, R.D. (1972). Lateral root formation in *Vicia faba* L. I. The development of large primordia. *Chromosoma*, **39**: 341-350.

McNally, S.F., Hirel, B., Gadal, P., Mann, A.F. and Stewart, G.R. (1983). Glutamine synthetases of higher plants. *Plant Physiology*, **72**: 22-25.

MacRae, S. (1991). *Agrobacterium*-mediated transformation of eucalypts to improve rooting ability. *IUFRO Symposium on Intensive Forestry: The Role of Eucalyptus. Durban, South Africa, 2-6 September.*

MacRae, S. and Van Staden, J. (1993). *Agrobacterium rhizogenes*-mediated transformation to improve rooting ability of eucalypts. *Tree Physiology*, **12**: 411-418.

Maher, E.P. and Martindale, S.J.B. (1980). Mutants of *Arabidopsis thaliana* with altered responses to auxins and gravity. *Biochemical Genetics*, **18(11/12)**: 1041-1053.

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P.N. and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes and development*, **14**: 2938-2943.

Malamy, J.E. and Benfey, P.N. (1997a). Down and out in *Arabidopsis*: the formation of lateral roots. *Trends in Plant Science*, **2(10)**: 390-396.

Malamy, J.E. and Benfey, P.N. (1997b). Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development*, **124(1)**: 33-44.

Marchant, A., Kargui, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M.J. (1999). AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *The EMBO Journal*, **18(8)**: 2066-2073.

Marques, C.M., Araujo, J.A., Ferreira, J.G., Whetten, R., O'Malley, D.M., Liu, B.-H. and sederoff, R. (1998). AFLP genetic maps of *Eucalyptus globulus* and *E. tereticornis*. *Theoretical and Applied Genetics*, **96(6-7)**: 727-737.

Martinez, M.C., Jorgensen, J.-E., Lawton, M.A., Lamb, C.J. and Doerner, P.W. (1992). Spatial pattern od *cdc2* expression in relation to meristem activity and cell proliferation during plant development. *Proceedings of the National Academy of Sciences of the United States of Amenrica*, **89(16)**: 7360-7364.

Marty, I. and Meyer, Y. (1992) cDNA nucleotide sequence and expression of a tobacco cytoplasmic ribosomal protein L2 gene. *Nucleic Acids Research*, **20**: 1517-1522. *Nucleic Acids Reseach*, **20(7)**: 1517-1522.

Masucci, J.D. and Schiefelbein, J.W. (1996). Hormones act downstream of *TTG* and *GL2* to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *The Plant Cell*, **8**: 1505-1517.

Mau, S.-L., Chen, C.-G., Pu, Z.-Y., Moritz, R.L., Simpson, R.J., Bacic, A. and Clarke, A.E. (1995). Molecular cloning of cDNAs encoding the protein backbones of

arabinogalactan-proteins from the filtrate of suspension-cultured cells of *Pyrus communis* and *Nicotiana alata*. *The Plant Journal*, **8(2)**: 269-281.

Maurel, C. (1997). Aquaporins and water permeability pf plant membranes. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**: 399-429.

Mayer, U., Ruiz, R.A.T., Berleth, T., Miséra, S. and Jürgens, G. (1991). Mutations affecting body organisation in the *Arabidopsis* embryo. *Nature*, **353**: 402-407.

Meindl, U., Lancelle, S. and Harper, P.K. (1992). Vesicle production and fusion during lobe formation in *Micrasterias* visualized by high-pressure freeze fixation. *Protoplasma*, **170**: 104-114.

Mellon, J.E. and Helgeson, J.P. (1982). Interaction of a hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. *Plant Physiology*, **70**: 401-405.

Meng, S-Y. and Bennett, G.N. (1992). Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. *Journal of Bacteriology*, **174(8)**: 2659-2669.

Michael, A.J. (1996). A cDNA from pea petals with sequence similarity to pollen allergen, cytokinin-induced and genetic tumour-specific genes: identification of a new family of related sequences. *Plant Molecular Biology*, **30**: 219-24

Migge, A., Carrayol, E., Hirel, B. and Becker, T.W. (2000). Leaf-specific overexpression of plastidic glutamine synthetase stimulates the growth of transgenic tobacco seedlings. *Planta*, **210**: 252-260.

Momany, C., Ernst, S., Ghosh, R., Chang, N-L. and Hackert, M.L. (1995). Crystallographic structure of a PLP-dependent ornithine decarboxylase from *lacto bacillus* 30a to 3.0 Å resolution. *Journal of Molecular Biology*, **252**: 643-655.

Monro, J.A., Bailey, R.W. and Penny, D. (1974). Cell wall hydroxyproline-polysaccharide associates in *Lupinus* hypocotyls. *Phytochemistry*, **13**: 375-382.

Moore, I., Diefenthal, T., Zarsky, V., Schell, J. and Palme, K. (1997). A homolog of the mammalian GTPase Rab2 is present in *Arabidopsis* and is expressed predominantly in pollen grains and seedlings. *Proceedings of the National Academy of Sciences of the United States of America*, **94**: 762-767.

Moore, P.J. and Staehelin, L.A. (1988). Immunogold localization of the cell-wall-matix polysaccharides rhamnogalacturonan I and xyloglucan during cell expansion and cytokinesis in *Trifolium pratense* L.; implication for secretory pathways. *Planta*, **174**: 433-445.

Morey, K.J. and Sengupta-Gopalan, C. (1998). Soybean gene coding for a nodule specific glutamine synthetase (Accession No. AF091456) (PGR 98-194).

Muday, G.K., Lomax, T.L. and Rayle, D.L. (1995). Characterisation of the growth and auxin physiology of roots of the tomato mutant, *diageotropica*. *Planta*, **195**: 548-

Müller, E.-C. and Wittmann-Liebold, B. (1997). Phylogenetic relationship of organisms obtained by ribosomal protein comparison. *Cellular and Molecular Life Sciences*, **53**: 34-50.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 473-497.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M. and Reed, J.W. (2000). *AXR2* encodes a member of the Aux/IAA protein family. *Plant Physiology*, **123(2)**: 563-573.

Nakjima, K., Sena, G., Nawy, T. and Benfey, P.N. (2001). Intercellular movement of the putative transcription factor SHR in root patterning. *Nature*, **413**: 307-311.

Nelson, D.E., Glaunsinger, B. and Bohnert, H.J. (1997) Abundant accumulation of the calcium-binding molecular chaperone calreticulin in specific floral tissues of *Arabidopsis thaliana*. *Plant Physiology*, **114**: 29-37.

Nielsen, T.H. and Wischmann, B. (1995). Quantitative aspects of the in vivo regulation of pyrophosphate:fructose-6-phosphate 1-phosphotransferase by fructose-2,6-bisphosphate. *Plant Physiology*, **109**: 1033-1038.

Nishi, R., Hashimoto, H., Kidou, S., Uchimiya, H. and Kato, A. (1993). Isolation and characterisation of a rice cDNA which encodes a ubiquitin protein and a 52 amino acid extensin protein. *Plant Molecular Biology*, **22(1)**: 159-161.

Nothnagel, E.A. (1997). Proteoglycans and related compounds in plant cells. *International Review of Cytology*, **174**: 195-291.

O'Brien, T., Beall, F.D. and Smith, H. (1985) De-etiolation and plant hormones. In Pharis, R.P. and Reid, D.M. (eds). *Hormonal Regulation of Development III: Role of Environmental Factors*. Springer-Verlag, Berlin, pp. 282-307.

Oeller, P.W., Keller, J.A., Parks, J.E., Silbert, J.E. and Theologis, A. (1993). Structural characterization of the early indoleacetic acid-inducible genes, *PS-IAA4/5* and *PS-IAA6* of pea (*Pisum sativum* L.). *Journal of Molecular Biology*, **233**: 789-798.

Oh, B., Balint, D.E. and Giovannoni, J.J. (1995). A modified procedure for PCR-based differential display and demonstration of use in plants for isolation of genes related to fruit ripening. *Plant Molecular Biology Reporter*, **13(1)**: 70-81.

O'Mahony, P.J. and Oliver, M.J. (1999). The involvement of ubiquitin in vegetative dessication tolerance. *Plant Molecular Biology*, **41(5)**: 657-667.

Okazaki, K., Okayama, H. and Niwa, O. (2000). The polyubiquitin gene is essential for meiosis in fission yeast. *Experimental Cell Research*, **254**: 143-152.

Parmentier, Y., Durr, A., Marbach, J. Hirsinger, C., Criqui, M.-C., Fleck, J. and Jamet, E. (1995). A novel wound-inducible extensin gene is expressed early in newly isolated protoplasts of *Nicotiana sylvestris*. *Plant Molecular Biology*, **29(2)**: 279-292.

Patton, E.E., Willems, A.R. and Tyers, M. (1998). Combinational control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends in Genetics*, **14(6)**: 236-243.

Paul, M., Sonnewald, U., Hajirezaei, M., Dennis, D. and Stitt, M. (1995). Transgenic tobacco plants with strongly decreased expression of pyrophosphate-fructose-6-phosphate 1-phosphotransferase do not differ significantly from wildtype in photosynthate partitioning, plant growth or their ability to cope with limiting phosphate, limiting nitrogen and suboptimal temperatures. *Planta*, **196(2)**: 277-283.

Pelham, H.R.B. (1990). The retention signal for soluble proteins of the endoplasmic reticulum. *Trends in Biochemical Sciences*, **15**: 483-486.

Pelosi, A., Chow, E.K.F., Lee, M.C.S., Chandler, S.F. and Hamill, J.D. (1995a). Effects of phytohormons on lateral root differentiation in *Eucalyptus globulus* and transgenic *Nicotoana tabacum* seedlings. In Terzi, M., Cella, R., Falavigna, A. (eds). *Current Issues in Plant Molecular and Cellular Biology*. Kluwer Academic Publishers, Dordrecht.

Pelosi, A., Lee, M.C.S., Chandler, S.F. and Hamill, J.D. (1995b). Hormonal control of root primordia differentiation and root formation in cultured explants of *Eucalyptus globulus* seedlings. *Australian. Journal of Plant Physiol.*, **22**: 409-415.

Pérez-García, A., de Vicente, A., Cantón, F.R., Cazorla, F.M., Codina, J.C., García-Gutiérrez, A. and Cánovas, F.M. (1998). Light-dependent changes of tomato glutamine synthetase in response to *Pseudomonas syringae* infection or phosphinothricin treatment. *Physiologia Plantarum*, **102**: 377-384.

Peterson, R.L. and Peterson, C. (1986). Ontogeny of lateral roots. In Jackson, M.B. (ed). *New Root Formation in Plants and Cuttings*. Martinus Nijhoff Publishers, Dordrecht.

Pfitzner, U.M. and Goodman, H.M. (1987). Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. *Nucleic Acids Research*, **15(11)**: 4449-4465.

Pickett, F.B., Wilson, A.K. and Estelle, M. (1990). The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. *Plant Physiology*, **94**:1462-1466.

Pieterse, C.M.J., Risseeuw, E.P. and Davidse, L.C. (1991). An *in planta* induced gene of *Phytopthora infestans* codes for ubiquitin. *Plant Molecular Biology*, **17**: 799-811.

Pryer, N. K., Wuestehube, L.J. and Schekman, R. (1992). Vesicle-mediated protein sorting. *Annual Review of Biochemistry*, **61**: 471-516.

Pysh, L.D., Wysocka-Diller, J.W., Camilieri, C., Bouchez, D. and Benfey, P.N. (1999). The GRAS gene family in *Arabidopsis*: sequence characterisation and basic expression analysis of the *SCARECROW-LIKE* genes. *The Plant Journal*, **18(1)**: 111-119.

Ramos, J.A., Zenser, N., Leyser, O. and Callis, J. (2001). Rapid degredation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. *The Plant Cell*, **13**: 2349-2360.

Rashotte, A.M., Brady, S.R., Reed, R.C., Ante, S.J. and Muday, G.K. (2000). Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*. *Plant Physiology*, **122**: 481-490.

Rayle, D.L. and Cleland, R.E. (1992). The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiology*, **99**: 1271-1274.

Reinhardt, D., Wittwer, F., Mandel, T. and Kuhlemeier, C. (1998). Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. *The Plant Cell*, **10**: 1427-1437.

Reynolds, G.J. and Hooley, R. (1992). cDNA cloning of a tetraubiquitin gene, and expression of ubiquitin-containing transcripts, in aleurone layers of *Avena fatua*. *Plant Molecular Biology*, **20(4)**: 753-758.

Richards, , S.A., Carey, K.L. and Macara, I.G. (1997). Requirement for guanosine triphosphate-bound ran for signal-mediated nuclear export. *Science*, **276**: 1842-1844.

Rose, J.K.C., Lee, H. and Bennett, A.B. (1997). Expression of a divergent expansin gene is fruit-specific and ripening-regulated. *Proceedings of the National Academy of Sciences of the United States of America*, **94(11)**: 5955-60.

Rougier, M. (1981). Secretory activity of the root cap. In Tanner, W. and Loewus, F.A. (eds). *Encyclopedia of Plant Physiology, vol. 13B, Plant Carbohydrates. II. Extracellular Carbohydrates*. Springer-Verlag, Berlin, pp. 542-574.

Rouse, D., Mackay, P., Stirnberg, P., Estelle, M. and Leyser, O. (1998). Changes in auxin response from mutations in an *AUX/IAA* gene. *Science*, **279**: 1371-1373.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Brown, D., Bernasconi, P., Turner, J., Muday, G. and Estelle, M. (1997). Reduced naphthylphthalamic acid binding in the *tir3* mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. *The Plant Cell*, **9(5)**: 745-757.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J. and Estelle, M. (1998). The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast Grr1p. *Genes and Development*, **12**: 198-207.

Russell, R.S. (1977). *Plant Root Systems: Their Function and Interaction with the Soil*. McGraw-Hill Book Company, Great Britain.

Saito, Y., Ihara, Y., Leach, M.R., Cohen-Doyle, M.F. and Williams, D.B. (1999). Calreticulin functions *in vitro* as a molecular chaperone for both glycosylated and non-glycosylated proteins. *The EMBO Journal*, **18(23)**: 6718-6729.

Sakurai, N., Hayakawa, T., Nakamura, T. and Yamaya, T. (1996). Changes in the cellular localization of cytosolic glutamine synthtase protein in vascular bundles of rice leaves at various stages of development. *Planta*, **200**: 306-311.

Sakurai, H. and Ishihama, A. (1997). Gene organization and protein sequence of the small subunits of *Schizosaccharomyces pombe* RNA polymerase II. *Gene*, **196**: 165-174.

Sakurai, H., Miyao, T. and Ishihama, A. (1996). Subunit composition of RNA polymerase II from the fission yeast *Schizosaccharomyces pombe*. *Gene*, **180**: 63-67.

Sandmeier, E., Hale, T.I. and Christen, P. (1994). Multiple evolutionary origin of pyridoxal-5'-phosphate-dependent amino acid decarboxylases. *European Journal of Biochemistry*, **221**: 997-1002.

Sasse, J. and Sands, R. (1995). Root system development in cuttings of *Eucalyptus globulus*. *Eucalypt Plantations: improving Field Yield and Quality. CRC for Temperate Hardwood Forestry-IUFRO, Hobart* Theme 4: Breeding and Selection Strategies-Sub Theme: Propagation, 299-303.

Schena, M. and Davis, R.W. (1992). HD-Zip proteins: Members of an *Arabidopsis* homeodomain protein superfamily. *Proceedings of the National Academy of Sciences of the United States of America*, **89**: 3894-3898.

Schena, M., Lloyd, A.M. and Davis, R.W. (1991). A steroid-inducible gene expression system for plant cells. *Proceedings of the National Academy of Sciences of the United States of America*, **88**: 10421-10425.

Scheres, B., Di Laurenzio, L., Willemsen, V., Hauser, M.-T., Janmaat, K., Weisbeek, P. and Benfey, P.N. (1995). Mutations affecting the radial organisation of the *Arabidopsis* root display specific defects throughout the embryonic axis. *Development*, **121**: 53-62.

Scheres, B., McKhann, H.I. and ven den Berg, C., Willemsen, V., Wolkenfelt, H., de Vrieze, G. and Weisbeek, P. (1996). Experimental and genetic analysis of root development in *Arabidopsis thaliana*. *Plant and Soil*, **187**: 97-105.

Schiefelbein, J.W. and Benfey, P.N. (1991). The development of plant roots: new approaches to underground problems. *The Plant Cell*, **3**: 1147-1154.

Schiefelbein, J.W. and Somerville, C. (1990). Genetic control of root hair development in *Arabidopsis thaliana*. *The Plant Cell*, **2**: 235-245.

Schlesinger, M.J. and Bond, U. (1987). Ubiquitin genes. *Oxford Surveys on Eukaryotic Genes*, **4**: 77-89.

Schultz, C. Gilson, P., Oxley, D., Youl, J. and Bacic, A. (1998). GPI-anchors on arabinogalactan-proteins: implications for signalling in plants. *Trends in Plant Science*, **3**: 426-431.

Schultz, C.J., Johnson, K.L., Currie, G. and Bacic, A. (2000). The classical arabinogalactan protein gene family of *Arabidopsis*. *The Plant Cell*, **12(9)**: 1751-1767.

Shcherban, T.Y., Shi, J., Durachko, D.M., Guiltinan, M.J., McQueen-Mason, S.J., Shieh, M. and Cosgrove, D.J. (1995). Molecular cloning and sequence analysis of expansins-a highly conserved, multigene family of proteins that mediate cell wall extension in plants. *Proceedings of the National Academy of Sciences of the United States of America*, **92(20)**: 9245-9249.

Shi, L., Gast, R.T., Gopalraj, M. and Olszewski, N.E. (1992). Characterization of a shoot-specific, GA_3- and ABA-regulated gene from tomato. *The Plant Journal*, **2(2)**: 153-159.

Shieh, M.W. and Cosgrove, D.J. (1998). Expansins. *Journal of Plant Research*, **111(1101)**: 149-157.

Shimizu, Y., Aotsuka, S., Hasegawa, O., Kawada, T., Sakuno, T., Saki, F. and Hayashi, T. (1997). Changes in levels of mRNAs for cell wall-related enzymes in growing cotton fibre cells. *Plant and Cell Physiology*, **38(3)**: 375-378.

Short, K.C. and Torrey, J.G. (1972). Cytokinins in seedling roots of pea. *Plant Physiology*, **49**: 155-160.

Showalter, A.M. (1993). Structure and function of plant cell wall proteins. *The Plant Cell*, **5**: 9-23.

Showalter, A.M., Zhou, J., Rumeau, D., Worst, S.G. and Varner, J.E. (1991). Tomato extensin and extensin-like cDNAs: structure and expression in response to wounding. *Plant Molecular Biology*, **16(4)**: 547-565.

Shpakovski, G.V., Acker, J., Wintzerith, M., Lacroix, J.-F., Thuriaux, P. and Vigneron, M. (1995). Four subunits that are shared by the three classes of RNA polymerase are functionally interchangeable between *Homo sapiens* and *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, **15(9)**: 4702-4710.

s, C., Migliaccio, F., Masson, P. Caspar, T. and Söll, D. (1995). A novel root vitropism mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. *siologia Plantarum*, **93(4)**: 790-798.

od, M., Beven, A., Donovan, N., Neill, S.J., Peart,J., Roberts, K. and Knox, J.P. 94). Localization of cell wall proteins in relation to the developmental anatomy the carrot root apex. *The Plant Journal*, **5(2)**: 237-246.

.L. and Fedoroff, N.V. (1995). *LRP1*, a gene expressed in lateral and adventitious t primordia of *Arabidopsis*. *The Plant Cell*, **7**: 735-745.

Webb, B. and Mougey, E.B. (1991). News from the nucleolus: rRNA gene pression. *Trends in Biochemical Sciences*, **16**: 58-62.

Knudsen, J., Bacic, A. and Clarke, A.E. (1998). Hydroxyproline-rich plant coproteins. *Phytochemistry*, **47(4)**: 483-497.

ac, L., Jane, S., Burn, T.C., Tenen, D.G. and Danna, K.J. (1995). Overcoming itations of the mRNA differential display technique. *Nucleic Acids Research*, **22)**: 4738-4739.

, Yamamoto, E. and Allen, R.D. (1995). Improved procedure for differential play of transcripts from cotton tissues. *Plant Molecular Biology Reporter*, **13(2)**: -181.

d, U., Lerchl, J., Zrenner, R. and Frommer, W. (1994). Manipulation of sink-rce relations in transgenic plants. *Plant, Cell and Environment*, **17**: 649-658.

n, S.G., Strauss, S.H., Olive, M.R., Harcourt, R.L., Decroocq, V., Zhu, X., Wellyn, D.J., Peacock, W.J. and Dennis, E.S. (1998). *Eucalyptus* has a functional ivalent of the *Arabidopsis* floral meristem identity gene *LEAFY*. *Plant Molecular logy*, **37(6)**: 897-910.

, J.P. and Sussex, I.M. (1992). Expression of a ribosomal protein gene in axillary s of pea seedlings. *Plant Physiology*, **100**: 1494-1502.

.C., Baumert, M., Perin, M.S. and Jahn, R. (1989). A synaptic vesicle membrane ein is conserved from mammals to Drosophila. *Neuron*, **2**:1475-1481.

M., Godoy, J.A., Kerk, N.M., Laskowski, M.J., Nusbaum, H.C., Welsch, J. and liams, M.E. (1995a). Cellular and molecular events in a newly organizing lateral meristem. *Philosophical Transactions of the Royal Society of London B.*, **350**: 3.

M., Godoy, J.A., Kerk, N.M., Laskowski, M.J., Nusbaum, H.C., Welsch, J.A. and liams, M.E. (1995b). Molecular and cellular events in the formation of new stems. In Terzi, M., Cella, R. and Falavigna, A. (eds). *Current Issues in Plant ecular and Cellular Biology*, Kluwer Academic Publishers, Dordrecht.

J., Ajoka, J., Eisen, H., Sanwal, B., Jacquemot, C., Browder, Z. and Buck, G. 8). The genomic organization and transcription of the ubiquitin genes of *anosoma cruzi*. *The EMBO Journal*, **7(4)**: 1121.

N. and Tabor, H.T. (1985). Polyamines in microorganisms. *Microbiol Reviews*, 81-99.

Taylor, B.H. and Scheuring, C.F. (1994). A molecular marker for lateral root initiation: The *RSI-1* gene of tomato (*Lycopersicon esculentum* Mill) is activated in early lateral root primordia. *Molecular General Genetics*, **243**: 148-157.

Temple, S.J., Vance, C.P. and Gantt, J.S. (1998). Glutamate synthase and nitrogen assimilation. *Trends in Plant Science*, **3(2)**: 51-56.

Terryn, N., Arias, M.B., Engler, G., Tiré, C., Villarroel, R., Van Montagu, M. and Inzé, D. (1993a). *rha1*, a gene encoding a small GTP binding protein from Arabidopsis, is expressed primarily in developing guard cells. *The Plant Cell*, **5**: 1761-1769.

Terryn, N., Van Montagu, M. and Inzé, D. (1993b). GTP-binding proteins in plants. *Plant Molecular Biology*, **22**: 143-152.

Thamarus, K.A., Groom, K., Murrell, J., Byrne, M. and moran, G.F. (2002). A genetic linkage map for *Eucalyptus globulus* with candidate loci for wood, fibre, and floral traits. *Theoretical and Applied Genetics*, **104**: 379-387.

Theodorou, M.E., Cornel, F.A., Duff, F.A., Duff, S.M. and Plaxton, W.C. (1992). Phosphate starvation-inducible synthesis of the alpha-subunit of the pyrophosphate-dependent phosphofructokinase in black mustard suspension cells. *Journal of Biological Chemistry*, **267(30)**: 21901-21905.

Theologis, A. (1986). Rapid gene regulation by auxin. *Annual Review of Plant Physiology*, **37**: 407-438.

Theologis, A., Huynh, T.V. and Davis, R.W. (1985). Rapid induction of specific mRNAs by auxin in pea epicotyl tissue. *Journal of Molecular Biology*, **183**: 53-68.

Tibbits, W.N., White, T.L., Hodge, G.R. and Joyce, K.R. (1997). Genetic control of rooting ability of stem cuttings in *Eucalyptus nitens*. *Australian Journal of Botany*, **45**:203-210.

Timpte, C., Lincoln, C., Pickett, F. B., Turner, J. and Estelle, M. (1995). The AXR1 and AUX1 genes of Arabidopsis function in separate auxin-response pathways. *The Plant Journal*, **8(4)**: 561-569.

Timpte, C., Wilson, A. K. and Estelle, M. (1994). The *axr2-1* mutation of *Arabidopsis thaliana* is a gain-of-function mutation that disrupts an early step in auxin response. *Genetics*, **138**: 1239-1249.

Todd, J.F., Blakeley, S.D. and Dennis, D.T. (1995). Structure of the genes encoding the α- and ß-subunits of castor phyrophosphate-dependent phosphofructokinase. *Gene*, **152**: 181-186.

Tokuyama, Y. and Takeda, J. (1995). Use of ^{33}P-labeled primer increases the sensitivity and specificity of mRNA differential display. *BioTechniques*, **18(3)**: 424-425.

Torrey, J.G. (1962). Auxin and purine interactions in lateral root initiation in isolated pea root segments. *Physiologia Plantarum*, **15**: 177-185.

Trentmann, S.M., van der Knaap, E. and Kende, H. (1995). Alternatives to ^{35}S as a label for the differential display of eukaryotic messenger RNA (Trentmann *et al.*). *Science*, 267: 1186.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. (1997). ARF1, a transcription factor that binds to auxin response elements. *Science*, **276**: 1865-1868.

Ulmasov, T., Hagen, G. and Guilfoyle, T.J. (1999). Activation and repression of transcription by auxin-response factors. *Proceedings of the National Academy of Sciences of the United States of America*, **96(10)**: 5844-5849.

Vassilakos, A., Michalak, M., Lehrman, M.A. and Williams, D.B. (1998). Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. *Biochemistry*, **37**: 3480-3490.

Vera, P., Lamb, C. and Doerner, P.W. (1994). Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristems. *The Plant Journal*, **6(5)**: 717-727.

Vollbrecht, E., Veit, B., Sinha, N., Hake, S. (1991). The developmental gene *knotted-1* is a member of a maize homeobox gene family. *Nature*, **350**: 241-243.

von der Kammer, H., Albrecht, C., M[illegible]us, M., Hoffmann, B., Stanke, G. and Nitsch, R.M. (1999). Identification [illegible]nes regulated by muscarinic acetylcholine receptors: application of an improved and statistically comprehensive mRNA differential display technique. *Nucleic Acids Research*, **27(10)**: 2211-2222.

von Kampen, J., Wettern, M., and Schulz, M. (1996). The ubiquitin system in plants. *Physiologia Plantarum*, **97**: 618-624.

Vuylsteker, C., Dewaele, E. and Rambour, S. (1998). Auxin induced lateral root formation in chicory. *Annals of Botany*, **81(3)**: 449-454.

Wang, J., Jiang, J. and Oard, J.H. (2000). Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.) *Plant Science*, **156**: 201-211.

Waterhouse, P.M., Wang, M.-B. and Finnegan, E.J. (2001). Role of short RNAs in gene silencing. *Trends in Plant Science*, **6(7)**: 297-300.

Wiesman, Z. and Lavee, S. (1995). Relationship of carbohygdrate sources and indole-3-butyric acid in olive cuttings. *Australian Journal of Plant Physiology*, **22**: 811-816.

Wightman, F., Schneider, E.A. and Thimann, K.V. (1980). Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. *Physiolagia Plantarum*, **49**: 304-314.

Wightman, F. and Thimann, K.V. (1980). Hormonal factors controlling the initiation and development of lateral roots. I. Sources of primordia-inducing substances in the primary root of pea seedlings. *Physiologia Plantarum*, **49**: 13-20.

Williams, M.E. and Sussex, I.M. (1995). Developmental regulation of ribosomal protein L16 genes in *Arabidopsis thaliana*. *The Plant Journal*, **8(1)**: 65-76.

Wilson, A.K., Pickett, F.B., Turner, J.C. and estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. *Molecular and General Genetics*, **222**: 377-383.

Worley, C.K., Zenser, N., Ramos, J., Rouse, D., Leyser, O., Theologis, A. and Callis, J. (2000). Degradation of Aux/IAA proteins is essential for normal auxin signalling. *The Plant Journal*, **21(6)**: 553-562.

Wu, Y., Sharp, R.E., Durachko, D.M. and Cosgrove, D.J. (1996). Growth maintenance of the maize primary root at low water potential involves increases in cell-wall

extension properties, expansin activity, and cell wall susceptibility to expansins. *Plant Physiology*, **111**: 765-772.

Wycoff, K.L., Powell, P.A., Gonzales, R.A., Corbin, D.R., Lamb, C. and Dixon, R.A. (1995). Stress activation of a bean hydroxyproline-rich glycoprotein promoter is superimposed on a pattern of tissue-specific developmental expression. *Plant Physiology*, **109(1)**: 41-52.

Xia, X. and Mahon, J. (1998). Pea polyubiquitin genes: (I) structure and genomic organization. *Gene*, **215**: 445-452.

Xu, N., Hagen, G. and Guilfoyle, T. (1997a). Multiple auxin response modules in the soybean *SAUR 15A* promoter. *Plant Science*, **126**: 193-201.

Xu, N., Johns, B., Pullman, G. and Cairney, J. (1997b). Rapid and reliable differential display from minute amounts of tissue: mass cloning and characterization of differentially expressed genes from loblolly pine embryos. *Plant Molecular Biology Reporter*, **15**: 377-391.

Xu, D.-P., Sung, S.-J.S., Loboda, T., Kormanil, P.P. and Black, C.C. (1989). Characterization of sucrolysis via the uridine diphosphate and pyrophosphate-dependent sucrose synthase pathway. *Plant Physiology*, **90**: 635-642.

Yamamoto, Y.T., Cheng, C. and Conkling, M.A. (1990). Root-specific genes from tobacco and *Arabidopsis* homologous to an evolutionarily conserved gene family of membrane channel proteins. *Nucleic Acids Research*, **18(24)**: 7449.

Yamamoto, Y.T., Taylor, C.G., Acedo, G.N., Cheng, C. and Conkling, M.A. (1991). Characterization of *cis*-acting sequences regulating root-specific gene expression in tobacco. *The Plant Cell*, **3**: 371-382.

Yamamoto, M. and Yamamoto, K.T. (1998). Differential effects of 1-naphthaleneacetic acid, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on the gravitropic response of roots in an auxin-resistant mutant of *arabidopsis*, *aux1*. *Plant and Cell Physiology*, **39**: 660-664.

Yamamoto, M. and Yamamoto, K.T. (1999). Effects of natural and synthetic auxins on the gravitropic growth habit of roots in two auxin-resistant mutants of *Arabidopsis*, *axr1*, and *axr4*: Evidence for defects in the auxin influx mechanism of *axr4*. *Journal of Plant Research*, **112(1108)**: 391-369.

Yan, T.F and Tao, M. (1984). Multiple forms of pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase from wheat seedlings. Regulation by fructose 2,6-biphosphate. *Journal of Biological Chemistry*, **259**: 5087-5092.

Yang, Z. and Watson, J.C. (1993). Molecular cloning and characterization of rho, a ras-related small GTP-binding protein from garden pea. *Proceedings of the National Academy of Sciences of the united States of America*, **90**: 8732-8736.

Youl, J.J., Bacic, A. and Oxley, D. (1998). Arabinogalactan-proteins from *Nicotiana alata* and *Pyrus communis* contain glycosylphosphatidylinositol membrane anchors. *Proceedings of the National Academy of Sciences*, **95(4)**: 7921-7926.

Zegzouti, H., Marty, C., Jones, B., Bouquin, T., Latché, Peeh, J. and Bouzayen, M. (1997). Improved screening of cDNAs generated by mRNA differential display enables the selection of true positives and the isolation of weakly expressed messages. *Plant Molecular Biology Reporter*, **15**: 236-245.

Zenser, N., Ellsmore, A., Leasure, C. and Callis, J. (2001). Auxin modulates the degradation rate of Aux/IAA proteins. *Proceedings of the National Academy of Sciences of the United States of America*, **98(20)**: 11795-11800.

Zhang, K., Letham, D.S. and John, P.C.L. (1996). Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of $p34^{cdc2}$-like H1 histone kinase. *Planta*, **199**: 2-12.

Zhang, H., Zhang, R. and Liang, P. (1996). Differential screening of gene expression difference enriched by differential display. *Nucleic Acids Research*, **24(12)**: 2454-2455.

Zhao, S., Ooi, S.L. and Pardee, A.B. (1995). New primer strategy improves precision of differential display. *BioTechniques*, **18(5)**: 842-850.

Zhu, J.-K., Bressan, R.A. and Hasegawa, P.M. (1993). Loss of arabinogalactan-proteins from the plasma membrane of NaCl-adapted tobacco cells. *Planta*, **190**: 221-226.

Zobel, R.W. (1972). Genetics of the diageotropica mutant in tomato. *Journal of Heredity*, **63**: 94-97.

Zobel, R.W. (1973). Some physiological characteristics of the ethylene-requiring tomato mutant diageotropica. *Plant Physiology*, **52**: 385-389.

Zobel, B.J. (1993). In Ahuja, M.R. and Libby, W.J. (ed). *Clonal Forestry: Genetics, Biotechnology and Application*. Springer-Verlag, Berlin, pp. 139-148.