MONASH UNIVERSITY

THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ON..... 1 March 2002 .....

.....

for Sec. Research Graduate School Committee

H24/3115

ADDENDUM

Abbreviations: include the terms "PITAIRE (CDKC), PPTALRE (CDKB1), and PPTTLRE (CDKB2) – variations of the PSTAIRE motif and the CDK class with which they are associated"
p2 section heading: "1.1.3 Cyclin Dependent Kinases" for "1.1.3 CDKs"
p25 section 2.2.1, line 2: centrifugation was at 3,000g
p26 section 2.2.2, line 5: centrifugation was at 5,000g
p28 line 2: centrifugation was at 15,000g
p34 line 3: incubation conditions were under lighting (Sylvania Gro-Lux, 25 $\mu E/m^2/s$) for a 16 h photoperiod
ff p34 Figure 3.4: add to legend "Arrows are used to indicate regions with the strongest staining."
p35 line 1: "1cm in diameter" for "1cm x 1cm"
p62 Section 4.4.5: Replace first sentence with "In yeast, the Cdc2-1w enzyme is not responsive to Wee1 inhibition and shows higher activity than Cdc2. This might also be the case in plants, which may result in narrower root meristems containing smaller cells due to accelerated entry into mitosis."
p70 section 5.3.4.1: remove last sentence
ff p73 Figure 5.2: add to legend "* denotes significant variation from a 3:1 ratio."
ff p75 Figure 5.7 key: the solid colour block of each pair should read "–2 mg/l"
ff p78 Figure 5.14: add to legend "Arrow indicates the difference in mean mitotic cell size with and without treatment."
p99 insert the reference: Inze, D., Gutierrez, C. and Chua, N.H. (1999) Trends in plant cell cycle research. *Plant Cell*, 11, 991-994.
p101 Labbé *et al.* (1994) is from pages 5155-5164
p102 insert the references: MacNeill, S.A., Creanor, J. and Nurse, P. (1991) Isolation, characterisation and molecular cloning of new mutant alleles of the fission yeast p34cdc2+ protein kinase gene: identification of temperature-sensitive G2-arresting alleles. *Molecular & General Genetics*, **229**, 109-118.
Marcote, M.J., Knighton, D.R., Basi, G., Sowadski, J.M., Brambilla, P., Draetta, G. and Taylor, S.S. (1993) A three-dimensional model of the Cdc2 protein kinase: localization of cyclin- and Suc1-binding regions and phosphorylation sites. *Molecular & Cellular Biology*, **13**,5122-5131.
p109 Schena *et al.* (1991) is from the journal *Proceedings of the National Academy of Sciences of the United States of America*
p109 Skoog and Miller (1957) is from volume **11**, 118-131.

ERRATA

Abstract line 1: "fission yeast" for "fissions yeast"
Abstract line 11: "Cdc2 kinase" for "Cdc 2 kinase"
p15 section 1.3.1 line 6: space between "*cdc2*" and "have"
p23 para 2, line 1: "in" for "inn"
p26 section 2.2.2, line 2: "containing" for "coontaining"
p28 section 2.2.5, 4th line from the bottom: "*E. coli*" for "E. coli"
p30 section 2.3.5.1, line 3: "RNAses" for "Rnases"
p30 section 2.3.5.1, line 5: "LiCl" for "Li Cl"
p30 section 2.3.5.1, 3rd last line: "sodium acetate" for "NaAcetate"
p33 section 2.4.1.1, line 2: "3%(w/v) sucrose" for "3% sucrose"
p34 line 5: "5 µg/ml of" for "of 5 µg/ml"
p34 section 2.4.2, line 3: "from Professor" for from by Professor"
p34 section 2.4.2, line 7: 'Plant Cell" for " plant Cell"
p35 section 2.4.3 heading: "lateral root primordia" for "Lateral Root Primordia"
p35 section 2.4.3, line 9: " 2%(w/v) chromium" for "2% Chromium"
p41 3rd para, line 1: "Tc-inducible" for "Tc-repressible"
ff p45 Figure 3.7, *y*-axis and figure legend: "pmol MU/min/mg" for "p/mol/MU/min/mg"
p47 section 3.4.2.4, 3rd para, line 2: "over 2 days" for "over 4 days"
p48 section 3.4.2.6, line 8: "resulting from" for "resulting in from"
p60 section 4.4.3, 2nd para, line 4: "10^{-4} M K-IBA" for "10^{-4} K-IBA"
p62 section 4.4.5, line 3: "condition" for "conditions"
p65 section 5.2, line5: "also" for "Also"
p65 last line: "reviewed by" for "reviewed y"
p69 section 5.3.2.2, line 6: "dithiothreitol" for "Dithiothreitol"
p70 section 5.3.4.1, line 3: "buffer pH" for "bufferpH"
p79 section 5.5, para 2, line 4: "Northern" for "Northrn"
p80 line 6: "earlier" for "earier"
p83 2nd para, line 11: "Cdc25 enzyme" for "Cd25 enzyme"
p97 line 5: "*Schizosaccharomyces*" for "Schizosaccharomyces"

Effects of Yeast Cell Cycle Gene Expression in Transgenic *Nicotiana tabacum*

A Thesis submitted for the degree of Doctor of Philosophy

by

Penelope Webb BSc.(Hons)

Department of Biological Sciences

Monash University

February 2001

Table of Contents

Abstract

Precise control of cell cycle events is essential in all eukaryotes. In budding and fissions yeasts, progression through the cell cycle is controlled largely by a kinase (CDK) interacting closely with specific cyclin proteins. Recent studies with multicellular organisms have revealed some apparently universal aspects of eukaryotic cell cycle control. In higher plants, cell division occurs in defined meristematic zones and requires sophisticated mechanisms to control growth and development. The aim of the experimental program described in this thesis was to investigate whether cell division in plant roots could be influenced by the introduction of genes encoding cell cycle regulators from yeast. Experiments utilised a tetracycline inducible version of the powerful CaMV35S promoter to provide controlled overexpression of key yeast cell cycle genes. Expression of *cdc2-1w*, a dominant mutant form of Cdc 2 kinase, was observed to reduce the capacity of transgenic tobacco seedlings to form lateral root primordia (LRP) in response to auxin. Conversly, expression of *cdc25*, a key phosphatase that activates Cdc2 by tyrosine dephosphorylation, was observed to increase the sensitivity of seedlings to auxin with respect to LRP formation. Furthermore, the average size of mitotic cells expressing *cdc25* was smaller than in tetracycline treated controls. Together, these observations suggest that basic mechanisms governing control of cell division are shared, at least to some extent, between plants and yeast.

Declaration

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

Penelope Webb

Penelope Webb

Acknowledgements

I wish to express my gratitude to the many people who made the completion of this thesis possible. Many thanks to Prof John Hamill for his patience and perseverance and to Dr Pete John for sharing his extensive knowledge. Thanks to both of you for the effort when the end was nigh.

I would like to extend thanks and appreciation to the Genetics community in the Department of Biological Sciences. Your friendship, support and technical assistance have been invaluable. Also to the folk of Plant Cell Biology, RSBS, ANU – thankyou so much for your help, especially Frank Sek and Owen Schwartz for so willingly sharing their expertise.

This manuscript would not have been possible without the able assistance of Tanya, Chris, Helene, Alex, Cecilia and Karen. Your beer is waiting.

To my wonderful family and friends, thankyou for being there from the bottom of heart, you are much appreciated.

For your love and your faith, many hugs to Dad, Alex & Anna, Helen, Jude. With a very special thank you to Chris & Trevor, David & Sonia.

Midway along the journey of our life,
I woke to find myself in a dark wood,
Where the straight way was lost.

Ah, how hard it is to speak of it –
That wild, rough and stubborn forest
(the thought of it brings back all my old fears),

A bitter place! Death could scarce be bitterer.
Yet there I gained such good, that, to convey
The tale, I'll write what else I found therewith.

Dante
The Divine Comedy
Canto I

Genetic Abbreviations

I. For all genes italic characters are used.
eg. *cdc2* is the cell division cycle gene identified in fission yeast. The equivalent gene in budding yeast is *CDC28*. In humans and *Arabidopsis* it is *CDC2* (also named *CDK1* by some authors).
Mutant forms of these genes are indicated by a superscript e.g. $cdc2^{ts}$ signifies a temperature sensitive form of *cdc2*.

II. Gene products are described as $p34^{cdc2}$ (signifying with the gene and the size of the protein in kDa) or in title case non-italicised e.g. Cdc2. Human and *Arabidopsis* gene products are in upper case non-italicised eg. CDC2.

III. Generic descriptions of widely conserved genes are lowercase and non-italicised e.g. cdc2

IV. Plant gene families are expressed by abbreviation of species name;gene name;member number e.g. *Zm;CycA1;1*

Abbreviations

ADC	arginine decarboxylase
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumen
CAK	cdk or $p34^{cdc2}$ activating kinase
cDNA	complementary deoxyribonucleic acid
cdc	cell division cycle
CDI	cdk inhibitory protein
ClTc	chlor-tetracycline
CTAB	cetyltrimethylammonium bromide
DAPI	4,6-diamidino-2-phenylindole
DIC	differential interference contrast
DMFA	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescent detection
EDTA	ethylenediamine tetracetic acid
GUS	β-glucuronidase
IBA	indole-3-butyric acid
IgG	immunoglobulin G
IPTG	isopropyl-B-D-thiogalactoside

kDa	kilodalton
kb	kilobase
LB	Luria-Bertani medium
LRP	lateral root primordia
MAPK	mitogen-activated protein kinase
MPF	maturation or M-phase promotion factor
mRNA	messenger ribonucleic acid
NLS	nuclear localization siganl
NP40	NONIDET P-40, non-ionic detergent
OD	optical density
ODC	ornithine decarboxylase
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PPB	preprophase band
pRb	retinoblastoma protein
PSTAIRE	EGVPSTAIREISLLKE amino acid residues
Ri	root inducing
RIPA	multi-component protein extraction buffer
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tc	tetracycline
TCE	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylene diamine
Tris	tris(hydroxymethyl)aminoethane
Tween 20	polyoxyethylenesorbitan monolaurate

Chapter 1
Introduction

1.1 THE CELL CYCLE

Cell division in eukaryotes is governed by mechanisms and genes that are highly conserved. The process between one cell division and the next is called the cell cycle. The classic view of the cell cycle is one where it is composed of four main parts: G1 (Gap1) in which the cell and organelles grow prior to division, S phase in which DNA replication occurs, G2 (Gap2) the phase before nuclear division, M the mitotic phase when nuclear division takes place. G0 is also commonly used to denote a period of cell quiescence that may be terminated by entry into G1 phase (Murray and Hunt, 1993).

1.1.1 Cdc2 is associated with checkpoints

The sequence of cell cycle events is tightly regulated, and generally assures that cells have grown adequately since the previous division and that later events are not initiated until earlier events have been successfully completed. This ensures a cell divides to produce a pair of genetically identical daughters. Maintaining the integrity of the genome through the cell division cycle is achieved through feedback controls and distinct checkpoints, beyond which the cell cycle will not proceed unless conditions are appropriate. The major control points in the cell cycle at which growth and completion of prior events can operate checkpoints are at the G1-S transition and at the G2-M boundary (Murray, 1992). Additionally, a checkpoint for successful alignment of chromosomes on the spindle operates at the metaphase-anaphase progression.

In *Saccharomyces cerevisiae* (budding yeast), the *CDC28* gene was isolated after recovery of a mutant which arrested cells at G1-S (or START). *cdc2* was isolated in *Schizosaccharomyces pombe* (fission yeast) as a gene required at both G1 and G2 checkpoints (Nurse and Thuriaux, 1980; Nurse and Bisset, 1981). Only much later was the requirement for *CDC28* at mitosis recognized. A demonstration of the unity of cell cycle controls was made by Beach *et al.* (1982) with the finding that *cdc2* could complement a *cdc28* mutation. Both *cdc2* and *CDC28* genes encode a 34kDa protein with specific serine-threonine kinase activity (Simanis and Nurse, 1986).

In addition to cell cycle arrest at either G1 or G2, caused by loss of function at restrictive temperatures (Nurse and Bisset, 1981; Piggot *et al.*, 1982), some dominant missense alleles of *cdc2* were identified which could also accelerate progress through the cell cycle. This indicated the activity level of Cdc2 was rate limiting and therefore provided a control point to regulate cell cycle timing (Nurse and Thuriaux, 1980).

$p34^{cdc2}$ activity is high in proliferating yeast with a peak during M phase. Conversely, its activity is low in interphase cells or non-dividing cells (Simanis and Nurse, 1986; Booher *et al.*, 1989), providing further evidence that this enzyme has a role in mitosis.

1.1.2 Homologues of *cdc2* in higher eukaryotes

Though *S. pombe* and *S. cerevisiae* have been separated in evolution for 1000 million years, the similarity of their Cdc2 and CDC28 gene products indicates that eukaryotic cell cycle mechanisms evolved very early, probably as a key component of the evolution of a eukaryotic cell (Draetta, 1990). This stimulated attempts to identify homologous genes in higher eukaryotes (Lee and Nurse, 1987). It has since been demonstrated that *cdc2* homologues are an important cell cycle regulator for many diverse species, ranging from yeast to humans. Significantly, the basic mechanism for the control of M phase is retained in all eukaryotic cells, including higher plants (for reviews, see Nurse, 1990; Jacobs, 1993).

A *cdc2* homologue can be identified by its ability to complement a yeast defective in *cdc2/CDC28* (Pines and Hunter, 1991). Functional *cdc2* homologues have been isolated in many eukaryotic species, some examples of which are shown in Table 1.1. In $p34^{cdc2}$ regions of amino acid have been identified that are common to all protein kinases, namely the ATP-binding site and the Thr167 phosphorylation acceptor site, which is phosphorylated to produce active kinase. There are also regions conserved and unique to cdc2, indicating important functional domains (Figure 1.1). In particular, $p34^{cdc2}$ has the so-called PSTAIRE region, a stretch of 16 amino acids which appears in every cdc2 (Lee and Nurse, 1987). PSTAIRE is an abbreviation for the full sequence of EGVPSTAIREISLLKE (Glu Gly Val Pro Ser Thr Ala Ile Arg Glu Ile Ser Leu Leu Lys Glu).

1.1.3 CDKs

To date, yeasts are the only organisms where the same Cdc2 protein has been identified as the controlling factor at both G1-S and G2-M checkpoints. Of the several CDKs expressed during the plant cell cycle, only *cdc2a* is transcribed throughout and is probably involved in both S phase and M phase events (reviewed by Mironov *et al.*, 1999). In animal cells, a family of cdc2 related proteins has been found, each of which regulates different parts of the cell cycle (Pines, 1993). An association with specific proteins, termed cyclins, is obligatory for activity of $p34^{cdc2}$ kinase and related proteins, therefore these proteins are termed Cyclin Dependent Kinases (CDKs).

CDKs operate at many points during the cell cycle in all eukaryotes. Some CDK proteins (Cdk4, Cdk6), that are in a signal transduction pathway for some growth

Table 1.1 Examples of *cdc2* homologues identified in diverse taxa

TAXA	NAME	REFERENCE
Mammals	Human	(Draetta *et al.*, 1987; Lee and Nurse, 1987)
	Mouse	(Lee *et al.*, 1988)
	Rat	(Draetta *et al.*, 1988)
Birds	Chicken	(Krek and Nigg, 1989)
Amphibians	*Xenopus laevis*	(Dunphy *et al.*, 1988; Gautier *et al.*, 1988)
Invertebrates	Starfish	(Arion *et al.*, 1988; Labbé *et al.*, 1988)
	Sea urchin	(Meijer *et al.*, 1989)
	Clam	(Draetta *et al.*, 1989)
Plants	*Zea mays*	(Colasanti *et al.*, 1991)
	Arabidopsis thaliana	(Ferreira *et al.*, 1991)
	Alfalfa	(Hirt *et al.*, 1991)

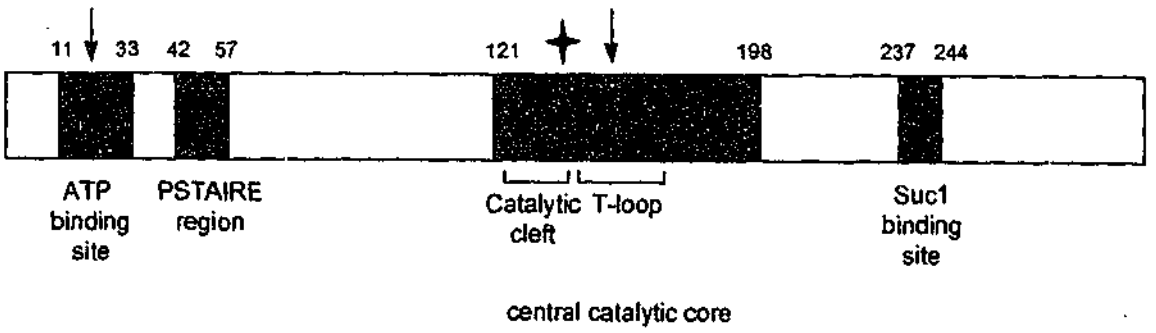


Figure 1.1 Schematic representation of the fission yeast p34^{cdc2} protein. The shaded regions are conserved functional domains. The arrows indicate residues that are phosphorylated. The star indicates the site of the substitution resulting in the *cdc2-1w* allele. (After MacNeill *et al.*, 1991; Marcote *et al.*, 1993).

A. The animal cell cycle

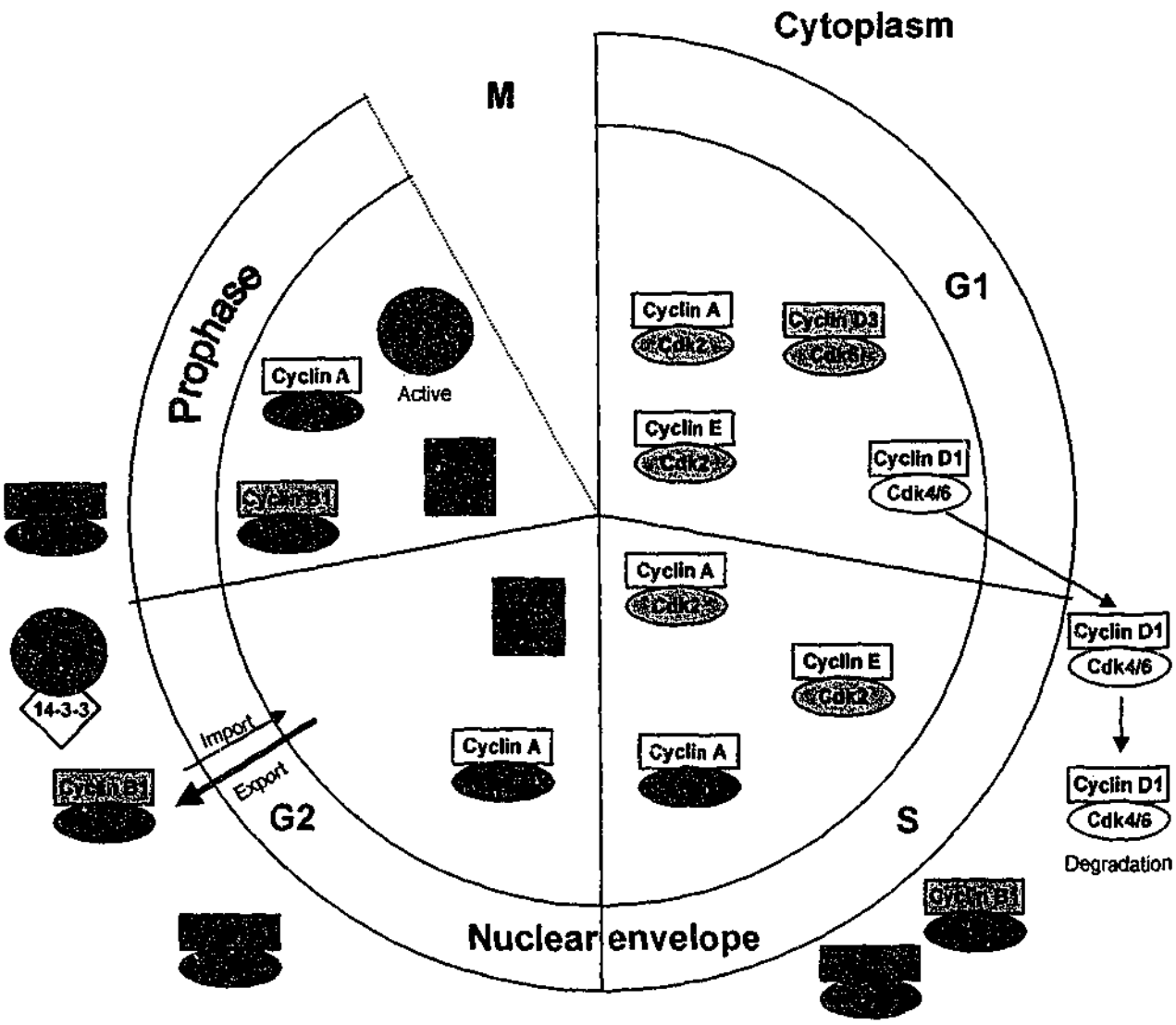
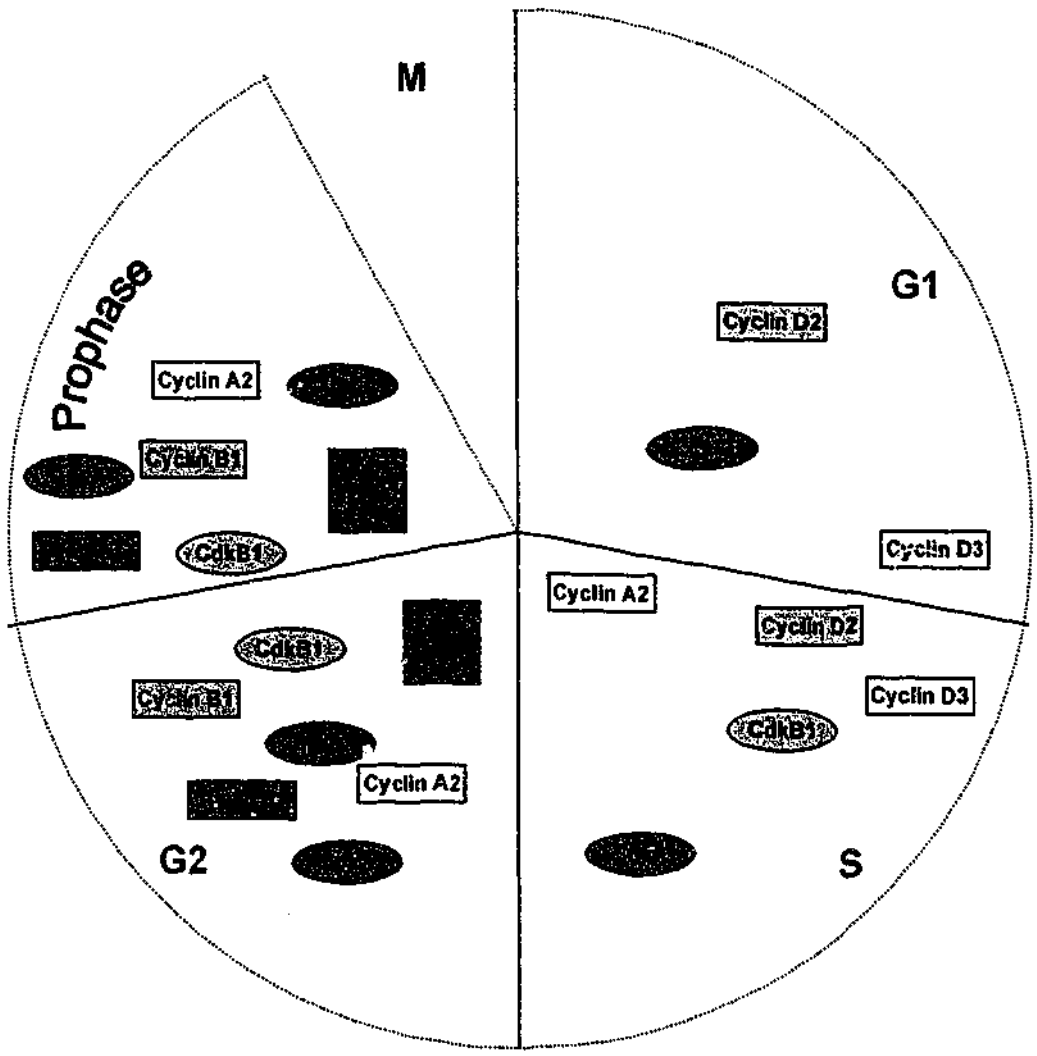


Figure 1.2 CDK-cyclins and their regulators – location during the cell cycle. A. In animals many CDK-cyclin pairs have been identified. Cdk2 is active in G1-S and Cdc2 operates in S-M with various cyclin counterparts. In G2, Cdc2-cylin B1 is mainly cytoplasmic due to a high export rate. Weel is a nuclear protein throughout the cell cycle, whereas Cdc25 is generally cytoplasmic and inactive. In some systems Cdc25C and cyclin B1 enter the nucleus just

B. The plant cell cycle



before the commencement of mitosis. **B.** In plants CDKs and cyclins have not been assigned partners, nor have subcellular localizations been entirely determined. CdkA is thought to be involved at both G1-S and G2-M as is Cdc2 in yeast. Not all identified regulators of the plant cell cycle have been assigned to a particular phase. (Modified from Yang and Kornbluth, 1999).

factors, act in early G1 to activate the transcription of genes required for S phase and continue in late G1 to initiate S phase itself (Ekholm and Reed, 2000). These proteins are replaced by Cdk2 to maintain S phase and in late G2 Cdk1 initiates mitosis (Reed, 1992). All CDKs are 35-40 kDa in size and have >40% sequence identity to each other (Morgan, 1995).

CDKs have been identified either by sequence homology, via PCR, or by functional complementation of yeast *CDC28* or *cdc2* mutants. In human cells, the human cdc2 homologue, CDK1 (also referred to as CDC2), is active only at mitosis. It is interesting to note that human CDK2 and human $p34^{CDC2}$ are each 65-67% similar to $p34^{cdc2}$ in *S. pombe*, yet they are only 66% identical to each other and have very different roles (Paris *et al.*, 1994). Other CDKs have been identified in human cells, most of which are thought to play a part in cell cycle regulation (Meyerson *et al.*, 1992; Morgan, 1995). Human CDK 1, 2 and 3 all contain the PSTAIRE motif, whilst the remaining members of the multigene family identified by PCR, contain variations in this region (Nigg, 1995).

1.2 CONTROL OF CDK ACTIVITY

1.2.1 Levels of CDK control

To become active, a CDK must associate with a cyclin and be phosphorylated on the threonine residue at position 161 (Thr161). These two events are the rate-limiting factors in the activation of Cdk4-cyclinD and Cdk6-cyclin D in G1 phase. Additionally, CDK-mitotic cyclin complexes are subject to inhibitory phosphorylation, especially at the tyrosine residue at position 25 (Tyr15), and must be dephosphorylated at that residue while retaining Thr161 phosphorylation to become active. This dephosphorylation is the rate-limiting event for Cdk2-cyclin E, Cdk1-cyclin A and Cdk1-cyclin B activity. CDK-cyclins may also be subject to regulation by binding inhibitory proteins (CKI) or accessory proteins (CKS) or by changes in their subcellular location (Pines, 1995). These controls are briefly described in the following sections.

1.2.2 The cyclin association

MPFs (Maturation-Promoting Factors) were first identified in *Xenopus* demonstrating an activity which oscillated during the cell cycle and peaked at M-phase. When MPF from an egg was injected, it could induce oocytes to enter M phase (Masui and Markert, 1971). Meanwhile, cyclin proteins were identified by their cyclical pattern of accumulation and destruction during the synchronous divisions that characterize early embryonic development in marine invertebrates (Evans *et al.*, 1983). Their involvement in the cell cycle was not understood until the activation of MPF, controlling the onset of

mitosis, was recognized as being concurrent with cyclin accumulation (Minshull, 1993). When purified, MPF activity was found to be associated with two proteins, one the equivalent of $p34^{cdc2}$ and the other a larger component that was a cyclin (Labbé *et al.*, 1989).

Further biochemical analysis has established that the catalytic subunit requires phosphorylation at Thr161 to be enzymically active (reviewed by Morgan, 1995) and to attain activities of physiological significance it must be in complex with cyclin. The importance of CDK-cyclin complexes in influencing the plant cell cycle is indicated by the stimulation of plant mitosis which follows micro-injection of active plant CDK-cyclin into plant cells (Hush *et al.*, 1996). Cyclins function by binding proteins that can become substrates of CDK (Peeper *et al.*, 1993), a significant role in cell cycle progression. Changing populations of cyclins throughout the cell cycle allow CDK activity to be directed to different sets of proteins. The presence of multiple cyclins and CDKs enables the fine regulation of cell cycle controls and checkpoints at many sites within the cell cycle (Ohi and Gould, 1999).

At the G2-M transition, the total amount of intracellular phosphate which is protein-bound increases dramatically. This is consistent with phosphorylation constituting a major mechanism for bringing about the profound structural change that accompanies the entry of cells into mitosis. Many CDK-cyclin complexes act preferentially to phosphorylate serine or threonine residues that are followed by a C-terminal proline (Nigg *et al.*, 1996).

CDK activity can be directed, by complexing with different cyclins, to specific substrates or subcellular locations (Kong *et al.*, 2000). Most G1-S and S phase substrates appear to be either transcriptional regulators involved in controlling the expression of S phase genes or components of the DNA synthesis machinery itself (Nigg, 1995). M phase phosphorylation is concentrated on components required for the organization of division, structural proteins and regulatory enymes (Pines, 1995). Localization is particularly important, for instance, Cdc2-cyclin B must have access to the microtubules for spindle formation to occur (Pines, 1999). Subsequent sections will discuss those aspects of cyclin function.

1.2.2.1 Cyclins at M phase

Mitotic initiation requires cyclin synthesis although, in all cells except oocytes, the overriding control of activity is the phosphorylation state of Tyr15. In oocytes, this phosphorylation does not occur (Minshull *et al.*, 1989). Both MPF activity and mitosis cease upon cyclin degradation (Murray, 1989). Following the isolation of a 47K subunit

of purified MPF that was found to be cyclin (Labbé *et al*., 1989), two mitotic cyclins were described and were named A and B, reflecting the sequence in which they accumulated (Minshull *et al*., 1990).

The first mutant isolated due to a mutation in a cyclin gene, was *cdc13* in fission yeast. Subsequently recognised as a mitotic cyclin gene, *cdc13* mutation prevented $p34^{cdc2}$ activation at mitosis. The encoded protein, $p56^{cdc13}$, was shown to be required both before and during mitosis (Moreno *et al*., 1989) and destroyed at the end of mitosis (Hagan *et al*., 1988). Cyclin B association with the $p34^{cdc2}$ complex at M phase has subsequently been identified in many eukaryotes, including humans and the role of Cdk1-cyclin B in mitosis appears to be universal (Pines, 1995).

1.2.2.2 Cyclins Throughout the Cell Cycle

Cyclins have been grouped according to the stage of the animal cell cycle when they are expressed, with early G1, late G2, mitotic A and mitotic B cyclins having been described (Morgan, 1995). At least 10 classes of cyclin (A to J), have been described to date in different organisms, with some organisms possessing multiple members of the same class (Pines, 1995). Plant cyclins do not precisely match animal cyclins in structure and have been difficult to classify as cyclins A and B because so many plant cyclins share features of both animal cyclin A and animal cyclin B (Renaudin *et al*., 1996). Data on the function of plant cyclin proteins are scarce and while some share properties with animal cyclin B in showing initial cytoplasmic retention before nuclear migration, others have unique properties such as persistance to telophase and association with the phragmoplast (Mews *et al*., 1997).

Cyclin protein level is determined by a combination of transcriptional induction and repression and by timed proteolytic destruction and is best understood in yeasts (reviewed by Nasmyth, 1996). A direct relationship between mRNA and protein levels has also been reported for cyclin A, B and E in mammals (Müller, 1995; Dohna *et al*., 2000).

All cyclins share two structural features although elsewhere in the protein they are structurally diverse. All contain a "cyclin box", a conserved region of amino acid sequence of about 100 residues, which is the defining feature of all cyclins. It has the function of binding to the cyclin's CDK partner (Noble *et al*., 1997). The second common trait is that all cyclins are rapidly degraded by the ubiquitin-dependent proteolysis system and possess ubiquitin ligase recognition motifs that differ between G1-cyclins and mitotic cylins (Pines, 1995).

The archetypal cyclins A and B of animals also possess a conserved 250-amino acid domain referred to as the cyclin core which may have evolved by duplication of the cyclin box. The cyclin box is located in the first fold of a rigid tertiary structure organized into two folds of five helices that comprise the cyclin core. The cyclin core of cyclin A is sufficient for binding and activation of Cdk1 and Cdk2 (Noble *et al.*, 1997).

1.2.2.3 The CDK-cyclin partnership

The diversity of cyclins and their replacement by other cyclins during the cell cycle provides a means by which very different cell cycle events can be catalysed. In particular, G1 cyclins regulate cdc2 activity at the late G1 event called START at which DNA replication is comitted to and mitotic (B-type) cyclins regulate cdc2 at mitosis (Pines, 1993).

The order of cell cycle events is maintained by the programmed synthesis and destruction of each cyclin, assuring an appropriate temporal window for the activation of their CDK partners. In *S. cerevisiae,* G_1 cyclins (CLNs) CLN1 and CLN2 activate CDC28 which, in turn both stimulates *CLN* transcription and represses mitotic cyclin (CLB) degradation. While S phase is underway, CLB levels are consequently rising. CLB stimulates both its own transcription and represses that of *CLN.* CDC28 is mitotically activated when CLB levels are high, the completion of mitosis then leading to CLB breakdown (Amon *et al.*, 1993). In vertebrates, several of these partnerships are well-defined as illustrated in Figure 1.2. At the G2-M transition, CDK-cyclin complexes consist of Cdc2-cyclin A, Cdc2-cyclin B1 and Cdc2-cyclin B2. Cdk2-cyclin A are paired through S phase progression. In G1, Cdk4-cyclin D and Cdk6-cyclin D are active, with Cdk2-cyclin E being prominent at G1 exit (Yang and Kornbluth, 1999). The situation in plants is not so well understood as will be noted in later sections.

1.2.3 CDK Regulation

CDKs and their cyclin regulatory subunits are the core complexes that govern the progress through the cell cycle. As a combination, CDK-cyclins can be regulated at the levels of transcription, translation and post-translation, ultimately providing responsive and flexible control. Identifying the proteins which antagonize and promote CDK-cyclin activity is essential to understanding their regulation. Phosphorylation of specific sites within the protein is a mechanism used to both inhibit and promote CDK activity. Inhibition of CDK activity is also achieved by inhibitory proteins (CKI) which bind to CDKs, precluding access of other regulatory proteins to essential regions.

1.2.3.1 CAKs -activating CDKs

The conserved residues Thr167 (in yeast) and Thr161 (other eukaryotes) must be phosphorylated to produce active Cdc2 (Gould *et al.*, 1991; Krek and Nigg, 1991). CAK (Cdc2 Activating Kinase) has been identified as the protein kinase that performs this phosphorylation and brings about $p34^{cdc2}$ activation. Thr167/161 phosphorylation is not observed in the absence of cyclin binding (Solomon *et al.*, 1992).

Phosphorylation of CDK at Thr161 stabilizes the association of the CDK catalytic subunit and cyclin. Once the stable complex has formed, the Thr161 site becomes resistant to dephosphorylation (Labbé *et al.*, 1994). The Thr161 site is in a region known as the T-loop of the kinase structure that blocks the protein substrate binding site in free Cdk2. It has been observed, by X-ray crystallography, that cyclin binding and Thr161 phosphorylation conforms the protein to render the substrate binding site accessible (De Bondt *et al.*, 1993; Morgan and De Bondt, 1994). In the absence of cyclin, Thr161 is no longer a substrate for CAK and the site becomes exposed again to phosphatase action.

1.2.3.2 CKIs - inhibiting CDKs

A family of proteins that inhibit CDK-cyclin catalytic activity when bound to the CDK are called the CDK Inhibitory Proteins (CKIs). CKIs inhibit kinase activity by preventing the CDKs from binding cyclins or by binding to the Thr160/161 phosphorylated complex. The main function of CKIs may be to order cell cycle events and they may also arrest the cycle under certain conditions. The list of CDK inhibitors includes members from both budding and fission yeasts ($p40^{SIC1}$ and Rum1 respectively) and two protein families in mammals (Hunt and Sherr, 1994). Crystal structure analysis reveals that CKI protein binds to the cyclin and inserts into the catalytic cleft of the CDK thereby inactivating it (Russo *et al.*, 1996).

One possible significance of CKI's is that they require substantial activity of CAK to have accumulated before cell cycle progression can occur. For instance, in *S. cerevisiae* $p40^{SIC1}$ binds and inhibits CDC28-CLB5 in G1 until the G1-S transition, then releasing a supply of active enzyme complex to initiate S phase (Schwob *et al.*, 1994). Significantly, the release of Cdc28-Clb5 activity occurs when the Sic1 protein has been proteolysed because it has been phosphorylated by Cdc28-cLN. This appears to be a universal mechanism since CDK/G1 cyclin activity removes Rum1 from fission yeast and $p27^{Kip1}$ from mammalian cells at the initiation of S phase. Sic1, Rum1 and $p27^{Kip1}$ are all inhibitors of CDK/mitotic cyclin complexes and only mitotic cyclins can drive DNA synthesis (Nasmyth, 1996). G1 cyclins are perfectly suited to catalyze the

phosphorylation of CKI and its proteolysis since Cdk/G1 cyclins are not inhibited by G1 accumulated CKI (Martín-Castellanos *et al.*, 2000). The presence of CKI prevents activity of CDK-mitotic cylin in G1 phase and prevents catastrophic premature entry into mitosis (Correa-Bordes and Nurse, 1995; Correa-Bordes *et al.*, 1997; Martín-Castellanos *et al.*, 2000).

In higher eukaryotes there are two distinct families of CKIs, the $p21^{Kip}/p27^{Cip}$ family and the Ink4 family of inhibitors. The Ink4 proteins inhibit Cdk4 or Cdk6 specifically whereas the Cip/Kip family have a broader effect on CDK-cyclin complexes. Both are thought to be involved in G1 checkpoint control in response to environmental signals (Nakayama and Nakayama, 1998). The suppression of S phase entry by these proteins seems to be brought about by inhibiting interaction with the pRb pocket-protein family. CDK-cyclin D and E are currently thought to promote entry into S phase by their ability to phosphorylate pRb-E2F and release E2F transcription factors essential for S phase progression (Vidal and Koff, 2000). Proteolysis of p27, after its phosphorylation by Cdk4,6-cyclin D (Pagano *et al.*, 1995), releases sufficient CDK-mitotic cyclin activity to ensure that Rb is phosphorylated and that S phase can occur (Harbour *et al.*, 1999).

1.2.3.3 Suc1/Cks1

The *suc1* (suppressor of cell block) gene was first identified in fission yeast by its ability to rescue temperature sensitive *cdc2* alleles (Hayles *et al.*, 1986). Its homologue *CKS1* (cyclin-dependent kinase subunit) was correspondingly recognised in budding yeast (Hadwiger *et al.*, 1989). These small proteins are known to bind to CDKs and are essential for cell cycle progression but do not have a fully defined biochemical role. The interaction between Suc1/Cks1 proteins and CDKs is strong and has been utilized in affinity chromatography purification (John *et al.*, 1991). A recent two-hybrid screen has observed that *S. cerevisiae* Cks1 to interact directly with all three forms of cyclin B from budding yeast (Uetz *et al.*, 2000). This explains the strong binding of Cks to CDK.

suc1/CKS1 overexpression leads to a delay of mitotic onset, whereas deletion of *suc1/CKS1* results in cells arresting within mitosis (Moreno *et al.*, 1989). Many higher eukaryotes contain homologous genes (De Veylder *et al.*, 1997a) and the Suc1/Cks1 protein may participate in the docking of CDK complex regulators (Bourne *et al.*, 1996). Binding of Cks to CDK-cyclin extends the range of substrates which may be phosphorylated by CDKs (Patra and Dunphy, 1998). High levels of the Suc1/Cks1 protein may interfere with dephosphorylation of CDK by Cdc25 consequently delaying the onset of mitosis (Patra *et al.*, 1999), whereas low levels may prevent the targeting of cell cycle proteins for degradation required for mitotic exit (Sudakin *et al.*, 1997).

1.2.4 Inhibitory phosphorylation in CDK regulation

Genetical, and subsequently biochemical, evidence in fission yeast has indicated that $p34^{cdc2}$ phosphorylation on Tyr15 down-regulated the kinase activity in *S. pombe*, and mitosis occurs when this phosphorylation declines and $p34^{cdc2}$ activity rises. Mutation of Tyr15 to a non-phosphorylatable phenylalanine residue was also observed to cause cells to prematurely enter mitosis, suggesting that the phosphorylation of this residue was crucial for maintaining $p34^{cdc2}$ inactive prior to mitosis (Gould and Nurse, 1989). In higher eukaryotes an additional negative regulatory phosphorylation site, Thr14, has been identified. Dephosphorylation of both Tyr15 and Thr14 at the G2-M transition is required for full activation of the $p34^{cdc2}$ protein kinase (Krek and Nigg, 1991; Norbury *et al.*, 1991; Solomon *et al.*, 1992). In human CDK2, crystal structure analysis has shown Tyr15 and Thr14 to be located in the ATP binding site which is subsequently blocked upon phosphorylation (De Bondt *et al.*, 1993).

The extent of Tyr15-phosphorylation in $p34^{cdc2}$ is determined by the opposing activities of Wee1 tyrosine kinases and Cdc25 protein phosphatases (Figure 1.3). The first suggestion that both enzymes have an interacting and opposing role in regulating the cell cycle *in vivo* was based upon the observations of Russell and Nurse (1986). It was observed that deletion of *cdc25* did not prevent progression into mitosis if *wee1* was also inactive. The importance of their interaction in controlling the onset of mitosis was also indicated by the results of experiments with yeast in which *cdc25* was overexpressed in cells lacking the *wee1* gene. The effect of altering both genes was additive and resulted in catastrophic early initiation of mitosis (Russell and Nurse, 1986).

1.2.4.1 *wee1*

When grown in rich medium *S. pombe* cells are particularly large on entering mitosis. If mitotic control is disabled by the $wee1^-$ mutation, cells undergo division at approximately half the length of wild-type *S. pombe* cells, hence their designation as having a "wee" phenoptype. Whereas cells that have extra copies of $wee1^+$ undergo division at longer cell lengths that are directly related to $wee1^+$ gene dose (Russell and Nurse, 1987).

Fission yeast Wee1 is a 107 kDa tyrosine kinase which regulates mitosis by carrying out the inhibitory Tyr15 phosphorylation of $p34^{cdc2}$ (Russell and Nurse, 1987). Wee1 is itself regulated by phosphorylation. In *S. pombe*, Nim1 serine/threonine protein kinase contributes to the induction of mitosis by inhibitory phosphorylation of Wee1 (Wu and Russell, 1993). In *Xenopus*, a second form of inhibitory phosphorylation is performed either directly or indirectly by Cdc2-cyclin B kinase.

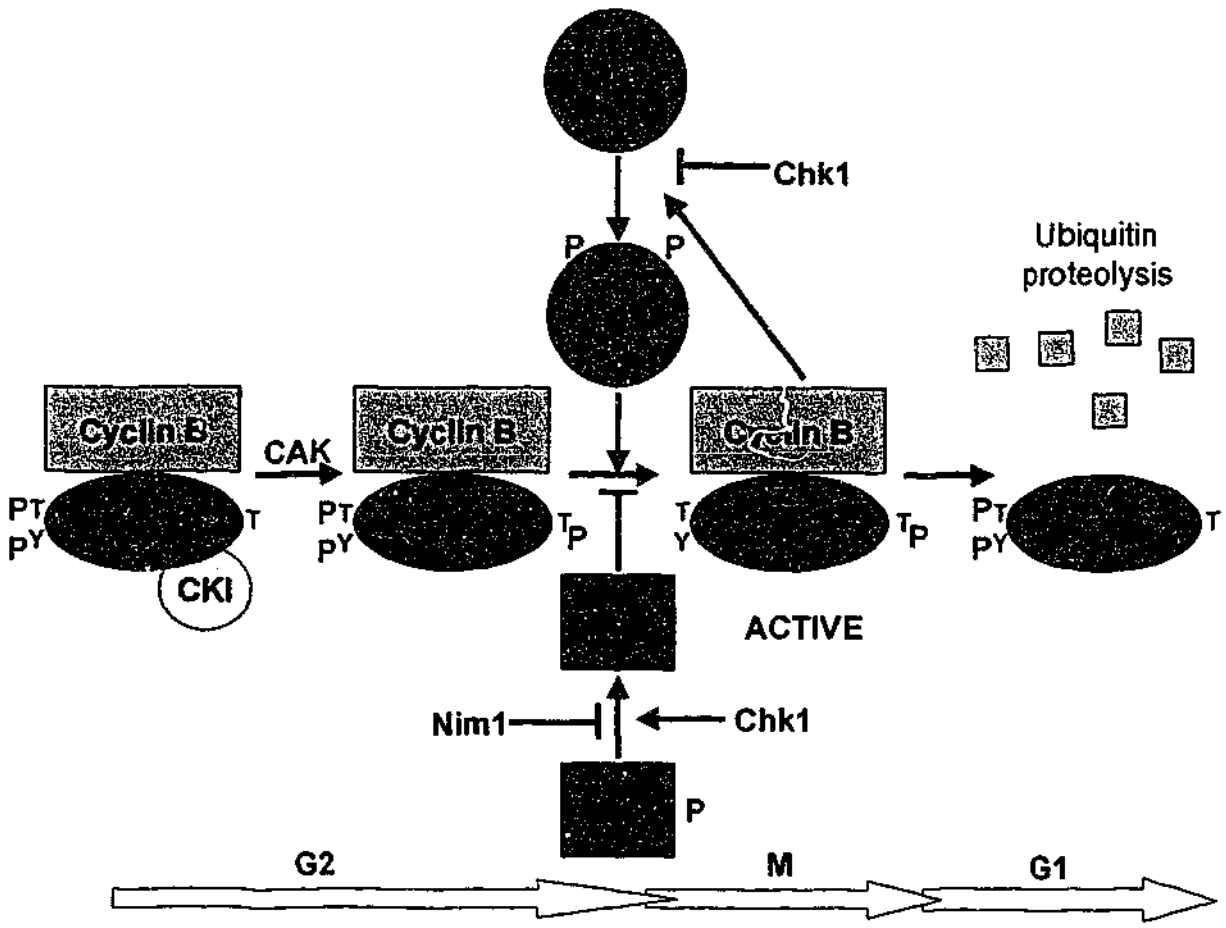


Figure 1.3 Regulation of CDK activity at G2-M. To be fully active $p34^{cdc2}$ requires phosphorylation on Thr161 by CAK, dephosphorylation on Tyr15 (and Thr14 in mammals) by Cdc25 and no association with CKI proteins. The DNA damage checkpoint is mediated through Chk1, in yeast this operates through both Cdc25 and Wee1.

The protein kinase catalytic domain of Wee1 is located in the C-terminus of the protein. Cdc2-cyclin B is much more actively phosphorylated by Wee1 than is Cdc2 alone and it seems some part of the N-terminal domain of Wee1 is important for substrate interaction (Parker *et al.*, 1992). It seems most likely to be the 363-408 amino acid sequence (Aligue *et al.*, 1997). The N-terminal region also probably contains domains of inhibitory regulation, since the specific activity of Wee1 is increased by truncation of this region. *wee1* mRNA levels have been shown to be stable during the cell cycle, but some Wee1 protein degradation does take place in M phase (Aligue *et al.*, 1997).

In fission yeast, exposure to DNA damaging agents delays entry into M phase until the DNA is repaired. Here, Wee1 is phosphorylated by Chk1 *in vivo*, and the consequent increase in Wee1 enzyme activity results in maintenance of Tyr15 phosphorylation and hence G2 delay (O'Connell *et al.*, 1997). The damage checkpoint operates in yeast by simultaneously upregulating Wee1 and downregulating Cdc25 (Raleigh and O'Connell, 2000).

1.2.4.2 *mik1*

Wee1 phosphorylates Cdc2 *in vitro* at Tyr15 but not Thr14, supporting the notion that a separate Thr14 kinase also exists. In *S. pombe*, Mik1 also performs Tyr15 phosphorylation, as does Myt1 in animal cells, but Wee1 has the dominant role. Myt1 also phosphorylates Thr14 (Campbell *et al.*, 1995; Mueller *et al.*, 1995a; Mueller *et al.*, 1995b).

Mik1 protein accumulates in S phase and may create a link between S phase and mitosis that ensures S phase is complete before mitosis occurs (Christensen *et al.*, 2000). A possible mechanism for this was postulated when it was found that Mik1 is required for DNA damage checkpoint response in strains that lack Cdc25 (Baber-Furnari *et al.*, 2000). A role for Myt1 (the animal equivalent of Mik1), has also been suggested in the DNA replication checkpoint (Winkler *et al.*, 2000).

1.2.4.3 *cdc25*

The *cdc25* gene, which was cloned first from *S. pombe*, encodes a 67 kDa phosphoprotein [(Russell and Nurse, 1986; Gould *et al.*, 1990; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991). This threonine-tyrosine phosphatase directly activates $p34^{cdc2}$ (Dunphy and Kumagai, 1991; Millar and Russell, 1992) (The Cdc25 protein is known as $p80^{cdc25}$, as mobility on SDS gels corresponds to an unphosphorylated protein of about 80 kDa rather than 67 kDa).

Temperature sensitive lethal *cdc25*^{ts} mutants of *S. pombe* become blocked in G2 upon a shift to the restrictive temperature (Fantes, 1979). Also, overexpression of *cdc25* in yeast causes the initiation of mitosis at a reduced cell size relative to wild-type cells. These observations indicate that *cdc25* is both necessary and rate limiting for entry into mitosis in fission yeast. As noted earlier, *cdc25* functions to counteracts the activity of the mitotic inhibitor kinase *wee1* upon Tyr15 (Russell and Nurse, 1986). *cdc25* deletion mutants are viable as another protein, Pyp3, can also dephosphorylate Tyr15. Like Mik1, Pyp3 is normally a minor player in cell cycle control but it becomes more significant when Cdc25 is inactivated (Millar *et al.*, 1992).

In human cells, cdc25 proteins are encoded by a multigene family, consisting of *cdc25A*, *cdc25B* and *cdc25C*. Cdc25A plays a crucial role at the G1-S phase transition, Cdc25C is involved in the dephosphorylation and activation of the mitotic kinase Cdc2-cyclin B, whereas Cdc25B may contribute to both processes (Lammer *et al.*, 1998). Some data suggest a role in the initiation of mitosis (Gabrielli *et al.*, 1996; Nishijima *et al.*, 1997; Karlsson *et al.*, 1999). A constant feature of these observations is the shuttling of each Cdc25 between the nucleus and cytoplasm at different stages of the cell cycle. To add to the complexity of this aspect of cell cycle control, *CDC25B* has multiple splicing variants which may be differently regulated with respect to localization (Woo *et al.*, 1999).

1.2.4.4 Potential activation loop of Cdc2/Cdc25 and cyclin B recognition

In fission yeast, protein levels of Cdc25 increase as cells proceed through G2, reaching a peak just before the onset of mitosis (Moreno *et al.*, 1990). Studies in mammals record that Cdc25 levels do not vary significantly during the cell cycle (Girard *et al.*, 1992), but it appears that within the nucleus levels do oscillate (Lopez-Girona *et al.*, 1999). Once Cdc25 accumulates inside the nucleus, MPF activity increases rapidly as it becomes de-phosphorylated on Tyr15 and Thr14. In *S. pombe*, Cdc25 is degraded by the ubiquitin pathway at the end of mitosis. *pub1* is a gene which regulates this event, possibly by directly ubiquitinating Cdc25 *in vivo* (Nefsky and Beach, 1996).

The C-terminal catalytic domain for the Cdc25 phosphatases is highly conserved. However, the N-terminal regulatory domain is specific to each Cdc25 isoform and varies between species (Yang *et al.*, 1999). Activation of Cdc25 catalytic activity in late G2 occurs through its phosphorylation. The active form of human Cdc25C is phosphorylated on Thr48, Thr67, Ser122, Thr130, Ser214 (Hoffmann *et al.*, 1993).

According to kinetic studies undertaken by Aguda (1999), MPF and Cdc25 activities rise together during the cell cycle. As active Cdc25 is obtained *in vitro* after

phosphorylation by MPF itself (Izumi and Maller, 1995), as well as by Plx (polo-like kinase) kinase (Kumagai and Dunphy, 1996), a potential feedback loop is created between $p80^{cdc25}$ and MPF leading to rapid $p34^{cdc2}$ kinase dephosphorylation. Conversely if either total MPF or Cdc25 concentrations fall below a critical threshold, then both activities of MPF and Cdc25 are expected to decline (Aguda, 1999).

A two step model has been proposed for the Cdc2 positive feedback loop. In step one, Cdc25 has a basal activity which stimulates a linear activation of Cdc2 that, in turn, partially phosphorylates Cdc25. Plx kinase also phosphorylates Cdc25 in a positive manner in *Xenopus* oocyte extracts, when activated by Plkk1. Both Plx and Plkk1 (polo-like kinase kinase) are activated in step one by cdc2 (Karaiskou *et al.*, 1999).

Full phosphorylation and activation of Cdc25 occurs at step 2, thereby initiating the amplification loop. Unlike step one, step two is conditional upon several assisting factors. It requires Plx activity, the inhibition of PP2A and Suc1 association with Cdc25/Cdc2 (Karaiskou *et al.*, 1999). Cdc25 is dephosphorylated and hence inactivated by PP2A a type 2A phosphatase (Clarke *et al.*, 1993).

The positive feedback model, noted above, can incorporate the action of Wee1 as Cdc2 appears to have some role in its regulation. Activation of a small fraction of Cdc2-cyclin B can be rapidly amplified into total activation by a process in which Cdc2-cyclin B catalyzes stimulatory phosphorylation of Cdc25 plus inhibitory phosphorylation of Wee1 (Dunphy, 1994). These elegant models are, however, difficut to verify in live cells.

In mammalian cells, protein levels of each of the three *cdc25* homologues are relatively constant through the cell cycle. Therefore it is probably the activity of each Cdc25 protein, rather than their quantities *per se*, that is critical for cell cycle control. Different CDK-cyclin complexes are known to activate different Cdc25 homologues, possibly following a similar model of activation to that proposed for mitotic entry (Hoffmann and Karsenti, 1994; Izumi and Maller, 1995).

1.2.5 Checkpoint response through Cdc25

In response to DNA damage and incomplete replication, Cdc25 regulation delays mitosis through the failure of the Cdc25 phosphatase to activate Cdk1 (Martinho *et al.*, 1998; Furnari *et al.*, 1999). The mechanism involved includes both phosphorylation and spatial control of the protein. While coupling of Cdc25 activation of the completion of DNA replication and repair is almost universal, the budding yeast is clearly different in coupling the activation to successful bud growth (Lew and Reed, 1993).

During interphase, Cdc25 is located in the cytoplasm (Dalal *et al.*, 1999) and is phosphorylated by CTAK1 in human cells (Peng *et al.*, 1998). During entry into mitosis, Cdc25 collects in the nucleus. When mitosis is delayed, in response to a checkpoint control such as DNA damage, Cdc25 is again phosphorylated and excluded from the nucleus.

Two proteins have been identified that phosphorylate Cdc25 in a checkpoint dependent manner, the Chk1 and Cds1 protein kinases (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997; Kumagai *et al.*, 1998; Matsuoka *et al.*, 1998; Blasina *et al.*, 1999). A conserved serine residue is the single phosphorylation target in human and *Xenopus* Cdc25C (Peng *et al.*, 1997; Kumagai *et al.*, 1998), whereas *S. pombe* Cdc25 appears to have several phosphorylation sites (Zeng and Piwnica-Worms, 1999).

Phosphorylation of Cdc25 promotes 14-3-3 protein binding to form a complex with Cdc25. In yeast, Cdc25 is actively exported from the nucleus when phosphorylated. Rad24, a 14-3-3 protein homologue with a Nuclear Export Signal (NES), binds to Cdc25 and the protein complex is rapidly transported to the cytoplasm (Lopez-Girona *et al.*, 1999; Zeng and Piwnica-Worms, 1999). In humans and *Xenopus*, 14-3-3 proteins also bind to Cdc25. But in this case they act to obscure the Nuclear Localisation Sequence NLS) so the Cdc25 is retained in the cytoplasm (Kumagai *et al.*, 1998; Dalal *et al.*, 1999; Kumagai and Dunphy, 1999; Yang *et al.*, 1999).

Chk1 and Cds1 are unrelated protein kinases but unusually both phosphorylate the same substrate, Cdc25, at the same site (Ser99 in yeast)(Furnari *et al.*, 1999). In fission yeast, Cds1 appears to be S phase specific and respond to incompletely replicated DNA, thereby providing a checkpoint for successful completion of S phase. Chk1 operates at G2-M in response to damaged DNA, but can also be shown to respond to replication disruption in the absence of Cds1 (Brondello *et al.*, 1999). Cdc25 is therefore controlled by a similar regulatory mechanism conveying different cellular signals at different phases of the cell cycle, with some apparent redundancy.

Recently, it has been shown that the region of the Cdc25 protein which interacts with 14-3-3 proteins also interacts with cyclins through a motif similar to a P-box (Morris *et al.*, 2000). A P-box was first identified as a domain in cyclin B1 required for Cdc25 activation (Zheng and Ruderman, 1993). Thus, at the G2-M checkpoint, Cdc25C is excluded from the mitotic process by 14-3-3 binding in two ways. Firstly, by interference with transport to the nucleus and secondly, by blocking the interaction with cyclin necessary for Cdc25 activation.

As well as being susceptible to DNA irregularities, Cdc25 may also serve to ensure that mitosis does not occur before a minimum cell size is attained. Daga and Jiminez (1999) have ascertained that *S. pombe cdc25* mRNA has a 5' untranslated leader which ensures that initiation of translation is a limiting step. Cdc25 is particularly affected by the reduced production of translation initiation factors. If Cdc25 fails to accumulate due to low biosynthetic activity in a cell, cycle arrest ensues. This means that entry into mitosis is sensitive to reduced protein synthesis in the fission yeast cell, providing a link between cell cycle progression and growth. The translation of Cdc13 cylin B is also limited by this mechanism. The rate of expression of *cdc25* (*String*) in *Drosophila* is influential in M phase initiation (Daga and Jimenez, 1999), suggesting that this translational control mechanism may operate more generally in eukaryotes. It is possible however that the cell size mechanism may operate in another way and activation of Cdc25 is a secondary consequence of size attainment.

1.3 THE CELL CYCLE IN PLANTS

The conservation of basic cell cycle mechanisms amongst eukaryotes has enabled the cloning of plant CDK and cyclin by homology (for review see Mironov *et al.*, 1999). One of the first plant cell cycle genes, cloned by homologous PCR amplification, was *cdc2* in pea (Feiler and Jacobs, 1991). It seems the basic mechanism of cell cycle catalysis and its control points has been retained in plants but with additional sophistication, possibly due to the complexities of plant cell structure, development and architecture.

Plants are unique amongst multicellular eukaryotes in their immobility, structural formation and their plasticity. Their fixed position means they need to be adaptable and be able to respond to a vast array of environmental conditions. Cell division in a plant occurs in meristems and plant cells have strong cell walls. There is no cell migration as is seen in animals.

The elaborate cell cycle control mechanism in plants reflects the need to respond to a wide variety of input signals and indicates the intricate regulation of the timing and placement of division. Spatial control is required at the time of division for correct tissue and organ formation and here a unique cytoskeletal structure makes a vital contribution. In the root, quiescent meristematic cells are able to resume division, as are pericycle cells which give rise to lateral root primordia. In addition, differentiated cells have an adaptive potential to dedifferentiate and are capable of re-entering the cell cycle (Skoog and Miller, 1957). They are able to form proliferating callus tissues from which shoots and/or roots may be recovered by manipulation of phytohormones such as auxin and cytokinin (for review see Hamill, 1993).

It is becoming increasingly clear that the plant cell cycle has more in common with the mammalian control systems than with yeast. Fundamental mechanisms are well conserved between plants and animals but are designed to respond to different growth regulators in diverse growth and development contexts. However plants differ from mammals in having multiple CDKs active later in the cell cycle and a greater diversity of cyclins as well as unique cyclin locations (for review, see Huntley and Murray, 1999).

1.3.1 Plant *cdc2* Homologues

As has been noted, the first plant homologues of yeast *cdc2* were cloned in 1991 and many typical $p34^{cdc2}$ features were found to be highly conserved. Homologues containing the characteristic PSTAIRE motif are designated A-type CDKs (reviewed by Burssens *et al.*, 1998). Plant Cdk1 genes, as from all other eukaryotes, can rescue *cdc2* mutations in yeast (reviewed by Jacobs, 1995).

Homologues to *cdc2*have been isolated from many species: *Pisum sativum* (Feiler and Jacobs, 1991); *Medicago sativa* (Hirt *et al.*, 1991; Magyar *et al.*, 1997); *Zea mays* (Colasanti *et al.*, 1991); *Oryza sativa* (Hata, 1991; Hashimoto *et al.*, 1992); *Arabidopsis thaliana* (Ferreira *et al.*, 1991; Hirayama *et al.*, 1991); *Glycine max* (Miao *et al.*, 1993) and *Antirrhinum majus* (Fobert *et al.*, 1996). Plant CDKs now represent nearly 50 different sequences from more than 20 species and Joubès *et al.* (2000) recently attempted to unify the plant nomenclature by dividing them into 5 classes, A-E.

At the protein level, the A-type plant CDKs show 60-65% identity to the yeast Cdc2 protein (Sundaresan and Colasanti, 1998) and several species contain two *CdkA* genes. Amongst themselves the plant CDKAs isolated are highly conserved showing 89% similarity at the amino acid level. However, they show much less homology to any of the other classes of plant CDK, appearing more closely related to human Cdk1, Cdk2 and Cdk3 (Joubès *et al.*, 2000). This reflects the high level of conservation of this component of the cell cycle control mechanism between eukaryotes.

The plant B-type CDKs do not have a PSTAIRE motif but rather the unique plant motifs PPTALRE (CdkB1 –sub class 1) or PPTTLRE (CDKB2 –sub class 2) (Mironov *et al.*, 1999). The plant CDK-B class is unusual amongst non-PSTAIRE CDKs in participating in G2-M control and could be responsible for plant specific functions. Transcripts and protein from these genes accumulate during the S-M phase in contrast to all other eukaryotic CDKs which maintain cycle-independent levels (Fobert *et al.*, 1996; Magyar *et al.*, 1997). CdkB2 expression is more specific to the G2-M phase

indicating the CDKB class contains two effectively discrete gene groups. Interestingly, plant CDKB are unable to complement yeast *cdc2* mutants (Fobert *et al.*, 1996).

Class C is defined by the PITAIRE motif and homology to human CDK-related CHED kinase but its participation in cell division is uncertain. Class D is also referred to as the CAK kinases that are involved in activating the other CDKs during the cell cycle and are discussed in a later section. The cell cycle credentials of class E are unknown, it consists of a unique alfalfa sequence unrelated to any other plant gene. The broadest range of CDKs found in one species characterized to date are the six *cdc2* homologues identified in alfalfa (*Medicago sativa*) (Two A, two B, one C and one E-class CDKs). (Magyar *et al.*, 1997). Stals *et al.* (2000) reported that a total of five homologues exist in *Arabidopsis* (One A, one B, two C and one D-class CDKs). It is primarily around sequences isolated in these two species that the classes C, D and E have been outlined.

A recent, somewhat confusing development, is the claim of the identification of another *cdc2cAt* found only expressed in flowers and is most closely related to *cdc2MsC* (*Medsa;CdkC;1*). The sequence also shows homology to human CHED protein kinase, aligning it with Cdc2-like proteins associated with differentiation rather than cell division in general (Lessard *et al.*, 1999). However, the deduced sequence is completely lacking the PSTAIRE motif so it is unclear how this proposed CDK fits in the nomenclature system.

1.3.2 Plant Cyclins

Plant cyclins are much less well characterized than those of animals. There is little information concerning their CDK partners, when the cyclin proteins accumulate, or where most are located in the cell. Although it may be inappropriate to follow the classifications of mitotic cyclins into A-type and B-type, following the pattern in animal cells, this has been attempted for plant cyclins in an effort to bring order to the nomenclature. The designations are a little arbitrary since many plant cyclins have properties of animal cyclins A and also of animal cyclins B. Furthermore, plant cyclins have many unique functions in mitosis.

One of the unique features of the plant cell is the preprophase band (PPB) that forms as mitosis approaches. The microtubules (MTs) form a band that encircles the cell periphery in the region of the nucleus, which indicates where the new cell wall will form in telophase (Gunning and Sammut, 1990). Immunostaining using a PSTAIRE/Cdc2 type antibody has been used to show a possible localization of $p34^{cdc2}$ to the PPB (Mineyuki *et al.*, 1991; Colasanti *et al.*, 1993). By co-injecting fluorescently labelled tubulin and plant metaphase kinase, Hush and co-workers (Hush *et al.*, 1996)

were able to greatly accelerate PPB disassembly. Ths indicated a new role for Cdc2 in the plant cell at G2-M and a requirement for a unique affinity in a cyclin partner. Some candidates for targeting cdc2 in this manner are maize cyclins Zm;CycB1;2 and Zm;CycA1;1 which have been shown to associate with the PPB just prior to its breakdown (Mews *et al.*, 1997).

Given the unique features of the plant cell cycle, the alignment of plant cyclins with A-type or B-type may not be meaningful. With these reservations, plant CycA cyclins show a 37% similarity to the cyclin core of animal A-type cyclins at the amino acid level. Plant CycB cyclins show 30% homology to both A- and B-type cyclins, making it difficult to draw a direct parallel to animal classification. Motifs particular to each group are found in the variable N-terminus (Renaudin *et al.*, 1998).

Plants possess at least three distinct A-type groups of cyclins, two B-type groups and four D-type groups (Renaudin *et al.*, 1996 ; De Veylder *et al.*, 1999). H-type cyclins have also been recently identified in plants (Yamaguchi *et al.*, 2000) but so far no cyclin E-type equivalent has been found. The plant cyclins have been assigned classes based on partial homology with cyclin classes of other eukaryotes. According to plant specific structural features they have then been divided into groups unrelated to those found in animals which have been named CycA1, CycA2 etc (Renaudin *et al.*, 1996). Some functional similarity exists as they can induce meiotic maturation of immature *Xenopus* oocytes (Hata *et al.*, 1991; Ferreira *et al.*, 1994) but they are not necessarily outright functional homologues.

An additional level of complexity is that some plants have several cyclins within the A-type group, unlike animal cells (Nigg, 1995). There are three separate CycA2s in *Arabidopsis* (Ferreira *et al.*, 1994) and three CycA3s in tobacco (Reichheld *et al.*, 1996), making it difficult to determine the role of each individual cyclin. This intricacy compared to other metazoans implies unique plant-related roles for CycAs.

CycA genes are expressed mainly in S phase, the steady state levels of *CycA* RNAs increase at or after the onset of S phase, usually until G2 but occasionally until M phase. *CycA3* genes have been found to express earlier in S phase and are more restricted than *CycA1* and *A2* (for review, see Chaubet-Gigot, 2000). Even within the CycA3s different expression patterns can be observed (Reichheld *et al.*, 1996).

Plant cyclin Ds were isolated by their ability to rescue yeast mutants lacking a functional G1 cyclin gene and have been found to contain the retinoblastoma protein (Rb)-binding motif also found in mammals. CycD groups show clear differential expression patterns of mRNAs between tissues in *Arabidopsis* and show differential cell

cycle regulation and responses to sucrose and plant hormones in a cell culture system (Soni *et al.*, 1995). It appears that plants, somewhat unexpectedly, may be similar to mammals in utilizing the Rb and E2F *trans*-factor pathway to respond to environmental stimuli via growth regulators activating cyclin D (De Jager and Murray, 1999).

Cyclin levels are tightly regulated and influenced by periodic gene expression and proteolysis. Myb binding sites have been identifed as playing an important role in restricting CycB expression specifically to M phase They also contain a consensus sequence which has been found in many G2/M phase expressed genes (Ito *et al.*, 1998; Tréhin *et al.*, 1999).

Plant mitotic cyclins contain the destruction box motifs that marks animal mitotic cyclins for ubiquitin dependent-proteolysis at appropriate times through G2 phase and mitosis (Glotzer *et al.*, 1991). CycA and CycB cyclins exhibit different consensus sequences for each respective destruction box (Renaudin *et al.*, 1998) as is the case in animals and yeast, reflecting differences in mode or timing of degradation. Cyc D cyclins, in contrast, contain PEST sequences (Soni *et al.*, 1995) that are associated with rapid protein turnover signals, as do equivalent G1 cylins in yeast (Reed, 1991) and humans (Lew *et al.*, 1991). It is therefore likely that plant cyclin D are subjected to independent proteolytic controls from the mitotic cyclins, as occurs in animals.

1.3.2.1 Cyclin D and control of growth

Recent data indicate that cyclin D regulates the growth rate of plants by accelerating the cell cycle in response to external signals. Cockcroft and co-workers overexpressed *CycD2At* in tobacco and observed a shorter G1 and an increased rate of cell division resulting in an elevated growth rate and accelerated development. Cell size and meristem size were normal, however, leading to the conclusion that meristem activity and growth rate can be influenced by cell division as well as cell division being a consequence of growth (Cockcroft *et al.*, 2000). It was suggested that a unique relationship may exist between cell growth and cell division in plant cells, but a similar effect has been observed with CKI-knockout mice (Nakayama *et al.*, 1996). Also, it recently been observed in *Drosophila* wing that overexpression of cyclin D and its partner Cdk4 reduced the cell doubling time resulting in normal sized cells and a corresponding accelerated growth rate (Datar *et al.*, 2000; Foley and Sprenger, 2000). This acceleration of growth is however tissue dependent, as in other cell types it appeared Cdk4-CycD promoted growth rather than being a direct G1-S regulator.

The plant *CycD* genes are expressed at a constant level in actively dividing cells of *Arabidopsis* and at specific times during cell cycle re-entry (Dahl *et al.*, 1995; Soni *et*

al., 1995; Fuerst *et al.*, 1996; Sorrell *et al.*, 1999). Cyclins of the CycD2 type are activated in G1 and respond rapidly to increased sucrose levels (Riou-Khamlichi *et al.*, 2000). CycD3 cyclins are expressed near the S phase boundary and mediate spatial and hormone signals (Riou-Khamlichi *et al.*, 1999) in the presence of sucrose but also respond to sucrose directly (Riou-Khamlichi *et al.*, 2000). CycD4 is transcriptionally upregulated by increasing sucrose levels and appears to be involved in the resumption of cell division in a transitory and tissue specific manner; during the formation of lateral root primordia (De Veylder *et al.*, 1999). Two cyclin D genes show mitotic accumulation of transcripts, but this may be peculiar to the tobacco BY-2 cell culture (Sorrell *et al.*, 1999).

In *Arabidopsis*, Northern analysis and *in situ* hybridization have shown that *CycD1* is expressed in flowers and leaves, *CycD2* in leaves and roots, *CycD3* in roots and *CycD4* is expressed during vascular tissue development, embryogenesis and the formation of lateral root primordia (Soni *et al.*, 1995; De Veylder *et al.*, 1999). *Arabidopsis* CycD4;1 and CycD1;1 both bind to Cdc2aAt and Cdc2bAt *in vitro*. The co-regulation of Cdc2aAt and CycD4 indicates a likely partnership (De Veylder *et al.*, 1997b; De Veylder *et al.*, 1999).

Elevated B cyclin levels, as a result of expression under the *cdc2aAt* promoter, are also observed to increase growth and accelerate development in roots without altering meristem or root morphology. No extra lateral root primordia were observed, indicating cyclin B is a limiting factor in root growth but not in lateral root initiation (Doerner *et al.*, 1996).

1.3.2.2 Retinoblastoma protein

The retinoblastoma (Rb) protein has a role in regulating cell cycle, differentiation and apoptosis in many metazoans (Durfee *et al.*, 2000). In animals, G1 exit is controlled to a large degree by the level of cyclin D and the phosphorylation of Rb protein. Active cyclin D participates in phosphorylating Rb, which is subsequently unable to bind the transcription factor E2F. The release of E2F transcription factors activates genes required to proceed into S phase. One of these genes is cyclin E, which also contributes to the phosphorylation of Rb. Hyperphosphorylated pRb persists until M phase, where it encounters Protein Phosphatase type I. (for review, see Dyson, 1998; Harbour *et al.*, 1999).

Some components of the Rb control pathway appear to be conserved in plants (Gutiérrez, 1998). Rb protein is important in pathogenesis since Rb function is a restraint on DNA replication and geminiviruses promote their replication by

sequestering plant Rb proteins (Gutiérrez, 2000). Both Rb and E2F homologues have been cloned in plants. Maize ZmRb1 and tobacco NtRb1 interact with cell cycle components in a manner concordant with mammalian Rb counterparts (Huntley *et al.*, 1998; Nakagami *et al.*, 1999). ZmRb1 has been shown to bind all plant Cyc D cyclins *in vitro* (Ach *et al.*, 1997).

Plant E2F has been cloned from tobacco (*NtE2F*) and the protein shown to bind tobacco Rb protein (Sekine *et al.*, 1999). Wheat E2F protein also shows binding to maize Rb (ZmRb1), however the interaction of the two proteins requires alternative residues compared to those that are essential for animal E2F binding (Ramirez-Parra *et al.*, 1999).

A Cdc2Nt1-CycD3 complex was capable of phosphorylating Rb-related protein *in vitro*, unlike Cdc2Nt1 alone (Nakagami *et al.*, 1999). This suggests that Cdc2 and cyclin D may form a complex that controls the Rb-related protein via phosphorylation furthering the parallels with the mammalian G1/S control mechanisms. Rb genes are expressed in all plant tissues at some level as they are in mammals, and in contrast to yeast which does not have an Rb homologue. The three Rb-related genes found in maize exhibit complex regulatory patterns that as yet are incompletely understood (Durfee *et al.*, 2000).

1.3.3 Control of *cdc2* in plant development

1.3.3.1 Plant *suc1*

Suc1 may expedite the docking of CDKs regulatory partners. The distribution of $p13^{suc1}$ in living plant cells was shown not to associate with any particular mitotic apparatus, however, some similarity in localization was observed with the Cdc25 protein in fibroblasts (Girard *et al.*, 1992; Hepler *et al.*, 1994). An *Arabidopsis* homologue of *Suc1/Cks1* has been identified and designated *Cks1At*. Cks1At binds to both forms of Cdc2 present in *Arabidopsis* (De Veylder *et al.*, 1997a). It expresses quite extensively in cycling cells and is also shown to be present in endoreduplicating tissue which may indicate that Cks modifies the mitotic cycle (Jacqmard *et al.*, 1999).

1.3.3.2 CKIs

Arabidopsis contains at least four CKIs (Inzé, 1999). The *Arabidopsis* ICK1 (Inhibitor of Cyclin Dependent Kinase) protein acts to inhibit plant Cdc2 kinase *in vitro*, but is ineffective on the human Cdc2 homologue. A consensus sequence shows similarity to $p27^{Kip1}$ CDK-binding domain but otherwise the rest of the deduced

sequence is novel (Wang *et al.*, 1997). ICK1 can interact with Cdc2a and CycD3 and is induced by abscissic acid (Wang *et al.*, 1998).

ICK2 shares some features with ICK1: it interacts with Cdc2aAt but not Cdc2bAt; is also a potent inhibitor of cyclin-dependent kinase activity; and contains the same CKI-like putative binding domain. The remainder of the ICK2 sequence diverges from that of ICK1 and the mRNA accumulates in different tissue types. ICK1 is found primarily in leaves whereas ICK2 is more prevalent in stems and inflorescence apices and in other tissues both are at comparable levels (Lui *et al.*, 2000). Given the location of the mRNA during development, they may be arresting or preventing cell division, a possibility supported by their induction by ABA (Wang *et al.*, 1998). It is possible that individual CKIs are required in different tissue, varying environmental signals or separate CDK types.

1.3.3.3 *wee1*

A *wee1* homologue has been cloned from maize (*Zea mays* L.) and it interacts with Cdc2 in *S. pombe*. When overexpressed it inhibited progress through the cell cycle (Sun *et al.*, 1999). At the amino acid level, 50% identity was observed between the protein kinase domain of human Wee1 and the maize Wee1 homologue. Homology at the N terminus was reduced, which is consistent with previous observations showing divergence in this region of the protein in eukaryotic Wee1 (Aligue *et al.*, 1997). Molecular phylogeny shows plant Wee1 to be more closely related to animal Wee1 homologues than those of fungi (Sun *et al.*, 1999).

At mitosis in plants, there is a marked increase in activity of $p34^{cdc2}$-like histone H1 kinase activity, particularly at prophase (John *et al.*, 1993). Simultaneously a decline in Tyr-P can be observed (Zhang *et al.*, 1996). There is evidence that plants use inhibitory tyrosine phosphorylation of $p34^{cdc2}$ to regulate onset of mitosis (John, 1996) and the cloning of *wee1* from plants further supports this view of the conserved nature of mitotic control in eukaryotes.

1.3.4 Response of cell division to hormonal signals

Plant growth regulators and environmental factors affect the division activity of plants. In particular auxin and cytokinin alteration cause profound changes in cellular organization and growth (Hamill, 1993). Cell cycle components are also directly affected by exogenous stimuli, but with as yet incompletely defined intermediate signals. As previously discussed (section 1.3.2.1), numerous nutritional and hormonal stimuli influence the cell cycle through D-type cyclins. In particular, *Arabidopsis* cyclin D3 is induced by cytokinin in suspension culture and seedlings and to a lesser extent by

brassinosteroid, auxin, gibberellin (Soni *et al.*, 1995; Riou-Khamlichi *et al.*, 1999). Plants and callus displaying constitutive expression of cycD3 bypassed the need for exogenous cytokinin. Thus cytokinin activation of cell division at G1-S seems to be via CycD3 mRNA induction (Riou-Khamlichi *et al.*, 1999).

Cyclin A and Cdc2 activity are also influenced by phytohormone signals. The *Arabidopsis cdc2* promoter is inducible by auxins and less inducible by cytokinins, as detected by GUS fusion (Hemerly *et al.*, 1993). CycA1 and Cdc2 are co-ordinately regulated by giberellin in rice (Fabian *et al.*, 2000). CycA2;1 is upregulated in roots by auxin and in the shoot apex by cytokinin (Burssens *et al.*, 2000a).

The importance of tyrosine phosphorylation of Cdc2 as a downstream regulation target in plants has been supported by experiments in tobacco. A high level of tyrosine phosphorylation was detected in $p34^{cdc2}$-like kinase from G2-arrested tobacco pith and cell suspension culture. Addition of auxin and cytokinin allowed resumption of the cell cycle and stimulated dephosphorylation of $p34^{cdc2}$. The plant Cdc2-like kinase isolated by $p13^{suc1}$ purification could be activated *in vitro* by yeast Cdc25 phosphatase (Zhang *et al.*, 1996). Also, in tobacco suspension cells, *cdc25* from *S. pombe* could substitute for a cytokinin requirement to progress through the G2-M checkpoint (Zhang, personal communication), further substantiating a role for phytohormone mediated control via control of the phosphorylation state.

Thus, while much remains to be learned about cell cycle control in plants, several rate-limiting events have been identified and it is appropriate to investigate whether their manipulation has detectable effects in the plant.

1.4 AIM OF THIS THESIS

The regulation of CDK-cyclin by tyrosine phosphorylation has been shown to be a key element in regulating the progress of cell division, especially at entry into mitosis. Through Cdc25 phosphatase Tyr15 dephosphorylation in Cdk1 is coupled to completion of repair to DNA damage, completion of DNA replication, growth rate and probably to attainment of adequate cell size. How plant CDK-cyclin is influenced by checkpoint signals on a molecular level is unknown. Whether the mechanism of CDK-cyclin regulation by Tyr15 phosphorylation is conserved in plants is the question under consideration.

It is known that the plant cell cycle does contain checkpoint controls, since mitosis can be blocked by inhibitors which prevent completion of prior DNA replication, yet the possible involvement of Tyr15 phosphorylation in Cdc2 (Cdk1) as part of this mechanism has not been established. Of more general possible significance, is the role

of Tyr15 phosphorylation as a rate-limiting event in mitotic initiation. Also important is the possibility of consequences in plant growth or morphology if this control can be manipulated by raising CDK-cyclin activity. This might be done through the introduction of an additional copy of Cdk1, or by a gene likely to alter the Tyr-phosphorylation of Cdk1.

To address these issues inn this project a tetracycline –inducible promoter was used to overexpress key cell cycle genes in *Nicotiana tabacum* plants and transformed roots cultured axenically. It was found to be necessary to first investigate the tetracycline induction system in detail to allow optimization of induction conditions to suit the model systems under investigation (Chapter 3).

The relationship between plant and yeast regulatory mechanisms was investigated in this work by overexpressing yeast *cdc2* with a view to test for any response that would indicate corresponding function and regulation within the cell cycle (Chapter 4). In addition to the wild-type *cdc2* from *S. pombe*, a dominant activated allele *cdc2-1w* was expressed since inability to respond to *wee1* inactivation in yeast (Russell and Nurse, 1987) made it a suitable candidate to possibly highlight particular events occurring in plant CDK regulation.

Additionally, yeast *cdc25* was also overexpressed to ascertain if plant CDK-cyclin activity could be regulated by a Cdc2 Tyr15 specific phosphatase (Chapter 5). In *S. pombe cdc25* overexpression accelerates the cell cycle by dephosphorylating Cdc2 and precipitating early mitosis (Russell and Nurse, 1986). Mitotically active plant tissue, in which *cdc25* was induced by tetracycline, was therefore examined for any evidence of alterations in cell division patterns.

Chapter 2
Materials & Methods

2.1 MEDIA AND REAGENTS

2.1.1 Reagents

2.1.1.1 General

Ampicillin: 25 mg/ml in dH_2O, filter-sterilized with 0.2 µm sterile disposable filters.

Cefotaxime: 25 mg/ml in dH_2O, filter-sterilized

Chlortetracycline: 5 mg/ml in dH_2O, filter-sterilized

Hygromycin: 50 mg/ml in dH_2O, filter-sterilized

IPTG: 100 mM stock in dH_2O, filter-sterilized.

Kanamycin: 25 mg/ml in dH_2O, filter-sterilized

Rifampicin: 40 mg/ml in ethanol

Tetracycline: 5 mg/ml in dH_2O, filter-sterilized

X-Gal: 40 mg/ml in DMFA (Dimethylformamide).

Phenol: 500 g of phenol crystals dissolved in 1.5 ml 10 M NaOH, 3 ml Tris HCl pH 7.5, 125 ml dH_2O.

Ethidium bromide: 10 mg/ml EtBr in dH_2O.

2.1.1.2 Buffers and solutions

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

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

20x SSC: 3 M NaCl, 0.3 M Na_3Citrate.

20x SSPE: 3 M NaCl, 0.02 M Na_2EDTA, 0.2 M Na H_2PO_4.

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

10x TBE buffer: 89 mM Tris, 89 mM Boric acid, 25 mM Na_2EDTA, pH 8.

TE buffer: 10 mM TrisHCl, 1 mM EDTA, pH 8.

2.1.2 Media

2.1.2.1 Bacterial media

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.

Yeast mannitol broth (YMB): 0.05%(w/v) K_2HPO_4, 0.02%(w/v) $MgSO_4$, 0.01%(w/v) NaCl, 0.04%(w/v) yeast extract, 1%(w/v) mannitol; pH 7.0. Solid media contained 1.5% agar.

Tryptone yeast broth (TY): 0.5%(w/v) tryptone, 0.3%(w/v) yeast extract; pH 7.0. Solid media contained 1.5% agar.

Mannitol L-glutamic acid broth (MG): 0.5%(w/v) mannitol, 0.5%(w/v) tryptone, 0.25%(w/v) yeast extract, 0.1%(w/v) L-Glutamic acid, 0.025%(w/v) KH_2PO_4, 0.01%(w/v) NaCl, 0.01%(w/v)$MgSO_4$, 1 µg/l biotin.

2.1.2.2 Plant media

B5 (Gamborg's B5): 0.3875%(w/v) B5 plant medium (ICN-Biomedical), 3%(w/v) sucrose, pH 5.8-6.0 after autoclaving. Solid media contained 0.18%(w/v) phytagel (Gamborg *et al.*, 1968).

MS (Murashige and Skoog): 0.471%(w/v) MS plant medium powder (ICN-Biomedical), 3% (w/v) sucrose, pH 5.8-6.0 after autoclaving. solid media contained 0.18%(w/v) phytagel (Murashige and Skoog, 1962).

Modified MS: In a final volume of 1 litre; 50ml of 20x modified Macro elements, 1 ml of 1000x Micro elements, 1 ml of 100x Organic nutrients , 0.5 ml 200x Fe-EDTA solution, 1 g myo-inositol, 30 g sucrose and was adjusted to pH 5.8-6.0. Solid media contained 0.18%(w/v) phytagel.

20x modified Macro elements: 33 g NH_4NO_3, 38 g KNO_3, 8.8 g $CaCl_2.2H_2O$ and 3.4 g KH_2PO_4 in a final volume of 1 l.

1000x Micro elements consisted of 0.622 g H_3BO_3, 2.23 g $MnSO_4.4H_2O$, 0.86 g $ZnSO_4.7H_2O$, 83 mg KI, 25 mg $Na_2MoO_4.2H_2O$, 2.5mg $CuSO_4.5H_2O$, 2.5 mg $CoCl_2$ in a final volume of 100 ml.

100x Organic solution: 5 mg nicotinic acid, 5 mg pyridoxine-HCl, 1 mg thiamine-HCl, 20 mg glycine in a final volume of 100 ml.

200x Fe-EDTA solution contained 0.671 g Na_2EDTA, $FeCl_3.6H_2O$ in final volume of 100 ml.

2.2 BACTERIAL TECHNIQUES

2.2.1 Transformation of competent cells with plasmid DNA

E. coli DH5α cells were grown to early log phase (OD_{600}=0.5) in LB broth. Cells were pelleted by centrifugation at 4.5K for 5 minutes, resuspended in 1/10 volume of 0.1M $MgCl_2$ and then incubated on ice for 10 minutes. Cells were pelleted as above,

resuspended in 1/20 the original volume of cold 0.1M $CaCl_2$ and incubated on ice for 2 hours. For the transformation 200 µl aliquots of cells were pipetted into cold 1.5 ml eppendorf tubes and mixed with 50 ng of plasmid DNA. Cells were incubated on ice for 30 minutes, before a heat shock at 42°C for 2 minutes and returned to ice for a further 30 minutes. After the addition of 200 µl LB broth the cells were incubated at 37°C for 1 hour. The cells were plated onto antibiotic-containing agar plates for the selection of transformants. In the case of blue-white selection, cells were plated onto agar plates containing 0.5 mM IPTG, 80 µg/ml X-gal and 75 mg/ml Ampicillin.

2.2.2 Large scale isolation of plasmid DNA

High quality plasmid DNA was obtained using a protocol adapted from Sambrook *et al.* (Sambrook *et al.*, 1989). 500 ml of LB broth coontaining the appropriate antibiotic(s) was inoculated with a loopful of cells and grown overnight at 37°C. The bacterial suspension was poured into two 250 ml centrifuge pots and centrifuged in a Sorvall GSA rotor at 6,000 rpm for 30 minutes at 4°C. The pellets were resuspended in a few drops of the supernatant and combined. The pellet was added to 10 ml of plasmid extraction buffer (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA) containing 50 mg of lysozyme and chilled on ice for 5 minutes. The sample was gently mixed after the addition of 20 ml of 0.2M NaOH/1%(w/v) SDS and chilled on ice for a further 10 minutes. To remove cell chromosomal DNA and bacterial debris 15 ml of ice-cold 5 M potassium acetate was added and the sample centrifuged in the Sorvall SS-34 rotor at 9,000 rpm at 4°C for 15 minutes. The supernatant was filtered through nylon mesh and then transferred to clean Oakridge tubes. Isopropanol, 66% by volume, was added mixed gently and allowed to stand for 15 minutes before being centrifuged in the Sorvall SS-34 rotor for 8,000rpm at room temperature for 30 minutes. The pellets were washed in 70% ethanol, dried briefly in a vacuum desiccator and dissolved in 4 ml TE buffer. The duplicate suspensions were combined in 10 polypropylene tubes. The solution was adjusted to pH 8 using 2 M Tris and then made up to 10 ml with TE. Caesium chloride 1 g/ml and 500 µl of ethidium bromide, was added to the samples. The solution was carefully transferred to Beckman quick-seal tubes (used according to the manufacturer's instructions) and then centrifuged in the ultracentrifuge, Beckman model L8-80M, using an 80 Ti rotor at 40,000rpm for 48 hours at 18°C. The DNA bands were visualized under long-wave ultra-violet light and the plasmid DNA extracted using a 2 ml syringe. Ethidium bromide was removed from the DNA with the addition of H_2O saturated n-butanol. This step was carried out three times. After the removal of all ethidium bromide, the DNA solution was transferred to glass corex tubes, 2 volumes of sterile water, 10% by volume of sodium acetate pH 6 and 2 volumes of 95% ethanol was added. The DNA was recovered by centrifugation in the Sorvall SS-34

rotor at 10,000 rpm at 4°C for 30 minutes. The supernatant was discarded and the pellet dissolved in 0.5 ml of TE buffer and transferred to an 1.5 ml eppendorf tube. The DNA was reprecipitated and pelleted by centrifuging at 14,000 rpm for 10 minutes at 4°C. the pellet was then washed with ice-cold 70% ethanol, dried in a vacuum desiccator and dissolved in 200 µl of TE buffer.

2.2.3 Rapid *E.coli* plasmid miniprep

Plasmid DNA was isolated from 1 ml cultures of *E.coli* grown overnight at 37°C in LB broth containing the appropriate antibiotic. The cells were pelleted by centrifuging for 20 seconds and then resuspended in 100 µl of GTE plasmid isolation buffer (50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl pH 8). After the cells were incubated for 5 minutes at room temperature 200 µl of fresh 0.1 M NaOH/1% SDS was added and mixed gently by inverting the tubes several times. The tubes were incubated on ice for 5 minutes and after centrifugation for 1 minute, 400 µl of the supernatant was transferred to new 1.5 ml eppendorf tubes. The plasmid DNA was precipitated by adding 400 µl of isopropanol and incubating at room temperature for 2 minutes. The DNA was pelleted by centrifuging for 1 minute, washed in 70% ethanol, dried and resuspended in 15 µl. 10 µl was used per restriction digest. After digestion 2 µl of RNase A at 10 mg/ml was added and incubated for 5 minutes.

Plasmid DNA from pBin19 derived plasmids was isolated using Wizard Minipreps (Promega) following the manufacturer's instructions.

2.2.4 Alkaline-lysis/PEG precipitation for the isolation of plasmid DNA for automated sequencing

This method was recommended by Applied Biosystems, *Inc.* for the isolation of high quality plasmid DNA for automated sequencing. Cultures were incubated overnight at 37°C in LB broth containing the appropriate antibiotic. Between 1 and 4 ml of cells were pelleted by centrifuging for 1 minute at 14,000 rpm. The cells were resuspended in 200 µl of GTE plasmid isolation buffer (50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl, pH 8). 300 µl of freshly prepared 0.2 M NaOH/1% SDS was added, mixed by inversion until the solution cleared and then incubated on ice for 5 minutes. The tubes were incubated for a further 5 minutes on ice after the addition of 300 µl of 3 M potassium acetate pH 4.8. Cellular debris was pelleted by centrifuging for 10 minutes at room temperature and supernatants transferred to clean tubes. 2 µl of RNase A (10 mg/ml) was added and incubated for 20 minutes at 37°C. After RNase treatment the supernatants were extracted twice with 400 µl of chloroform. The samples were mixed for 30 seconds and centrifuged for 1 minute to separate the phases. The aqueous phases

were transferred to clean tubes. DNA was precipitated by adding an equal volume of isopropanol and centrifuging immediately for 10 minutes at room temperature. The pellets were washed with 500 µl of 70% ethanol, dried and resuspended in 32 µl of water. Plasmid DNA was then precipitated by adding 8 µl of 4 M NaCl and 40 µl of 13% PEG_{8000}. After incubating on ice for 20 minutes the plasmid DNA was pelleted by centrifugation for 15 minutes at 4°C. The supernatants were removed and pellets washed in 500 µl of 70% ethanol. The pellets were air dried and resuspended in 20 µl H_2O Plasmid DNA was quantified and 500 ng was used per sequencing reaction.

2.2.5 Conjugation of recombinant plasmids into *Agrobacterium* (Triparental Mating)

The conjugation of recombinant plasmids into *Agrobacterium* was based on the method by Van Haute *et al.* (1983). A single colony of the donor strain (the *E.coli* strain containing the correct construct) and the *E.coli* helper strain pRK2013 were inoculated into 10 ml LB broths containing the appropriate antibiotic and grown overnight at 37°C. A single colony of the recipient strain, wild-type *Agrobacterium,* was inoculated into a 10ml YMB or TY broth and grown at 28°C for 16 hours. 100µl of each of the three strains to be mated were spread onto either a 1/2 LB/YMB or 1/2 LB/TY, depending on the strain of *Agrobacterium* used, and incubated overnight at 28°C. Using a sterile loop 2 streaks of cells were resuspended in 500 µl YMB or TY broth. The cells were streaked out onto YMB or TY plates containing selective antibiotics for transformed *Agrobacterium* and 100 mg/l of rifampicin to select against E.coli helper and donor cells. Plates were incubated for 2 days at 28°C. Single colonies were selected and restreaked onto plates as above. After restreaking twice single colonies were streaked onto YMB or TY containing selective antibiotics for *Agrobacterium* but no rifampicin.

2.2.6 Miniprep for the isolation of total DNA from *Agrobacterium*

Total DNA was isolated from *Agrobacterium* using a modified version of the protocol described by Slusarenko (1990). A 5 ml broth of MG containing the appropriate antibiotic was inoculated with a single colony and grown at 28°C for 16 hours. The cells were pelleted by centrifugation at 5000 rpm for 5 minutes and resuspended in 900 µl of lysis buffer (125 µg/ml proteinase K, 8.3 mg/ml lysozyme, 1.25%(v/v) sarkosyl) and incubated at 37°C for 1 hour. After centrifugation at 14,000 rpm for 15 minutes, the supernatant was transferred to a clean 1.5 ml eppendorf tube and 0.5 volume of 7.5 M ammonium acetate was added, mixed well and incubated on ice for 20 minutes. The tubes were centrifuged twice at 14000 rpm for 20 minutes, after each spin the supernatant was transferred to a clean eppendorf. An equal volume of cold isopropanol was then added to the supernatant and the tube incubated on ice for 30 minutes. The

precipitated DNA was removed by twisting it around a pipette, DNA was washed in 70% ethanol, air dried and dissolved in 50 µl of water. 10 µl was used for restriction digests and Southern blotting.

2.3 MOLECULAR TECHNIQUES

2.3.1 Restriction digestion

Restriction digests were carried out with approximately 1-10 µg of DNA using restriction endonucleases purchased from Promega or New England Biolabs, under conditions recommended by the manufacturer. DNA was digested in the presence of 1 unit/µg of the specific restricition enzyme and the appropriate 1x enzyme buffer. The digests were incubated at 37°C for 3-5 hours. The reaction was stopped by heating to 65°C for 2 minutes and chilling on ice or by the addition of a 1 in 6 volume of loading dye (15%(w/v) Ficoll, 25%(w/v) Xylene cyanol, 25% (w/v) Bromphenol blue).

2.3.2 Gel electrophoresis

DNA samples were run on a 0.8%(w/v) agarose/TBE gel containing ethidium bromide 3 µl/100 ml of agarose. Accurate size measurements of linearised fragments were determined by running 250 ng of a λ *BstEII* standard ladder, λ *HindIII* 1 kb ladder or a ϕ *HaeIII* standard ladder . Minigels were run for approximately 2 hours at 40 mA in 1x TBE buffer. Larger gels were run for 16 hours at 25 mA in 1x TBE buffer.

RNA samples were run on a formaldehyde denaturing gel (1.5% agarose, 5% formaldehyde, 1x MOPS and 20 µl ethidium bromide). RNA was prepared as in Sambrook *et al.* (1989), 20-50 µg of total RNA was added to a solution of 50%(v/v) deionised formamide, 15%(v/v) formaldehyde and 1x MOPS. The samples were heated for 15 minutes at 65°C and then quickly chilled on ice before the addition of 2 µl of stop solution. The samples were run overnight at 25 mA so that the dye front had moved 8 cm down the gel. The gels were examined on an ultraviolet transilluminator and photographed with a Polaroid camera using Polaroid 667 black and white instant pack film.

2.3.3 DNA fragment isolation

The DNA fragments used in standard molecular biology techniques were extracted from 0.8% agarose gels after enzyme digestion and gel electrophoresis. The appropriate bands were identified under UV illumination and recovered by cutting the bands from the gel. After all excess agarose was removed, the gel fragment was thoroughly crushed and 0.5 ml phenol added. Samples were homogenized by vortexing and then incubated

at -20°C overnight or -70°C for 30 minutes. The samples were then centrifuged for 30 minutes at 14,000 rpm to separate the phases. The top layers were removed and an equal volume of (24:1) chloroform:isoamylalcohol(v/v) was added. The samples were centrifuged again for 10 minutes and the top layer removed as above. DNA was recovered from this aqueous phase by overnight ethanol precipitation at -20°C and subsequent centrifugation for 30 minutes at 14,000 rpm. Pellets were washed with 70% ethanol, air-dried at 37°C and resuspended in 10 µl of sterile distilled water.

2.3.4 Ligations

Linearised vector DNA was treated with Shrimp Alkaline Phosphatase (United States Biochemical) according to the manufacturer's instructions and then heat treated at 65°C for 15 minutes to inactivate the phosphatase. For blunt-ended ligations insert DNA was treated with Klenow 5'-to3'- DNA polymerase (Promega). Ligations were carried out according to the manufacturer's instructions (Promega). 50-100 ng of linearized phosphatased vector DNA and DNA insert at equimolar, 1:3 and 3:1 ratios were used per reaction. For blunt-ended ligations ratios of vector DNA to DNA insert used were 1:3, 1:6, 1:9 and 1:12. Ligations were incubated at 15°C for 3 hours and the incubated at 4°C overnight. 3 µl of the ligation reaction was used to transform into *E.coli*.

2.3.5 RNA and DNA Extractions

2.3.5.1 A small-scale procedure for the rapid isolation of plant mRNA

All equipment reserved for RNA extractions was cleaned with 1%(w/v) SDS, absolute ethanol and oven sterilized. Solution were autoclaved wherever possible to exclude Rnases. RNA was isolated from 0.1-0.5 g of plant tissue as in Verwoerd *et al.* (1989). The tissue was ground to a fine powder in liquid nitrogen. The frozen powder was transferred to a mixture of of TLES (100 mM Tris-HCl pH 8, 100 mM Li Cl, 10 mM EDTA, 1% SDS) and phenol pH 8 (750 µl of each) which had been preheated to 80°C in Oakridge tubes. The mixtures were homogenized by vortexing for 30 seconds, 750 µl of chloroform:isoamylalcohol (24:1) (v/v) was added and the mixture again vortexed. The samples were equally divided into 2 eppendorf tubes and centrifuged for 5 minutes at 14,00 rpm in a bench centrifuge. After centrifugation, the supernatant was transferred to a clean eppendorf and mixed with an equal volume of 4 M lithium chloride. RNA was allowed to precipitate overnight at 0-4°C and collected by centrifugation. The pellets were dissolved in 250 µl of sterile Milli-Q water and precipitated with 10% by volume 2.5 M NaAcetate pH 6.0.and 2 volumes of 100% ethanol. After centrifugation the RNA pellets were washed with 70% ethanol, dried and resuspended in 50-100 µl of sterile Milli-Q water.

2.3.5.2 Plant DNA miniprep for PCR

DNA was isolated from 0.05-0.1g fresh weight tissue in a 1.5ml eppendorf tube. The tissue was crushed in 500µl of extraction buffer (0.14 M Sorbitol, 0.22 M Tris-HCl (pH 8), 0.022 M EDTA, 0.8 M NaCl, 0.8%(w/v) CTAB, 1%(w/v) N-laurylsarcosine) with a mini pestle. An extra 200 µl of extraction buffer and 300 µl of chloroform:isoamylalcohol (24:1) (v/v) was added and the tube inverted several times to mix contents thoroughly. Samples were incubated at 65°C for 30 minutes with the lids open in the fumehood and again mixed well before being centrifuged for 10 minutes at 14,00 rpm in a bench centrifuge. The upper 600ml of the aqueous phase was carefully removed and transferred to a fresh tube containing 600 µl isopropanol, sharply inverted several times and centrifuged for 15 minutes. The supernatant was decanted and the pellet washed carefully with 200 µl of 70% ethanol. The pellet was dried at 37°C for 30 minutes and resuspended in 25 µl of TE buffer. In each PCR reaction 2.5 µl of this plant DNA solution was used as template.

2.3.6 Southern and Northern analysis

2.3.6.1 Southern analysis

Southern analysis was based on the procedure described in Amersham Co. methods booklet. Gels were soaked in 0.25 M HCl for 10 minutes, denatured for 40 minutes in 1.5 M NaCl, 0.5 M NaOH, pH>12 and neutralized for 30 minutes in 1.5 M NaCl, 1 M Tris-HCl, pH 7.2. Capillary blots were prepared as in Sambrook *et al.* (1989). Transfers occurred over 24-48 hours. The Hybond N^+ membranes were alkali-fixed in 0.4 M NaOH for 5 minutes, by placing the filter DNA side up on 3 pieces of presoaked Whatman paper. Membranes were briefly rinsed in 5x SSC with gentle agitation for 1 minute and then allowed to air dry between Whatman paper.

Prehybridization and hybridization conditions were suggested by Amersham Co. The filters were incubated in a 50 ml solution of prehybridization mix (5xSSC, 5xDenhardt's solution, 0.5%(w/v) SDS and 1mg/ml of denatured salmon sperm DNA) for 3 hours at 65°C.

Filters were put through a series of 15 minute washes. All filters were initially rinsed in a low stringency wash (3x SSC, 0.5% SDS) and then given one 15 minute low stringency wash at room temperature. Filters were then given 1 or 2 medium stringency washes (1xSSC, 0.5% SDS) at 60°C for 15 minutes and subsequent high stringency (0.1x SSC, 0.5% SDS) washes at 60°C if required. DNA probes were removed from nylon membranes by washing them in 100 ml of 0.4 M NaOH at 45°C for 30 minutes,

followed by a second wash in 100 ml of 0.1x SSC, 0.1%(w/v) SDS, 0.2 M Tris-HCl, pH 7.5 for 30 minutes at 45°C.

2.3.6.2 Northern analysis

Formaldehyde denaturing gels were rinsed twice in 10x SSC for 20 minutes to remove the formaldehyde. Blots were set up in the same way as for Southern blots, however the equipment used was first washed thoroughly in 1%(w/v) SDS and absolute ethanol and the Whatman paper was autoclaved and dried at 65°C. The gel was turned upside down and blotted overnight. The Hybond N^+ membranes were alkali-fixed in 0.05 M NaOH for 5 minutes and then rinsed in 2x SSPE.

Prehybridization and hybridizations were carried out in ExpressHyb solution from Clontech containing no more than $6x10^6$ cpm/ml of P^{32}-labelled DNA probe. Filters were initially given 2x 20 minute low stringency washes (1x SSC, 0.5% SDS) at room temperature, followed by 2x 20 minute high stringency washes (0.1x SSC, 0.1% SDS) at 65°C.

All filters were placed in an X-ray cassette and covered with a piece of Fuji X-ray film and intensifying screen. Cassettes were then put at -70°C for between 3 hours and 7 days depending on the filter's radioactivity. The X-ray films were developed using an Agfa-Gevaert Gevmatic 60 X-ray machine, under NX-914 safety lights.

To determine that each lane contained an approximately equal amount of total RNA, filters were reprobed with the coding sequence of the ubiquitin gene from *Antirrhinum majus* (in pJAM293, kindly supplied by Dr Cathie Martin, John Innes Institute, Norwich, UK). This gene had been shown to be constitutively expressed in leaf tissue and found not to be affected by wounding or circadian rhythm (Lidgett *et al.*, 1995). The radioactive emissions of all filters were quantified using a Storm PhosphorImager (Molecular Dynamics) and ImageQuant software. Histogram graphs constructed to represent the relative level of transcript standardized against the level of ubiquitin in that sample. The sample with the highest relative level of transcript was designated 100%, all other samples were adjusted accordingly.

2.3.6.3 Radioactive probe preparation for Southern and Northern hybridization

^{32}P ATP-labelled DNA fragments were prepared using GIGAPRIME labeling kit from Bresatec, according to the manufacturer's instruction. Approximately 100ng of DNA fragment was labeled per reaction. Labeled DNA fragments were separated from the unincorporated nucleotides using a Sephadex G-50 column. Columns were equilibrated

with TE. Labeling reactions were added to the column and then 8 drop fractions were collected in series of eppendorf tubes. The radioactivity of each eppendorf tube was monitored, the eppendorf tubes containing the P^{32}-labeled DNA were detected and used in subsequent probings. A Scinitillation Counter was used to determine the radioactivity of the probe in cpm/ml.

2.3.7 PCR

2.3.7.1 PCR conditions

All PCR reactions were carried out using a standard PCR mix containing 10mM dNTPs, 3mM MgCl, 1x reaction buffer, 1.5 units of *Taq* polymerase (Promega) and between 50-100ng of primer per reaction. The PCR regime is as follows: 30 cycles, 92°C 1 mins, 63°C 1min 30 secs, 72°C 2 mins.

2.3.8 DNA sequencing

All sequencing reactions were performed on double-stranded plasmid DNA using an Applied Biosystems Prism sequencing kit. Sequencing determination was carried out with an ABI373A automated sequencer by the Microbiology department at Monash University.

2.4 TISSUE CULTURE TECHNIQUES

2.4.1 Root material

2.4.1.1 General maintenance

Transformed root cultures of *Nicotiana tabacum* L. (var Wisconsin 38) *tetR* containing TX constructs were grown in 50 ml of MS media pH 5.7-6.0 containing 3% sucrose and 125 µg/ml of ampicillin in 250 ml sloped pots. New root cultures were initiated every 3 weeks by sub-culturing approximately 0.2-0.5 grams of root tissue into fresh media. All root cultures were shaken at 80 rpm and grown in the dark at 22°C±2°C.

2.4.1.2 The generation of transformed root lines

Transformations were carried out using fresh cultures of *A. rhizogenes* containing binary vector and wild type *A. rhizogenes* 9402 strains. On the day of infection sterile young healthy leaves were cut from *N. tabacum* L. (var Wisconsin 38) *tetR* grown *in vitro*. Leaf sections 5cm in length and 3-4cm in width containing the midrib were used in the infections. The midrib region was wounded by stabbing several times with a syringe and then infected with a few drops of bacterial culture. The culture was allowed

to penetrate the wound site before all excess culture was removed. The infected leaves were placed petiole first into pots containing solid B5 medium. After 3 to 4 weeks incubation at 22°C hairy roots appeared from the wound sites. The wound sites containing a number of hairy roots were excised from the leaf and transferred to liquid B5 medium containing 500 µg/ml of ampicillin and of 5 µg/ml hygromycin in the case of transformed lines. The roots that grew well were subcultured from a single root tip and used to derive transformed root lines.

2.4.1.3 Induction of cultured roots with tetracycline

TX transformed cultured root lines to be induced were subcultured by transferring approximately 0.2-0.5 grams of root tissue into MS media pH 5.7-6.0 containing 3% sucrose and 250 µg/ml of ampicillin in 250 ml sloped pots. After 5 days rapidly growing cultures were transferred from MS into Mg free liquid media containing Tc. Typical induction was with 1 mg/l Tc or Cl-Tc for 1 to 4 days.

2.4.2 Plant material

Nicotiana tabacum L. (var Wisconsin 38) containing the *E.coli* transposon Tn*10* tetracycline repressor protein gene *tetR* expressed at high levels. Seeds were obtained from by Professor C. Gatz (Institut für Pflanzenphysiologie, University of Göttingen, Germany) (Gatz *et al.*, 1991; Gatz *et al.*, 1992).

Nicotiana tabacum L. (var Wisconsin 38) wild type seeds were obtained from Dr D.S. Letham, plant Cell Biology group, Research School of Biological Sciences ANU, Canberra Australia.

2.4.2.1 Growing and harvesting conditions

Nicotiana tabacum plants were grown at 25°C in a temperature controlled greenhouse supplemented with fluorescent lighting (Sylvania Gro-Lux, Thorn, Germany wave band 360-720nm, photon flux density 150 umol $m^{-2}s^{-1}$, 16 hour photoperiod, lights switched on at 06:00). All plants were grown in a soil mix composed of 2 parts seedling mix, 1 part perlite or vermiculite and 100 g per 6 l of slow release complete fertilizer (Osmocote).

2.4.2.2 The generation of transformed plants

Transformations were carried out using fresh cultures of *A. tumefaciens* strain 4404 plus binary vector and wild-type *A. tumefaciens* strain 4404. On the day of infection, young healthy leaves were harvested from 4-10 week old *N. tabacum var.* Wisconsin 38 tetR plants. These leaves were sterilized by immersion in 10% Domestos for 15 minutes.

The leaves were then thoroughly rinsed three times in sterile water. Leaf discs 1cm x 1cm containing the midrib were scratched with a scalpel and immersed in 1:25 dilution of *A. tumefaciens* strain 4404 for 2 minutes (1 ml culture and 25 ml MS medium). All excess liquid was removed from the leaf discs by blotting them on sterile Whatman paper. The leaf discs were then cultured for 2 days at 22°C on MSR1 plates (solid MS media containing 1 mg/l BAP and 2 mg/l IAA). The leaf discs were then transferred to selective MSR1 plates containing 250 mg/l cefotaxime and 40 mg/l hygromycin in the case of transformants containing the binary vector. The leaf discs were incubated for 1 week and then turned over and incubated for another week at 22°C. After 2-3 weeks shoots appeared on the leaf discs. The shoots were excised and cultured on MS plates containing 250 mg/l cefotaxime and 40 mg/l hygromycin until roots appeared. Once the roots appeared the seedlings were transferred to vermiculite and water with 1/5 strength MS media. After 2 weeks the established seedlings were transferred to soil and grown in a growth cabinet.

2.4.2.3 Induction of seedlings with tetracycline

Tobacco seeds containing the TX construct were surface sterilized in 10% Domestos for 30 minutes and germinated in MS liquid in petri dishes on a shaker at 80 rpm at 25±2°C in dull light. When the seeds had germinated, on day 11 they were placed in small groups in jars containing Mg-free MS liquid media with Tc. Typical induction was with 1 mg/l Tc or Cl-Tc for 1 to 4 days. If longer, on day 4 after transfer, seedlings were placed in media containing fresh Tc. Tissue was harvested into liquid nitrogen and stored at -70°C for use in RNA isolation and GUS assays.

2.4.3 Measuring the frequency of Lateral Root Primordia

Several seedling lines pure breeding for hygromycin resistance were selected. F2 seeds were sterilized in 10% hyperchlorite for 30 minutes and rinsed thoroughly in sterile MilliQ H_2O and germinated in the vertical position on solid MS medium containing 10 g/l Difco Agar. Plates were incubated in darkness at 23°C for 16 hrs and at 20°C for 8 hrs each day. After 11 days the germinated seedlings were transferred to solid media containing various concentrations of IBA: 0, 10^{-6}, 10^{-5} or 10^{-4} M (K-IBA dissolved in H_2O) ± tetracycline. Modified solid MS media without Mg was used containing 10 g/l Difco Agar. After 72 hours the seedlings were harvested and immersed for 12 hours in 2% Chromium trioxide (Cr_2O_3) (see Pelosi *et al.*, 1995) The lateral root primordia appear as darker spots or bumps on the root due to the concentration of small cells and can be easily counted. The entire primary root length was measured and lateral root primordia counted and due to some variation in primary root length it was considered more accurate to express this measurement as a frequency Measurements were

calculated in terms of frequency of lateral root primordia per cm of root length. At least 8 seedlings were recorded for each data point.

Chapter 3
Inducible Gene Expression in Cultured Roots and Seedlings

3.1 AIMS

In this chapter the investigation of the overexpression of genes from an inducible promoter in tobacco is reported. Suitable tissue types in which to stimulate candidate gene expression are analyzed. Conditions are also optimized for gene induction; these comprise the concentration of the inducing agent, the suitability of media, mode of application and length of exposure.

3.2 INTRODUCTION

The overexpression of genes encoding proteins of importance in cell cycle control, has been a valuable approach in determining functionality. The value of overexpressing cell cycle genes is well documented in yeast, where in addition to the null mutant phenotype, the overexpression phenotype has proven useful in determining the role of a protein (Nurse, 1990).

In plants overexpression has been used widely and effectively to determine the effect of gene action upon growth and development, by identifying traits resulting from a gain of function. Some recent examples include classifying the effects of expressing microbial genes affecting plant growth, such as genes from *A. rhizogenes* TL-DNA (Lemcke and Schmülling, 1998), or alternatively determining how plant organ development is regulated in *Arabidopsis* by *AINTEGUMENTA (ANT)* (Mizukami and Fischer, 2000).

At the inception of this project, cell cycle genes had not been overexpressed in plants, but since then much progress has been made in the field, as has been noted in the General Introduction. Crucial to experimental success is the choice of gene sequence, the type of promoter driving it and the plant system in which to employ the construct.

3.2.1 Inducible promoters in plants

3.2.1.1 The advantages and properties of inducible promoters

In this study it was decided to use an inducible promoter to ectopically express cell cycle genes as it was speculated that constitutive expression of cell cycle genes in plant tissues, particularly *cdc25,* might be deleterious to growth. If the genes impair the regeneration of transformants, only poorly-expressing transgenic lines might be isolated. Use of an inducible gene has the potential advantage that it may provide a

clearer correlation between induction of the gene and occurrence of an altered phenotype. Specific processes might also be studied, by inducing expression at a particular stage of development, for example, the effects of an increased CDK activity on the initiation of lateral root primordia.

An effective inducible promoter needs to be tightly regulated; in particular it should be completely repressed in the absence of the inducer. Leaky expression may interfere with the comparison between induced and uninduced systems. The inducing treatment should ideally be convenient, inexpensive, rapidly perceived by the plant and cause a swift promoter induction response. The inducer should have minimal effect on the plant of its own accord.

3.2.1.2 Inducible promoter design in plants

Two main types of promoter have been developed to regulate the expression of transgenes in plants. The first type is reliant upon endogenous plant promoters, the second on regulatory elements identified and imported from other organisms (for review, see Gatz, 1998). The risk in utilizing gene induction mechanisms of plant origin is that of the inducing stimuli provoking a general physiological response in the plant, in addition to the possible effects of the transgene. Inducing stimuli include heat-shock, stress signaling molecules, elicitors, safeners (compounds which increase plant tolerance to herbicides), wounding (for review see Gatz, 1997) or heavy metals (Mett *et al.*, 1993; McKenzie *et al.*, 1998). Given the possible side-effects of induction, this type of promoter was unsuitable for investigating plant cell growth division.

A feature of non-plant induction mechanisms is fidelity between a promoter sequence and a specific chemical, as induction stimuli provoke no other plant gene responses. The main shortcomings of this second type of scheme appear to be penetration of the agent into intact tissues or unwanted side effects. Tetracycline (Tc) (Gatz *et al.*, 1992; Weinmann *et al.*, 1994), glucocorticoids (Aoyama and Chua, 1997), ecdysone (Martinez *et al.*, 1999) and ethanol (Caddick *et al.*, 1998) are all agents currently in use. These compounds have all been adapted for use in plants, in conjunction with the appropriate *cis* reponse elements: a bacterial repressor, fungal regulatory elements and animal steroid receptors. Although low background effect is reported, ethanol is a plant metabolite which could risk inducing secondary physiological responses.

An early version of a steroid-inducible promoter available at the commencement of this project, employed the mammalian glucocorticoid receptor (GR). Dexamethasone and GR form an activator which was shown to induce *CAT* (chloramphenicol acetyltransferase) gene expression in transiently transformed tobacco cells containing a

promoter with GR-binding sites (Schena *et al.*, 1991). This system of induction did not function, however, in whole *Arabidopsis* plants (Lloyd *et al.*, 1994; Gatz, 1997), presumably due to spurious interaction of the transcription factor with endogenous plant promoters which caused stunted growth. Subsequent versions of the inducible promoter were shown to work well in tobacco but an alternative inducible promoter was selected for the present work.

Tetracycline (Tc) is a favoured chemical inducer because it readily enters eukaryotic cells. Both a Tc-repressible and a Tc-inducible promoter are available and have been used in plants. A Tc-repressible system developed for plants utilizes 7 Tn*10* operators from *Escherichia coli*. The activation domain of *Herpes simplex* VP16 protein fused to the Tn*10*-encoded Tet repressor protein is used to propel transcription (Weinmann *et al.*, 1994). To silence the transgene, constant tetracycline presence is required, which may be a disadvantage when cultivating plants, especially as tetracycline degrades on exposure to light.

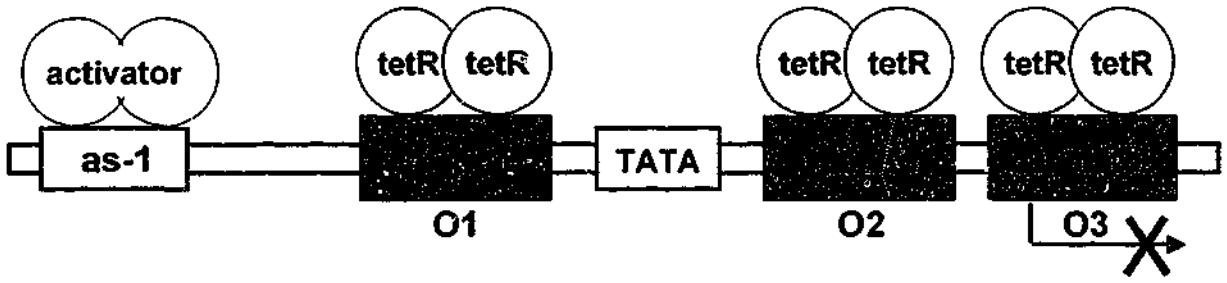
The Tc-inducible system appeared to have a promising reported success rate and also several favourable characteristics, including reports that the uptake of Tc for induction of gene expression was more rapid than inactivation of Tc to enable gene induction (Gatz, 1997; Sommer *et al.*, 1998; Love *et al.*, 2000)

3.2.2 The tetracycline inducible promoter

The tetracycline inducible Triple-X promoter is a tailored CaMV35S promoter that incorporates three *tet* operator sites at which the Tc repressor can bind as illustrated in Figure 3.1 (Gatz *et al.*, 1992). Initially the target plant is transformed with the *E.coli* transposon Tn*10 TetR* repressor gene. It must be under the control of a strong promoter constitutively expressed in plants in order to express TetR protein at a high enough level to cause promoter deactivation. Introduced into this background is the Triple-X promoter attached to the candidate transgene, which is under negative control since the repressor protein binds to the promoter and prevents transcription (Gatz *et al.*, 1991; Gatz *et al.*, 1992). When Tc is introduced into the cell it binds to the repressor protein which undergoes a conformational change and is released from the promoter. Consequently transcription is de-repressed as the multifactorial initiation complex can assemble and transcribe the gene (Hillen *et al.*, 1984).

One advantage of this particular system is that Tc has no naturally occurring analogue in plant cells, which might otherwise interfere with promoter regulation. The Tc induction system has been effectively used in tobacco to enable inducible expression of several *Agrobacterium rhizogenes* genes: *rolB* (Röder *et al.*, 1994), *rolC* (Faiss *et al.*,

A. without tetracycline inducer



B. with tetracycline inducer

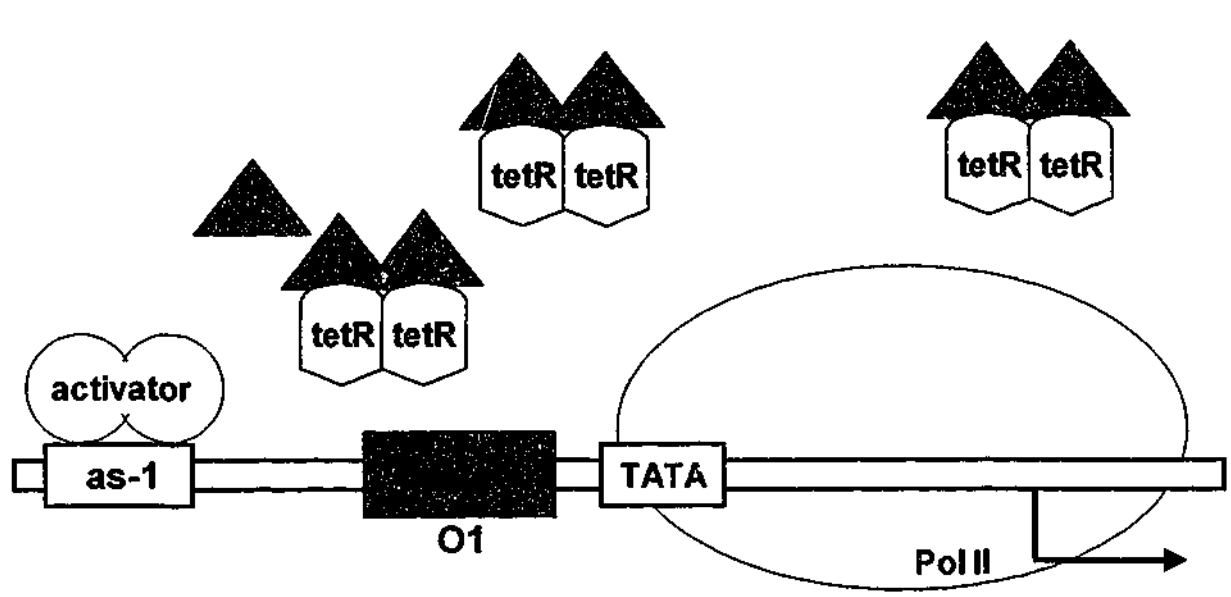


Figure 3.1 Region –90 to +17 of the Triple-X promoter. The promoter contains plant enhancer modules upstream of the TATA-box as well as operator sequences in the vicinity of the TATA-box (one upstream, two downstream). **A.** In the absence of Tc, repressor molecules (tetR) interfere with transcription initiation **B.** In the presence of Tc, the repressor disengages from the operator allowing the transcription initiation complex to assemble. The arrow marks the start of transcription. (After Gatz, 1997; Gatz *et al.*, 1992).

1996) and TLDNA ORF13 (Lemcke and Schmülling, 1998). Several genes from other plants have been successfully expressed in tobacco under the control of this promoter including; Oat arginine decarboxylase (Masgrau *et al.*, 1997); *Arabidopsis* auxin-binding protein 1 (ABP1) (Jones *et al.*, 1998); pea isopentenyl transferase (ipt) (Motyka *et al.*, 1996) and microbial genes such as, *E. coli ubiC* (Sommer *et al.*, 1998). The system was also effective in potato for expressing S-adenosylmethionine decarboxylase (SAMDC) (Kumar *et al.*, 1996).

3.2.3 Plant systems for investigating cell division and growth

The choice of plant system in which to use an inducible promoter is quite limited. Unlike tobacco or potato, and for reasons as yet unclear, *Arabidopsis* cannot sustain the production of high levels of the TetR repressor protein (Gatz, 1996) and neither can tomato (Corlett *et al.*, 1996). This phenomenon has also been reported in mammalian cells (Gossen and Bujard, 1993). Tobacco is a suitable experimental plant as it can produce the repressor to a level that blocks transcription of the Triple-X promoter, without detrimental effects.

In evaluating the consequences of gene expression the choice of plant tissue is very important as it must be accessible and allow examination of any resulting phenotypic variations. Roots have features that are suitable for studying the effects of a disrupted cell cycle on plant development. Seedlings and young plants can be grown *in vitro* or in hydroponics and effects upon root system growth and development, due to altering gene expression, can be ascertained. Axenic root cultures offer the advantage of not having the structural complexity of the whole plant but are considerably more advanced in organization than a plant cell suspension culture. Root cultures have the same accessibility to inducing agents as cells in culture, but nevertheless, are fully organized into tissues with many of the constraints upon cell division that are inherent in an organ system but are absent in a cell suspension system. Transformed roots do not require added hormones due to the incorporation of Ri T-DNA and may offer advantages to studies of root metabolism and development (Hamill and Lidgett, 1997). The need to add phytohormones to the roots of many species, including tobacco, to promote growth may make the assessment of phenotype difficult after induction of gene expression.

3.2.3.1 Systems effective with Triple-X inducible promoter

Several different methods can be used to induce promoter activity in tobacco plant tissue. To induce transient promoter activity or gene expression in leaves, vacuum infiltration of Tc into detached leaves or placing leaf discs in buffer containing Tc is sufficient (Gatz *et al.*, 1992; Röder *et al.*, 1994; Masgrau *et al.*, 1997).

To observe a phenotypic or cellular growth response as a result of induced gene expression, Tc needs to be introduced into intact plants. Simple methods include spraying Tc solution onto the leaves of whole plants with or without detergent, or simply submersing plants once a day in Tc solution. These methods gave mediocre results (Gatz *et al.*, 1992) (Masgrau *et al.*, 1997). Similarly growing explants on solid media was not effective as the Tc could not be replenished without physical disturbance of the roots during movement to fresh medium (Gatz *et al.*, 1992).

The optimal method of Tc delivery is in solution via the roots. By growing the plants hydroponically or on vermiculite and other substrates, Tc can be applied by watering with buffer or feed solution (Gatz *et al.*, 1992; Röder *et al.*, 1994; Masgrau *et al.*, 1997). A detached root system cultivated in liquid with plant hormones has also been successfully induced (McKibbin *et al.*, 1998).

A *GUS* reporter gene driven by the Tc-repressible promoter, was used to assess the regulation of gene expression, with a view to using the system to express cell cycle genes in tobacco and examine cell division in roots. The aims of the experiments reported in this section were to address the following questions:

1. Can the *GUS* reporter gene be induced in tobacco tissue suitable for examining cell division characteristics, namely seedlings, cultured roots and stem pith?

2. What are the kinetics of induction in these tissue types? Under what conditions is gene expression maximized?

3. What are the limitations of the Tc induction system in the context of the assessing cell cycle variation in root meristems?

3.3 MATERIALS & METHODS

3.3.1 Reporter constructs and plant transformation

DNA manipulations were carried out generally as described in Chapter 2 Materials & Methods and Sambrook *et al.* (1989). pTetR and pBINHygTX-GUS (pBTX-GUS) (Figure 3.2) were kindly supplied by Professor C. Gatz (Institut für Pflanzenphysiologie, University of Göttingen, Germany) (Gatz *et al.*, 1991; Gatz *et al.*, 1992). The *GUS* gene was under control of a Tc-inducible CaMV35S promoter including the triplicated *tet* operator motif and *nos* terminator. The pBIN19 based pBTX-GUS plasmid was introduced into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* via triparental mating procedures. These transgenic

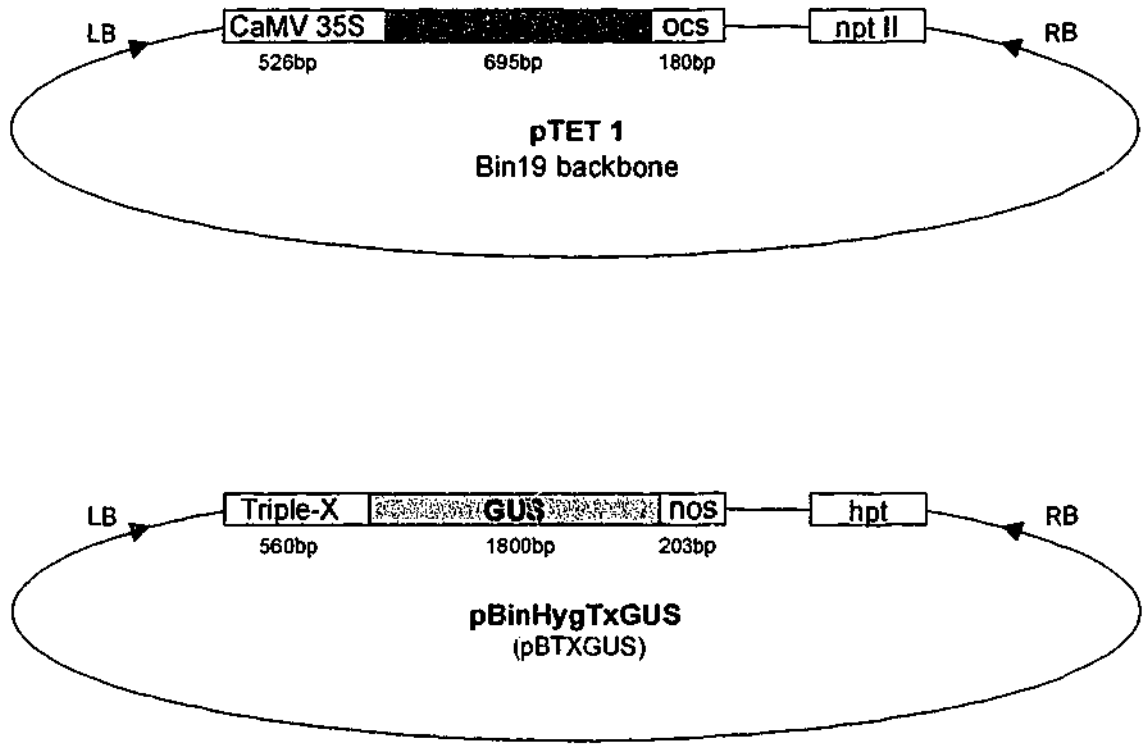


Figure 3.2 Structure of plasmid constructs pTET1 and pBTXGUS (Gatz *et al.*, 1992; Gatz *et al.*, 1991). The *tetR* gene is between the CaMV 35S promoter and the octopine synthase (*ocs*) polyadenylation signal. The β-glucuronidase (*GUS*) gene was inserted between the Triple-X promoter and the nopaline synthase (*nos*) polyadenylation signal. The resistance genes shown are neomycin phosphotransferase (*npt*) and hygromycin phosphotransferase (*hpt*). RB, right border; LB, left border. Not to scale.

agrobacteria were then used to generate plants and root cultures respectively, as described in Chapter 2, in a background of highly expressing TetR repressor protein. For this purpose *Nicotiana tabacum* L. (var Wisconsin 38) constitutively expressing the TetR repressor was used (seeds also kindly supplied by Professor Gatz).

3.3.2 Plant and root culture methods

3.3.2.1 Measuring root growth

Transformed root cultures were established and maintained as described in Chapter 2 Materials & Methods by Hamill and Lidgett (1997). Transformed TXGUS1 roots were grown in 50 ml of MS medium, pH 5.8 containing 3% sucrose and 250 µg/ml of ampicillin. At sub-culturing, 0.2 grams of fresh tissue was weighed and transferred into 250 ml sloped pots. For the first five days after subculturing magnesium was included in the media without tetracycline. At day five 0, 0.1, 0.2, 0.5, 0.75 or 1 mg/l tetracycline was added to the experimental pots in MS without Mg. Pots with complete MS were also treated ±Tc (1 mg/l). Tc can form insoluble complexes with Mg and it was important to discover if there was any effect on root growth if Mg was excluded from the induction media to maximize promoter induction. The 0.2 g of tissue used to set up the growth curves came from actively growing 15-day-old root cultures. Extreme care was taken to keep all factors such as the pH of the media and the state of the initial starting material constant. The pots were randomly arranged on the same shaker and shaken at 80 rpm at 25±2°C in darkness. The Tc and media were changed every five days. On the 12th and 19th day roots were harvested, at each time point three or four replicas of each root line were harvested. They were weighed after all excess liquid was removed, and their mass recorded and used to plot accurate growth curves of the roots. Material was dried at 65°C to obtain the dry weight as a more accurate measure of the tissue weight. Dry weight was found to correspond directly to fresh weight measurements.

3.3.2.2 Induction of dedifferentiation in stem pith tissue of tobacco

To prepare stem pith tissue of *N. tabacum*, plants were grown according to conditions recorded in Chapter 2 Materials & Methods for about 4 months. Sections of stem about 60 cm long were harvested and segments 15 cm long were cut from these and surface sterilized by immersion for 20 minutes in 20% sodium hypochlorite then rinsed twice in sterile water. All xylem and internal phloem were cut off the sterilized stem pieces and then 10 mm long cylindrical segments of central pith tissue were cut. Each segment was split longitudinally into four quadrant blocks, which were dispersed to sterile solid MS media. Pith blocks were incubated at 25°C with 8 hours of light and 16 hours of

darkness. Concurrent induction of dedifferentiation and *GUS* expression was achieved by the inclusion of 5.4 μM NAA and 0.56 μM BAP for ten days (Zhang *et al.*, 1996) in the presence of varying levels of Tc.

3.3.3 GUS methods

3.3.3.1 GUS staining

Histochemical detection of glucuronidase was made by vacuum infiltration of roots, pith and seedlings with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium) in 50 mM NaPO4 pH 7, and incubation overnight at 37°C in a 24-well plate to ensure tissue penetration of the substrate as described by Jefferson *et al.* (1987).

3.3.3.2 GUS quantification

The fluorimetric assay of GUS enzyme activity procedure is based upon that described by Jefferson *et al.* (1987). 0.1g tissue was homogenized with 500 μl GUS extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 0.1% TritonX, 0.1% sarkosyl, 10mM β-mercaptoethanol) and incubated with the substrate MUG (1mM 4-methylumbelliferyl-β-glucoronide) at 37°C. Duplicate reactions were set up with 50 μl each sample. Samples were diluted with 0.2 M sodium carbonate to stop the reaction and maximize the fluorescence of the product MU. Quantification of the fluorescence was measured at 455 nm and converted to pmoles MU produced using a calibration curve. Linearity of reaction rate was observed over an hour and the rate of the reaction was calculated in terms of pmoles MU/min. The protein content of the extract was measured using Bradford's reagent and OD at 595 nm to determine specific activity in pmoles MU/min/mg protein.

3.4 RESULTS

3.4.1 Introduction of *GUS* into plants and root cultures

3.4.1.1 Transformation of plants with *A. tumefaciens*

Leaf disc transformation was performed on *N. tabacum* (var Wisconsin 38) tetR plants, with *A. tumefaciens* containing the *GUS* gene under the Triple-X promoter. By selection on hygromycin five transgenic plants were obtained that were healthy and normal in appearance. These independent TXGUS transformants, designated A to E were self-pollinated and the seed collected. By Tc induction and GUS staining of the seedlings, one line (TXGUSA) was selected as giving the strongest staining response and subjected to further analysis. The F1 generation segregation analysis was on medium

containing 50 µg/ml hygromycin. 121 seeds were germinated, some showing clear signs of hygromycin sensitivity (small size, bleaching, little or no root growth) (Figure 3.3). The ratio of resistant to non-resistant seedlings did not deviate significantly from 3:1 (χ^2=0.22, 0.7>*P*>0.5). After germination on hygromycin all subsequent experiments with TXGUSA F1 seedlings were carried out with a minimum of 20 seedlings (a mixture of 2:1 ratio hemizygous:homozygous plants).

3.4.1.2 Detecting promoter induction in seedlings with GUS staining

In order to test the newly created lines for presence of an expressible *GUS* gene, hygromycin resistant TXGUSA F1 seedlings were treated with ±1 mg/l Tc for 3 hours or 18 hours in petri dishes with shallow H_2O. The staining patterns observed in line TXGUSA can be seen in Figure 3.4. The blue stain appearing upon treatment with Tc indicates the transformation with *TXGUS* was effective and it can be successfully induced in seedlings.

Although the seedlings have not been cleared by ethanol or bleach treatment, blue staining is visible in the leaves. After 3 hours quite extensive gene induction could be observed in the leaves and was just starting to appear in the meristems of the root system. Following overnight Tc treatment GUS activity was evident throughout the root system as well as the leaves.

In the untreated (0 Tc) sample there was some blue staining in the vascular system of the seedling root. Potential causes of this are discussed later.

3.4.1.3 Transformation of root cultures with *A. rhizogenes*

Following leaf inoculations performed on *N. tabacum* (var Wisconsin 38) tetR plants, a number of root lines resulting from the co-transfer of *A. rhizogenes* binary vector T-DNA and the *GUS* gene (showing hygromycin resistance) were established from single root tips. After induction three root lines were selected on the basis of the strength of staining response – TXGUS1, 13 and 21.

3.4.1.4 Detecting promoter induction in roots with GUS staining

Three cultured root lines transformed with the inducible *GUS* gene, TXGUS1, 13 and 21, were treated with 1 mg/l Tc. Figure 3.5 shows the presence of indigo dye illustrating that the *TXGUS* gene is present in the hygromycin resistant root lines and can be induced by Tc in these cultured roots. Of the three separate lines TXGUS1 showed the strongest induction response, following concurrent overnight staining, and was selected for further experiments. In all lines intensity of staining appears to have increased over

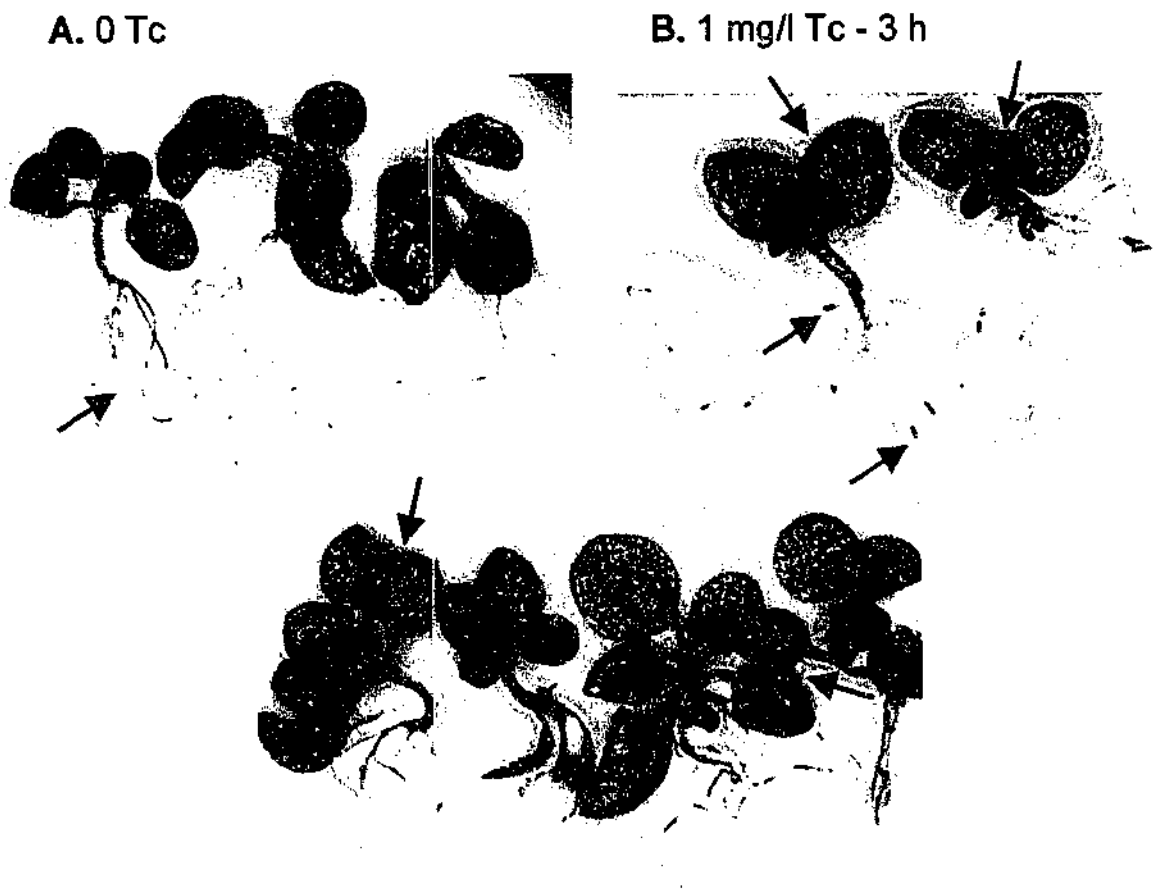


Figure 3.4 Tc inducible *GUS* expression in TXGUSA seedlings. F1 seedlings were treated with: **A.** H_2O overnight **B.** 1 mg/l Tc for 3 hours **C.** 1 mg/l Tc for 18 hours. Staining with X-gluc was used to visualize *GUS* expression.

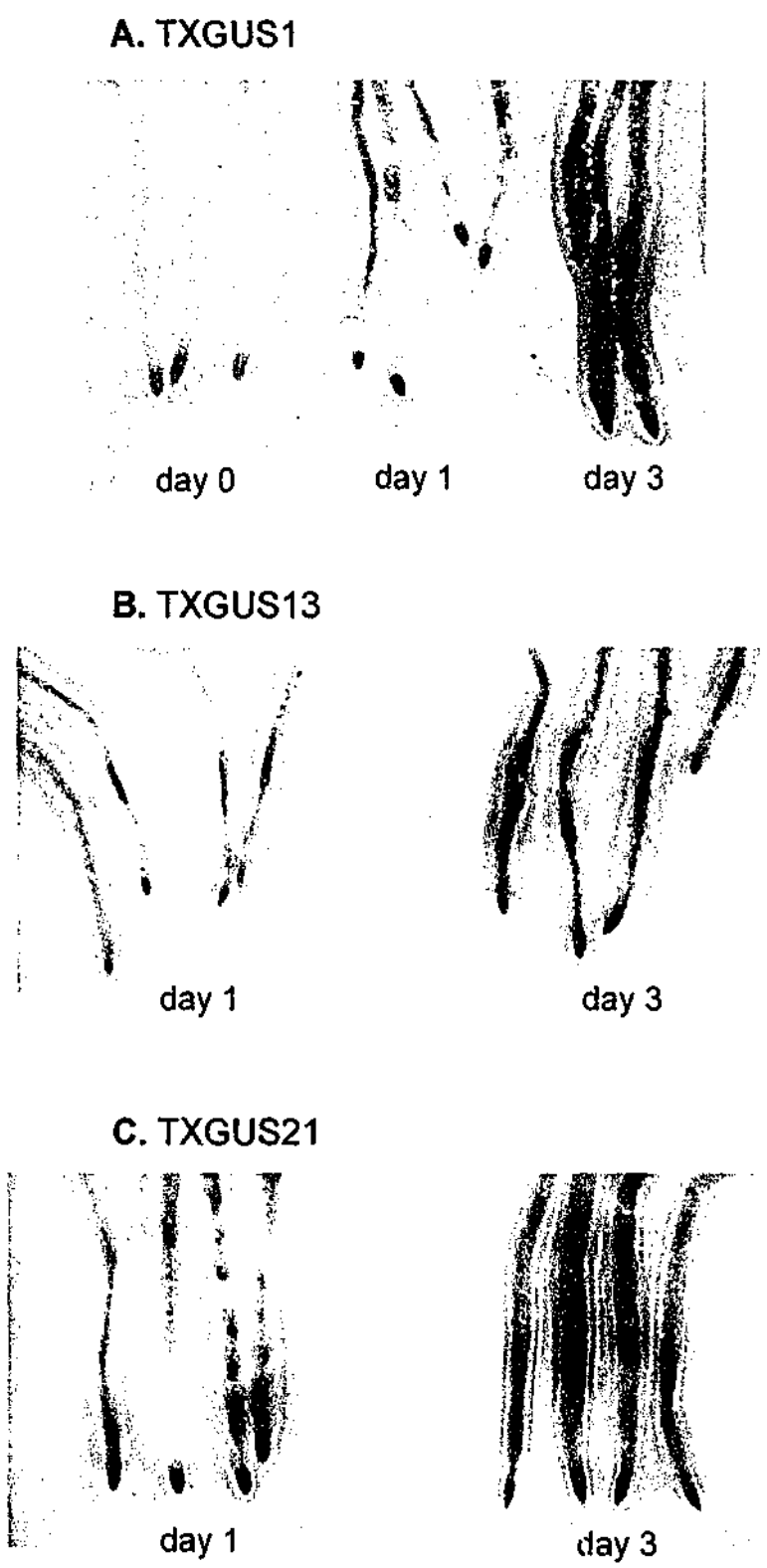


Figure 3.5 Tc inducible *GUS* expression in TXGUS cultured roots. Transformed cultured root lines were treated with 1 mg/l Tc for 0, 1 and 3 days in liquid MS without magnesium and stained with X-gluc to detect *GUS* expression. **A.** Line TXGUS1 **B.** Line TXGUS13 **C.** Line TXGUS21.

the longer induction period, this was investigated in 3.4.4.2. No *GUS* expression was evident in the established root in the absence of the inducer, however, just as the cultures were becoming established some of the single root tips which were excised showed blue staining in the meristem region (data not shown). The significance of this observation is explored later.

3.4.2 Induction of GUS activity in transgenic plants and root cultures

3.4.2.1 Media modifications for optimum promoter induction

Initially *GUS* induction was undertaken in TXGUS cultured roots in complete Murashige and Skoog medium containing 1 mg/l Tc. Low levels of expression were achieved. However, as Tc may become complexed to divalent cations in the media (Budavari, 1996) the effective levels available for repressor binding may be reduced. By using modified media lacking magnesium, much more efficient induction was seen with 1 mg/l Tc (Figure 3.6). Even with concentrations as low as 0.2 mg/l Tc high levels of induction were observed.

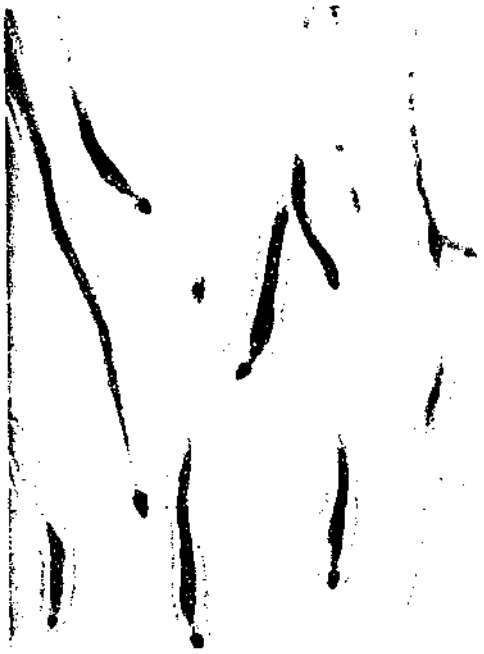
3.4.2.2 GUS quantification

Visible levels of GUS staining show if the Triple-X promoter has been induced, and indicate a level of expression. However, histochemical staining is an inaccurate guide to relative expression levels as small changes in GUS concentration can sometimes result in a profound difference in the intensity of the blue colour (Shaul *et al.*, 1999). To provide a guide to achieving maximum cell cycle transgene expression with the Tc-inducible system a quantitative study was made. In TXGUSA F1 seedlings, induction levels of GUS activity in response to different concentrations of Tc over time were measured.

Three days after germination commenced, seedlings were transferred to modified MS liquid media containing different concentrations of Tc ranging from 0 to 1 mg/l. Treated seedlings were harvested at time points over a week, with the Tc being refreshed on day four. Seedling tissue was homogenized and assayed for GUS activity. Figure 3.7 portrays the GUS specific activity induced in tissues exposed to varying concentrations of Tc over time.

The GUS activity of transformed seedlings analyzed in the absence of Tc was approximately 1130 U (U = pmol 4-MU produced, min^{-1}, mg $protein^{-1}$). This is almost ten times the background level of 129 U which was detected in untransformed control seedlings. Thus there was a low but consistent level of Triple-X promoter leakage in this seedling line where the TetR mechanism was not achieving full repression.

A. +Mg 1 mg/l Tc



B. -Mg 1 mg/l Tc

C. -Mg 0.2 mg/l Tc

Figure 3.6 Effect of magnesium on the Tc inducible *GUS* expression in TXGUS1 roots. Line TXGUS1 roots were induced in MS with or without Mg, for 3 days, then stained with X-gluc to detect and compare levels of *GUS* expression **A.** +Mg 1 mg/l Tc **B.** –Mg 1 mg/l Tc **C.** –Mg 0.2 mg/l Tc.

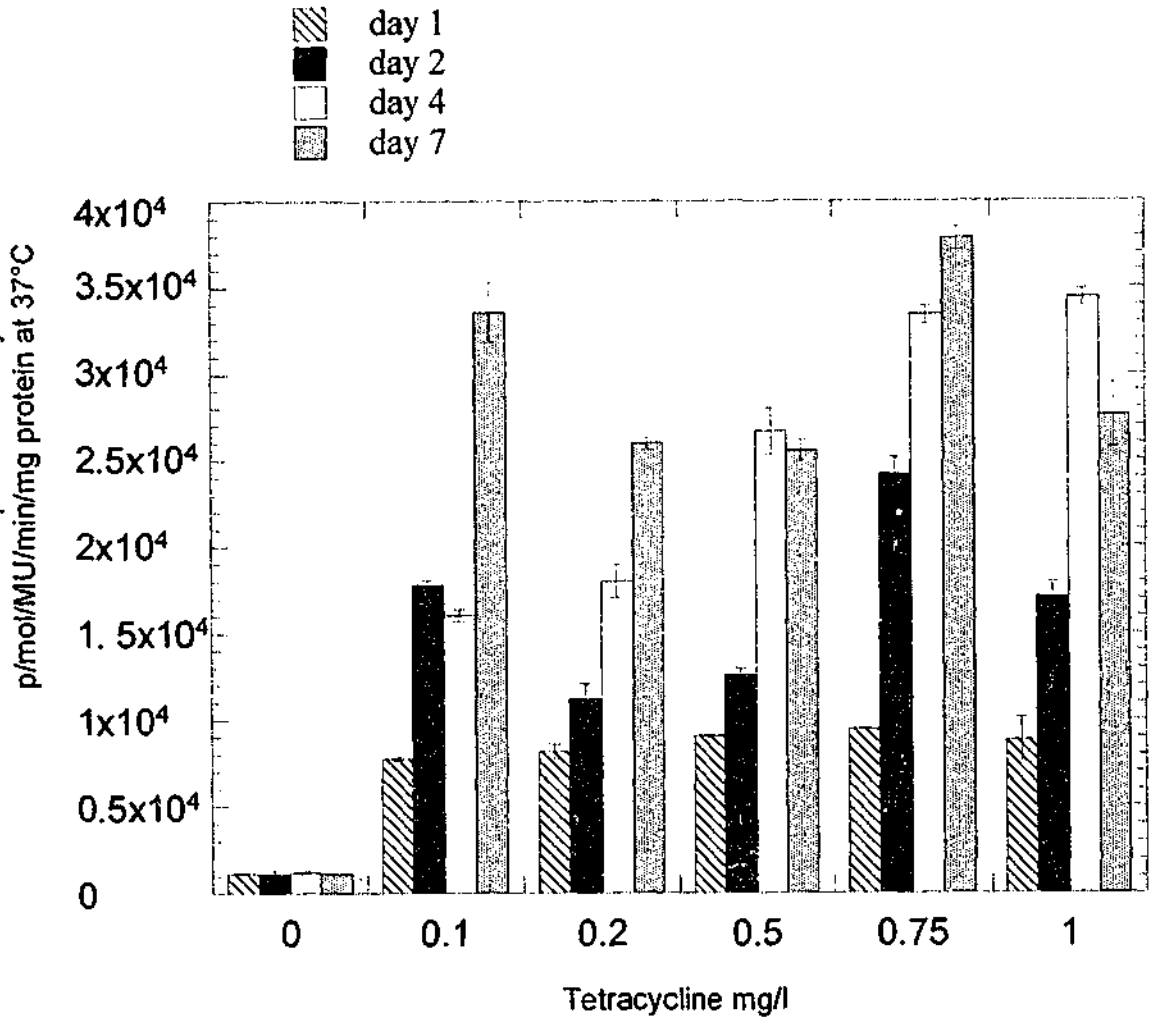


Figure 3.7 Assay of Tc induction of GUS activity in whole TXGUSA seedlings. Transgenic 11 day old tobacco seedlings carrying the *TetR* gene and the *TXGUS* reporter gene were transferred to modified MS containing varying Tc levels and harvested on days 1, 2, 4 and 7. Specific GUS activity (p/mol/MU/min/mg protein at 37°C) plotted against Tc concentration (mg/l).

Induction was detectable at the lowest concentration tested, 0.1 mg/l Tc. At day one, about the same level of induction was observed irrespective of whichever level of Tc was administered. Day four shows a consistent rise in expression as the Tc levels were increased. On days two and four at each Tc concentration a reasonable correlation was observed between time of exposure and the level of induction. However, on day seven wide variation was observed in induction levels, independent of the amount of Tc present. A maximum level of induction is reached after seven days of exposure to 0.75mg/l Tc, but at the same time point both 0.5 and 1 mg/l Tc exhibited reduced GUS activity. There are several possible explanations for this wide range in values.

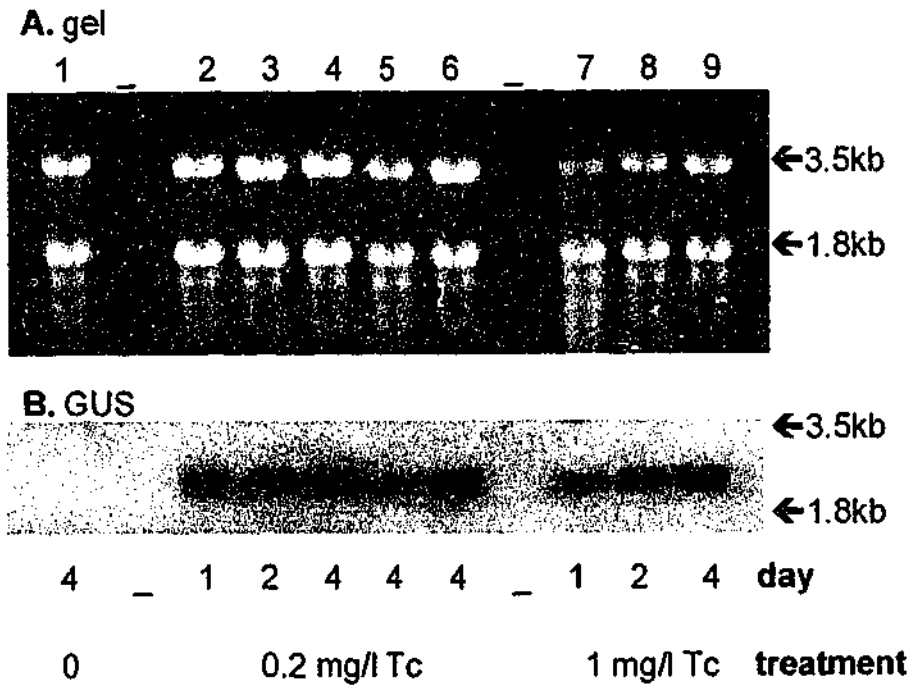
3.4.2.3 Kinetics of transcriptional induction by Tc in seedlings

The histochemical GUS assay by itself is insufficient for estimating the levels of GUS expression and may be particularly misleading in some cells (Shaul *et al.*, 1999). Although GUS activity is easy to measure, it does not accurately portray the possible changing kinetics of gene expression over a short time scale because the GUS enzyme is a relatively stable protein. Weinmann *et al.* (1994) report that GUS protein can have a half-life in green tobacco plants of up to 3-4 days although this may be reduced in smaller plants.

To obtain more direct information on the kinetics of induction at the molecular level, total RNA was prepared and subjected to Northern blot analysis. In these experiments hygromycin resistant TXGUSA F1 seedlings were treated with different concentrations of Tc. One sample was treated in MilliQ water containing Tc, in another the sample was transferred to Tc free media after two days. Tissue was harvested on days one, two and four. Total RNA was prepared from 0.5g of seedlings (not less than 20 plants) and subjected to Northern blot analysis.

No *GUS* mRNA was detected after four days without tetracycline induction (Figure 3.8), but in 0.2 mg/l and 1 mg/l Tc at days 1 and 2 there was detectable and concentration dependent induction. After four days the difference between 0.2 and 1 mg/l induction was less marked, which may be due to an accumulation in the seedlings of both Tc and *GUS* mRNA, as it is quite stable (Jefferson *et al.*, 1987; De Rocher *et al.*, 1998). This view is supported by the persistence of *GUS* mRNA observed in the sample in lane 6 which had been removed from 0.2 mg/l Tc after two days but had a very strong signal on day four.

0.2 mg/l Tc appears to be a useable level of inducer that achieves an equivalent level of *GUS* induction to 1 mg/l as long as exposure is not less than four days. Where 0.2 mg/l Tc has been applied to the seedling in water the *GUS* induction observed is markedly



Lanes
1. 0 Tc control day 4
2. 0.2 mg/l Tc day 1
3. 0.2 mg/l Tc day 2
4. 0.2 mg/l Tc day 4
5. 0.2mg/l Tc in H_2O day 4
6. 0.2 mg/l Tc day 4 (2 days Tc + 2 days 0 Tc)
7. 1 mg/l Tc day 1
8. 1 mg/l Tc day 2
9. 1 mg/l Tc day 4

Figure 3.8 Northern blot analysis of the induction of GUS mRNA in GUSA seedlings treated with Tc. Seedlings were treated with 0, 0.2 and 1 mg/l Tc for 1, 2 and 4 days. The sample in lane 5 was treated with 0.2 mg/l Tc for 4 days in H_2O; sample in lane 6 was exposed to 0.2 mg/l Tc for 2 days followed by 2 days with 0 Tc. **A.** equal loadings of 20 µg RNA per lane **B.** probed with *BamHI-SacI* full-length fragment of the 1.8kb *GUS* coding region.

lower than in media. There appears to be no advantage in applying the inducer in water to avoid cations which complex with Tc. In water, the less favorable growth conditions for the seedling seem to impair transgene transcription, modified MS medium without Mg was therefore adopted.

3.4.2.4 Kinetics of transcriptional induction by Tc in cultured roots

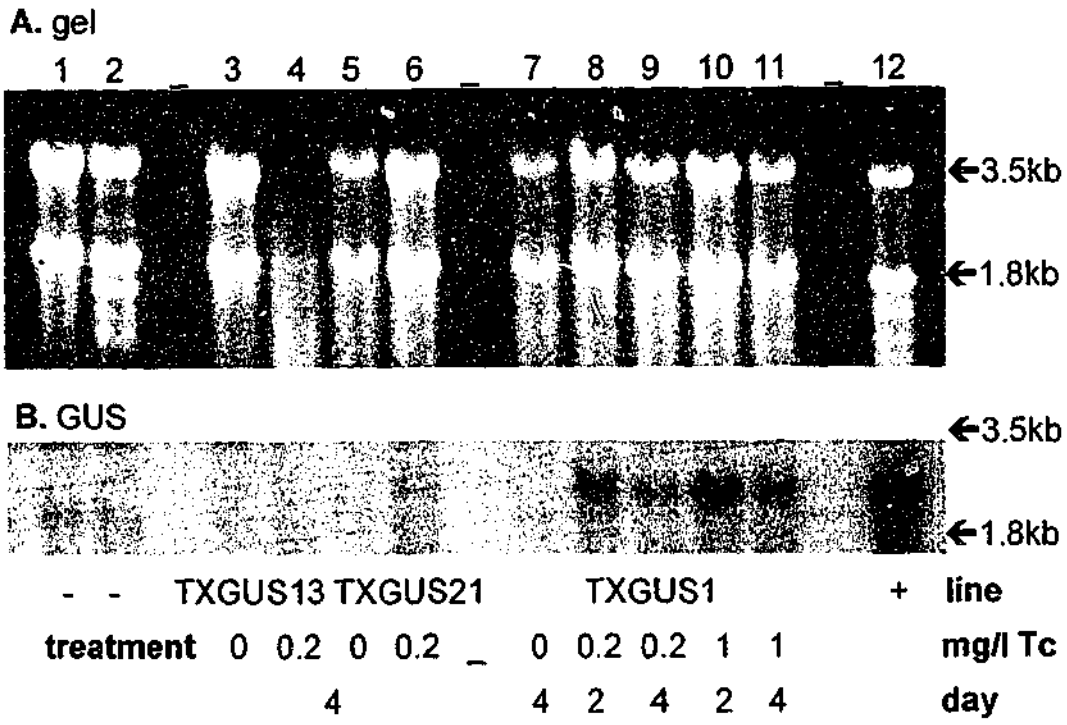
Triple-X promoter induction kinetics were analyzed in root cultures, to take into account any differences between the tissue systems. Total RNA from root culture TXGUS1 was prepared and subjected to Northern blot analysis. Rapidly growing cultures were transferred from MS into Mg-free liquid media containing different concentrations of Tc. Tissue was harvested after a further two and four days of growth. Also, cultured roots and seedlings that do not contain *GUS* were included as a negative control and induced TXGUSA seedlings as a positive control.

None of the 0 Tc samples contained detectable *GUS* mRNA produced without Tc induction in any of the lines (Figure 3.9). This indicates that if any promoter leakage occurs in the three root cultures at this stage, it is not readily detectable with Northern blot technology. By comparing 0.2Tc day four mRNA levels it can be seen that rootline TXGUS1 is expressing at a much higher level than rootline TXGUS21. Due to sample degradation relative expression levels for rootline TXGUS13 could not be included in this comparison.

Strength of expression was highest using 1 mg/l Tc over 2 days, followed by 0.2 mg/l Tc induction over 4 days. Lower again was the level reached with 1 mg/l Tc for 4 days, then 0.2 mg/l Tc for four days which gave the weakest expression. As induction levels are declining by day four, it appears that after four days the roots are starting to react to the presence of the Tc. A comparison of roots and seedlings shows that Tc induced message does not persist in roots as it does in seedlings, possibly because of different tolerance by the tissue of Tc.

3.4.2.5 Optimizing the expression system

From observation of induction in root cultures it became apparent that Tc was inhibiting growth. In order to quantify the negative effect Tc was having on the roots, cultures were exposed to different levels of Tc for 7 and 14 days. Rapidly growing cultures of TXGUS1 were subcultured and allowed to establish for five days before being transferred to Tc treatment conditions. Upon harvesting both fresh weight and dry weight were measured, and were found to change in parallel, thus only fresh weight measurements are shown.



Lanes
1. *GUS* negative root control
2. *GUS* negative seedling control
3. TXGUS13 - 0 Tc day 4
4. TXGUS13 - 0.2 mg/l Tc day 4
5. TXGUS21 - 0 Tc day 4
6. TXGUS21 - 0.2 mg/l Tc day 4
7. TXGUS1 – 0 Tc control
8. TXGUS1 - 0.2 mg/l Tc day 2
9. TXGUS1 - 0.2 mg/l Tc day 4
10. TXGUS1 - 1 mg/l Tc day 2
11. TXGUS1 - 1 mg/l Tc day 4
12. TXGUSA control – 0.2 mg/l Tc day 4

Figure 3.9 Northern blot analysis of *GUS* mRNA induction in TXGUS cultured rootlines. Root culture TXGUS1 was treated with 0, 0.2 and 1 mg/l Tc for 0, 2 and 4 days. Root cultures TXGUS 13 and 21 were treated with 0 and 0.2 mg/l Tc for 4 days. Negative controls include untransformed cultured roots and seedlings. Positive control is TXGUSA seedlings treated with 0.2 Tc for 4 days. **A.** 20 µg RNA loaded per lane **B.** probed with *BamHI-SacI* full-length fragment of the 1.8kb *GUS* coding region.

Root cultures grow well over 19 days with or without Mg, slightly better in Mg medium (Figure 3.10). Probably because sufficient Mg is carried over in the roots subcultured to sustain healthy growth. Tc is clearly deleterious to growth at 0.5 mg/l Tc and above, in the absence or presence of Mg. Exposure to 0.1 mg/l Tc does not cause a reduction in growth rate and 0.2 mg/l Tc is slightly deleterious to growth over 14 days.

3.4.2.6 GUS – induction in pith

The root culture system used here showed slightly different properties to seedlings and plants used in previously published experiments. As a result, it was considered prudent to test expression in excised stem pith, as another tissue culture system that may be useful in assessing the effects of cell cycle genes. Excised pith tissue from tobacco, when treated with auxin and cytokinin, forms callus tissue (Skoog and Miller, 1957). This method was used by Zhang *et al.* (1996) and enabled the assaying of Cdc2-like kinase activity in tobacco. By adapting this method to the Tc induction system it may be possible to detect any changes in Cdc2-like kinase activity resulting in from the overexpression of *cdc* genes. TXGUSA transformed plants were grown to maturity in the greenhouse and the pith excised. Pith blocks were incubated on modified MS agar with 5.4 μM NAA and 0.56 μM BAP for ten days (Zhang *et al.*, 1996) in the presence of varying levels of Tc. After treatment it was observed that callus tissue has not developed at all where the Tc treatment is highest at 1 and 5 mg/l Tc (Figure 3.11), and callus is reduced by 0.5 mg/l Tc. This is despite the fact the Tc has little effect on whole plants (Gatz *et al.*, 1992), although concentration in cells of excised tissue may have been far higher than obtained in whole plants. The relatively high level of Tc required to achieve *GUS* induction throughout the callus tissue, and the loss of callus production as a consequence, suggests that it might be very difficult to find an induction level that allows successful measurements of Cdc2-like kinase activity to be made in this system.

3.5 DISCUSSION

The attributes of the strong 35S CaMV promoter without the potentially negative effects of constitutive expression meant the Tc inducible promoter seemed ideally suited to this project. In the course of the project, it became clear that the situation was more complicated than originally thought. Especially in the case of roots, tetracycline had detrimental effects on growth and it was necessary to discover a Tc level which allowed maximum induction with minimum negative effects on plant growth. These detrimental side-effects have subsequently been documented in tobacco and other species (Corlett *et al.*, 1996; Gatz, 1997).

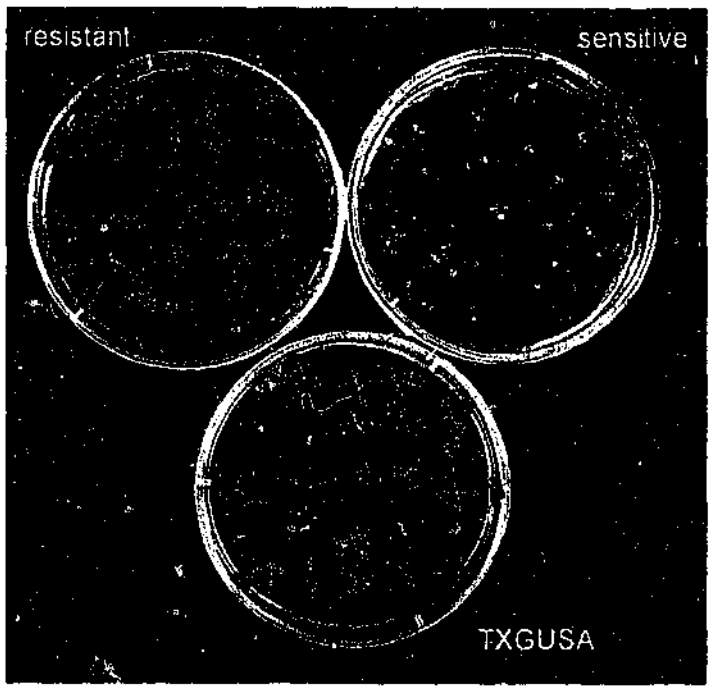


Figure 3.3 Hygromycin selection of seedlings. Selection of TXGUSA F1 transgenics on 50 µg/ml hygromycin. After 12 days a 3:1 ratio of resistant:sensitive seedlings was clear. Resistant and sensitive lines were also grown on 50 µg/ml hygromycin as controls.

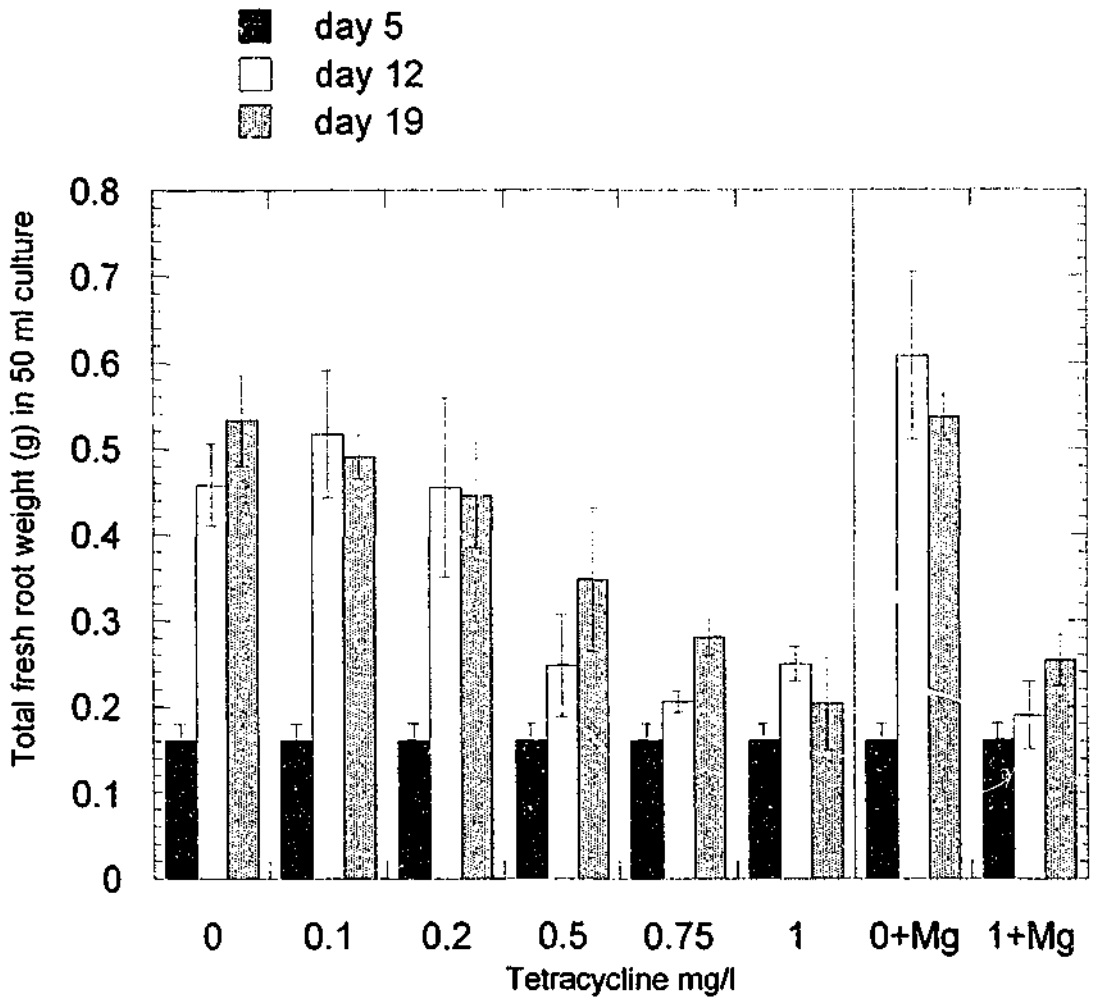
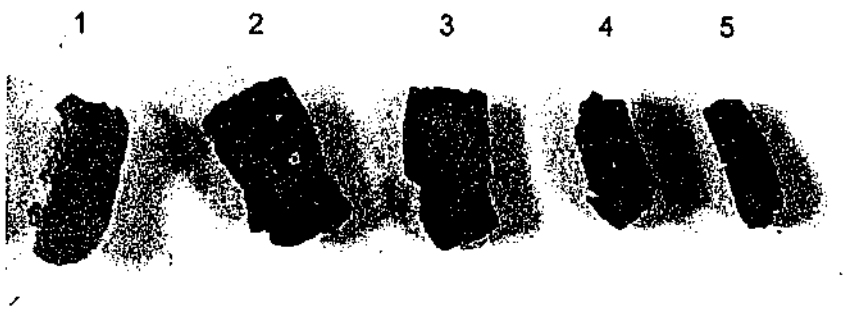


Figure 3.10 Growth in Tc of TXGUS root cultures. Established TXGUS1 cultures were treated with 0, 0.1, 0.2, 0.5, 0.75, and 1 mg/l Tc over 7 and 14 days in modified MS without Mg. Samples on the right of the graph were cultivated in media containing normal levels of $MgSO_4$.



Legend
1. 0 mg/l Tc control
2. 0.2 mg/l Tc
3. 0.5 mg/l Tc
4. 1 mg/l Tc
5. 5 mg/l Tc

Figure 3.11 The production of callus and *GUS* expression in Tc induced TXGUSA tobacco plant stem pith. Approximately equal sized pith segments were excised and treated with 0, 0.2, 0.5, 1, 5 mg/l Tc. Tissue was incubated on modified MS agar media and 5.4 µM NAA and 0.56 µM IBA hormones for 10 days. Staining with X-gluc was used to visualize *GUS* expression.

Another possible limitation of the Tc inducible system was the slight leakiness of the promoter. In the TXGUSA non-induced seedling root there was some blue staining in the vascular system and in the tips of newly emerging TXGUS cultured roots, suggesting incomplete repression of the Triple-X promoter. Interestingly DeVeylder *et al.* (2000) have recently published a paper questioning the efficacy of the Triple-X promoter for expression of genes likely to perturb plant growth. To date it appears that no truly toxic genes have been successfully expressed at high levels using this promoter. Nevertheless, it has been used successfully without apparent detrimental effects, to express genes affecting growth and development. Though, root phenotype may not have been examined in some studies.

3.5.1 Induction conditions

Using the coding sequence of *uidA* (*GUS*) reporter gene to study the efficacy of the induction system, transgene induction under the Triple-X promoter can be achieved in cultured transformed roots, seedlings and pith of tobacco. However it appears that the Tc possibly has too great an inhibitory effect on the callus development of the treated pith in that context to be a useful system.

From GUS specific activity measurements, the highest level of GUS expression in the most responsive line TXGUSA was measured at approximately 38,000 pmol MU/min/mg protein at 37°C (U) (0.75 mg/l Tc after 7 days exposure). According to Gatz (1997) the induction factor of TXGUS in tobacco can be up to 500-fold. High expressing plants have background levels of 2000 U that can be induced to 180,000 U. Low expressing plants, with almost zero background, elevate to between 1000 and 2000 U. The plants recovered in this study have a relatively high background of 1130 U and a low 34-fold induction factor compared to other reports. However, it is known that expression levels differ between individual transgenic plants and may also be different in seedlings compared to mature plants. CaMV35S promoter activity is reported to range from 321 U in one plant (Jefferson *et al.*, 1987) to 113,000 U as an average of 10 plants (Benfey *et al.*, 1989). It is therefore difficult to directly compare the TX promoter efficiency from these results with that of the CaMV35S in the literature.

In general GUS activity increases over time with Tc concentration. From RNA and protein studies the seedlings appear to tolerate 1 mg/l Tc well to achieve good levels of induction but seven days is too long an induction period for a reliable response floating in liquid. One possibility is that the submersion in higher levels of Tc had some effect on the seedling. In roots, Tc levels of 0.5 mg/l and above have a measurable impact over time, in negatively affecting growth in culture and *GUS* mRNA levels. There is therefore a need to strike a balance between retarding growth of the roots and using the inducing

treatment long enough for the induced mRNA to manifest itself at the cellular level in a measurable way. For short treatments, high levels of Tc are required for rapid induction. 1 mg/l Tc was the lowest concentration to achieve significant expression. However, for a lower level of induction over a longer term 0.2 mg/l Tc was more suitable for minimum growth inhibition. Also, modifying the MS media to be magnesium free provided better Tc induction conditions than complete MS, with a mild negative effect on root culture growth over extended periods.

3.5.1.1 The effect of Tc on root growth

Though the Tc-inducible promoter has been used widely to control the expression of foreign genes in plants without apparent toxicity, closer inspection of the literature does reveal some reports of toxicity in the 0.1 – 1 mg/l range required for induction. In concurrent studies performed elsewhere, Tc has been reported to cause a significant reduction in root growth in tomato (Corlett *et al.*, 1996) and Gatz notes some otherwise unpublished data to that effect in tobacco (Gatz, 1997). This was unexpected at the concentrations that have been used as the lethal concentration for the tetracycline-sensitive prokaryote *Bacillus subtilis* is >0.5 mg/l (Geissendörfer and Hillen, 1990).

Tc-like compounds that might be gene effectors with fewer side-effects have been screened for their potential use with tetR based gene switches (Chrast-Balz and Hooft van Huijsduijnen, 1996). The close homologues anhydro-tc and doxycycline were shown to strongly inhibit DNA binding by the Tet repressor. Doxycycline has a much longer half-life than Tc which is a disadvantage when it is desirable to have tightly regulatable induction of a foreign gene in transgenic tissues. Anhydro-Tc efficiently induces TetR regulated expression (Gossen and Bujard, 1993) and is claimed to be less toxic for plants than Tc. It is inconvenient to use in hydroponics, however, as its instability requires much higher levels to be used e.g. Jones *et al.* (1998) required 12 µg/ml to induce for 48 hours.

A possible alternative appears to be chlor-Tc. The root phenotype in *Nicotiana tabacum* (var Wisconsin 38) was reported to be less affected by chlor-Tc than by Tc, and was shown in hydroponic culture to cause less browning of the roots and a reduction of root growth. Tc and chlor-Tc also affected photosystem II efficiency by 5-15% and 3.7-8.6% respectively (Böhner *et al.*, 1999). Although not directly related to roots these measurements might indicate relative toxicity to the plant energy systems. The potential to reduce the toxic effects of the inducer led to the investigation of equivalent concentrations of Cl-Tc being used to express cell cycle genes in plants (see Chapter 4).

3.5.2 Limitations of the TX-GUS system

These studies have assisted in determining approximate induction conditions in root cultures and seedlings but, as both the *GUS* mRNA and protein is likely to persist longer than that of the *cdc* genes, some adjustment may be required. Luciferase has a half life of approximately 3 hours (Thompson *et al.*, 1991) and has been used in the assessment of other inducible promoters (Aoyama and Chua, 1997) and could be used for any further characterization of experimental systems.

The basal uninduced expression that was detected in the newly establishing root cultures (data not shown) and in uninduced seedlings may have been due to persisting bacteria or endogenous GUS activity of the tissue. The GUS activity levels, however, indicate there is a level of background expression of the transgene. Gatz had reported some basal level of Triple-X activity in the absence of activator (Gatz *et al.*, 1992).This is a limitation of the Tc-inducible system which may prevent lines highly expressing *cdc* genes from regenerating. It might also reduce the longevity of root cultures and the fertility of whole plants.

Despite these potential limitations, the TXGUS experimental system shows promise of allowing inducible expression of cell cycle genes with the Triple-X promoter. Possible side effects have been identified and this information allowed the experimental work undertaken in Chapters 4 and 5 to be designed to take account of these effects.

Chapter 4
Overexpression of the *S. pombe cdc2* gene in roots and seedlings of *N. tabacum*.

4.1 AIM

The aim of the experiments detailed in this chapter was to overexpress wild-type and dominant mutant forms of the *cdc2* gene from *S. pombe*, under the Tc-inducible promoter in plants and cultured roots. Since plant *cdc2* genes function successfully in yeast (Colasanti *et al.*, 1991; Ferreira *et al.*, 1991), it is interesting to test for the effects of the yeast *cdc2* gene when expressed in plants. In particular, the dominant activated allele *cdc2-1w* (Russell and Nurse, 1987) could provide indications of *cdc2*-dependent cell cycle events in plants. In order to determine the conserved elements of *cdc2* regulation and activity in plants, cell division in root meristems of transformed tissue was examined. The experiments also aim to served as a valuable comparison relevant to expressing other yeast cell cycle genes in plants, such as *cdc25*.

4.2 INTRODUCTION

CDKs are central regulators of the cell cycle and highly conserved between all eukaryotes. cdc2 (also called Cdk1) is particularly conserved and the homologue from many species is functional in yeast (Nurse, 1990). Thus, information gathered from many different organisms may be relevant to how CDKs function in plants. Much mutational analysis of CDKs has been done in yeast (Carr *et al.*, 1989; MacNeill and Nurse, 1993) and the crystal structure of the enzyme has been determined, in the form of the close variant Cdk2 from human (De Bondt *et al.*, 1993). While many of the functional aspects and structural features found are retained in plants, there is a greater complexity of the plant cell cycle. In plants a successive deployment of four cytoskeletal systems, appears to involve a rich array of Cdks and cyclins are more reminiscent of animal than yeast cells.

4.2.1 Conserved motifs in cdc2

The *cdc2* gene from *S. pombe* is 1.69kb long, contains four introns (Hindley and Phear, 1984) and codes for a 297 amino acid protein of approximately 34 kilodaltons (Booher and Beach, 1986). The cdc2 protein has many domains important in catalytic activity and binding substrates and regulators (see Figure 1.1 in Chapter 1 Introduction).

Analysis of human Cdk2-cyclinA crystal structure (Jeffrey *et al.*, 1995) shows the most extensive binding occurs within the α1 helix containing the PSTAIRE motif and the T-loop. These cyclin-binding site residues are conserved also in yeast and plants, strongly

suggesting functional similarity (Dudits *et al.*, 1998). In addition to cyclin, Cdc2 interacts with many other components of the cell cycle control apparatus such as: activators (e.g. Cdc25, CAK); docking factors (e.g.Suc1/Cks1); CDK inhibitors (e.g.Wee1, CKIs) and numerous substrates (Morgan, 1995; Nigg, 1995; Stals *et al.*, 2000).

The theoretical structure of alfalfa CdkA;1 kinase, deduced from the gene sequence is very similar to that of human Cdk2, suggesting that many of the protein interactions are also conserved (Dudits *et al.*, 1998). Thirty-eight amino acid residues are exposed to solvent in free human Cdk2 and are therefore available for binding regulatory proteins. These sites have an identical sequence in *S. pombe* Cdc2 and have only three changes in the plant CdkA consensus sequence (of 31 sequences) (De Bondt *et al.*, 1993; Joubès *et al.*, 2000). *Arabidopsis* CdkA in particular has four or five changes depending on the alignment (Lessard *et al.*, 1999).

4.2.2 Identifying *cdc2* function by expression in yeast

4.2.2.1 Complementation with plant *cdc2* in fission yeast

The plant *cdc2* homologue from *Arabidopsis*, *cdc2aAt*, has 63% amino acid identity and 82% similarity with fission yeast Cdc2 protein. When the plant gene is introduced, the plant enzyme can partially complement (for two rounds of cell division) the temperature sensitive mutant *cdc2-33* of *S. pombe* (Hirayama *et al.*, 1991; Imajuku *et al.*, 1992). The plant kinase can therefore perform most, but not all, of the tasks required of yeast Cdc2. In a detailed study of plant *cdc2* mutants expressed in yeast, Porceddu *et al.* (1999) have suggested that Cdc2aAt kinase interacts inefficiently with yeast cell cycle regulators.

4.2.2.2 Overexpression of plant cdc2

It has been illustrated that plant CdkA has functional activity in yeast by expressing both wild-type and mutant forms of the plant *cdc2* homologue. Cdc2aAt (CdkA;1) is capable of binding yeast Cdc2 regulators, but Cdc2bAt (CdkB1) shows its more distant relationship by being unable to show functional activity in yeast and binding regulatory proteins less efficiently (Porceddu *et al.*, 1999).

Wild-type Cdc2aAt, when overexpressed in fission yeast gives a wee phenotype, i.e. advancement of mitotic initiation indicative of incomplete control (Hemerly *et al.*, 1995). Budding yeast *cdc2* overexpressed in fission yeast also gives a wee phenotype (Fleig and Nurse, 1991). Based on residues identified in yeast *cdc2* as being essential for regulation of activity (Beach *et al.*, 1982; Durkacz *et al.*, 1986), mutant forms of plant *cdc2* were generated to provide information regarding the importance of

equivalent residues in the plant enzyme. *cdc2aAt.A14F15* was mutated to abolish sites of potential inhibitory phosphorylation on Thr14 and Tyr15. Expression of this plant mutant in yeast results in abnormally varied cell sizes, indicating that a comparable control mechanism may regulate the plant kinase via phosphorylation (Hemerly *et al.*, 1995; Porceddu *et al.*, 1999).

Expression of another mutant version of the gene, *cdc2aAt.N147* (designated dominant negative (*DN*) or *N146*) inactivated the kinase by disrupting Mg-dependent ATP binding and caused yeast cell cycle arrest (Hemerly *et al.*, 1995). Three separate mutations in *cdc2aAt* (*DL41, DL36*, and *DL50*) also showed similar phenotypes to the corresponding yeast *cdc2* mutants which interfere quite specifically with progress at different points in the cell cycle. However, none produce active kinases that can complement an *S. pombe* null mutant (Porceddu *et al.*, 1999) and thus show homologous regulator binding, but not a gain of function as the equivalent yeast mutant does.

In contrast to *Cdc2aAt*, *Cdc2bAt* is clearly not an orthologue of yeast *cdc2*. *Cdc2bAt* mutated in analogous sites to those of *Cdc2aAt*, behaves as an inactive allele, except for *Cdc2bAt.DL50*, which causes mitotic catastrophe. This is again not due to extra kinase activity, but possibly due to a more profound interference with the normal workings of the cell than the other alleles (Porceddu *et al.*, 1999).

4.2.3 Identifying *cdc2* function by overexpression in plants

The overexpression of wild-type Cdc2aAt in both tobacco and *Arabidopsis* results in no phenotypic effect despite a 2-fold increase of extractable Cdc2 histone H1 kinase activity (Hemerly *et al.*, 1995; De Veylder *et al.*, 2000). A tendency to lose apical dominance in some *Arabidopsis* plants is the only consequence of overexpressing Cdc2aAt.AF in both *Arabidopsis* and tobacco, despite this mutant having an obvious phenotype in yeast (Hemerly *et al.*, 1995). This finding is consistent with the diagnosis that cytokinin promotes removal of inhibitory phosphate from Cdc2 (Zhang *et al.*, 1996). It indicates that release from apical dominance can be obtained from copying the effect that cytokinin has on Cdc2, as well as by application of cytokinin. For obtaining an even stronger effect, one or several of the regulatory proteins required for activation may be the limiting factor and this illustrates that division in plants is regulated and not simply a direct result of *cdc2aAt* expression (De Veylder *et al.*, 1998).

A dominant allele of plant *cdc2* that has been overexpressed in tobacco, *cdc2aAt.N147* (*DN* or *N146*), has Asn147 instead of the highly conserved Asp147 therefore producing an inactive kinase (Hemerly *et al.*, 1995). A decreased number of

cell divisions is caused, resulting in 5-10-fold larger cells, due to an excess of growth relative to the cell number available. This has no effect on the morphogenesis of the plant other than a marked stunting. The morphogenic shaping of organisms is therefore seen to involve determinants other than assembling components cells. The transgenic tobacco seedlings expressing DN *cdc2* have shorter roots with a reduced number of lateral roots, in comparison with wild-type seedlings. The shoot apical meristem had a reduced size with normal sized cells, but the root apical meristem was completely disorganized (Hemerly *et al.*, 1995). The phenotype was most likely caused by inactive kinase titrating out cyclin and other regulatory proteins and reducing the overall CDK activity. When expressed under an embryo specific promoter in *Arabidopsis*, it resulted in a range of distortions in embryo formation (Hemerly *et al.*, 2000).

4.2.4 Expressing yeast genes in plants

4.2.4.1 *Cdc2-1w* mutation

The dominant *cdc2-1w* allele of *S. pombe* encodes a protein with an Asp residue at position 146, rather than the wild-type Gly146. It contains a single base pair change resulting in the codon GAC replacing GGC (Carr *et al.*, 1989). This single substitution causes the advancing of mitosis relative to growth and hence cells smaller (wee) at mitosis (Booher and Beach, 1986). The *cdc2-1w* mutation renders cells largely insensitive to *wee1* mitotic inhibition, though they still require *cdc25* phosphatase induction (Russell and Nurse, 1987).

In all functional homologues of *cdc2* in eukaryotes, except plants, the residue equivalent to *S. pombe* 146 is conserved as a glycine. At the corresponding *Arabidopsis* residue 139 is a threonine; Thr139 is also the consensus sequence for plants, calculated from 31 CDKAs (Joubès *et al.*, 2000). As a result of *Arabidopsis* gene complementation studies in yeast, it was suggested that this threonine substitution can be tolerated because the conformational change that is produced in the structure is minor (Ferreira *et al.*, 1991), unlike the substitution to aspartic acid in *cdc2-1w* (Booher and Beach, 1986). The *cdc2-1w* substitution could therefore function analogously in plants as in yeast, particularly as this residue is located in the middle of a highly conserved region.

4.2.4.2 The role of an inducible promoter

Hemerly and coworkers (1995) found that under the control of the CaMV 35S promoter, *A. thaliana* plants expressing the dominant negative *CDC2aAt.N147* could not be regenerated whilst only three tobacco transformants were obtained. This maybe because the mutant *Arabidopsis* protein reacts a little less specifically in the tobacco cell cycle system than it does in *Arabidopsis* (De Veylder *et al.*, 1998). If overexpressing

cdc2, particularly in mutant form, may interfere with the regeneration of transgenics plants then using an inducible promoter could to overcome this limitation and create lines capable of strong expression.

In this study the assumption has been made that as plant CdkA, namely Cdc2aAt, can operate in yeast, yeast Cdc2 might be able to perform many plant functions. For yeast CDK to be active cyclin binding is required, as is phosphorylation of Thr167 in the T-loop, dephosphorylation of catalytic cleft residue Tyr15 and absence of CKI binding (for review see Ohi and Gould, 1999). The activity of yeast Cdc2 in plants could help determine the importance of inhibitory phosphorylation in plant cell cycle control and the possible significance of Cdc25- and Wee1-like regulation in particular.

The experiments reported in this section were intended to address the following questions:

1. Can *S. pombe cdc2* and *cdc2-1w* genes be expressed at high levels under the Triple-X promoter in plants and cultured roots?

2. What is the phenotype of plants overexpressing yeast *cdc2* and *cdc2-1w*?

3. Is Cdc2 regulated by similar mechanisms in yeast and plants? In particular, is there any evidence from the transgenic phenotypes that Tyr15 phosphorylation conserved?

4.3 MATERIALS & METHODS

4.3.1 Constructs

The 894bp coding sequence of the *S. pombe* wild-type *cdc2* and mutant *cdc2-1w* genes with introns removed were obtained as pUC18 based pcdc2-39 and pcdc2-40 respectively, kindly supplied by Dr Robert Booher. To add compatible sites for insertion into pBTX the genes were subcloned into pBluescriptIISK+ as *NdeI-EcoRI* fragments where the *NdeI* site is located at the initiating methionine. Sequence analysis confirmed the correct identity of both DNA fragments. The *cdc2* genes were placed under the control of the tetracycline inducible Triple-X promoter and *ocs* terminator by insertion in the Triple-X expression cassette on a *SmaI-SalI* fragment (see Figure 4.1). The genes *cdc2* and *cdc2-1w* were introduced into roots and plants by transformation with the pBIN19 derived pBinHygTx (pBTX) vector.

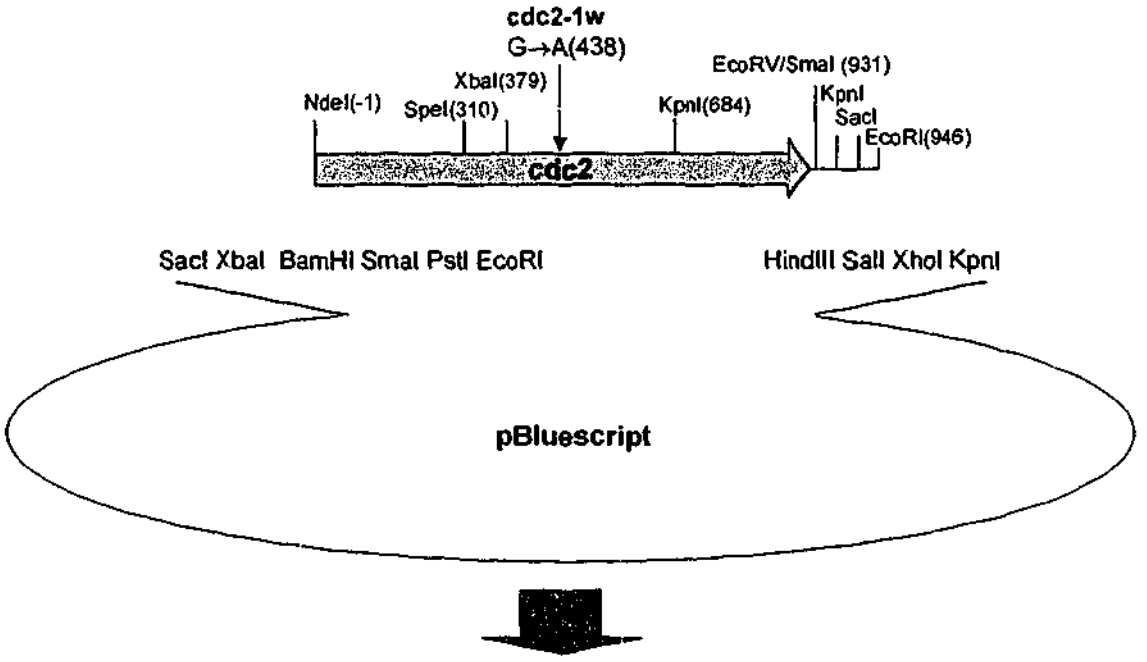


Figure 4.1 Structure of plasmid constructs pBTXcdc2 and pBTXcdc2-1w. The *cdc2* and *cdc2 -1w* genes were inserted between the Triple-X promoter and the octopine synthase (*ocs*) polyadenylation signal. The selectable marker gene shown is hygromycin phosphotransferase (*HPTII*). RB, right border; LB, left border. Not to scale.

4.3.2 PCR

4.3.2.1 Primers

The two oligonucleotides used as PCR primers for *cdc2* were:
cdc2 Forward Primer (2F): 5'-GCATTCTAGAAGAATCATTCATCGC G-3'
cdc2 Reverse Primer (2R): 5'-GCGATGGGCAGGGTCATAAACAAGC-3'
This primer pair amplifies a 471bp fragment from positions 374 to 844 in the *cdc2* gene.

4.4 RESULTS

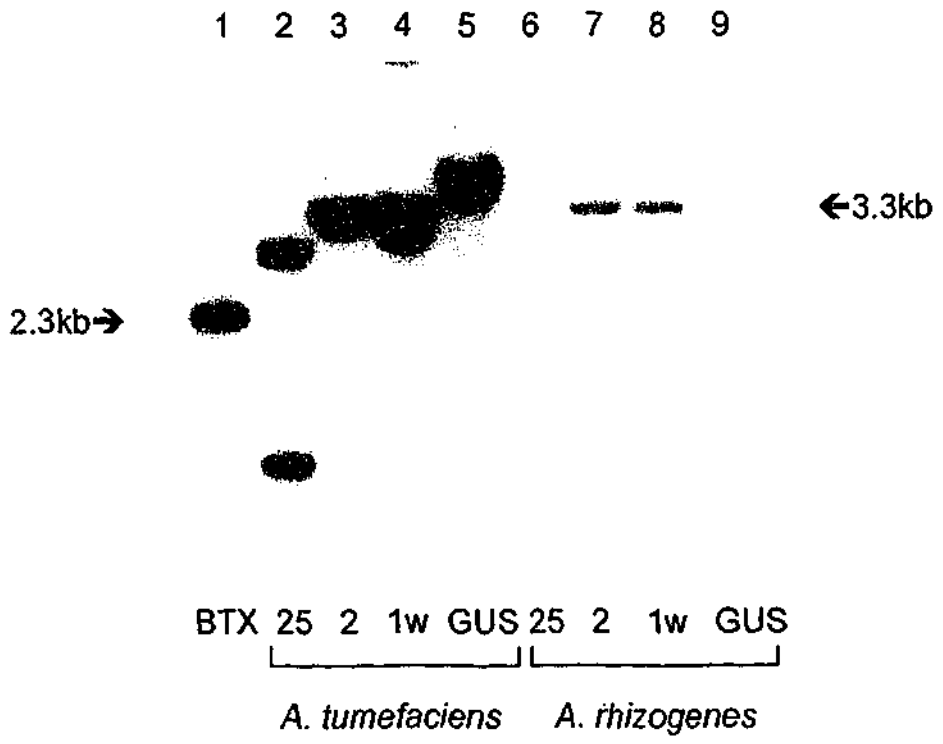
4.4.1 Induction of *cdc2* in transgenic plants and root cultures

4.4.1.1 Transformation of *Agrobacterium* sp.

The pBTXcdc2 and pBTXcdc2-1w plasmids were introduced into *A. tumefaciens* and *A. rhizogenes* by triparental mating. This transformation was confirmed by Southern blot (Figure 4.2). Agrobacterial DNA was probed with a fragment containing the empty Triple-X cassette to show the presence of the Tc-inducible promoter and the size of the 973bp insert it contained.

4.4.1.2 Transformation of plants with *A. tumefaciens*

Leaf disc transformation was performed on *N. tabacum* (var Wisconsin 38) tetR plants with *A. tumefaciens* containing the wildtype and mutant *cdc2* genes under control of the Triple-X promoter. Selection from more than 30 leaf discs on 50 µg/ml hygromycin medium yielded four *cdc2* and three *cdc2-1w* transgenic plants. These independent TXcdc2 and TXcdc2-1w transformants were healthy and normal in appearance, although the TXcdc2-1w callus was slightly slower to regenerate shoots. Each transformant was transferred to soil, self-pollinated and the seed collected from the seven lines. Seeds of F1 were surface sterilized and germinated on medium containing 50 µg/ml hygromycin. The ratio of resistant to non-resistant seedlings did not deviate significantly from 3:1 for lines A and D of TXcdc2-1w, A and B of TXcdc2. Txcdc2.C and G showed a significant deviation with ratios approaching 6:1, perhaps indicative of multiple insertion(Figure 4.3A). The observed numbers of sensitive seedlings are lower than expected, which may be due to them not germinating at all on such a high level of hygromycin or not developing sufficiently to be counted (Figure 4.3B). Line TXcdc2-1w.B showed 100% hygromycin sensitivity.



Lanes
1. pBTX plasmid only control
2. BTXcdc25 in *A. tumefaciens BglII-SacII* digest
3. BTXcdc2 " "
4. BTXcdc2-1w " "
5. BTXGUS " "
6. BTXcdc25 in *A. rhizogenes BglII-SacII* digest
7. BTXcdc2 " "
8. BTXcdc2-1w " "
9. BTXGUS " "

Figure 4.2 Southern blot of transformed *A. tumefaciens* strain AGLO and *A. rhizogenes* 9402. Agrobacterial DNA digested with *BglII-SacII* (*SstII*) was probed with 790bp *EcoRI-HindIII* fragment of BTX containing Triple-X promoter and *ocs* terminator.

(A) Segregation analysis of F1 seedlings

LINE	SENSITIVE	RESISTANT	TOTAL	*P*
TXcdc2.A	14	61	75	>0.05
TXcdc2.B	10	58	68	>0.05
TXcdc2.C	12	70	82	<0.05*
TXcdc2.G	13	78	91	<0.02*
TXcdc2-1w.A	27	86	113	>0.05
TXcdc2-1w.B	84	0	84	-
TXcdc2-1w.D	11	57	68	>0.05

(B) Hygromycin sensitivity of seedlings

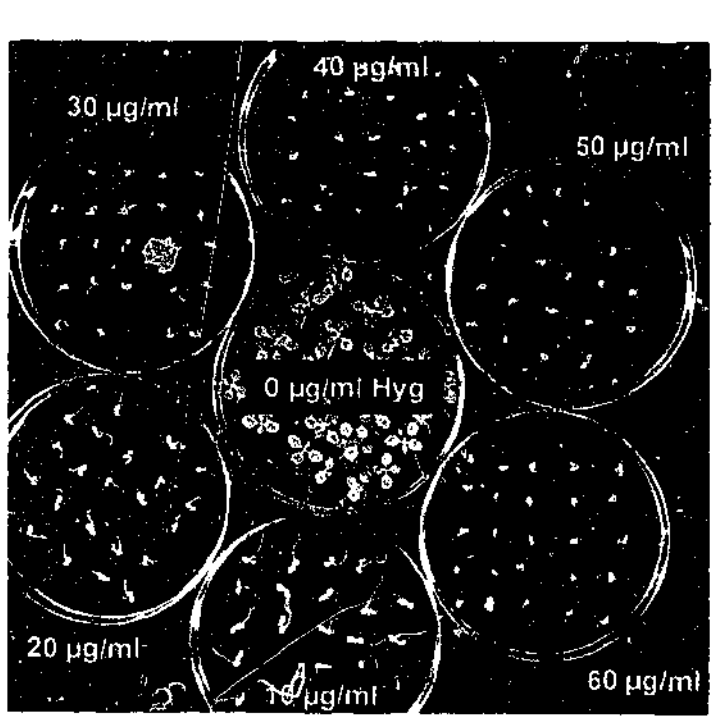


Figure 4.3 Hygromycin selection of seedlings: **(A)** Segregation analysis of self fertilized TXcdc2 and TXcdc2-1w transformants on 50 µg/ml Hyg. Proposed ratio of 3:1 resistant:sensitive seedlings. Levels of significance are indicated by the Contingency $\chi 2$ test. **(B)** Hygromycin kill curve. 17 day old hygromycin sensitive seedlings germinated on MS containing between 0 to 60 µg/ml Hyg. Centre plate contains no antibiotic.

4.4.1.3 Transformation of root cultures with *A. rhizogenes*

Leaf inoculations, performed on *N. tabacum* (var Wisconsin 38) tetR plants, with *A. rhizogenes* resulted in the co-transfer of binary vector T-DNA and the transgenes into emerging roots. A number of root lines showing hygromycin resistance were established from single root tips derived from separate transformation events. Nine lines containing *cdc2* and seven containing *cdc2-1w* under the control of the Triple-X promoter were derived.

4.4.1.4 Genetic confirmation of transgenic lines

To determine that plants and the rootlines contained the *cdc2* genes they were subject to PCR analysis (Figure 4.4). All seven of the original hygromycin selected plants showed the presence of the *cdc2* and *cdc2-1w* genes, including the hygromycin sensitive line TXcdc2-1w.B which did not segregate and had been included as a control. Of the nine original TXcdc2 rootlines, seven flourished and were shown to contain the transgene. Of the seven original TXcdc2-1w rootlines only five flourished, with four of those containing the transgene. Transgene-negative lines presumably integrated only the selectable marker.

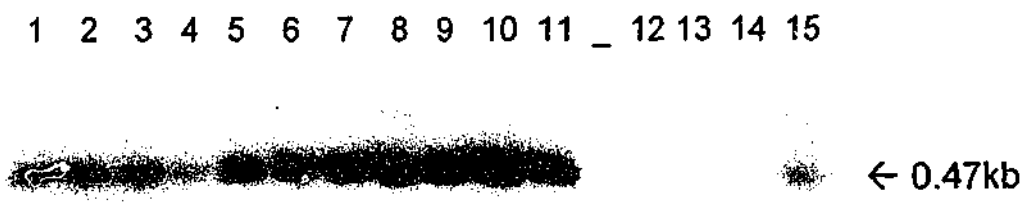
4.4.2 Transcriptional induction of cdc2 in seedlings

After confirmation of the presence of the *cdc2* transgenes Northern blot analysis was undertaken to determine which of the lines chosen were expressing the transgene and at what relative levels. In this experiment TXcdc2 and TXcdc2-1w F1 seedlings germinated in liquid MS media were transferred to modified MS liquid media containing 0.2 mg/l Tc for 4 days before harvesting. Total RNA was prepared from approximately 1 g of tissue and was subjected to Northern blot analysis.

Some *cdc2* mRNA was detected in all 7 lines tested after Tc induction (Figure 4.5). Correction for variation in the loading was made using the ubiquitin internal standard and relative levels can be seen in the histogram in Figure 4.5. The strongest level of expression is in lines TXcdc2.B and C. Lower levels of expression are observed in lines TXcdc2.A and G and TXcdc2-1w.A and B followed by TXcdc2-1w.D. None of the TXcdc2-1w lines was highly expressing. Although a small sample size, this indicates that the slight leakage of the TX promoter is having a noticeable effect. Possibly some expression of the dominant Cdc2-1w protein interfered with the regeneration of highly expressing transgenic lines and therefore they are not represented.

Though the ubiquitin-probed blot and the ribosomal RNA on the Ethidium bromide stained gel reveal tight bands of RNA, and thus good quality RNA, the *cdc2* bands

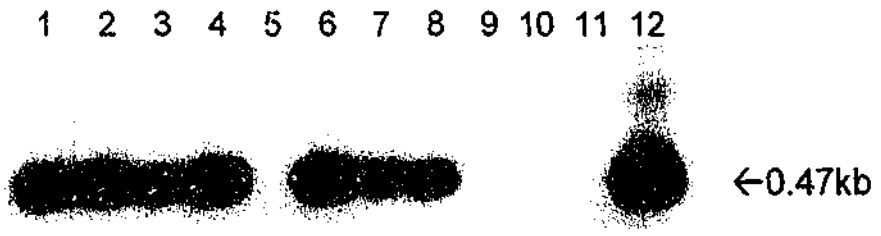
A. TXcdc2



Lanes

seedling lines	root lines	controls
1. TXcdc2.A	5. TXcdc2.2	12. ϕ*HaeIII* standard
2. TXcdc2.B	6. TXcdc2.2.3	13. TXGUS13
3. TXcdc2.C	7. TXcdc2.4	14. No template
4. TXcdc2.G	8. TXcdc2.9	15. BScdc2
	9. TXcdc2.10.2	
	10. TXcdc2.13	
	11. TXcdc2.14	

B. TXcdc2-1w



Lanes

seedling lines	root lines	controls
1. TXcdc2-1w.A	4. TXcdc2-1w.2.1	9. TXGUS13
2. TXcdc2-1w.B	5. TXcdc2-1w.2.2	10. No template
3. TXcdc2-1w.D	6. TXcdc2-1w.4	11. ϕ*HaeIII* standard
	7. TXcdc2-1w.10	12. BScdc2
	8. TXcdc2-1w.11	

Figure 4.4 PCR analysis of hygromycin resistant root lines and plants for the presence of the (A) *cdc2* and (B) *cdc2-1w* genes. DNA was extracted as described in Section 2.3.5.3 with TXGUS13 seedlings as a plant control. Using the 2F and 2R primer pair a band of 472bp indicated the presence of the *cdc2* gene. This was confirmed by a Southern blot of the PCR gel probed with a 1kb *BamHI-XhoI* fragment of the full-length *cdc2* coding region.

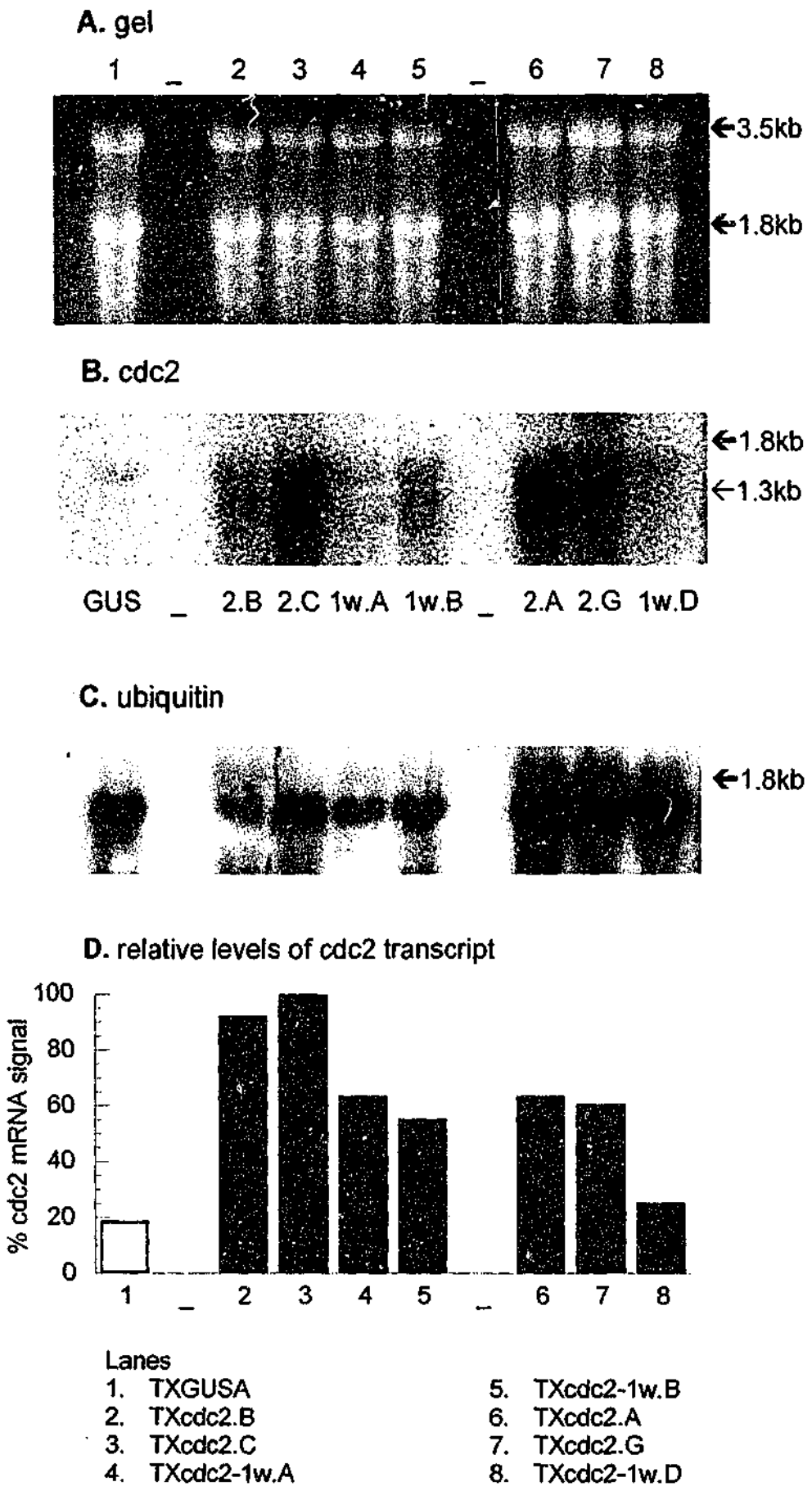


Figure 4.5 Northern blot analysis of the induction of *cdc2* mRNA in TXcdc2 and TXcdc2-1w seedlings. Seedlings were treated with 0.2 mg/l Tc for 4 days in modified MS: **A.** gel with 40 µg RNA loaded per lane **B.** probed with 1kb *BamHI-XhoI* fragment containing the full-length *cdc2* coding region **C.** probed with the ubiquitin coding sequence from *Antirrhinum majus* **D.** relative level of *cdc2* transcript standardised against the ubiquitin level in that sample.

observed are smearing a little down the blot indicating some degradation. The *cdc2* mRNA appears less stable in the plant cell than ubiquitin mRNA or ribosomal RNA.

There is a faint tight band that can be seen in the control TXGUSA sample lane. The *Arabidopsis cdc2* homologue gives a 1.5kb message, so the observed band would be the right size to correspond to tobacco *cdc2*, as both species have *cdc2* cDNA of identical length (Hirayama *et al.*, 1991; Setiady *et al.*, 1996). The yeast *cdc2* probe has limited regions of high DNA sequence homology with the tobacco *cdc2* homologue. This may have allowed for some slight detection of the endogenous CdkAs, even though the filter was hybridized and washed at high stringency.

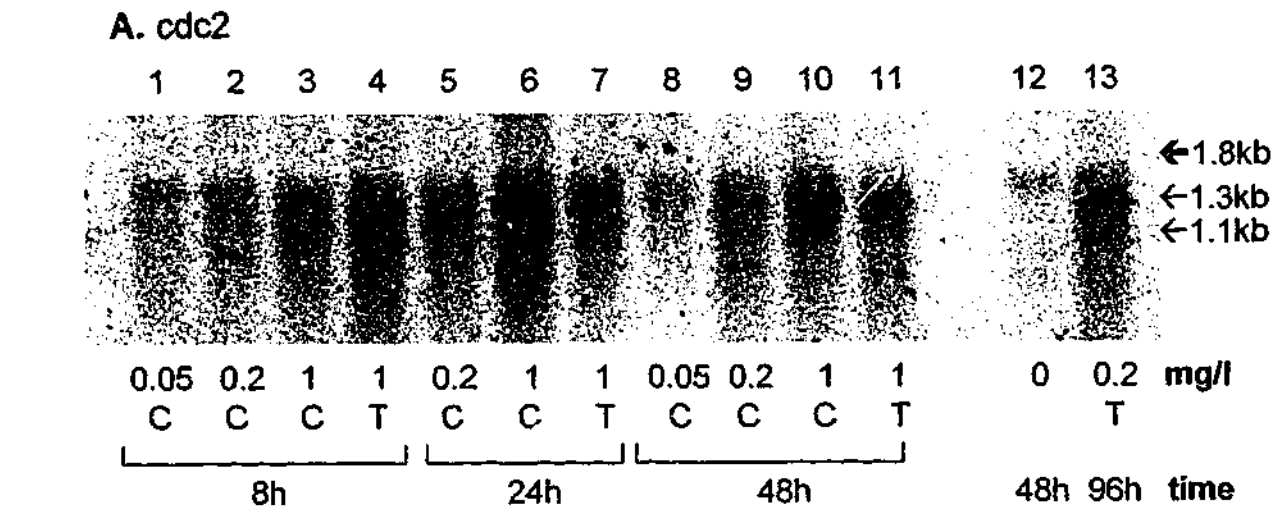
4.4.2.1 Comparative kinetics of transcriptional induction by Cl-Tc in seedlings

Chlor-tetracycline (Cl-Tc) is a relatively stable analogue of the Tc inducer which has been reported to be less toxic to plants (Böhner *et al.*, 1999). In order to compare the Tc treatment used in previous experiments, one line of TXcdc2 seedlings was selected for induction with Cl-Tc and subjected to Northern blot analysis. In these experiments TXcdc2C F2 seedlings were treated with various concentrations of Cl-Tc over time. For comparison, Tc levels were used that had previously achieved maximum expression levels in transgenic TXGUS seedlings (Figure 4.6).

After 8 hours of induction mRNA accumulation is already apparent in the seedlings. Very low levels of *cdc2* mRNA are produced with 0.05 mg/l Cl-Tc which show no increase over time. 0.2 mg/l Cl-Tc shows a moderate level of induction that reaches a maximum by 24 hours that is maintained thereafter. This indicates little or no toxic side effects for the seedlings over 48 hours at this level. In contrast, induction by 1 mg/l Cl-Tc is still strong at 48 hours but has dropped from the peak at the 24 hour time-point.

Unexpectedly, the 1 mg/l Tc treated seedlings have reached the peak levels of induction by 8 hours; *cdc2* mRNA levels then dropped over time. TXGUS experiments carried out over a similar timescale, in chapter 3 (Figure 3.8), showed mRNA induction to be quite low after 1 mg/l Tc treatment for 24 hours but had increased markedly by 48 hours. Quantification of *TXGUS* mRNA showed 0.2 mg/l Tc treatment for 4 days gave an equivalent signal to 1 mg/l Tc induction after 2 days. This is also the case with *TXcdc2* mRNA with the kinetics of induction over 2 days varying somewhat from a straightforward accumulation over time.

The negative control shows a slightly larger band than 1.3kb. This maybe an indication of some promoter leakage but as a TXGUSA control sample on the previous Northern blot (Figure 4.5) has shown a similar band, it could conceivably be the endogenous



Lanes

1. 0.05 mg/l Cl-Tc 8 hours
2. 0.2 mg/l Cl-Tc 8 hours
3. 1 mg/l Cl-Tc 8 hours
4. 1 mg/l Tc 8 hours
5. 0.2 mg/l Cl-Tc 24 hours
6. 1 mg/l Cl-Tc 24 hours
7. 1 mg/l Tc 24 hours
8. 0.05 mg/l Cl-Tc 48 hours
9. 0.2 mg/l Cl-Tc 48 hours
10. 1 mg/l Cl-Tc 48 hours
11. 1 mg/l Tc 48 hours
12. no treatment
13. 0.2 mg/l Tc 4 days

Figure 4.6 Northern blot analysis of the induction of *cdc2* mRNA in TXcdc2.C seedlings treated with Cl-Tc. Seedlings were treated with 0.05, 0.2 and 1 mg/l Cl-Tc (C) or 1 mg/l Tc (T) for 8, 24 and 48 hours. Sample in lane 12 received no treatment and lane 13 sample was exposed to 0.2 mg/l Tc for 4 days. 30 µg RNA loaded per lane: **A.** probed with 1kb *BamHI-XhoI* fragment containing the full-length *cdc2* coding region **B.** probed with the ubiquitin coding sequence from *Antirrhinum majus* **C.** relative level of *cdc2* transcript standardised against the ubiquitin level in that sample.

cdc2 homologue. Again some degradation of the *cdc2* mRNA can be seen from the smears on the gel below the 1.3kb band. A discrete second smaller band of about 1.1kb can also be seen in some samples, this shorter transcript may be due to early transcription termination. Both inducible bands were measured in the signal quantification as potentially able to produce protein.

Figure 4.6 shows that maximum induced transgene mRNA levels were achieved in seedlings with a treatment of 1 mg/l Cl-Tc and comparable with any induction achieved with Tc at these levels. Accordingly, Cl-Tc is a useful alternative to Tc as an inducer in this system.

4.4.3 Frequency of lateral root primordia

The establishment of new root meristems is a stage of development that is both sensitive and amenable to control by the cell cycle genes, as it is a time of rapid cell division. The presence of Cdc2 is required to initiate new roots but it does not appear to be a limiting factor. *cdc2* gene expression is found all along the pericycle but roots only develop at discrete sites (Hemerly *et al.*, 1993; Umeda *et al.*, 1999). If the limiting factor is rather the activation of Cdc2, then the dominant mutant *cdc2-1w* may stimulate pericycle division resulting in the emergence of more lateral roots.

To determine the effect the overexpression of *cdc2* and *cdc2-1w* on the root meristem, the frequency of emerging lateral root primordia (LRP) along the primary seedling root was assessed. 11 day old pure-breeding transgenic seedlings were treated for 72 hours with 10^{-4} K-IBA, found to be optimal for LRP production (see Section 5.4.3), with or without 5 mg/l Cl-Tc. measurements were calculated in terms of the frequency of lateral root primordia per cm of primary root length.

The data presented in Figure 4.7 show that in seedling lines transcribing the *cdc2-1w* gene there is a reduced frequency of lateral root primordia compared to the uninduced seedlings. The mean frequency of LRP in treated TXcdc2-1w.D seedlings were significantly different to the untreated (P=0.003) using the two-tailed Independent Samples T-test. Mean LRP/cm (±SE) were +Tc=10.96(±0.41) and –Tc=9.17(±0.37). In TXcdc2-1w.B the difference between mean frequency of LRP in treated and untreated seedlings was close to significant (P=0.051). The TXcdc2 lines show no significant difference in mean LRP/cm (P=0.832-0.986), similar to the TXGUSA control line (P=0.764). If 5 mg/l Cl-Tc treatment is having any effect on the seedlings it is not apparent in the LRP measurements.

The results obtained here were different from those which were expected. If the cdc2-1w protein is fully functional in plants, it may be predicted to increase the levels of

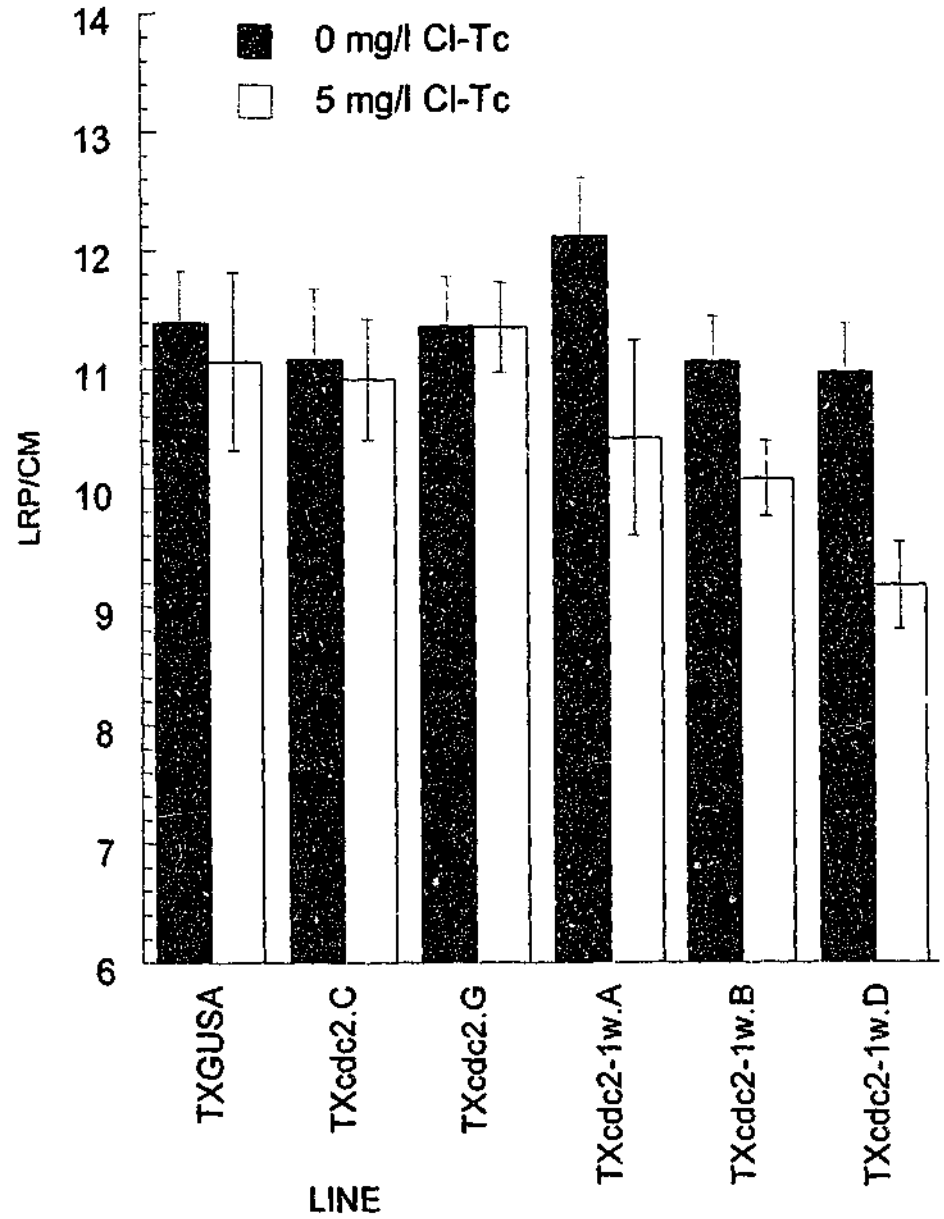


Figure 4.7 Mean frequency of lateral root primordia (±SE) in uninduced (■) and induced (□) seedlings. TXGUSA, TXcdc2 (lines C, G), and TXcdc2-1w (lines A, B, D) 11 day F2 seedlings, pure breeding for hygromycin resistance, were transferred to modified MS containing 10^{-4} M K-IBA ±5 mg/l Cl-Tc. After 72 hour treatment the seedlings were treated in 2% Chromium trioxide to reveal LRP (Pelosi *et al.*, 1995). The frequency of LRP per cm primary root length was measured (n≥10).

active kinase in the roots, causing cells to enter mitosis precipitately possibly resulting in extra, more rapidly dividing root meristems. This would occur if the effect was to trigger cell divisions in new regions rather than accelerate existing cell divisions. In fact, fewer lateral root primordia emerged along the length of the primary root in *cdc2-1w* transgenics. The mutant Cdc2 yeast protein is possibly interfering with mitosis in the plant cell early in the process of instigating a new meristem. In contrast, the presence of much higher levels of wild-type yeast cdc2 transcript doesn't appear to impede plant cell division at all.

4.4.4 Transcriptional induction of *cdc2* expression in roots

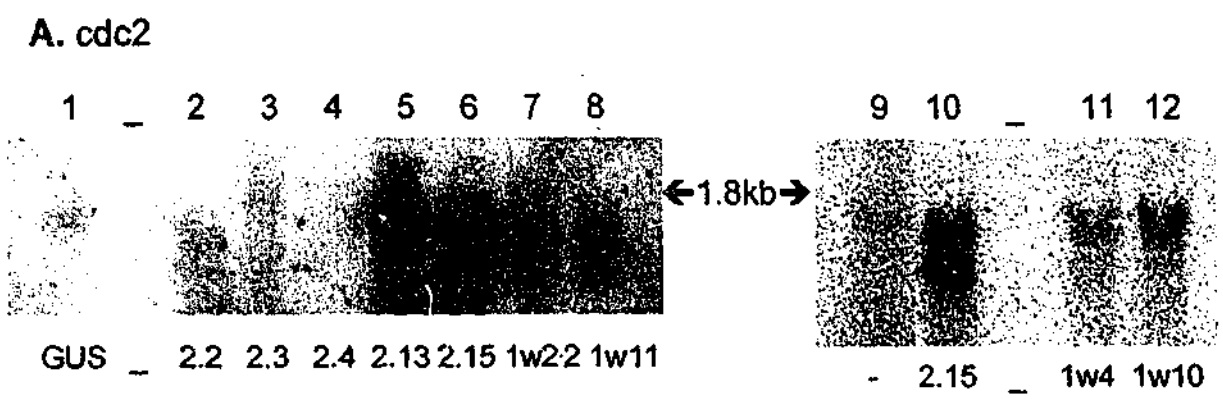
To determine if transformed rootlines were expressing *cdc2* mRNA when treated with inducer, total mRNA was prepared and a Northern blot performed. It was quite difficult to detect intact *cdc2* mRNA from the rootlines. From the data presented in Figure 4.8 it is clear that TXcdc2 lines 13 and 15 are expressing highly, whilst in TXcdc2-1w lines 2.2, 4, 10 and 11 *cdc2-1w* transcript is present at low levels. It is uncertain whether line TXcdc2.4 is expressing as the sample had degraded. TXcdc2.2 and 2.3 showed no expression. The TXcdc2.15 sample was run on both gels to allow a comparison of signal intensity, but no meaningful quantitative comparison could be made between the two separate blots, especially as some of the signals were diffuse.

4.4.4.1 Comparative kinetics of transcriptional induction by Cl-Tc in cultured roots

As root cultures are more sensitive to the negative growth effects of Tc than seedlings it was important to determine if equivalent derepression of the Triple-X promoter could be achieved using the less toxic Cl-Tc.

Figure 4.9 indicates that in cultured roots 1 mg/l Cl-Tc, 5 mg/l Cl-Tc and 1 mg/l Tc induce the *cdc2* mRNA at equivalent levels over a 24 hour treatment period. Perhaps complete derepression of the promoter was achieved. Despite Cl-Tc being less toxic than Tc, it still has possible negative effects on the roots when at high levels. As 5 mg/l showed little increase in induction over 1 mg/l Cl-Tc, growth inhibition due to toxicity of the higher concentration of Cl-Tc, may be countering the extra induction. Cl-Tc and Tc gave similar levels of induction at the concentration of 1 mg/l. Any difference in the toxicity of the two inducers was not evident over the timescale of this experiment.

Two discrete bands of approximately 1.3kb and 1.1kb were visible, probably originating from the *TXcdc2* gene. Two bands were also seen in seedling (Figure 4.6), this Northern shows more clearly that both bands are inducible.



Lanes
1. TXGUS1 control
2. TXcdc2.2
3. TXcdc2.3
4. TXcdc2.4
5. TXcdc2.13
6. TXcdc2.15
7. TXcdc2-1w.2.2
8. TXcdc2-1w.11
9. uninduced control
10. TXcdc2.15
11. TXcdc2-1w.4
12. TXcdc2-1w.10

Figure 4.8 Northern blot analysis of the induction of *cdc2* mRNA in TXcdc2 and TXcdc2-1w roots. Cultured roots were treated in modified MS with 1 mg/l Tc for 4 days (lanes 1-8, 10) or 1 mg/l Tc for 1 day. 40 µg RNA loaded per lane: **A.** probed with 1kb *BamHI-XhoI* fragment containing the full-length *cdc2* coding region **B.** probed with the ubiquitin coding sequence from *Antirrhinum majus*. No quantitation is presented as comparison of relative mRNA signal levels between two separate blots is inaccurate.

4.4.5 Root tip width comparison

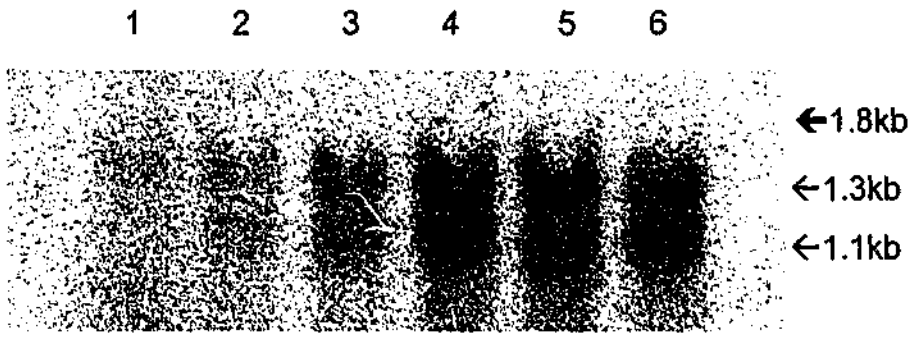
In plants, the Cdc2-1w enzyme may show higher activity , as it does in yeast, because it is not susceptible to inhibitory effects narrower root meristems may be expected, containing smaller cells due to accelerated entry into mitosis. This is on the conditions that cell size is not stabilized by a requirement for normal cell size at the G1-S progression and root morphogenesis does not compensate by accomodating a larger number of smaller sized cells in a root of normal diameter. In response to Tc treatment, TXcdc2-1w.11 shows very little size difference in the width of root tips (Figure 4.10). A tendency to a slightly narrower root tip may be a small effect due to the presence of the dominant mutant transgene, but it is not statistically clear. Neither TXGUS nor TXcdc2.13 showed any difference in root tip width due to Tc treatment. This experiment was a trial but nevertheless supports the suggestion that *cdc2-1w* may be obstructing cell division, in contrast to its effect in yeast. The experiment would be more meaningful if it was repeated with optimum Cl-Tc treatment and multiple lines or the line with the highest protein level.

4.5 DISCUSSION

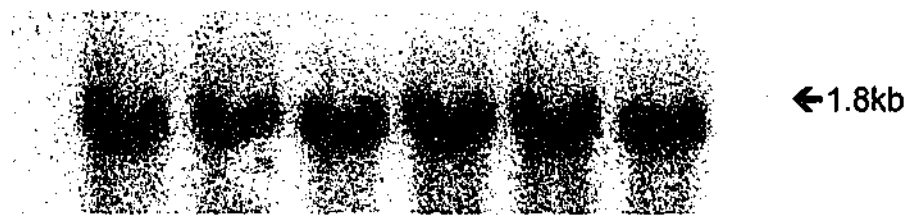
By inducing TXcdc2 seedlings and cultured roots, Cl-Tc was found to stimulate similar transcription levels to Tc. As it is reported as being less toxic to roots in particular (Böhner *et al.*, 1999), it was subsequently used as a preferable form of Tc inducer. Relative to *GUS*, only low levels of both forms of the *cdc2* mRNA were observed, indicating they are easily degraded. The lack of stability of the cell cycle gene mRNA may be a limiting factor in producing high levels of protein. An additional indicator of instability was the smears observed on *cdc2* probed Northern blots, although these may have been partly due to the second smaller transcript detected. In plants, RNA degradation products are rarely observed, although they are in yeast (Abler and Green, 1996), so the smears may indicate *in vitro* instability perhaps unrelated to *in vivo* properties. The discrete second smaller band of about 1.1kb seen in some samples is possibly due to early transcription termination. Plants respond to quite a range of poly(A) signals rather than one universal signal (Rothnie, 1996). The recognition of a signaling element in the 40bp of yeast sequence beyond the translation stop codon may have resulted in polyadenylation approximately 200bp prior to the *ocs* poly(A) signal and thus a shorter transcript.

Furthermore, only lines transcribing low levels of *cdc2-1w* in roots and seedlings were recovered, relative to those lines containing the TXcdc2 construct. Even a small amount of *cdc2-1w*, that is able to "leak" from the promoter, negatively affects regeneration. In

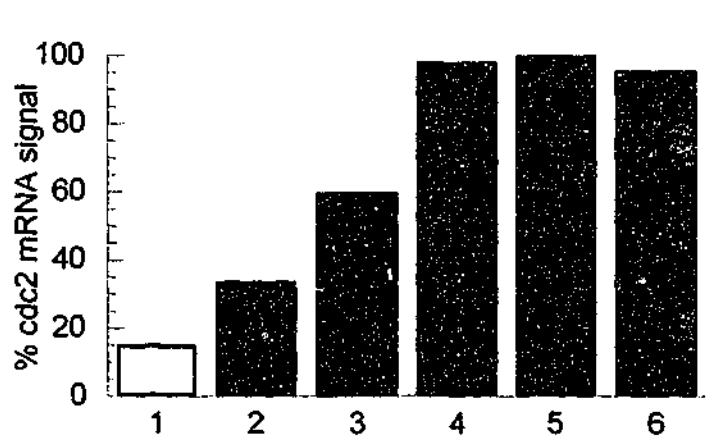
A. cdc2



B. ubiquitin



C. relative levels of cdc2 transcript



Lanes
1. no treatment
2. 0.05 mg/l Cl-Tc
3. 0.2 mg/l Cl-Tc
4. 1 mg/l Cl-Tc
5. 5 mg/l Cl-Tc
6. 1 mg/l Tc

Figure 4.9 Northern blot analysis of the induction of *cdc2* mRNA in the TXcdc2.15 rootline treated with Cl-Tc. Cultured roots were treated for 24 hours with 0, 0.05, 0.2, 1, 5 mg/l Cl-Tc and 1 mg/l Tc for comparison. Approximately 40 µg total RNA was loaded: **A.** probed with 1kb *BamHI-XhoI* fragment containing the full-length *cdc2* coding region **B.** probed with the ubiquitin coding sequence from *Antirrhinum majus* **C.** relative level of *cdc2* transcript standardised against the ubiquitin level in that sample.

A. Root tip width measurement

B. Mean root tip width ±Tc

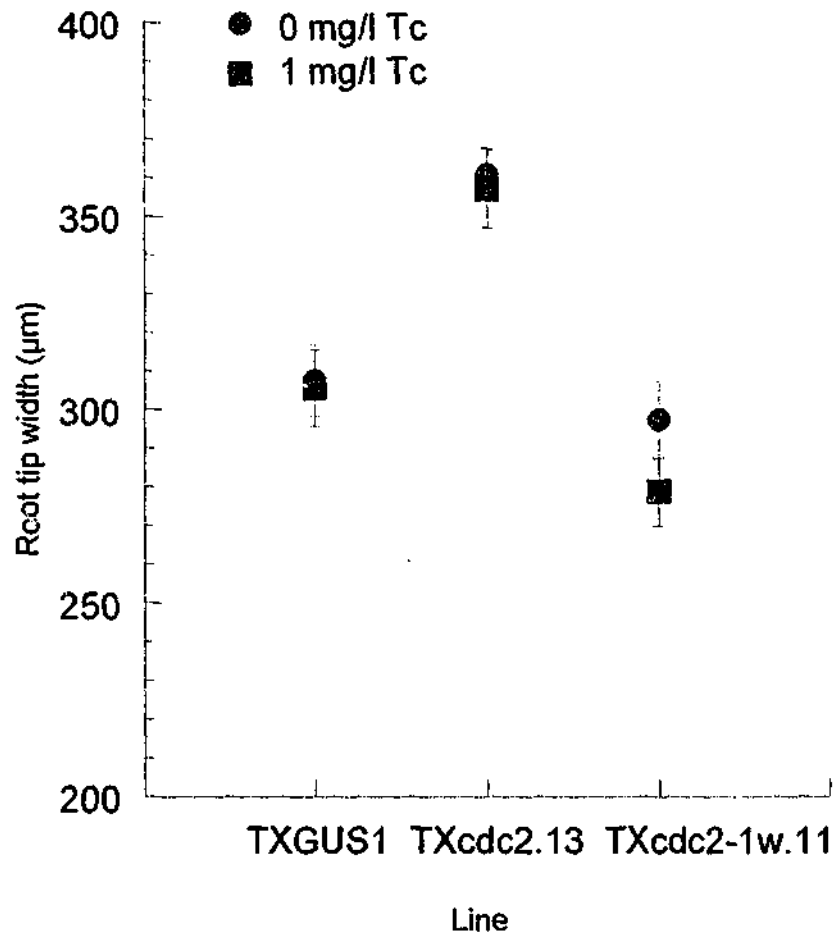


Figure 4.10 Mean (±SE) root tip width (µm) in uninduced (●) and induced (■) cultured roots. TXGUS1, TXcdc2.13 and TXcdc2-1w.11 actively growing 11 day old root cultures were treated with 1 mg/l Tc in modified MS for 4 days: **A.** measurements were made inside the perimeter of the region of dense cells at the base of the root tip **B.** duplicate cultures of each line were measured (n≥6) with a graticule at 64X magnification.

retrospect, evidence of such leakage may include slight symptoms of retarded regeneration, delayed seedling germination and difficult in maintenance of rootlines.

The phenotype of seedlings induced to ensure *cdc2-1w* overexpression is that of reduced LRP frequency in response to 10^{-4}M IBA, a trait not seen in lines overexpressing wild-type *cdc2* or *GUS* genes. Root meristem width in cultured roots remains unaffected by *cdc2-1w* expression. The TXcdc2-1w phenotype is analogous to those observed in tobacco plants overexpressing the dominant allele *cdc2aAT.N147*, a mutant form of *Arabidopsis* CdkA. In that study, shorter roots with reduced numbers of lateral roots and similar sized root tips with a disorganized cell structure were reported. Plant organ dimensions remained the same, despite the reduced cell number caused by blocking division (Hemerly *et al.*, 1995).

How *cdc2-1w* might then bring about these effects is unclear. The Cdc2-1w protein could be acting to disrupt mitosis or delay progression in root meristems by binding the regulatory proteins, thereby reducing plant CDK activity. Plant CDK proteins have been reported to behave this way in yeast (Porceddu *et al.*, 1999), as has Cdc2aAT.N146 in tobacco (Hemerly *et al.*, 1995). The yeast protein, based on literature sequence comparisons (Joubès *et al.*, 2000), presumably has enough homology to plant CdkA to have similar binding sites, if not full functionality. However, the conformation of the protein must also be interfering with mitosis in some way, rather than simply a titration effect, as the wild-type cdc2, Cdc2aAT and Cdc2aAT.AF do not have this phenotype (Hemerly *et al.*, 1995; De Veylder *et al.*, 2000). Perhaps, as the Cdc2-1w product is unresponsive to the wee protein, it is titrating all activating proteins and leaving an excess of unbound plant *wee1* homologue to retard the cell cycle. Although it is uncertain whether in yeast Cdc2-1w is not inactivated by Wee1 phosphorylation or is a poor substrate for the kinase (Russell and Nurse, 1987).

To understand the TXcdc2-1w phenotype, it is essential to determine the levels of Cdc-1w protein and CDK activity in induced plant tissue. However, it may be more illuminating to obtain more highly expressing lines in which to do this. Very recently it was reported that only lowly expressing tobacco plants containing the *cdc2aAt.N146* (*N147* or *DN*) under the Tc-inducible promoter could be recovered. As a consequence, CDK activity could be reduced, but not to a level sufficient to block cell division. It was suggested that counterselection against strongly inducible lines had occurred as a result of promoter leakage. And further suggested that the Triple-X promoter was particularly unsuitable for the overexpression of cell cycle genes due to its propensity to leak in the meristematic cells (De Veylder *et al.*, 2000).

While adverse interaction of the modified Cdc2-1w with cell cycle regulators is possible, it would be interesting to measure the sizes of equivalent cell types at mitosis in wild-type and transgenic roots. Mitosis is the likely time of maximum cell size difference if mitosis is advanced or retarded and McKibbin *et al.* (1998) were able to observe that mitosis was advanced relative to growth in roots of whole tobacco plants. It would also be interesting to measure, by sectioning, the number of cells that contribute to the root in wild-type and transgenic lines, since tobacco has large roots with an open organization of tissue layers that accommodate changes in the number of cells that comprise tissues. There might be a smaller number of larger cells in transgenic lines. Furthermore, if a suitable promoter became available, the *Arabidopsis* root, with its closed organization and defined tissue layers could more certainly give different root diameter with altered cell sizes.

Chapter 5
Overexpression of the *S. pombe cdc25* gene in roots and seedlings of *N. tabacum.*

5.1 AIM

In this section the introduction and inducible overexpression of the *cdc25* gene from *S. pombe* in *N. tabacum* (var Wisconsin 38) is reported. Effects on meristematic cell size and lateral root primordia are explored in order to assess the possible effects of the yeast gene on cell cycle progression in plants.

5.2 INTRODUCTION

For activation of Cdc2 kinase in all investigated organisms, the Tyr15 residue is required. This residue is located in the ATP-binding pocket of Cdc2 and is conserved in plant CDKs (Dudits *et al.*, 1998),. In metazoa the inhibitory phosphorylation is reinforced by an additional phosphorylation of the contiguous Thr14. This Thr14 is Also present in the plant enzyme but its phosphorylation has not been investigated.

5.2.1 The role of *cdc25* in Cdc2 regulation

The *cdc25* gene was first identified in *S. pombe* by temperature sensitive mutation that was found to be lethal due to cell cycle block at the non-permissive temperature (Fantes, 1979). *cdc25* has been overexpressed in fission yeast by two methods: by integrating multiple copies of the gene; and by fusing the gene to the strong alcohol dehydrogenase (*adh*) promoter of *S. pombe* (Russell and Nurse, 1986). Though both manipulations resulted in cell division at a smaller size than wild type due to mitotic advancement relative to cell growth, significantly neither was lethal.

Cdc25 is a tyrosine phosphatase which dephosphorylates Cdc2 Tyr 15 in late G2, in metazoa Cdc25C can also dephosphorylate Thr 14, to allowing progress into mitosis (Kumagai and Dunphy, 1991; Sebastian *et al.*, 1993). In *S. pombe* a significant contribution comes from another phosphatase Pyp3, the inactivation of which delays mitosis (Millar *et al.*, 1992). Negative regulation on Tyr15 in opposition to Cdc25 phosphatase activity is catalyzed by $p107^{wee1}$ (Russell and Nurse, 1986; Parker *et al.*, 1992) with a small but significant contribution from Mik1 in *S. pombe* (Lundgren *et al.*, 1991). *cdc25* and *wee1* act antagonistically to regulate mitotic timing through the phosphorylation of Cdc2 (reviewed yNurse, 1990).

5.2.1.1 Tyrosine phosphatases and plant Cdc2 regulation

The importance of tyrosine phosphorylation in Cdc2 regulation has been supported by experiments conducted with plant cells. Data suggest that Cdc25 phosphatase, or an enzyme with a similar function, may have a role to play in dephosphorylating Cdc2, as is the case in other eukaryotes. A high level of tyrosine phosphorylation was detected in Cdc2-like kinase isolated from G2-arrested tobacco pith and cell suspension culture lacking cytokinin. Cytokinin allowed resumption of the cell cycle and stimulated tyr-dephosphorylation. The inactive plant Cdc2-like kinase, isolated by $p13^{suc1}$ purification, could be activated *in vitro* by fission yeast Cdc25 phosphatase, suggesting that low activity without cytokinin was due to tyrosine phosphorylation (Zhang *et al.*, 1996).

In a similar experiment $p13^{suc1}$-isolated Cdc2-related kinase from alfalfa could be activated *in vitro* by recombinant *Drosophila* Cdc25. In alfalfa, fluctuation during the cell cycle was shown in the amount of phosphotyrosine residues on a protein with a molecular mass of Cdc2MsA/B (Cdc2A and B from *Medicago sativa* i.e. alfalfa). Reduced phosphotyrosine was associated at G2 with increased histone H1 activity indicating Cdc2 activity (Meszaros *et al.*, 2000).

A plant homologue of *wee1* has recently been identified in maize and shown to phosphorylate CDK *in vitro* (Sun *et al.*, 1999), strengthening the argument for the role of Tyr15 phosphorylation in plant CDK regulation.

5.2.2 *cdc25*

The *S. pombe cdc25* gene is 1.75 kb and encodes a 67 K phosphoprotein (Russell and Nurse, 1986). *cdc25* homologues have been recognized in many eukaryotes: *cdc25* in *Xenopus* (Kumagai and Dunphy, 1992), *MIH* in *S. cerevisiae* (Russell *et al.*, 1989), *string* and *twine* in *Drosphila* (Edgar and O'Farrell, 1989; O'Farrell *et al.*, 1989; Jiminez *et al.*, 1990) and *CDC25A*, *B* and *C* in both mice(Kakizuka *et al.*, 1992; Gautier, 1993) and humans (Sadhu *et al.*, 1990; Galaktionov and Beach, 1991; Millar *et al.*, 1991b; Nagata *et al.*, 1991). Human Cdc25 phosphatase is shown to be activated by N-terminal phosphorylation (Hoffmann *et al.*, 1993). Cdc25 phosphorylation can be catalyzed by Cdc2 thus a positive feedback loop is possible, as identified in *Xenopus* extracts, whereby both Cdc2 and Cdc25 are rapidly activated (Karaiskou *et al.*, 1999). However the initial impetus may come from other kinase activity acting on Cdc25, such as Polo kinase (Kumagai and Dunphy, 1996). Cdc25 is part of a checkpoint which delays entry into mitosis of cells containing damaged or unreplicated DNA in fission yeast and higher eukaryotes (Zeng and Piwnica-Worms, 1999). In operation of the checkpoint

Cdc25 activity is reduced, thus Cdc25 is a pivotal enzyme in cell cycle control, with complex patterns of phosphorylation and of binding associations with other proteins.

5.2.2.1 Cdc25 in plants

A plant *cdc25* gene has yet to be reported, although several sequences homologous to *cdc25* have been amplified in plants using PCR. A *cdc25*-like fragment appears to be present in both monocotyledonous and dicotyledenous plants and a small gene family, similar to that found in humans, has been suggested (Sabelli *et al.*, 1998). The overexpression of fission yeast *cdc25* in plants, may indicate if the regulation of plant Cdc2 via Tyr15 phosphorylation is rate limiting for initiation of mitosis, as found in yeast. When *cdc25* is overexpressed in yeast and human cells entry into mitosis is accelerated, resulting in cells that are smaller than normal (Russell and Nurse, 1986; Blomberg and Hoffmann, 1999; Karlsson *et al.*, 1999). If Cdc25 interacts with plant CDK in a similar fashion, a faster activation of CDK in the G2 phase would result in division taking place at a smaller cell size.

cdc25 from *S .pombe* has been expressed under a steroid inducible promoter in tobacco suspension cells (Zhang, personal communication). Upon induction with dexamethasone the culture was able to continue dividing independently of cytokinin, whereas, prior to transformation with *cdc25* the cell line had been both cytokinin and auxin dependent. This shows Cdc25 from yeast is functionally active in disorganized plant cell cultures and suggests that cytokinin acts through a protein phosphatase with function that overlap with Cdc25. In whole plants, yeast *cdc25* has been expressed in tobacco and has resulted in altered leaf morphology, smaller cells in the root meristem and precocious flowering (Bell *et al.*, 1993). A more detailed examination of the meristematic regions of a plant expressing *cdc25* would provide stronger evidence for the role of Tyr15 regulation of Cdc2 in plant cell division.

Previously it was reported that *cdc25* was expressed under a constitutive CaMV35S promoter. Low messenger RNA levels were observed (Bell *et al.*, 1993) and it is possible that only poorly expressing lines were recovered. In this study an inducible promoter was chosen to overexpress *cdc25*, as it was speculated that strong constitutive expression of *cdc25* may disrupt the capacity of transgenics to regenerate into plants. Thus transgenic plants containing an inducible promoter might have a stronger phenotype when induced.

The aims of the experiments reported in this chapter were to address the following questions:

1. Can *S. pombe cdc25* be expressed at high levels under the control of the Triple-X promoter in whole plants and in transformed cultured roots of *N. tabacum*?

2. What is the phenotype of root tissue overexpressing yeast *cdc25*? What impact does altering the cell cycle have on the root meristem?

3. Are plants amenable to the Cdc25 regulation of Cdc2? Is the control of Cdc2 by phosphorylation on Tyr15, that is observed in other eukaryotes, also detectable in plants?

5.3 MATERIALS & METHODS

5.3.1 Constructs

The 1.74kb coding sequence of the *S. pombe* wild-type *cdc25* gene was obtained as pMNS21 courtesy of Chris Norbury. To add compatible sites for insertion into pBTX the genes were subcloned into pBluescriptIISK+ on *NdeI-SmaI* where the *NdeI* site is at the initiating methionine. Sequencing confirmed the correct identity of the DNA fragments. The *cdc25* gene was placed under the control of the tetracycline inducible Triple-X promoter and *ocs* terminator by insertion in the Triple-X expression cassette on a *KpnI-XbaI* fragment (see Figure 5.1). The *cdc25* coding sequence was introduced by transformation into roots and plants in the pBIN19 based pBinHygTx (pBTX) vector.

5.3.2 PCR and RT-PCR

5.3.2.1 Primers

The oligonucleotides used as PCR primers for *cdc25* were:
cdc25 Forward Primer (25NF): 5'-CTTCTGGTGGACAAGCTACCCGCC-3'
cdc25 Reverse Primer (25NR): 5'-GCCTTGGTGCAGGTCATAACATGCG-3'
This primer pair amplifies a 1006bp fragment from positions 649-1655 in the *cdc25* gene.

The oligonucleotides used as control primers for RT-PCR were
nptII Forward Primer (nptIIA): 5'-GAGGCTATTCGGCTATGACTG-3'
nptII Reverse Primer (nptIIB): 5'-ATCGGGAGCGGCGATACCGTA-3'
This primer pair amplifies a 700bp fragment from positions 201-900 in the *nptII* gene.

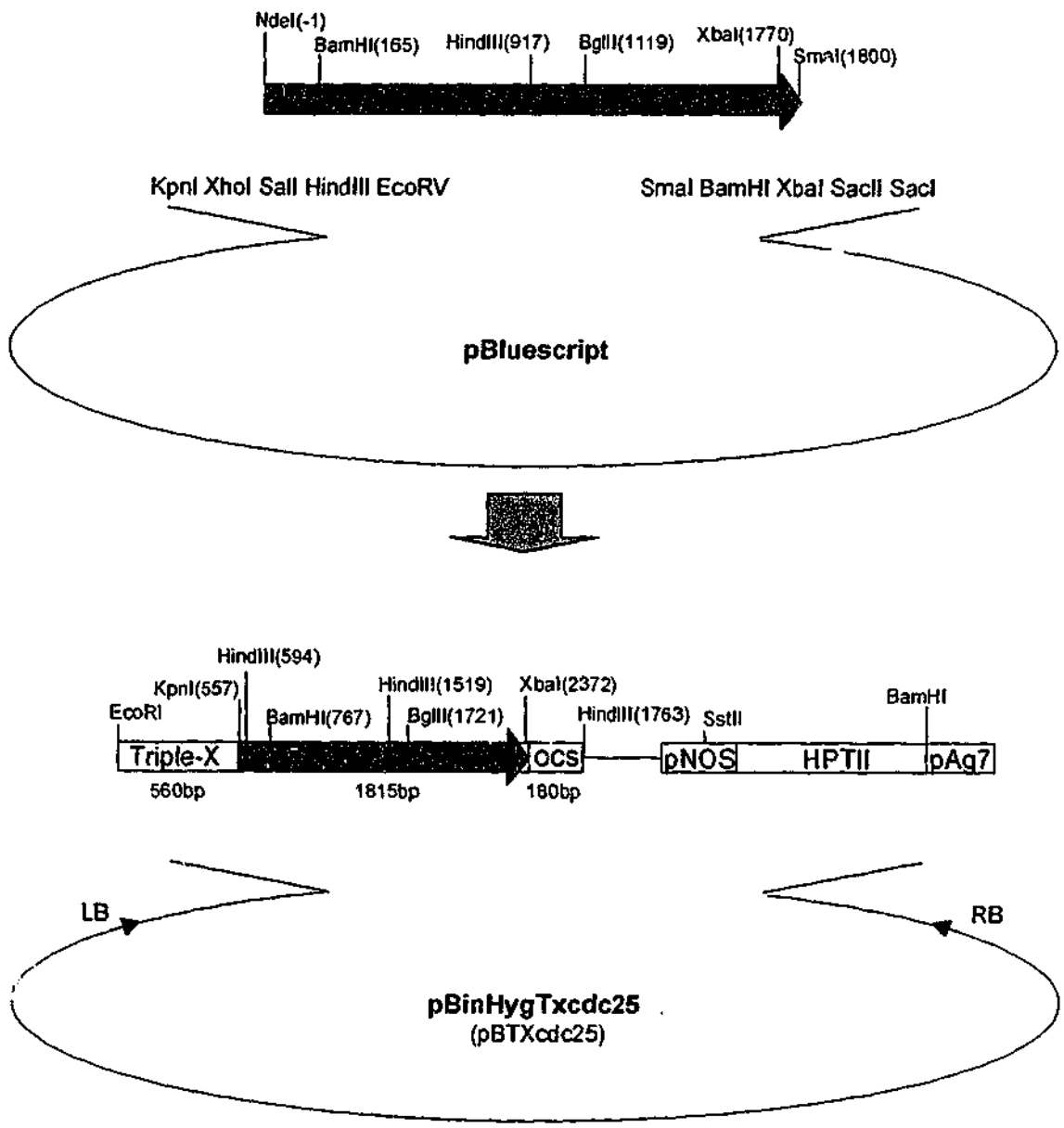


Figure 5.1 Structure of plasmid construct pBTXcdc25. The *cdc25* gene was inserted between the Triple-X promoter and the octopine synthase (*ocs*) polyadenylation signal. The selectable marker gene shown is hygromycin phosphotransferase (*HPTII*). RB, right border; LB, left border. Not to scale.

5.3.2.2 Solid phase cDNA libraries for RT-PCR

Solid state cDNA libraries were made from 15 µg of total RNA isolated from seedlings and cultured roots induced with 1 mg/l Tc for 1 day. 10 µl Dynabeads® oligo$(dT)_{25}$ were used to isolate mRNA for each sample according to the manufacturers instructions (Dynal).

First-strand cDNA was synthesized on to the beads in a final reaction mix of 1x buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 1 mM Dithiothreiotol, 10mM of each dNTP, RNase inhibitor and 200 units of SuperScript II RNase H$^-$ Reverse transcriptase. The 5x buffer, 0.1 M DTT and SuperScript II were purchased from GibcoBRL and used according to their instructions. Beads bound to mRNA were resuspended in final reaction mix. Reaction was incubated for 50 minutes at 42°C.

Reaction mix was removed from the beads using the magnet. 10 mM Tris, pH 7.5 was added and heated for 1 minute at 95°C to elute mRNA. Solid phase cDNA library was washed and stored in 50 µl TB. 25 ul of library was used per PCR reaction with the remaining 25 µl amplified with control primers. PCR was also performed on mRNA eluate to discount the possibility of DNA contamination.

5.3.2.3 RT-PCR

All PCR reactions were carried out using standard PCR mix containing 10 mM dNTPs, 3 mM MgCl, 1x reaction buffer, 1.5 units of *Taq* polymerase (Promega) and 50-100ng of primer per 50µl reaction. Solid-phase cDNA libraries were washed in 1x reaction buffer prior to addition of PCR mix and either removed after the first cycle for re-use or left for the duration.

The PCR regime consisted of:
For 25NF-25NR primer pair: 1 cycle, 92°C 1 min, 63°C 1.5 min, 72°C 5 min, 30 cycles, 92°C 1 min, 63°C 1.5 min, 72°C 2 min
For nptIIA-nptIIB primer pair: 1 cycle, 92°C 1 min, 57°C 1.5 min, 72°C 5 min, 30 cycles, 92°C 1 min, 57°C 1.5 min, 72°C 2 min

5.3.3 The isolation of genomic DNA

Genomic DNA was isolated from 5 g of plant material. The tissue was ground to a fine powder in liquid nitrogen. The frozen powder was then transferred to a Root Buffer (0.1 M EDTA, 3x SSC, 0.1 M Sodium Diethyldithicarbamate, pH 7) and 2 ml 10%(w/v) SDS which was heated to 65°C. The mixture was homogenized by vortexing for 30 seconds before the addition of 10 ml phenol:chloroform:isoamylalcohol (25:24:1). The

plant cell debris was pelleted by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 15 minutes at 4°C. 10 ml of chloroform:isoamylalcohol (24:1) was added to the supernatant and centrifuged as above. DNA was recovered from the aqueous phase by overnight ethanol precipitation at -20°C and subsequent centrifugation in the Sorval SS-34 rotor at 10,000 rpm for 20 minutes at 4°C. the pellets were dissolved in 9 ml of TE buffer and transferred to 10 ml polypropylene tubes. Caesium chloride 1 g/ml and 500 µl of ethidium bromide 10 mg/ml was added. The solution was carefully added into opaque Beckman quickseal centrifuge tubes and centrifuged in the ultracentrifuge, model L8-80M, using an 80Ti rotor at 40,000 rpm for 64 hours at 18°C. DNA bands were visualised under long-wave ultra-violet light, and plant DNA extracted using a 2 ml syringe. Ethidium bromide was removed from the DNA with the addition of an equal volume of water-saturated n-butanol. This step was usually carried out three times. The solutions were made up to 5 ml with sterile Milli-Q water and transferred to glass Corex tubes. 2 volumes of absolute ethanol and 10% by volume of 3M sodium acetate, pH 6 was added and the samples cooled to -20°C for 1 hour, before centrifugation in the Sorvall SS-34 rotor at 10,000 rpm for 20 minutes at 4°C. Pellets were dissolved in 0.5 ml of sterile Milli-Q water, ethanol-precipitated overnight at -20°C and DNA recovered by centrifugation at 14,000 rpm for 30 minutes. Pellets were given a final wash with 70% ethanol, before being dissolved in 100 µl TE.

5.3.4 Western Analysis

5.3.4.1 Protein extraction from cultured root tissue of tobacco

Roots were blotted dry and then ground in liquid nitrogen using a mortar and pestle. Protein was extracted from 0.1 g the resulting powder by vigorously mixing 100 µl of RIPA bufferpH 7.4 for 3 x 20 seconds at 0°C. RIPA buffer consisted of 20 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 0.1% Tween-20, 1 mM dithiothrietol (DTT), 10 µM lepeptin, 10 µM pepstatin, 10 µM NaF, 1 mM EGTA, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate to which was added immediately before use 1 mM sodium orthovanadate and 200 µM phenylmethylsulfonyl fluoride (PMSF). The protein extract was centrifuged at 4000 rpm for 5 minutes in a microfuge. For electrophoresis was used.

5.3.4.2 SDS-acrylamide gel electrophoresis

12% acrylamide gel contained 9 ml 40% acrylamide, 1.07% bis-acrylamide, 5.55 ml of H_2O, 15 ml 0.75 M Tris-HCl pH 8.8, 300 µl of 10% sodium dodecyl sulfate (SDS), 15 µl TEMED and 113 µl of 15%(w/v) ammonium persulphate.

4% stacking gel contained 1.5 ml 40% acrylamide, 1.07% bis-acrylamide, 11.4 ml of H_2O, 1.8 ml 0.75 M Tris-HCl pH 6.8, 150 µl of 10% sodium dodecyl sulfate (SDS), 15 µl TEMED and 37.5 µl of 15%(w/v) ammonium persulphate.

Sample was mixed with an equal volume of SDS sample buffer (Sx2), which contained 0.125 M Tris-HCL pH 6.8, 4%(w/v) SDS, 20%(v/v) glycerol, 2%(v/v) β-mercaptoethanol and 0.0002% bromophenol blue.

Electrophoresis buffer consisted of 25 mM Tris-HCl pH 8.3, 193 MM glycine and 0.1% SDS. Current was held at 25 mA per gel during stacking and increased to 35 mA per gel when the dye front reached the separating gel. Electrophoresis was stopped when the dye was within 5-10 mm of the bottom of the gel

5.3.4.3 Western blotting and immunodetection in blots

The conventional methods of western blotting were adopted (Towbin *et al.*, 1979).

Buffers:

Transfer buffer (TB) contained 25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3.

Antibody washing buffer (TBS 20) contained 10 mM Tris-HCl pH 7.4, 0.15 M NaCl and 0.05% Tween-20.

Blocking solution contained 10 mM Tris-HCl pH 7.4, 0.15 M NaCl and 5% skim milk powder.

Antibody dilution buffer (TBSBT) contained 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Tween-20, 1% bovine serum albumin (BSA), 0.05% sodium azide.

Alkaline phosphate substrate buffer contained 0.1M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2.6H_2O$.

Alkaline phosphate substrate was prepared by adding 5 ml alkaline phosphatase substrate buffer to 22 µl of nitroblue tetrazolium chloride (NBT) solution, which is 100 mg in 1.3 ml 70% dimethylformide, and 16 µl of 5-bromo-4-choro-3-indolylphosphate p-toluidine salt (BCIP) solution, which 100 mg in 2 ml 100% dimethylformamide

Proteins were transferred for 6 hours at 180 mA onto 0.45 micron nitrocellulose or PVDF membrane. The nitrocellulose was immersed in Ponceau S solution (0.4% Ponceau S in 3% TCA) for 15-30 minutes and unbound stain rinsed off with H_2O twice

until the background of the nitrocellulose was white then photographed using Tech-pan Kodak film with green filter.

Nitrocellulose was put in blocking solution for at least 1 hour with shaking then rinsed 3x 5 minutes in antibody washing buffer with shaking. After blocking nitrocellulose was overlaid at room temperature for 1-2 hours with anti-Cdc25 or anti-PSTAIRE antibody solution diluted to 1:500 relative to the concentration of the original serum. The unbound first antibody was rinsed off by 3x 10 min shaking in antibody washing buffer, which for the first two washes contained 0.05% Tween20 in TBS and for the last wash contained 0.2% Tween20 in TBS.

After reaction with the first antibody, the nitrocellulose was washed with TBS 20 and put incubated with ^{125}I anti-rabbit-IgG antibody produced in mouse (Amersham) diluted to 0.5 µci/ml with TBSBT for 2 hours. The unbound second antibody was washed off by 3x 10 min with 0.2% Tween20 in TBS with shaking. The nitrocellulose was dried and an image of the radioactivity was obtained by exposure in a phosphorImager (Molecular Dynamics).

5.3.5 Sectioning and Cell Size Measurement

5.3.5.1 Sectioning method

Root cultures of the lines TXGUS1 and TXcdc25.5 were treated with 1 mg/l Tc in modified MS media for 4 days as described in Chapter 2 Materials & Methods Section 2.4.1.3. Root tips were then excised, fixed in 4% paraformaldehyde overnight and embedded in PEG.

Specimens were allowed settle in gradually increasing concentrations of PEG. At room temperature PEG400: 10%, 20%, 30%, 40%, 50%, 70%, 90%, 100%(x3). Then at 50 C PEG1000(x4), PEG1450(x4). Then into 20% PEG1000/80% PEG1450. After embedding in moulds, samples were stored and desiccated at 0-4°C.

A ribbon of sections was cut to approximately 10 µm on a microtome at 16 C. The ribbon was mounted on glass slides shiny side down and dried sections were rinsed with distilled water before histochemical staining in 0.2 µg/ml DAPI. For detection of DNA via DAPI staining PEG was preferred to paraffin as embedding medium since paraffin solvents result in poor DAPI staining.

The series of sections constituting an entire root tip were viewed and photographed under fluorescence at 100x magnification with a Nikon Optiphot microscope using a standard UV filter set. Pictures were taken on Kodak Tmax 100 film. Each mitotic cell

identified was also photographed under 250x magnification with combined fluorescence/Differential Interference Contrast to visualise cell walls and cell areas were estimated from the resulting photograph. It was ensured that each mitotic cell was only measured once even if it appeared in more than one section. The most median section through each cell was selected for measurement and the average diameter was measured. Where the cell was an irregular shape the maximum and minimum diameters were measured and an average calculated. As cell wall thickness was negligible measurements were taken using the walls as the outer markers.

5.4 RESULTS

5.4.1 Introduction of *cdc25* into plants and root cultures

5.4.1.1 Transformation of plants with *A. tumefaciens*

Leaf disc transformation was performed on *N. tabacum* (var Wisconsin 38) tetR plants with *A. tumefaciens* containing the *cdc25* gene under the Triple-X promoter. Selection on hygromycin yielded nine putative *cdc25* transgenic lines These independent TXcdc25 transformants were healthy and normal in appearance, although the TXcdc25 callus, like TXcdc2-1w, was slightly slower to regenerate shoots than either the TXGUS or TXcdc2 callus. Each transformant was self-pollinated and the seed collected from the nine lines. Seeds of F1 were surface sterilized and germinated on medium containing 50 µg/ml hygromycin. Some lines showed low germination rates which is reflected in the total number of seedlings counted. The ratio of resistant to non-resistant seedlings did not deviate significantly from 3:1 for six of the nine lines (Figure 5.2). Line TXcdc25.G showed 100% hygromycin sensitivity and TXcdc25.H showed a 2:1 ratio so neither line was regarded as having a incorporated a single copy of *TXcdc25*. Line TXcdc25.P has a ratio of resistant to non-resistant seedlings that does not deviate significantly from 15:1 ($\chi 2$=0.002, P>0.90). A Mendelian ratio which suggests two copies of the gene were inserted. This line displayed low viability and fertility and no seed could be obtained in the F2 generation.

5.4.1.2 Transformation of root cultures with *A. rhizogenes*

Leaf inoculations, performed on *N. tabacum* (var Wisconsin 38) tetR plants, with *A. rhizogenes* resulted in the co-transfer of binary vector T-DNA and the transgene into emerging roots. Eight root lines showing hygromycin resistance were established from single root tips.

LINE	SENSITIVE	RESISTANT	TOTAL	*P*
TXcdc25.A	33	96	129	>0.05
TXcdc25.B	37	86	123	>0.05
TXcdc25.D	23	58	81	>0.05
TXcdc25.E	27	90	117	>0.05
TXcdc25.F	30	79	109	>0.05
TXcdc25.G	41	0	41	-
TXcdc25.H	40	82	122	<0.05*
TXcdc25.K	8	40	48	>0.05
TXcdc25.P	2	33	35	<0.01*

Figure 5.2 Hygromycin selection of F1 seedlings. Segregation analysis of self fertilized TXcdc25 transformants on 50 μg/ml Hyg. Proposed ratio of 3:1 resistant:sensitive seedlings. Levels of significance are indicated by the Contingency $\chi 2$ test.

5.4.1.3 Genetic confirmation of transgenic lines

To determine whether both the plants and the rootlines contained the *cdc25* gene they were subject to PCR analysis (Figure 5.3). Six of the nine original hygromycin selected plants and seven of the eight hygromycin selected root lines showed the presence of the *cdc25* gene.

5.4.2 Transcriptional induction of *cdc25* in seedlings

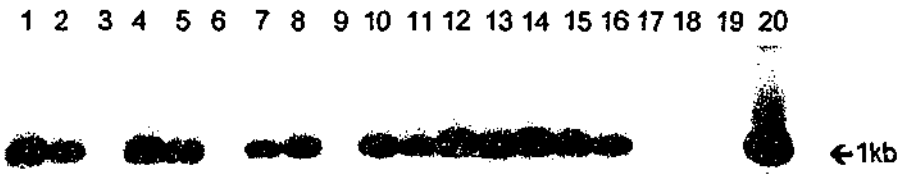
5.4.2.1 Southern blotting

A Southern blot was performed on the independently transformed TXcdc25plants. to determine that the transgene was present and potentially available for transcription. A *cdc25* fragment of approximately 1.8kb was observed in these lines (Figure 5.4B), indicated that these lines also had the full length *cdc25* gene integrated into their genomes. Figure 5.4 shows that these seedlings varied in the number of copies present in the genome. Allowing for slight variations in DNA loading and enzyme digestion, band number and band intensity indicate that lines TXcdc25.B and H contain a single insertion of cdc25 and lines A, E and F have two or more sites of insertion.

5.4.2.2 Northern Blot

Northern blots were also performed on the induced TXcdc25 seedling lines without success. No mRNA of the expected 2kb size could be detected, although from the gel of total RNA and the control probe used for the *ADC* gene it can be seen in Figure 5.5C there is a considerable amount of RNA present on the blot. This indicates that poor total RNA preparation was not responsible for a lack of detectable *cdc25* signal. The *ADC* gene is involved in polyamine synthesis and expressed in dividing cells so additionally confirmed the roots were metabolically active and undergoing division (Cohen *et al.*, 1982). Unlike *ODC* and *ubiquitin*, the transcript size of *ADC* was not similar to that expected from *cdc25* so any residual signal left after stripping would not obscure the results of *cdc25* reprobing.

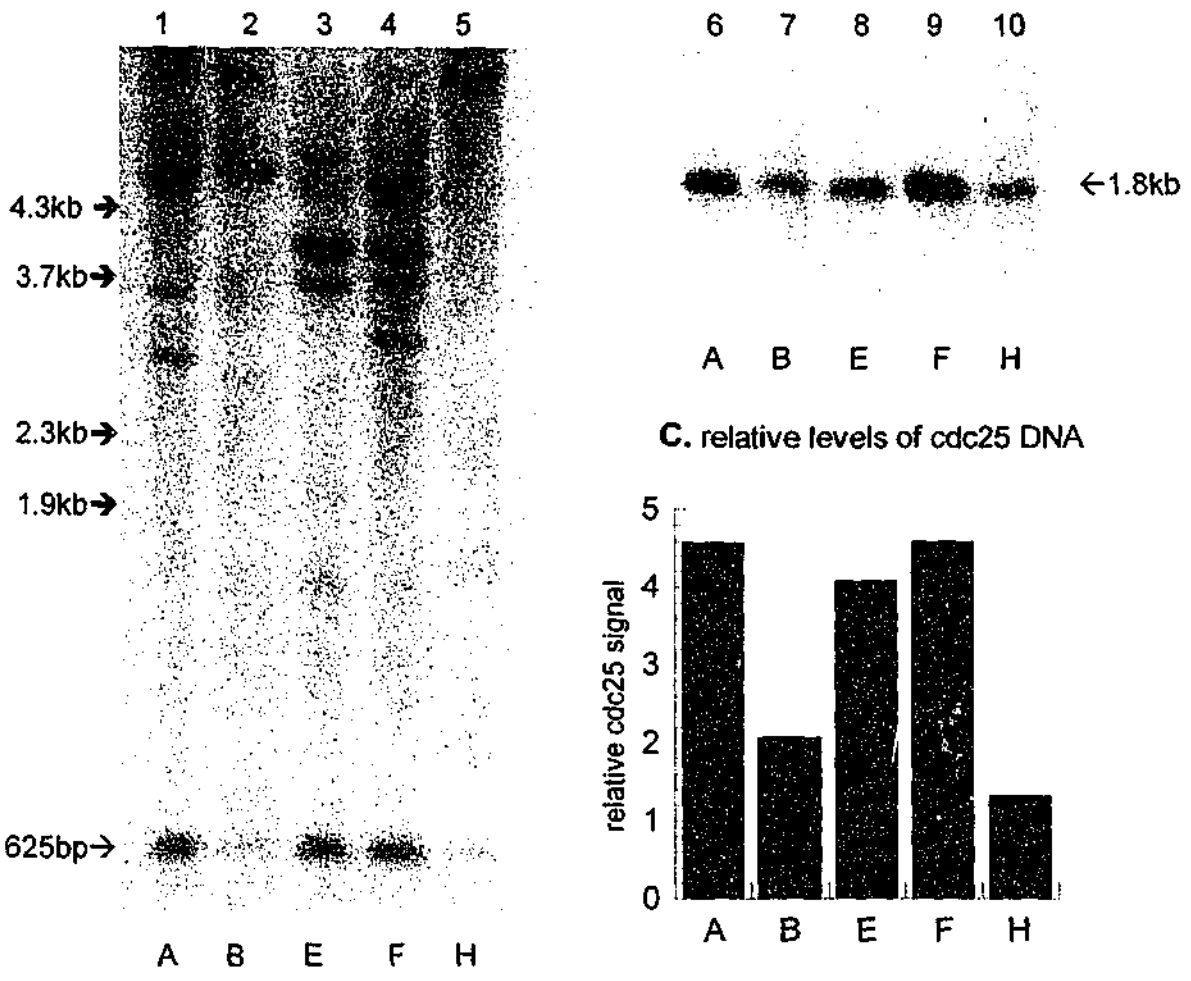
The reason why no *cdc25* transcript could be detected using the Northern hybridization technique appear likely to be due to the nature of the gene itself, rather than poor technique or choice of lines to analyze. Identical induction conditions were used to those that successfully induced both TXGUS and TXcdc2 rootlines. Variations in the induction conditions and RNA extraction techniques did not improve the Northern result. Millar et al. (Millar *et al.*, 1991b) note that in order to detect *cdc25* mRNA levels in *S. pombe* their autoradiographs were exposed 100 times longer than when detecting *adh* in the same samples. The fission yeast *cdc25* mRNA may therefore be very labile.



Lanes

seedling lines	root lines	controls
1. TXcdc25.A	10. TXcdc25.1	18. TXGUS.13
2. TXcdc25.B	11. TXcdc25.5	19. λ *BstEII* standard
3. TXcdc25.D	12. TXcdc25.5.2	20. BScdc25
4. TXcdc25.E	13. TXcdc25.10	
5. TXcdc25.F	14. TXcdc25.11	
6. TXcdc25.G	15. TXcdc25.12	
7. TXcdc25.H	16. TXcdc25.13	
8. TXcdc25.K	17. TXcdc25.14	
9. TXcdc25.P		

Figure 5.3 PCR analysis of hygromycin resistant root lines and plants for the presence of the *cdc25* gene. DNA was extracted as described in section 2.3.5.3 with TXGUS13 seedlings as a plant control. Using the 25NR and 25NF primer pair, a band of 1kb indicated the presence of the *cdc25* gene. This was confirmed by Southern blotting the PCR gel and probing with an 890bp *HindIII-BamHI* fragment from the 3'-end of the gene.

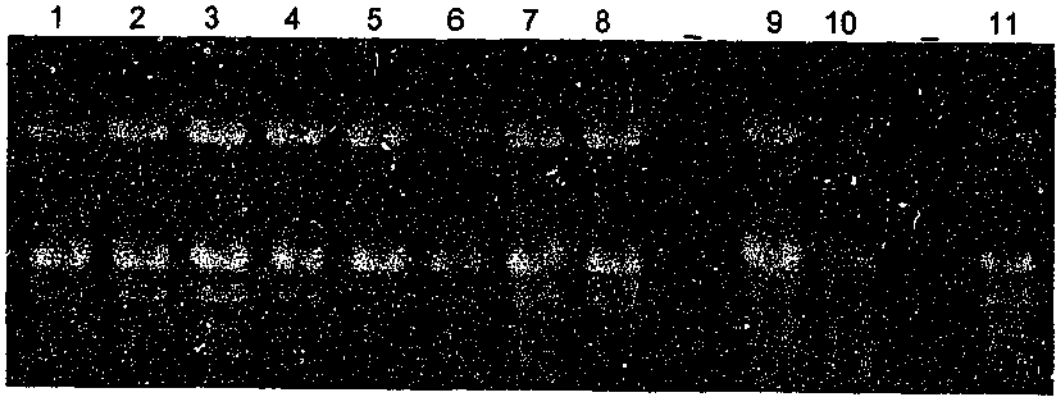


Lanes

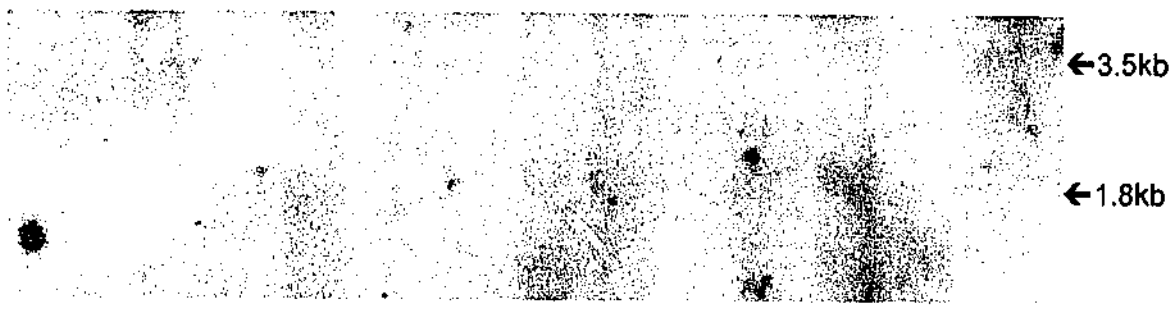
1. *BglII-XbaI* digest TXcdc25.A
2. *BglII-XbaI* digest TXcdc25.B
3. *BglII-XbaI* digest TXcdc25.E
4. *BglII-XbaI* digest TXcdc25.F
5. *BglII-XbaI* digest TXcdc25.H
6. *KpnI-XbaI* digest TXcdc25.A
7. *KpnI-XbaI* digest TXcdc25.B
8. *KpnI-XbaI* digest TXcdc25.E
9. *KpnI-XbaI* digest TXcdc25.F
10. *KpnI-XbaI* digest TXcdc25.H

Figure 5.4 Southern blot analysis of TXcdc25 seedlings. 20 μg of genomic DNA isolated from stable non-segregating F2 seedlings was loaded and the blot probed with a *KpnI-XbaI* fragment containing the full length *cdc25* coding region **A.** *BglII-XbaI* digest to determine the number of *cdc25* integrations **B.** *KpnI-XbaI* digest to determine *cdc25* insert size **C.** relative levels of *cdc25* DNA signal indicating copy number of *cdc25* gene.

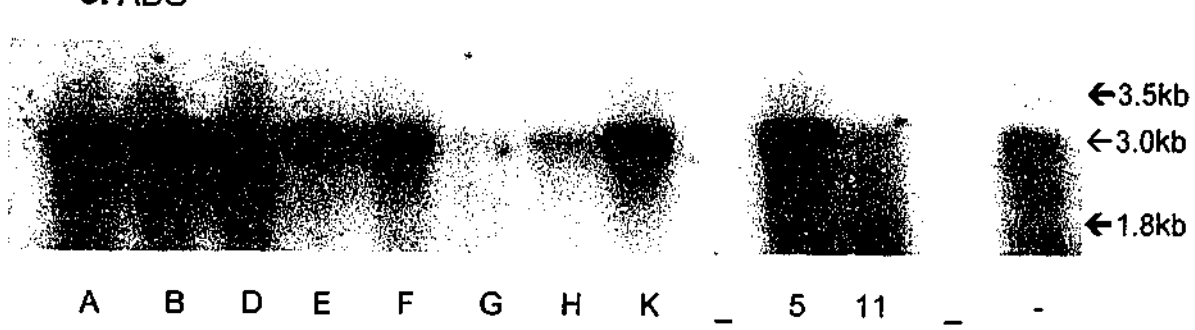
A. gel



B. cdc25



C. ADC



Lanes

1. TXcdc25.A
2. TXcdc25.B
3. TXcdc25.E
4. TXcdc25.F
5. TXcdc25.G
6. TXcdc25.H
7. TXcdc25.K
8. TXcdc25.5
9. TXcdc25.11
10. TXGUSA

Figure 5.5 Northern blot analysis of the induction of *cdc25* mRNA in TXcdc25 seedlings and cultured roots. 10 day old seedlings and rapidly growing root cultures were treated in 1 mg/l Tc for 24 hours in modified MS **A.** 40 µg total RNA was loaded **B.** probed with an 890bp *HindIII-BamHI* fragment from the 3'-end of the *cdc25* gene **C.** probed with 500bp *XbaI* fragment of the *ADC* gene from *N. tabacum*.

K. Zhang reports the ability to detect only a very faint *cdc25* mRNA signal from tobacco suspension cultures expressing the fission yeast gene driven by a dexamethasone-inducible promoter (personal communication). When *cdc25* is expressed in tobacco plants and roots, mRNA levels undetectable on a Northern blot have been reported (Bell *et al.*, 1993; McKibbin *et al.*, 1998).

5.4.2.3 RT-PCR

As induced *cdc25* mRNA levels appear to be very low, although the construct appeared intact and the induction system functional, the more sensitive RT-PCR was used to detect *cdc25* mRNA. Figure 5.6 shows four of the eight transgenic whole plants tested clearly expressing *cdc25* message. Southern blotting and probing of the RT-PCR gel confirms that the band shown is indeed *cdc25* and that two other samples may contain some *cdc25* mRNA. The control RT-PCR primers were for the *nptII* gene which should be present in all lines as the selection gene co-transformed with *tetR* (see Figure 3.2). These TX transformants were created in a tobacco line already highly expressing the *tetR* gene coding for the Tc repressor protein.

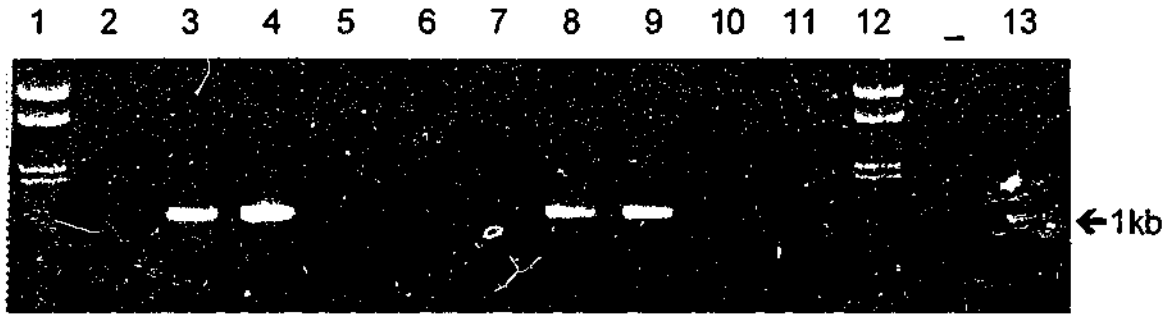
5.4.3 Frequency of Lateral Root Primordia

Lateral roots originate from the pericycle, the cells of which are expressing Cdc2, from evidence of promoter-GUS expression (Hemerly *et al.*, 1993), but the cells are believed to be paused in the cell cycle at G2 phase. If Cdc2 activation is required to initiate a new primordium, then the overexpression of Cdc25 in the pericycle may result in an increased activation of Cdc2 and a corresponding increase in lateral root primordia (LRP).

To determine the effect of the overexpression of *cdc25* on the initiation of root meristems, the frequency of emerging lateral root primordia along the primary seedling root was assessed. 11 day old homozygous transgenic seedlings were treated for 72 hours with the auxin K-IBA (indole-3-butyric acid): 0, 10^{-6}, 10^{-5} or 10^{-4} M ±2 mg/l Tc. Measurements were calculated in terms of lateral root primordia per cm of primary root length. The entire primary root length was measured and lateral root primordia counted and due to some variation in primary root length it was considered more accurate to express this measurement as a frequency.

In Figure 5.7 it can be seen that lines TXcdc25.B and TXcdc25.E showed growth responses at 10^{-4} IBA that were significantly different from those observed in the GUS control line, as determined by Tukey's HSD statistical test ($p>0.05$). Consequently line TXcdc25.B was selected for further experimentation. The frequency of lateral root primordia increased more markedly above control levels when a treatment of a high

A. gel



B. cdc25

C. nptII

St A B D E F G H K GUS -

Lanes

1. λ*BstEII* standard
2. TXcdc25.A
3. TXcdc25.B
4. TXcdc25.D
5. TXcdc25.E
6. TXcdc25.F
7. TXcdc25.G
8. TXcdc25.H
9. TXcdc25.K
10. Control TXGUSA
11. Control no RT
12. λ*BstEII* standard
13. Control BScdc25

Figure 5.6 RT-PCR analysis of TXcdc25 seedlings. A solid phase Dynabeads® cDNA library was prepared from total RNA of induced TXcdc25 seedlings. This library was used as a template in PCR reactions **A.** gel of PCR with *cdc25* NR and NF primers **B.** Southern Blot of the PCR gel probed with a 890bp *HindIII-BamHI* fragment from the 3'-end of the *cdc25* gene **C.** gel of PCR *nptII* A and B primers.

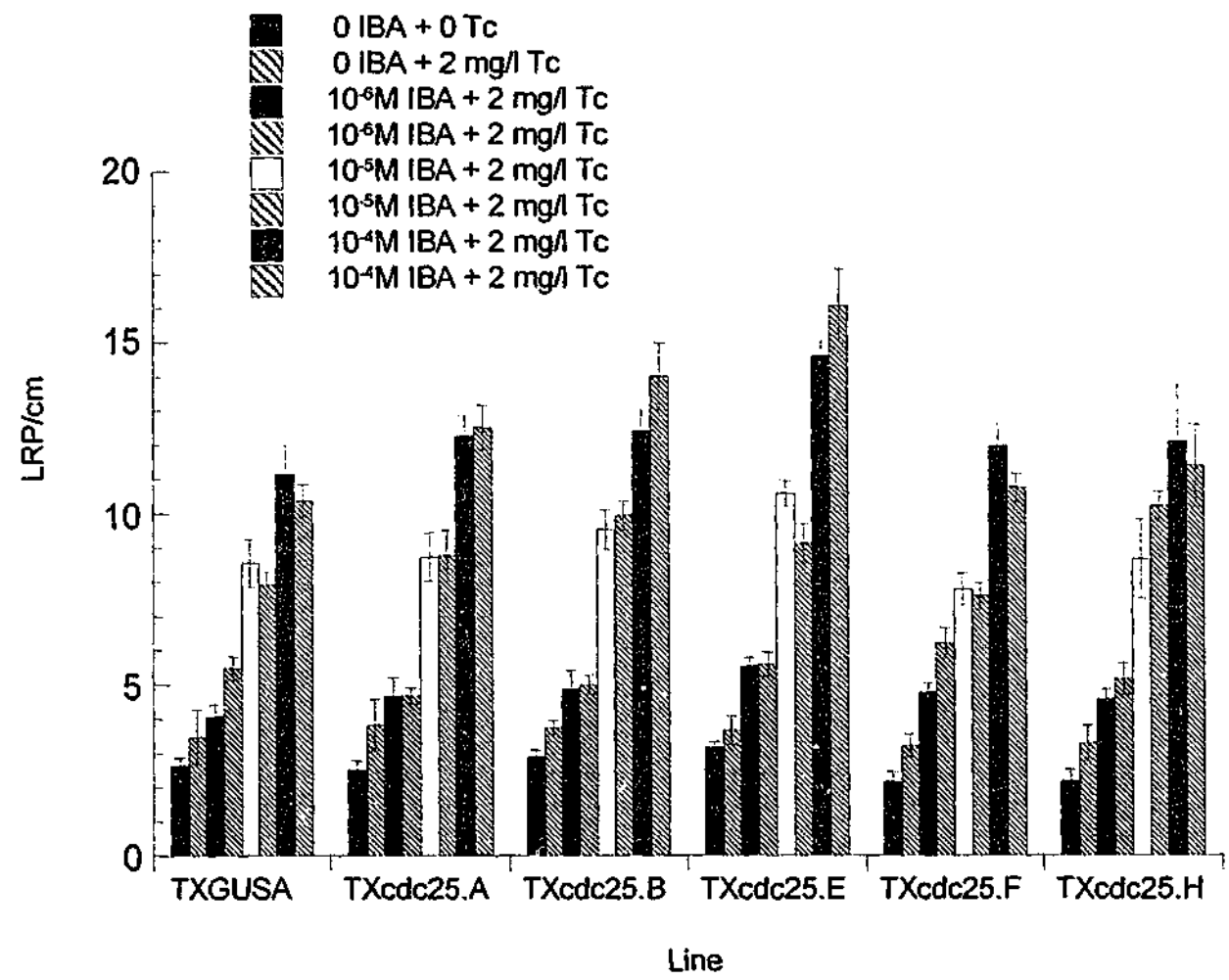


Figure 5.7 Mean frequency of lateral root primordia (±SE) in uninduced and induced seedlings. TXGUSA and TXcdc25 11 day F2 seed lines, pure breeding for hygromycin resistance, were transferred to modified MS containing 0, 10^{-6}, 10^{-5} and 10^{-4} M K-IBA ±2 mg/l Tc. After 72 hour treatment the seedlings were treated in 2% Chromium trioxide to reveal LRP (Pelosi *et al.*, 1995). The frequency of LRP per cm primary root length was measured (n≥8).

concentration of Tc (5 mg/l) was employed (Figure 5.8). In contrast TXGUSA control lines showed a 10% reduction in LRP in response to 5 mg/l Tc , possibly as a result of slight toxicity. No difference in the frequency of LRP was observed in roots treated with levels of K-IBA below 10^{-5}M. No root significant difference in length between treated and untreated roots was observed for the same auxin concentration. Cdc25 is possibly increasing the division events along the pericycle by increasing the level of active plant Cdc2. However, it has been frequently reported from the effects of exogenous auxin and the properties of the *SUPERROOT* mutation in *Arabidopsis* that the presence of auxin is also a limiting factor in the initiation of lateral roots (Boerjan, 1995).

5.4.4 Transcriptional induction of *cdc25* in cultured roots

As auxin stimulates or highlights the effects of Cdc25 in roots, independently cultured roots were investigated as a suitable experimental system to further ascertain effects of TXcdc25 on root meristems. Cultured transgenic roots are generated by transformation with Ri T-DNA from *A. rhizogenes* in addition to the Triple-X construct. Genes contained in the Ri T-DNA influence the plant metabolism to stimulate optimum root growth (Hamill and Lidgett, 1997). Thus reducing the possibility of root growth limiting factors obscuring the induced TXcdc25 phenotype.

5.4.4.1 Southern blot

To determine the size and copy number of the *cdc25* gene integrated into their genomes, a Southern blot was performed on TXcdc25 rootlines. A *cdc25* fragment of approximately 1.8kb was observed in these lines, the size of the full length coding sequence (Figure 5.9B). As all lanes contained an approximately equal amount of DNA. Estimation of total signal indicates relative numbers of the *cdc25* transgene (Figure 5.9C) and shows between 1 and 8 copies of *cdc25* are present in the transformed roots. There is a general correlation with the number of sites of integration (Figure 5.9A) except that TXcdc25.1 presumably has multiple insertions at a single site. Reports indicate that multiple copies of binary vector T-DNA and Ri- T-DNA may be present in transformed root lines (Hamill *et al.*, 1990).

5.4.4.2 RT-PCR

RT-PCR was used to detect *cdc25* mRNA in induced TXcdc25 rootlines. In Figure 5.10, five of the eight root lines tested showed the presence of *cdc25* mRNA – TXcdc25 lines 1, 5, 5.2, 11 and 12. Root line TXcdc25.11 is no longer expressing *nptII*, however the control was designed to show that isolation of cDNA was successful and this is apparent from the positive *cdc25* result.

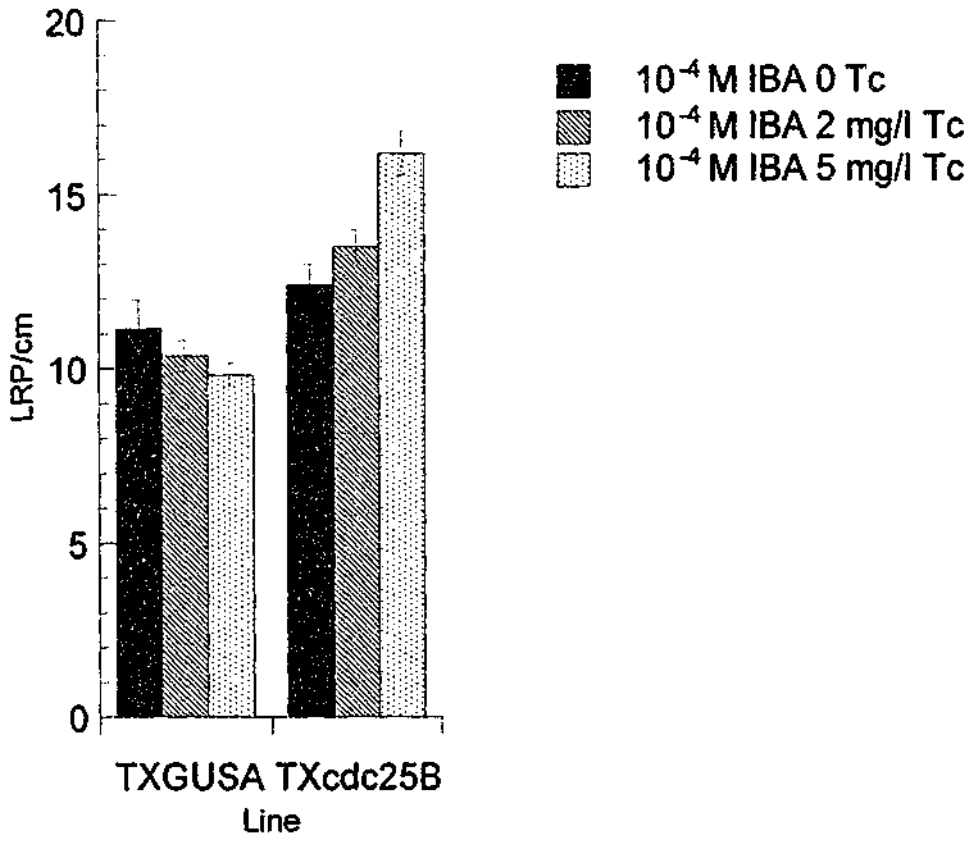
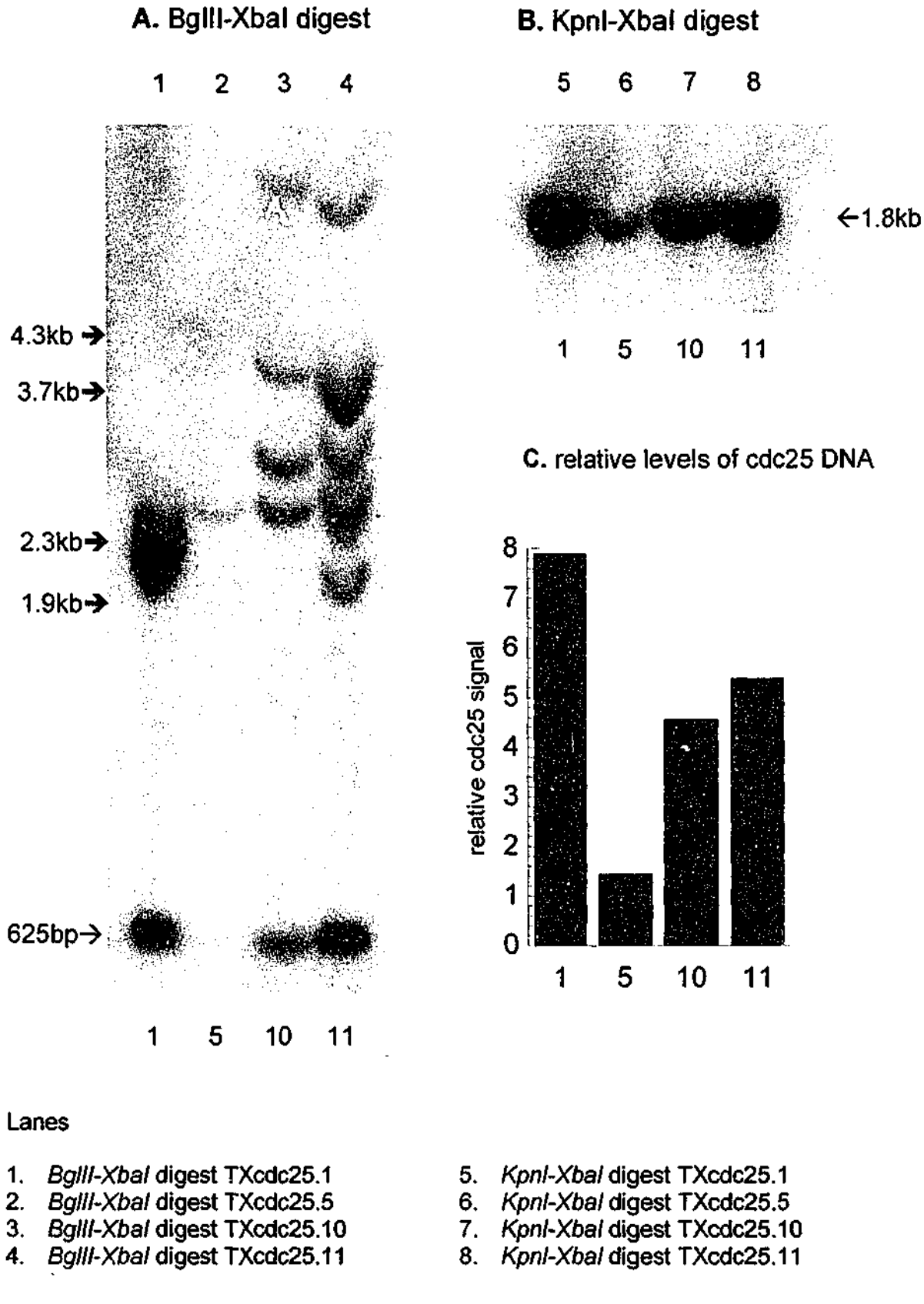


Figure 5.8 Mean frequency of lateral root primordia (±SE) in uninduced and induced seedlings. TXGUSA and TXcdc25.B 11 day F2 seedlings, pure breeding for hygromycin resistance, were transferred to modified MS containing 10^{-4} M K-IBA + 0, 2 or 5 mg/l Tc. After 72 hour treatment the seedlings were treated in 2% Chromium trioxide to reveal LRP (Pelosi *et al.*, 1995). The frequency of LRP per cm primary root length was measured (n≥10).

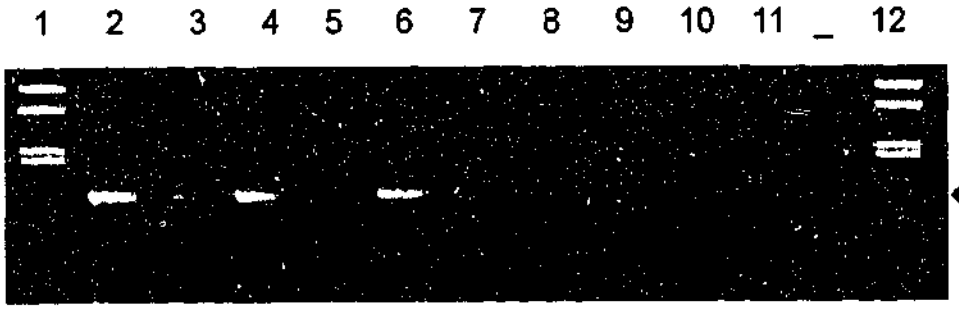


Lanes

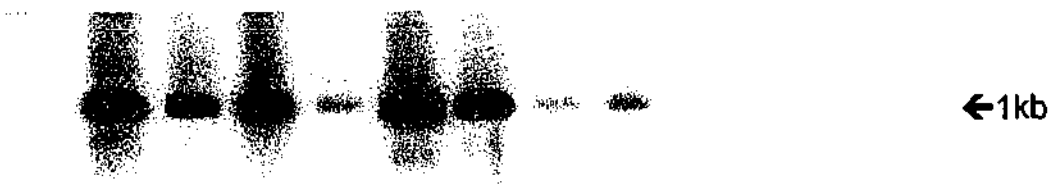
1. *BglII-XbaI* digest TXcdc25.1
2. *BglII-XbaI* digest TXcdc25.5
3. *BglII-XbaI* digest TXcdc25.10
4. *BglII-XbaI* digest TXcdc25.11
5. *KpnI-XbaI* digest TXcdc25.1
6. *KpnI-XbaI* digest TXcdc25.5
7. *KpnI-XbaI* digest TXcdc25.10
8. *KpnI-XbaI* digest TXcdc25.11

Figure 5.9 Southern blot analysis of TXcdc25 cultured roots. 20 µg of genomic DNA isolated from root cultures was loaded and blot probed with a *KpnI-XbaI* fragment containing the full length *cdc25* coding region **A.** *BglII-XbaI* digest to determine the number of *cdc25* integrations **B.** *KpnI-XbaI* digest to determine *cdc25* insert size **C.** relative levels of *cdc25* DNA signal indicating copy number of *cdc25* gene.

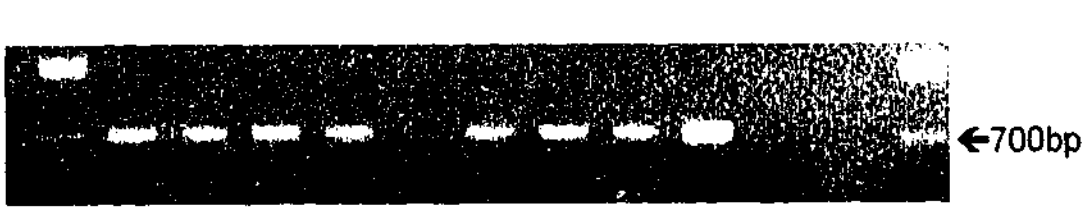
A. gel



B. cdc25



C. nptII



Lanes

1. λ*BstEII* standard
2. TXcdc25.1
3. TXcdc25.5
4. TXcdc25.5'2
5. TXcdc25.10
6. TXcdc25.11
7. TXcdc25.12
8. TXcdc25.13
9. TXcdc25.14
10. Control TXGUS
11. Control no RT
12. λ*BstEII* standard

Figure 5.10 RT-PCR analysis of TXcdc25 rootlines. A solid phase Dynabeads® cDNA library was prepared from total RNA of induced TXcdc25 cultured roots. This library was used as a template in PCR reactions **A.** gel of PCR with *cdc25* NR and NF primers **B.** Southern Blot of the PCR gel probed with a 890bp *HindIII-BamHI* fragment from the 3'-end of the *cdc25* gene **C.** gel of PCR *nptII* A and B primers.

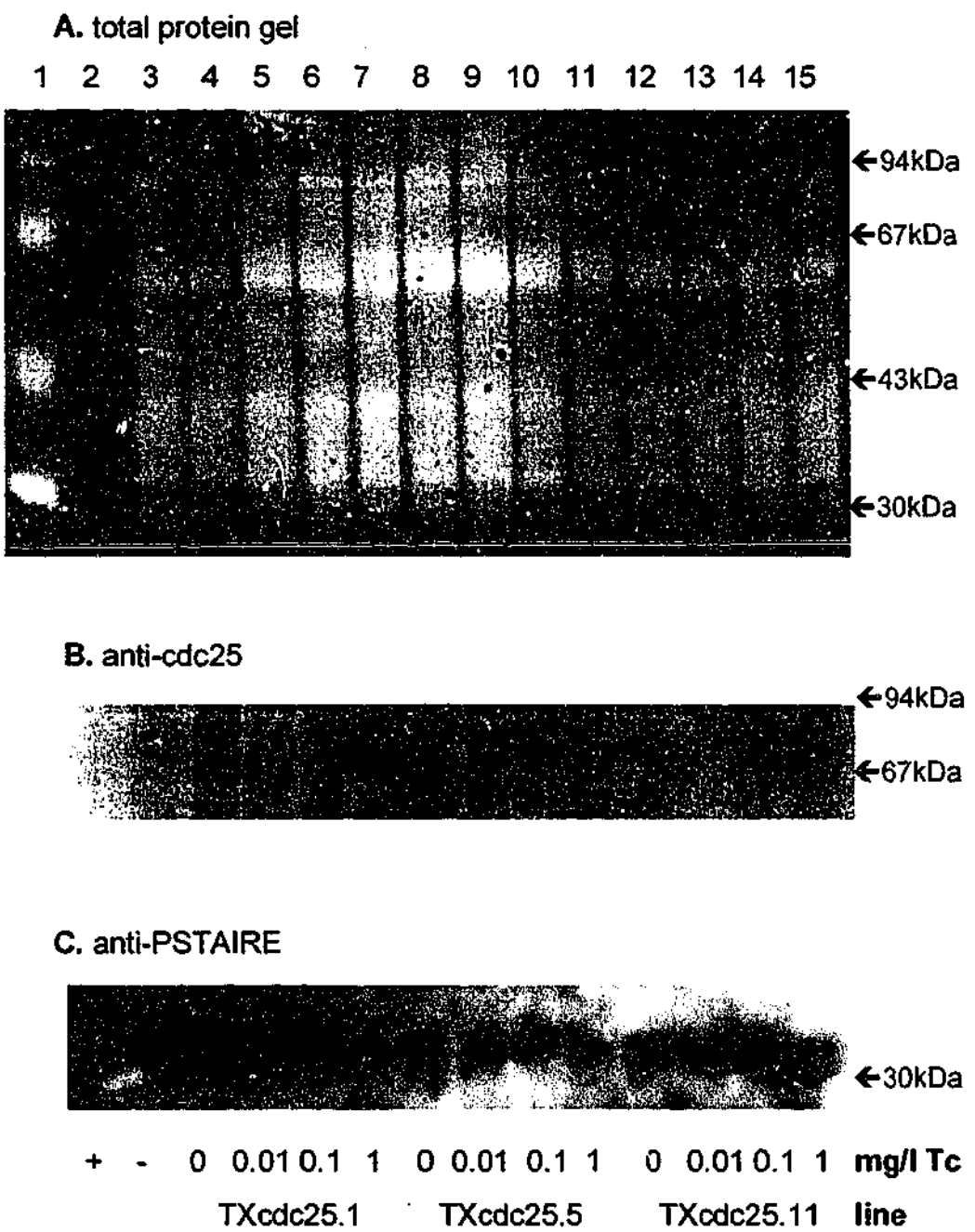
5.4.4.3 Western blotting

In order to determine which lines were capable of expressing the highest level of the Cdc25 protein Western blots were performed on selected rootlines following Tc induction, however negligible levels of protein could be detected (Figure 5.11). Upon application of Tc, the band detected by the anti-Cdc25 did not increase significantly above the level in control samples. The positive control, a GSTcdc25 fusion was clearly detected at 46kDa (not shown). The band detected by the anti-PSTAIRE antibody demonstrates relative protein loadings in each sample. Another antibody was successfully used by Zhang to detect Cdc25 protein in transgenic inducible tobacco suspension cultures following induction by dexamethasone (personal communication). He was able to synchronise the cell cycle of these cultures and this may have contributed to his success in isolating a detectable level of protein. This form of the steroid-inducible promoter system had not been demonstrated to function in whole plants, so was not selected for these experiments. Despite work being done with *cdc25* in plants (Bell *et al.*, 1993; McKibbin *et al.*, 1998) no detectable yeast Cdc25 protein in transgenic tobacco has been reported.

5.4.5 Root Tip Width Comparison

As was noted in section 4.2, it was possible that if mitotic cells were dividing at a smaller size under the influence of *cdc25* this may be apparent in the size of the root tip, if G2 was previously a prolonged phase of the cell cycle. Also, if the other major transition in the cell cycle, at G1/S, was not coupled to an unchanged critical minimum size, which would stabilize cell size in spite of earlier mitosis. Such dependence upon cell size from progression from G1 to S phase is usual and probably universal in yeast and mammalian cells (Jagadish and Carter, 1977; Nurse and Thuriaux, 1977). A smaller cell volume may lead to a smaller meristem volume, which could be observed as a narrower root. Southern blot and RT-PCR data from the root lines indicated that the TXcdc25 construct had been transformed intact and was producing messenger RNA in several of the root lines recovered. In the absence of Western blot or other protein data to confirm which of the root lines were expressing the Cdc25 protein root width was measured to determine whether any of the lines may be showing an altered phenotype when treated with Tc.

Some lines of TXcdc25 root culture showed a narrower root width measurement, relative to controls, when treated with Tc (Figure 5.12). Line TXcdc25.12 showed the largest difference in mean root tip width (±SE) +Tc=275±16 μm and -Tc=338±16 μm. Line cdc25-5 also shows a width difference, albeit not as marked +Tc=291±17 μm and



Lanes

1. standard
2. GST-Cdc25
3. TXGUS.1
4. TXcdc25.1 0 mg/l Tc
5. TXcdc25.1 0.01 mg/l Tc
6. TXcdc25.1 0.1 mg/l Tc
7. TXcdc25.1 1 mg/l Tc
8. TXcdc25.5 0 mg/l Tc
9. TXcdc25.5 0.01 mg/l Tc
10. TXcdc25.5 0.1 mg/l Tc
11. TXcdc25.5 1 mg/l Tc
12. TXcdc25.11 0 mg/l Tc
13. TXcdc25.11 0.01 mg/l Tc
14. TXcdc25.11 0.1 mg/l Tc
15. TXcdc25.11 1 mg/l Tc

Figure 5.11 Western blot analysis of Cdc25 protein induction in TXcdc25 cultured roots. TXcdc25 and TXGUS1 rapidly growing root lines were induced with 1 mg/l Tc for 24 hours in modified MS. Total proteins were extracted from 0.1g of frozen cell powder in RIPA buffer (w/v). **A.** Loadings of 50 µg protein were separated on a 12% SDS-PAGE gel and transferred **B.** probed with 1:500 polyclonal anti-Cdc25 antibody detected by I^{125} anti-rabbit IgG on phosphorimager. **C.** probed with 1:500 affinity purified polyclonal anti-EGVPSTAIREISLLKE antibody detected by ECL-anti-rabbit IgG.

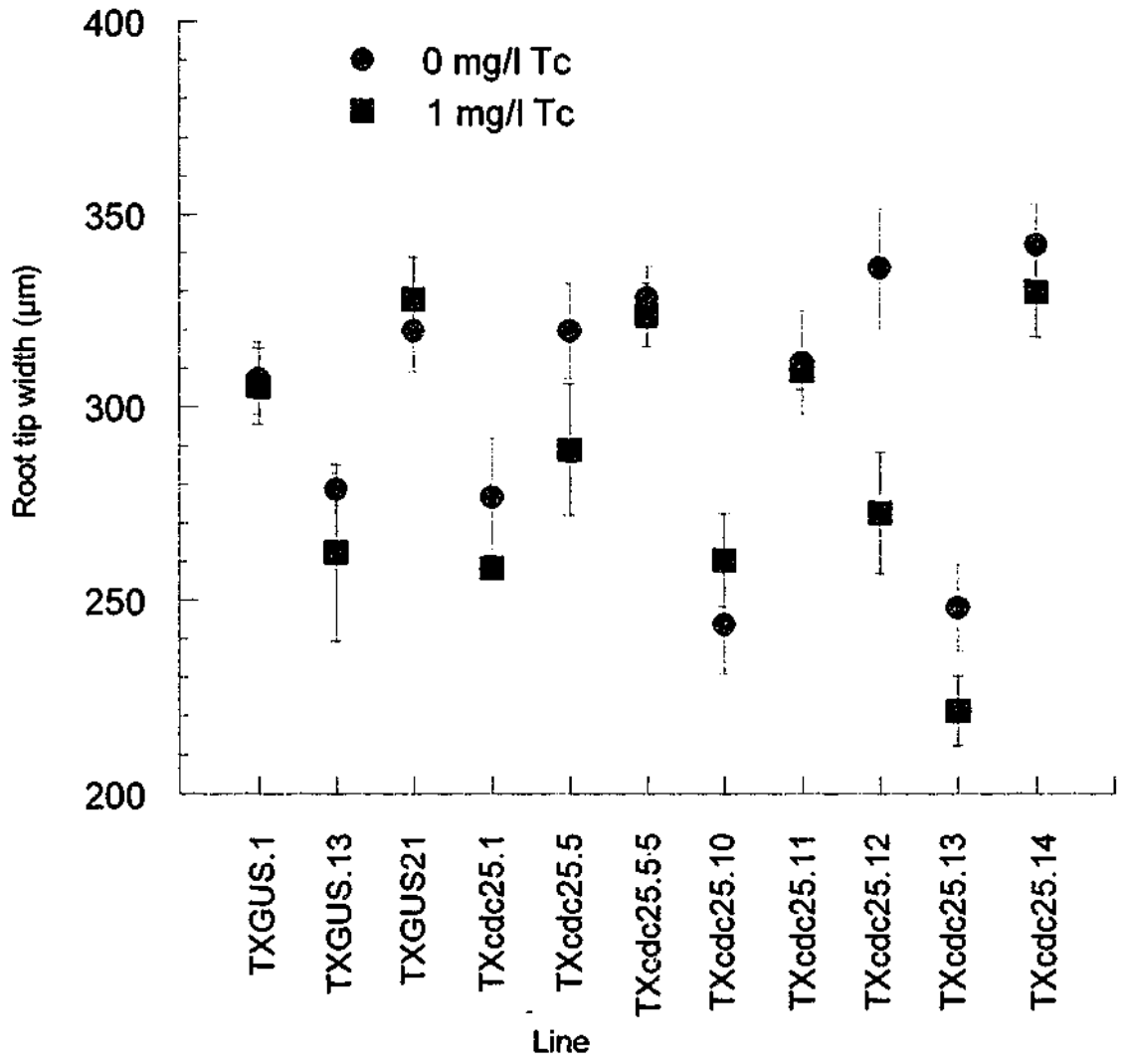


Figure 5.12 Mean (±SE) root tip width (µm) in uninduced (●) and induced (■) cultured roots. TXGUS and TXcdc25 actively growing 11 day old root cultures were treated with ±1 mg/l Tc in modified MS for 4 days. Duplicate cultures of each line were measured (n≥6) with a graticule at 64X magnification. Measurements were made inside the perimeter of the region of dense cells at the base of the root tip.

–Tc=322±12 μm. A small decrease in root width measurement translates to a substantial root tip volume reduction when considered in three dimensions.

The results of this experiment illustrate the variation between and within root cultures. The untreated root tip width ranged from 250±11 μm to 345±14 μm. In the control root lines Tc did not appear to have a consistent effect on root width in culture over the four day time span of the experiment. Line TXcdc25.13 shows a difference in root width under tetracycline treatment but is quite reduced in size compared to the controls even before treatment.

5.4.6 Mitotic Cell Size

As overexpression of *cdc25* in *S. pombe* results in a reduced cell size at mitosis and given the prolonged G2 phase in this yeast also in average sized cells (Russell and Nurse, 1986), a similar phenotype in plants may be observed even if final or differentiated cell size is governed by controls peculiar to the maintenance of plant structure. It may be possible to see variation in cell size at the time the mitotic cell actually divides within the meristem when *cdc25* is overexpressed, if the yeast protein product interacts in a similar manner with the plant cell cycle machinery.

Root cultures of the TXcdc25.5 line and control line TXGUS.1 were induced with Tc and the root tips were excised. Line TXcdc25.5 was selected for this experiment as line TXcdc25.12 proved difficult to propagate. Root tips were then fixed, embedded in PEG and sections were cut to 10 μm. Mitotic nuclei were stained with DAPI and viewed under fluorescence microscopy and cell walls were visualized using DIC optics. The series of sections containing an entire root tip were photographed and each mitotic cell was also photographed under higher magnification and measured (Figure 5.13). Care was taken to ensure each mitotic cell was only measured once even if it appeared in more than one section. The most median section through each cell was selected for measurement and the average diameter was measured. Where the cell was an irregular shape the maximum and minimum diameters were measured and an average calculated. As cell wall thickness was negligible measurements were taken using the walls as the outer markers.

The mean mitotic cell size was significantly reduced in TXcdc25.5 roots exposed to Tc (229±9 μm^2) compared to untreated roots (315±15 μm^2) (Figure 5.14). Uninduced roots containing both TXGUS.1 and TXcdc25.5 had a similar mean mitotic cell size, although the *cdc25* transformed line had fewer large dividing cells. TXGUS.1 control roots also show a reduced mitotic cell size when treated with Tc, but to a significantly smaller degree than the TXcdc25.5 roots. Induced TXcdc25.5 roots have no mitotic

A. Series of root tip sections with DAPI stained nuclei

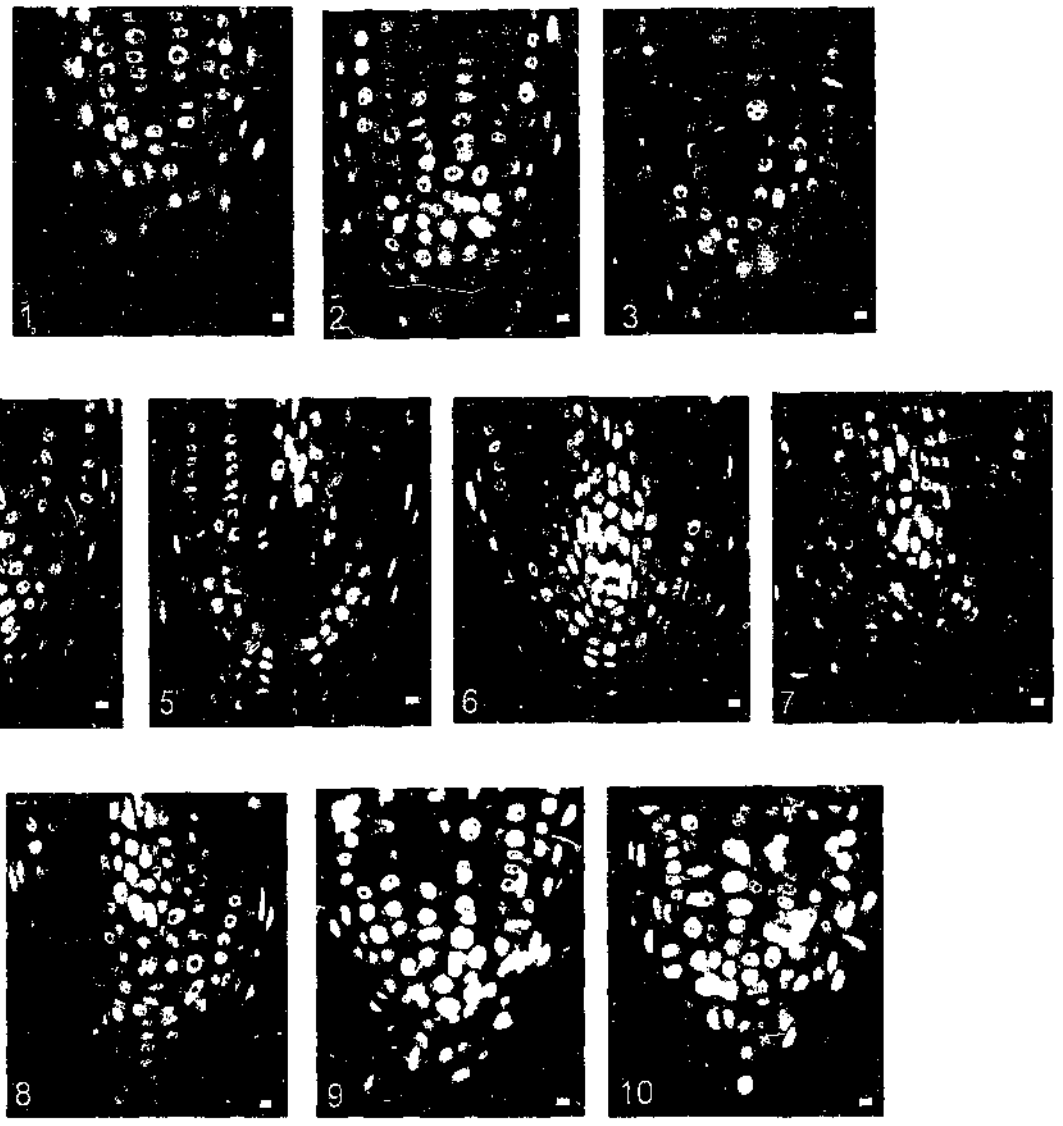
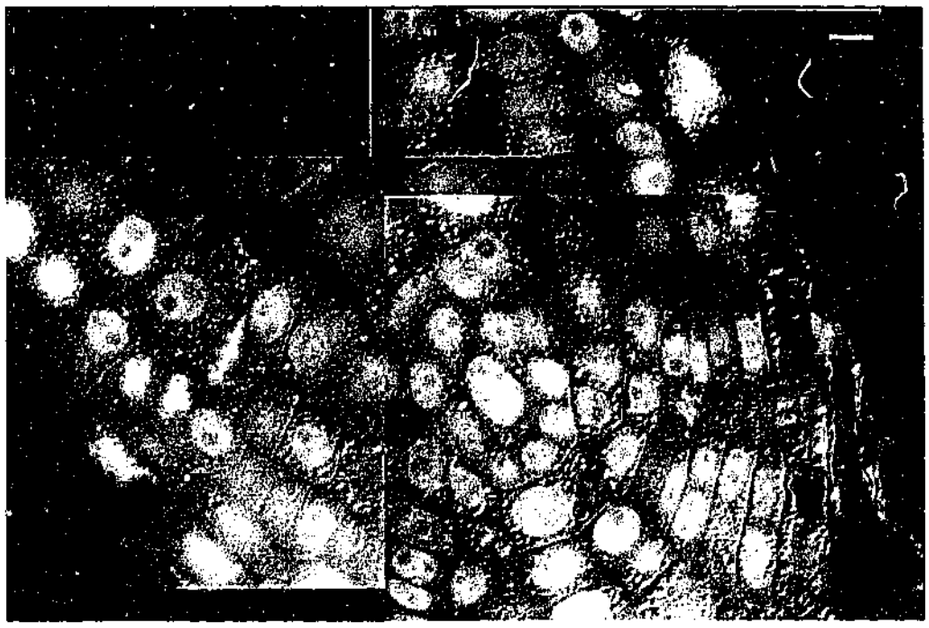


Figure 5.13 Series of sections encompassing the meristem of an induced TXcdc25.5 cultured root. Following induction in 1 mg/l Tc for 4 days cultured root tips were fixed in paraformaldehyde, embedded in PEG and 10 µm sections were cut. **A.** Sections were stained

B. Fluorescence/DIC



with DAPI and nuclei visualised by fluorescence in order to identify mitotic cells B. Fluorescence combined with DIC optics were used to measure the cell size of mitotic cells. Bar=10 μm.

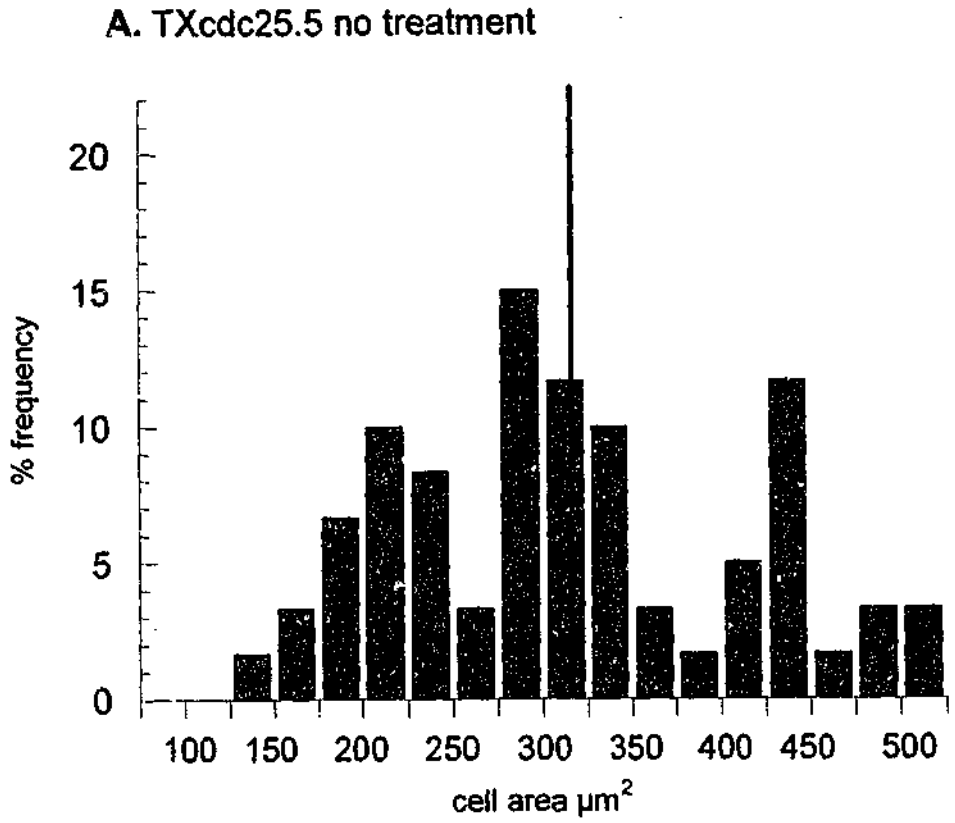
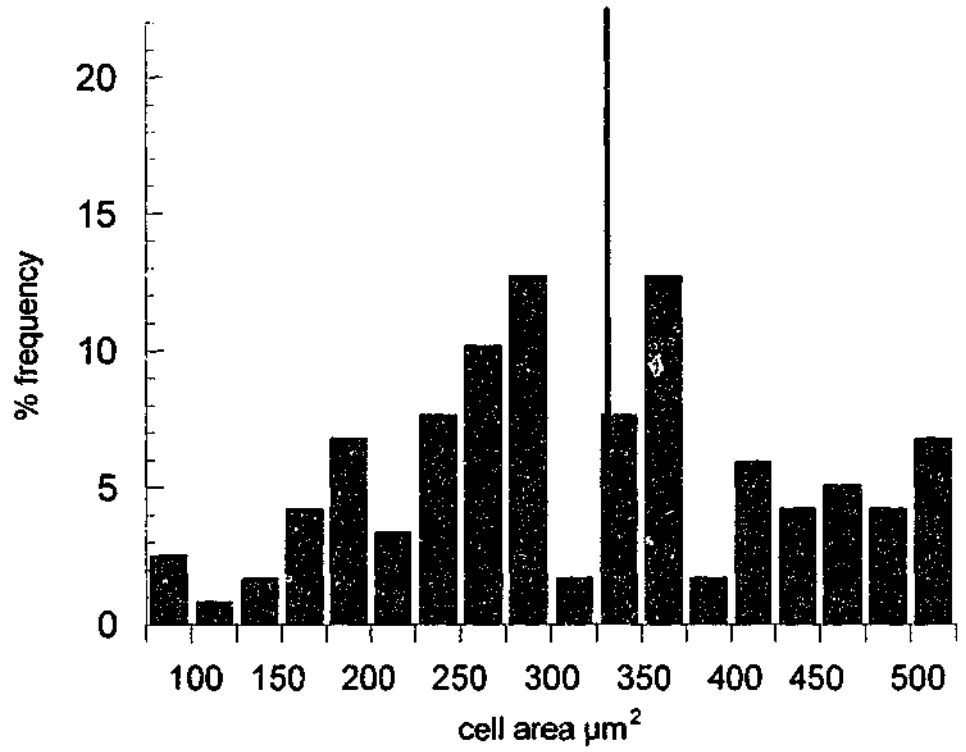
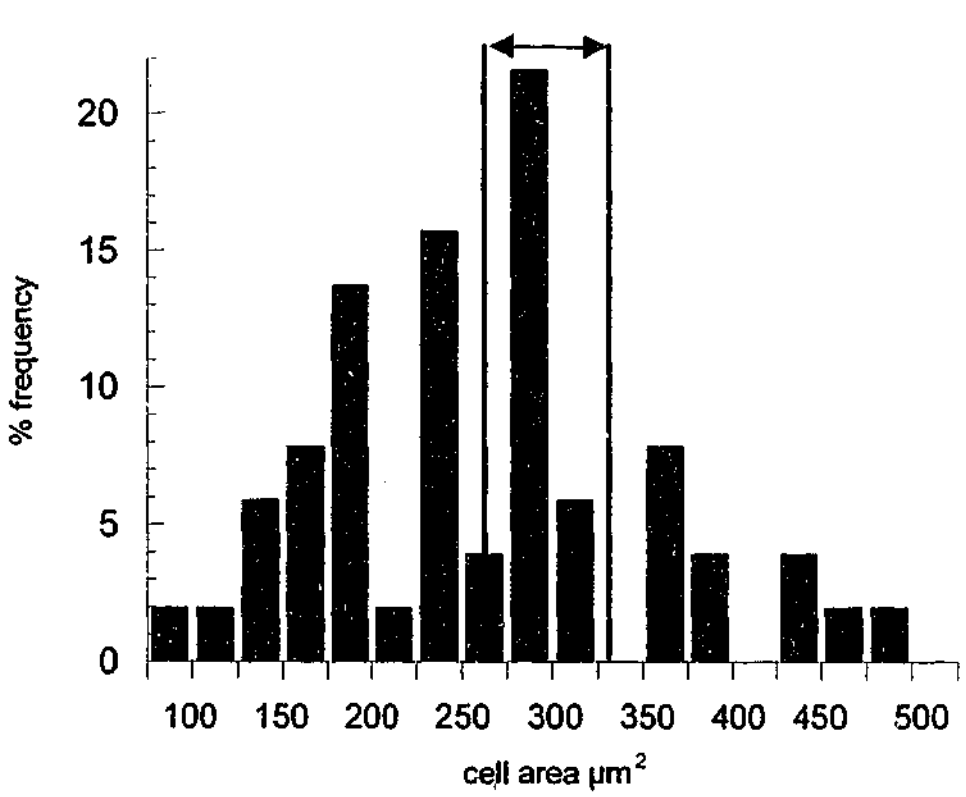


Figure 5.14 The percentage frequency of cell area (µm²) of mitotic cells in TXcdc25.5 and TXGUS.1 cultured roots. Following induction, root tips were excised, fixed in paraformaldehyde and embedded in PEG. 10 µm sections were cut and stained with DAPI.

C. TXGUS.1 no treatment

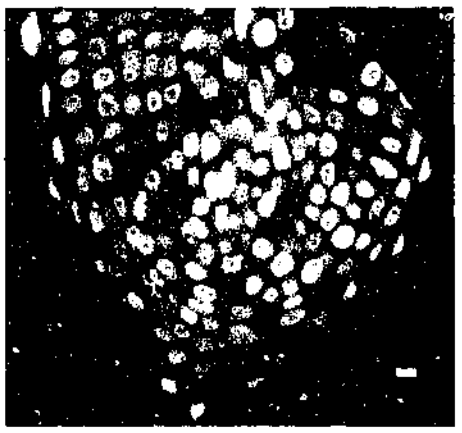


D. TXGUS.1 1 mg/l Tc 4 days



Photography at 100x magnification under fluorescence/DIC optics allowed the measurement of cell area (n>50) **A.** TXcdc25.5 no treatment **B.** TXcdc25.5 1 mg/l Tc 4 days **C.** TXGUS.1 no treatment **D.** TXGUS.1 1 mg/l Tc 4 days.

A. Tc treated TXGUS.1 root section



B. Tc treated TXcdc25.5 root section

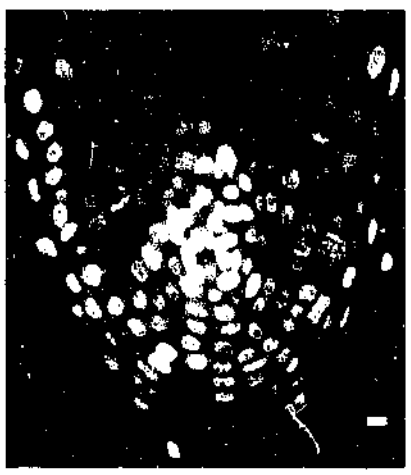


Figure 5.16 Median section of TXcdc25.5 and TXGUS.1 root meristems following Tc induction. Bar=10 μm.

cells over 400 μm² in size, a marked difference from the treated control line and indeed from the untreated root tips.

This mitotic cell size difference is most apparent in the cortical cells (for an example see Figure 5.15), those that are the largest in the frequency of mitotic cell size distribution. However, the majority of the smallest cells in all samples were found in the median sections of the root tip. The experimental design allowed observation on a cell-type basis and mean mitotic cell size appears to be reduced in all cell types, including those giving rise to the root cap and stele, rather than just a variation in cortical cell size. However, this observation was based on very few cells of some types so more data is needed for this type of analysis.

Comparing treated roots in control and TXcdc25 cultures, in each case mitotic cells appeared in 10 or 11 of the 10μm median sections (Figure 5.13A) indicating little variation in meristem size between the samples. The base of the meristem at its widest point is therefore a maximum of 110μm in diameter. Given that root tips are smaller in the induced samples containing *cdc25* (Figures 5.12 and 5.16) it seems that, under the influence of the transgene, a larger proportion of the cell population constituting these root tips is dividing. More data are needed to determine whether induced TXcdc25 roots have the same sized meristems as the controls, consisting of a greater number of smaller cells, or whether there is a meristem size difference.

5.5 DISCUSSION

The fission yeast *cdc25* gene was introduced into transgenic plants and root cultures under the control Triple-X Tc inducible promoter. *cdc25* transcript accumulation, following Tc induction, was clearly shown in transformed tissue by RT-PCR. When *cdc25* is overexpressed in plants the result is division at a reduced cell size. This agrees with the wee cell phenotype seen when *cdc25* is overexpressed in *S. pombe* (Russell and Nurse, 1986). This observation, and possibly consequent effects in reducing root tip width and increasing frequency of lateral root primordia, indicate that the fission yeast Cdc25 protein can interact with the plant cell cycle components in a positive manner to accelerate entry into mitosis.

These data also confirm and extend observations of other workers made in whole plants constitutively expressing *cdc25* (Bell *et al.*, 1993) and in excised roots containing inducible *cdc25* (McKibbin *et al.*, 1998). Interestingly, these authors also failed to demonstrate *cdc25* expression by Northen hybridization and utilized RT-PCR to confirm expression (McKibbin *et al.*, 1998). A decrease in primordium size of roots induced to express *cdc25* was also observed in a another study; as was an increase in the

frequency of LRP in an excised root system grown permanently in liquid media containing auxin (10^{-6} M 2,4-D) (McKibbin *et al.*, 1998). Bell et al. (1993) recorded a cell size, seen as smaller cell area in plan view, in root tip cells at all stages in spreads dissected from a whole plant overexpressing *cdc25*. McKibbin et al (1998) reports a reduced cell size at prophase from root cells dispersed into cell monolayers. A reservation about the methodology of this earier study is that in inducing TXcdc25 in this tissue with the high concentration of 5 mg/l Tc, the uninduced roots were used as the control. The effect of the inducer itself on cell size in the absence of *cdc25* was not mentioned. The present results take this into account but still show an effect of *cdc25* expression.

Cdc25 appears to be interacting with the plant cell cycle to influence cell division in two ways. In meristem cells that are already dividing, Cdc25 stimulates them to enter mitosis earlier relative to growth so that they are smaller at division. The control that allows cortical cells to grow to a large size before making their final division is overridden in induced roots transformed with TXcdc25. Dephosphorylation of Cdc2 therefore appears to be an element in controlling the entry of plant cells into mitosis, as it does in other eukaryotes. In the presence of optimum concentrations of auxin, Cdc25 incites cells in the pericycle, to divide and form LRP, cells that would otherwise have remained paused in G2. As well as regulating the onset of mitosis in the meristem, Cdc2 activation could also be a limiting factor in shaping plant development in the initiation of LRP.

A more marked phenotype may have been observed if the Cdc25 protein was expressed at a higher level in the plant tissue, than was the case in the plants produced in this study. Only very low levels of *cdc25* mRNA and protein could be detected after induction using the Triple-X promoter in tobacco. As previous experiments showed *GUS* and *cdc2* genes to be induced using this promoter, it seems likely that the low level of *cdc25* mRNA is due to a high level of lability within the plant cell. *cdc25* mRNA a is low level or unstable transcript in fission yeast (Millar *et al.*, 1991a), but this may be partly due to low activity of the *cdc25* promoter. Instability of the heterologous *cdc25* mRNA may be particularly high in plants due to its sequence. The AUUUA motif is an instability determinant in plants not recognized in yeast (for review see Gutiérrez *et al.*, 1999). A pattern search identifies five such elements in the *cdc25* transcript which, has a base composition of 58% AT, both features which may contribute to its instability. The AT% is somewhat high relative to that of many plant genes which often have a high GC content (Murray *et al.* 1989). Altering the sequence coding for the AUUUA motif and AU rich regions in BT toxin contributes to the stability of its mRNA in plants, as does changing the sequence to plant codon preferences (De Rocher *et al.*, 1998).

The construct used in these experiments contained the *cdc25* sequence from the initiating ATG plus 28bp 3'- of the stop codon. In leaving in a UTR of 5'-54bp and 3'-273bp McKibbin *et al.* (1998) were able to increase polypeptide production in an *in vitro* transcription/translation assay compared to the coding sequence alone. These UTRs either increased the stability of the *cdc25* mRNA and/or increased the efficiency of its transcription and/or translation. Nevertheless, even with this improvement these authors required RT-PCR to detect *cdc25* mRNA. Low levels of detectable Cdc25 may also be attributable protein instability, Cdc25B protein has been reported to have a short half-life in human cells (Baldin *et al.*, 1997; Nishijima *et al.*, 1997).

Low levels of expression may also be attributed to the leakiness of the Triple-X promoter in meristematic tissue (De Veylder *et al.*, 2000). As observed in this study with the *cdc2-1w* gene, if high levels of Cdc25 are detrimental to normal meristematic operation, counterselection would have occurred against highly expressing lines during root and shoot regeneration. The lack of large mitotic cells in uninduced TXcdc25 root tissue compared to the controls may be a symptom of promoter leakage in cultured root meristems.

An effect that required careful interpretation of mitotic cell size data, whilst overexpressing Cdc25, was the cell size reduction caused by Tc treatment itself. Tetracycline acts to interfere with prokaryotic ribosomal protein elongation by blocking the binding of aminoacyl-tRNA to the A-site of the ribosome. Mitochondria and plastids in plants cells have ribosomes with bacterial-like sensitivity to antibiotics. Mitochondria are particularly important in supplying energy for root growth so their sensitivity of organelles to Tc may be the cause of reduced root cell size in its presence. Roots typically show extra sensitivity to drugs such as kanamycin or streptomycin which interferes with the protein synthesizing of organelles (Hamill and Lidgett, 1997).

Chapter 6
General Discussion

Work described in this thesis has employed genes derived from the fission yeast that are well characterised in their cell cycle control functions. Experiments were designed to investigate whether the genes influenced cell cycle progression and organ growth in plants. Effects of the introduced genes could illuminate mechanisms of the plant cell cycle and additionally, could indicate the extent to which control of cell division influences plant development. It has remained contentious in all multicellular organisms whether cell division drives growth or merely reports on it, thus in plants, cell division might simply partition cytoplasm that has been created by growth. Evidence on this point is conflicting in both animal and plants systems (e.g. reviewed by Neufield and Edgar, 1998). The present study provides an intriguing hint that, at least in the initiation of new foci of growth by establishment of new lateral root primordia, manipulation of the cell cycle can influence plant growth and morphology.

This discussion will consider first the extent to which the introduced genes are equivalents of plant genes and therefore likely to influence biochemical events that are part of the normal plant cell division cycle. The transgenes will be more likely to illuminate normal developmental controls if they are closely related to plant genes. Then, expression of the transgenes in plants and roots will be discussed. The effects of the genes on cell division and development will be assessed in terms of the timing of cell division relative to cell growth. This is reflected in cell size at division and possibly in the overall dimensions of the apical meristem. Finally, the effects of altered cell cycle control on initiation of new lateral root meristems will be reviewed in relation to the possibility that cell division can influence growth.

The cell cycle genes *cdc2*, and *cdc25* of *S. pombe,* which were employed in this study, are likely to have homologues or close equivalents in plants. The *cdc2* and its budding yeast homologue *CDC28,* have both been shown, when inactivated by mutation, to be complemented by plant genes that were transferred from both monocotyledonous and dicotyledonous species, in several studies that were performed a decade ago (reviewed in Chapter 1). It is now known that the plant homologues of *cdc2* are structurally as close as the homologues found in other taxa. It is also known that *cdc2A.At* is expressed throughout the plant cell cycle (reviewed by Mironov *et al.*, 1999) and of similar significance in the plant cell cycle as in the yeast. Investigation of the effects of *cdc2* and its dominant activated mutant form *cdc2-1w* when introduced into plant cells is therefore likely to be of direct relevance to endogenous plant cell cycle controls.

No plant *cdc25* gene has yet been isolated, thus any transgenic study must currently employ a gene from another kingdom. This situation must raise the question of whether there is in plants a close equivalent of the *cdc25* gene. Sequencing of the *Arabidopsis* genome has not revealed a gene that has close structural equivalence to *cdc25* (The Arabidopsis Genome Initiative, 2000), but this is not conclusive evidence of its absence given the fallibility of intron recognition. Attempts to clone plant *cdc25* by complementation in yeast have also been unsuccessful and yielded phosphatases that are not *cdc25* (Ferreira *et al.*, 1993). This again is not conclusive negative evidence, since complementation is not a very specific approach when applied to this gene. It is known that other phosphatases can serve sufficiently well in place of Cdc25 to prevent cell death; indeed an early indication that Cdc25 is a phosphatase was obtained by complementation of a *cdc25* mutation with a non-cell cycle phosphatase (Gould et al 1990). Furthermore the fission yeast cell cycle employs two phosphatases acting on Cdc2 at mitotic initiation, and only the major one of these is Cdc25. A significant minor function is performed by the *pyp3* gene, which also encodes a protein phosphatase, but one with little structural similarity to Cdc25. If Pyp3 is inactivated then mitosis is significantly delayed even if Cdc25 activity is normal, thus indicating a significant contribution from Pyp3 in the normal fission yeast cell cycle (Millar *et al.*, 1992). Therefore, the effective plant Cdc25-like phosphatase will be Cdc2-activating, but it need not be close in structure to Cdc25. It may, in short, be a better functional homologue than structural homologue.

There is evidence that, in plants, mitotic initiation does require Cdc2 activation through removal of inhibitory tyrosine phosphorylation, as it does in yeasts and metazoa. Plant mitosis is dependent upon Cdc2 enzyme activity, from evidence of the accelerating effects of microinjected plant Cdc2 enzyme (Hush *et al.*, 1996) and also from the inhibitory effect of the dominant catalytically-inactive plant Cdc2 enzyme (Hemerly *et al.*, 1995). The increase in essential Cdc2 activity at mitosis comes from tyrosine dephosphorylation since plant cells arrested at the G2/M boundary have high tyrosine phosphorylation and low enzyme activity but on entry into mitosis phosphorylation declines and activity rises. Cdc2 enzyme extracted from cells at the G2/M boundary can be activated *in vitro* by yeast Cdc25 enzyme (Zhang *et al.*, 1996). A similar tyrosine phosphorylation in Cdc2 kinase and *in vitro* activation by Cd25 enzyme from *Drosophila*, has been observed in synchronised alfalfa cells (reviewed by Mészáros *et al.*, 2000). Furthermore, a *wee1* homologue has been cloned in maize (Sun *et al.*, 1999), which indicates Tyr15 phosphorylation of Cdc2 in plants, but does not establish its importance in governing cell division. Some evidence for control of activity through this phosphorylation comes from the finding that absence of Tyr15 and Thr14 phosphorylation does appear to increase plant Cdc2 enzyme activity when Cdc2At.AF,

in which non-phosphorylateable residues were substituted for Tyr15 and Thr14, is expressed in yeast (Porceddu *et al.*, 1999). However, caution should be exercised in interpreting results of *cdc25* expression, since yeast Cdc25, when present in the plant could be acting as a non-specific tyrosine phosphatase rather than behaving specifically as a cell cycle enzyme.

The present study sought to obtain inducible expression of the introduced cell cycle genes so that potential selection against strongly-expressing lines, due to disruption of regeneration from transgenic cells, might be avoided. It is known that over-expression of some cell cycle regulators can disrupt cell cycle progression to such an extent that division cannot be completed. The *cdc25* gene is one that can produce such effects (Berry and Gould, 1996).

While overexpression may be deleterious, evidence is needed that expression has been achieved before the possibility can be considered that the phenotype of transgenic plants can be attributed to the introduced gene. In the present study it was not possible to recover lines that were expressing at high level the *cdc25* and *cdc2-1w*, genes, although high induced expression of *cdc2* could be achieved, indicating that the problem was gene specific. The low expression is possibly attributable to leakiness of the Triple-X promoter and to deleterious effects of the *cdc25* and *cdc2-1w* genes on regeneration, as both were shown in this study to affect cell division when their expression was induced. Whereas the activity of Cdc2 is under posttranslational control by cyclin availability and by phosphorylation (Chapter 1), thus an increase in the pool of Cdc2 protein from expression of the transgene is not expected to have strong effects on total Cdc2 enzyme activity. The unmodified *cdc2* gene therefore acts as a control for other transformations. Plants expressing *cdc2* showed no change in phenotype, consistent with absence of a block to regeneration in lines that showed high-expression of the *cdc2* transgene.

On closer examination of the genes reported in the literature as successfully overexpressed under control of the Triple-X promoter that was used here, none were profoundly detrimental to growth. Therefore leakiness of the promoter may not previously have been stringently tested. Of the genes used previously, the one that appeared to be the most toxic, *SAMDC*, was quite deleterious to potato. Even so, experiments in tobacco with heterologous *SAMDC* gene from humans illustrated that low levels of SAMDC expression could be endured (Kumar *et al.*, 1996), possibly well enough to not reveal Triple-X promoter leakage. Further experiments, with potentially growth-disrupting cell cycle genes, would be better carried out with a promoter that did not leak when expressed in the meristematic regions of the plant (De Veylder *et al.*, 2000).

Several authors assert that stringent control is more likely to be achieved with a promoter under control by positive activation, than with one regulated by repression, because repressors must compete with endogenous transcription factors to bind (Gossen and Bujard, 1992; Weinmann *et al.*, 1994; Böhner *et al.*, 1999). Some of the more recently developed promoters could be effective. The steroid inducible system has been improved, a tripartite system was developed (Aoyama and Chua, 1997) and has been tested with a gene deleterious to *Arabidopsis* (McNellis *et al.*, 1998). A system which switches on with dexamethasone and off with Tc has only been used so far in tobacco (Böhner *et al.*, 1999). Love *et al.* (2000) have developed a stringent Tc-repressible system in *Arabidopsis* however ,as Tc was shown in the present study to affect cell size, an alternative inducer to Tc would be more suitable. A cyclin, CycD3 was successfully expressed by an alternative strategy in *Arabidopsis* after constitutive expressing plants failed to regenerate. A heat-shock promoter linked site-specific recombinase resulted in the widespread expression of CycD3 and disrupted growth (Riou-Khamlichi *et al.*, 1999).

A further factor that might have been taken into account to extend the present investigation is the attempted improvement of mRNA and protein yield from the *cdc25* gene. Some investigation of the translational efficiency may be required as UTRs have already been shown to affect protein yield (McKibbin *et al.*, 1998). Altering the yeast *cdc25* codon sequence to remove any AU-rich regions and conform to plant codon preferences may improve mRNA stability, if rapid degradation is a contributing factor to low mRNA and protein yields (De Rocher *et al.*, 1998). It should not be considered, however, that higher levels of expression would necessarily be advantageous since excess expression can cause cell cycle block, as discussed above.

Considering the effects of *cdc2-1w* expression there were two indications that *cdc2-1w* was able to interact with elements of the plant cell cycle. Compared to wild-type *cdc2*, the *cdc2-1w* gene gave only low-expressing lines. This indicates counter-selection against highly expressing lines may be taking place, which could occur if the presence of *cdc2-1w* interferes with regeneration. The second indication is that, when stimulated with auxin, the mean frequency of lateral root primordia was reduced. Cdc2-1w was therefore interfering with cell division in the pericycle that gives rise to LRP. In yeast, Cdc2-1w initiates early entry into mitosis because it is not responsive to Wee1 kinase (Booher and Beach, 1986). In plants, *cdc2-1w* retards entry into mitosis, possibly by binding cell cycle regulatory proteins. Plant Cdks are not fully functional in yeast (Porceddu *et al.*, 1999) and conversely, this study confirms that a yeast *cdc2* allele is not able to drive mitosis as it does in yeast. This may derive from the more diverse range of functions that are required from cyclin dependent kinases at mitosis in plant cells (Mews

et al., 1997). It does however, suggest that transgenically expressed *cdc2* homologues and endogenous plant regulatory proteins could interact substantially. This is further supported by the possible functional interaction seen here between *cdc25* and plant Cdks.

Expression of *cdc25* was also only obtained at low levels relative to expression of *cdc2*, so counter-selection of transgenic lines may again be an influence. In contrast to *cdc2-1w*, *cdc25* induction increased the mean frequency of LRP and decreased mean mitotic cell size in the root meristem. Cdc25 was therefore stimulating cells to divide, possibly by interacting with plant CDKs. Thus, while *cdc2-1w* reduced the amount of cell division by somehow obstructing the cell cycle process, on the other hand *cdc25* increased the rate of cell division by stimulating or overriding cell cycle regulation.

If the transgenes affected the timing of cell division relative to cell growth, as do genes that affect Cdc2 kinase activity in yeasts (reviewed by Nurse, 1999) this would be seen directly as a change in cell size at division and possibly seen by indirect changes in meristem size. Expression of *cdc25* resulted in smaller cell size at division. Nonetheless, roots were morphologically normal, indicating controls over morphogenesis that are not dependent upon cell size. A similar observation has been made by Hemerly *et al.* (1995) in roots expressing the negative effector *cdc2aAt.N147* which conversely resulted in large cells. However, when *cdc25* was induced, some narrowing of the root tip was observed. This is consistent with smaller daughter size but is difficult to interpret since the tip includes cells that have ceased division and have enlarged to their final size, which may not be related to size at division, and their enlargement may have been affected by the inducing agent.

One striking effect of cdc25 expression was an increase in lateral root primordium initiation. Auxin was an essential adjunct to the increase in LRP and auxin appears to have a direct effect on cell cycle mitotic components, being an inducer of Cdc2 (e.g. Gorst *et al.*, 1991; Martinez *et al.*, 1992). Cyclin A and *cdc2* are both upregulated by auxin in *Arabidopsis* (Hemerly *et al.*, 1993; Burssens *et al.*, 2000b). Thus it seems many of the rate-limiting events for cell cycle progression are stimulated by auxin, but other controls that are not operated by auxin are also involved and can be pinpointed experimentally when auxin is no longer limiting. There is therefore, evidence from expressing *cdc25* that activation of Cdc2 kinase is not fully attained by auxin and is limiting for initiation of lateral root primordia. This conclusion is fully consistent with the finding of Zhang *et al.* (1996) that auxin can induce Cdc2 in excised plant tissue but cytokinin is necessary for its tyrosine dephosphorylation and enzymic activation. This is precisely the predicted function of Cdc25.

The results of this study and that of McKibbin *et al.* (1998) both show increased lateral root initiation following *cdc25* expression, but both observations seem at first sight to be in contradiction to the observation that roots can initiate primordia even if cell division is blocked with colchicine (Foard *et al.*, 1965). Even in presence of the anti-microtubule agent foci of cytoplasmic growth develop in the pericycle region, where initiation of lateral primordia would occur. This localised growth produces a domed protuberance, like a developing primordium, from which a lateral primordium can indeed develop if the cytoskeletal inhibitor is removed. The localised growth cannot continue indefinitely without cell division, presumably because of adverse nucleus to cytoplasm ratios, has not initially been directed by formation of new cells (Foard *et al.*, 1965). In this situation localised growth was clearly not triggered by formation of new cells, but only the physical process of division was blocked here. The biochemical machinery of the cell cycle, such as the activation of cyclin dependent kinases which may also signal to growth processes, would presumably not have been affected. Thus it is not the physical process of division itself, but perhaps the underlying biochemical events such as activation of Cdc kinases that could accelerate growth.

The cell division machinery can therefore be seen to contribute to the development of plant form and also to the continuation of growth. This leaves open the question of whether in multicellular organisms cell division can play a part in driving growth. In plants, cell division could be the final stage in transduction of growth factor signals and newly formed daughter cells could be particularly active sinks for growth precursors.

A test of whether cell cycle progression drives growth can be made if cell division can be increased without directly altering other processes. If division is then able to stimulate growth there may be an increase in size of the organism, but if division has no effect on growth the average cell size will decline without change in growth. This form of test may have been performed in the present study by the expression of *cdc25*. The test has been performed in mice by knockout of the $p27^{kip1}$ inhibitor, which is widely expressed in nonproliferating cells and encodes a CKI that inhibits the activity of CDK2, which is responsible in mammals for progression into S phase. When both copies of the p27 gene were inactivated by targeted gene disruption there was a ten fold increase in CDK2 activity, a doubling of cell number and a 30% increase in size of the mouse, indicative of additional growth promoted by formation of additional daughter cells (Nakayama *et al.*, 1996). Thus additional cell division can lead to additional growth, even in animals, which have a determinate growth program.

The effect of *cdc25* expression on lateral root initiation may pinpoint a control mechanism that operates in the intact plant. It is striking that cells of the pericycle have been reported to be predominantly arrested in G2 phase (Blakely and Evans, 1979) and

within 6 hours of stimulation most can be in late mitosis. This is too short a time for them to have traversed S-phase, G2-phase and prophase, therefore all available data indicate that initiation of a primordium by the resumption of division therefore necessitates entry into mitosis by activation of Cdc2 kinase through tyrosine dephosphorylation. The point at which Cdc25-catalysed activation of Cdc2 kinase consistently impacts on the cell cycle is at the G2/M progression (Nurse, 1990). The present study suggests that this control mechanism acts as a control over plant development by regulating root branching.

References

Abler, M.L. and Green, P.J. (1996) Control of mRNA stability in higher plants. *Plant Molecular Biology*, **32**, 63-78.

Ach, R.A., Durfee, T., Miller, A.B., Taranto, P., Hanleybowdoin, L., Zambryski, P.C. and Gruissem, W. (1997) *RRB1* and *RRB2* encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. *Molecular and Cellular Biology*, **17**, 5077-5086.

Aguda, B.D. (1999) A quantitative analysis of the kinetics of the G2 DNA damage checkpoint system. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 11352-11357.

Aligue, R., Wu, L. and Russell, P. (1997) Regulation of *Schizosaccharomyces pombe* Wee1 tyrosine kinase. *Journal of Biological Chemistry*, **272**, 13320-13325.

Amon, A., Tyers, M., Futcher, B. and Nasmyth, K. (1993) Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell*, **74**, 993-1007.

Aoyama, T. and Chua, N.H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant Journal*, **11**, 605-612.

Arion, D., Meijer, L., Brizuela, L. and Beach, D. (1988) cdc2 is a component of the M phase specific histone H1 kinase: evidence for identity with MPF. *Cell*, **55**, 371-378.

Baber-Furnari, B.A., Rhind, N., Boddy, M.N., Shanahan, P., Lopez-Girona, A. and Russell, P. (2000) Regulation of mitotic inhibitor Mik1 helps to enforce the DNA damage checkpoint. *Molecular Biology of the Cell*, **11**, 1-11.

Baldin, V., Cans, C., Knibiehler, M. and Ducommun, B. (1997) Phosphorylation of human Cdc25b phosphatase by Cdk1-cyclin A triggers its proteasome-dependent degradation. *Journal of Biological Chemistry*, **272**, 32731-32734.

Beach, D., Durkacz, B. and Nurse, P. (1982) Functionally homologous cell cycle control genes in budding and fission yeast. *Nature*, **300**, 706-709.

Bell, M.H., Halford, N.G., Ormrod, J.C. and Francis, D. (1993) Tobacco plants transformed with *cdc25*, a mitotic inducer gene from fission yeast. *Plant Molecular Biology*, **23**, 445-451.

Benfey, P., Ren, L. and Chua, N.H. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue specific expression patterns. *EMBO Journal*, **8**, 2195-2202.

Berry, L.D. and Gould, K.L. (1996) Novel alleles of *cdc13* and *cdc2* isolated as suppressors of mitotic catastrophe in *Schizosaccharomyces pombe*. *Molecular & General Genetics*, **251**, 635-646.

Blakely, L.M. and Evans, T.A. (1979) Cell dynamics studies on the pericycle of radish seedling roots. *Plant Sci. Letts.*, **14**, 79-83.

Blasina, A., Van de Weyer, I., Laus, M.C., Luyten, W., Parker, A.E. and McGowan, C.H. (1999) A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. *Current Biology*, **9**, 1-10.

Blomberg, I. and Hoffmann, I. (1999) Ectopic expression of Cdc25A accelerates the G1/S transition and leads to premature activation of cyclin E- and cyclin A-dependent kinases. *Molecular & Cellular Biology*, **19**, 6183-6194.

Boerjan, W. (1995) *superroot*, a recessive mutation in *Arabidopsis* confers auxin overproduction. *Plant Cell*, **7**, 1405-1419.

Böhner, S., Lenk, I., Rieping, M., Herold, M. and Gatz, C. (1999) Transcriptional activator TGV mediates dexamethasone-inducible and tetracycline-inactivatable gene expression. *Plant Journal*, **19**, 87-95.

Booher, R., Alfa, C.E., Hyams, J.S. and Beach, D. (1989) The fission yeast Cdc2/Cdc13/Suc1 protein kinase:regulation of catalytic activity and nuclear localization. *Cell*, **58**, 485-497.

Booher, R. and Beach, D. (1986) Site-specific mutagenesis of $cdc2^{+}$, a cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *Molecular & Cellular Biology*, **6**, 3523-3530.

Bourne, Y., Watson, M.H., Hickey, M.J., Holmes, W., Rocque, W., Reed, S.I. and Tainer, J.A. (1996) Crystal structure and mutational analysis of the human CDK2 kinase complex with cell-cycle regulatory protein CksHs1. *Cell*, **84**, 863-874.

Brondello, J.M., Boddy, M.N., Furnari, B. and Russell, P. (1999) Basis for the checkpoint signal specificity that regulates Chk1 and Cds1 protein kinases. *Molecular & Cellular Biology*, **19**, 4262-4269.

Budavari, S. (ed.) (1996) *The Merck index: an encyclopedia of chemicals, drugs and biologicals*. Merck, Whitehouse Station, N.J.

Burssens, S., Engler, J.D., Beeckman, T., Richard, C., Shaul, O., Ferreira, P., Van Montagu, M. and Inzé, D. (2000a) Developmental expression of the *Arabidopsis thaliana CycA2;1* gene. *Planta*, **211**, 623-631.

Burssens, S., Himanen, K., van de Cotte, B., Beeckman, T., Van Montagu, M., Inzé, D. and Verbruggen, N. (2000b) Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in *Arabidopsis thaliana*. *Planta*, **211**, 632-640.

Burssens, S., Van Montagu, M. and Inzé, D. (1998) The cell cycle in *Arabidopsis*. *Plant Physiology & Biochemistry*, **36**, 9-19.

Caddick, M.X., Greenland, A.J., Jepson, I., Krause, K.P., Qu, N., Riddell, K.V., Salter, M.G., Schuch, W., Sonnewald, U. and Tomsett, A.B. (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. *Nature Biotechnology*, **16**, 177-180.

Campbell, S.D., Sprenger, F., Edgar, B.A. and Ofarrell, P.H. (1995) *Drosophila* Wee1 kinase rescues fission yeast from mitotic catastrophe and phosphorylates *Drosophila* Cdc2 in vitro. *Molecular Biology of the Cell*, **6**, 1333-1347.

Carr, A.M., MacNeill, S.A., Hayles, J. and Nurse, P. (1989) Molecular cloning and sequence analysis of mutant alleles of the fission yeast *cdc2* protein kinase gene: Implications for $cdc2^{+}$ protein structure and function. *Molecular & General Genetics*, **218**, 41-49.

Chaubet-Gigot, N. (2000) Plant A-type cyclins. *Plant Molecular Biology*, **43**, 659-675.

Chrast-Balz, J. and Hooft van Huijsduijnen, R. (1996) Bi-directional gene switching with the tetracycline repressor and a novel tetracycline agonist. *Nucleic Acids Research*, **24**, 2900-2904.

Christensen, P.U., Bentley, N.J., Martinho, R.G., Nielsen, O. and Carr, A.M. (2000) Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 2579-2584.

Clarke, P., Hoffmann, R., Draetta, G. and Karsenti, E. (1993) Dephosphorylation of Cdc25-C by a type-2A phosphatase:specific regulation during the cell cycle in *Xenopus* egg extracts. *Molecular Biology of the Cell*, **4**, 397-411.

Cockcroft, C., den Boer, B., Healy, J.M.S. and Murray, J.A.H. (2000) Cyclin D control of growth rate in plants. *Nature*, **405**, 575-579.

Cohen, E., Heimer, Y.M. and Mizrahi, Y. (1982) Ornithine decarboxylase and arginine decarboxylase activities in mersitematic tissue of tomato and potato plants. *Plant Physiology*, **70**, 544-546.

Colasanti, J., Cho, S.-O., Wick, S. and Sundarasen, V. (1993) Localization of the functional $p34^{cdc2}$ homolog of maize in root tip and stomatal complex cells: association with predicted division sites. *Plant Cell*, **5**, 1101-1111.

Colasanti, J., Tyers, M. and Sundaresan, V. (1991) Isolation and characterization of cDNA clones encoding a functional $p34^{cdc2}$ homologue from *Zea mays*. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 3377-3381.

Corlett, J.E., Myatt, S.C. and Thompson, A.J. (1996) Toxicity symptoms caused by high expression of Tet repressor in tomato (*Lycopersicon esculentum* Mill L) are alleviated by tetracycline. *Plant Cell & Environment*, **19**, 447-454.

Correa-Bordes, J., Gulli, M.P. and Nurse, P. (1997) $p25^{rum1}$ promotes proteolysis of the mitotic B-cyclin $p56^{cdc13}$ during G(1) of the fission yeast cell cycle. *EMBO Journal*, **16**, 4657-4664.

Correa-Bordes, J. and Nurse, P. (1995) $p25^{rum1}$ orders S phase and mitosis by acting as an inhibitor of the $p34^{cdc2}$ mitotic kinase. *Cell*, **83**, 1001-1009.

Daga, R.R. and Jimenez, J. (1999) Translational control of the Cdc25 cell cycle phosphatase: a molecular mechanism coupling mitosis to cell growth. *Journal of Cell Science*, **112**, 3137-3146.

Dahl, M., Meskiene, I., Bögre, L., Ha, D.T.C., Swoboda, I., Hubmann, R., Hirt, H. and Heberlebors, E. (1995) The D-Type Alfalfa cyclin gene *Cycms4* complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. *Plant Cell*, **7**, 1847-1857.

Dalal, S.N., Schweitzer, C.M., Gan, J.M. and DeCaprio, J.A. (1999) Cytoplasmic localization of human Cdc25C during interphase requires an intact 14-3-3 binding site. *Molecular & Cellular Biology*, **19**, 4465-4479.

Datar, S.A., Jacobs, H.W., de la Cruz, A.F.A., Lehner, C.F. and Edgar, B.A. (2000) The *Drosophila* cyclin D-Cdk4 complex promotes cellular growth. *EMBO Journal*, **19**, 4543-4554.

De Bondt, H.L., Rosenblatt, J., Jancarik, J., Jones, H.D., Morgan, D.O. and Kim, S.H. (1993) Crystal Structure of Cyclin-Dependent Kinase-2. *Nature*, **363**, 595-602.

De Jager, S.M. and Murray, J.A.H. (1999) Retinoblastoma proteins in plants. *Plant Molecular Biology*, **41**, 295-299.

De Rocher, E.J., Vargo-Gogola, T.C., Diehn, S. and Green, P. (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. *Plant Physiol.*, **117**, 1445-1461.

De Veylder, L., Beeckman, T., Van Montagu, M. and Inzé, D. (2000) Increased leakiness of the tetracycline-inducible Triple-Op promoter in dividing cells renders it unsuitable for high inducible levels of a dominant negative *CDC2aAt* gene. *Journal of Experimental Botany*, **51**, 1647-1653.

De Veylder, L., Engler, J.D., Burssens, S., Manevski, A., Lescure, B., Van Montagu, M., Engler, G. and Inzé, D. (1999) A new D-type cyclin of *Arabidopsis thaliana* expressed during lateral root primordia formation. *Planta*, **208**, 453-462.

De Veylder, L., Segers, G., Glab, N., Casteels, P., Van Montagu, M. and Inzé, D. (1997a) The *Arabidopsis* Cks1At protein binds the cyclin-dependent kinases Cdc2aAt Cdc2bAt. *FEBS Letters*, **412**, 446-452.

De Veylder, L., Segers, G., Glab, N., Van Montagu, M. and Inzé, D. (1997b) Identification of proteins interacting with the *Arabidopsis* Cdc2aAt. *Journal of Experimental Botany*, **48**, 2113-2114.

De Veylder, L., Van Montagu, M. and Inzé, D. (1998) Cell cycle control in *Arabidopsis*. In Francis, D., Dudits, D. and Inzé, D. (eds.), *Plant cell division*. Portland Press, London, pp. 1-19.

Doerner, P., Jørgensen, J.-E., You, R., Steppuhn, J. and Lamb, C. (1996) Control of root growth and development by cyclin expression. *Nature*, **380**, 520-523.

Dohrna, C.L.Z., Brandeis, M., Berr, F., Mossner, J. and Engeland, K. (2000) A CDE/CHR tandem element regulates cell cycle-dependent repression of cyclin B2 transcription. *FEBS Letters*, **484**, 77-81.

Draetta, G. (1990) Cell cycle control in eukaryotes: molecular mechanisms of cdc2 activation. *Trends In Biochemical Sciences*, **15**, 378-383.

Draetta, G., Beach, D. and Moran, E. (1988) Synthesis of p34, the mammalian homolog of the yeast cdc2+/CDC28 protein kinase, is stimulated during adenovirus-induced proliferation. *Oncogene*, **2**, 553-557.

Draetta, G., Brizuela, L., Potashkin, J. and Beach, D. (1987) Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by $cdc2^+$ and $suc1^+$. *Cell*, **50**, 319-325.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J. and Beach, D. (1989) Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell*, **56**, 829-838.

Dudits, D., Magyar, Z., Deák, M., Mészáros, T., Miskolczi, P., Fehér, A., Brown, S., Kondorosi, É., Athanasiadis, A., Pongor, S., Bakó, L., Koncz, C. and Györgyey, J. (1998) Cyclin-dependent and calcium-dependent kinase families: response of cell division cycle to hormone and stress signals. In Francis, D., Dudits, D. and Inzé, D. (eds.), *Plant cell division*. Portland Press, London, pp. 21-45.

Dunphy, W.G. (1994) The decision to enter mitosis. *Trends In Cell Biology*, **4**, 202-207.

Dunphy, W.G., Brizuela, L., Beach, D. and Newport, J. (1988) The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell*, **54**, 423-431.

Dunphy, W.G. and Kumagai, A. (1991) The cdc25 protein contains an intrinsic phosphatase activity. *Cell*, **67**, 189-196.

Durfee, T., Feiler, H.S. and Gruissem, W. (2000) Retinoblastoma-related proteins in plants: homologues or orthologues of their metazoan counterparts? *Plant Molecular Biology*, **43**, 635-642.

Durkacz, B., Carr, A. and Nurse, P. (1986) Transcription of the *cdc2* cell cycle control gene of the fission yeast *Schizosaccharomyces pombe*. *EMBO Journal*, **5**, 369-373.

Dyson, N. (1998) The regulation of E2F by pRB-family proteins. *Genes & Development*, **12**, 2245-2262.

Edgar, B. and O'Farrell, P. (1989) Genetic control of the cell division patterns in the *Drosophila* embryo. *Cell*, **57**, 177-187.

Ekholm, S.V. and Reed, S.I. (2000) Regulation of G1 cyclin dependent kinases in the mammalian cell cycle. *Current Opinion in Cell Biology*, **12**, 676-684.

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983) Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, **33**, 389-396.

Fabian, T., Lorbiecke, R., Umeda, M. and Sauter, M. (2000) The cell cycle genes *cycA1;1* and *cdc2Os-3* are coordinately regulated by gibberellin in planta. *Planta*, **211**, 376-383.

Faiss, M., Strnad, M., Redig, P., Dolezal, K., Hanus, J., Van Onckelen, H. and Schmülling, T. (1996) Chemically induced expression of the *rolC*-encoded Beta-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism - RolC does not hydrolyze endogenous cytokinin glucosides in planta. *Plant Journal*, **10**, 33-46.

Fantes, P. (1979) Epistatic gene interactions in the control of division in fission yeast. *Nature*, **279**, 428-430.

Feiler, H.S. and Jacobs, T.W. (1991) Cloning of the pea *cdc2* homologue by efficient immunological screening of PCR products. *Plant Molecular Biology*, **17**, 321-334.

Ferreira, P.C.G., Hemerly, A., Engler, J.D., Bergounioux, C., Burssens, S., Van Montagu, M., Engler, G. and Inzé, D. (1994) Three discrete classes of *Arabidopsis* cyclins are expressed during different intervals of the cell cycle. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 11313-11317.

Ferreira, P.C.G., Hemerly, A.S., Van Montagu, M. and Inzé, D. (1993) A protein phosphatase 1 from *Arabidopsis thaliana* restores temperature sensitivity of a *Schizosaccharomyces pombe* $cdc25^{ts}/wee1^{-}$ double mutant. *Plant Journal*, **4**, 81-87.

Ferreira, P.C.G., Hemerly, A.S., Villaroel, R., Van Montagu, M. and Inzé, D. (1991) The *Arabidopsis* functional homolog of $p34^{cdc2}$ protein kinase. *Plant Cell*, **3**, 531-540.

Fleig, U.N. and Nurse, P. (1991) Expression of a dominant negative allele of *cdc2* prevents activation of endogenous p34cdc2 kinase. *Molecular & General Genetics*, **226**, 432-440.

Foard, D.E., Haber, A.N. and Fishman, T.N. (1965) Initiation of lateral root primordia without completion of mitosis and without cytokinesis in uniseriate pericycle. *American Journal of Botany*, **52**, 580-590.

Fobert, P.R., Gaudin, V., Lunness, P., Coen, E.S. and Doonan, J.H. (1996) Distinct classes of *cdc2*-related genes are differentially expressed during the cell division cycle in plants. *Plant Cell*, **8**, 1465-1476.

Foley, E. and Sprenger, F. (2000) Cyclins: growing pains for *Drosophila*. *Current Biology*, **10**, R665-R667.

Fuerst, R., Soni, R., Murray, J.A.H. and Lindsey, K. (1996) Modulation of cyclin transcript levels in cultured cells of *Arabidopsis thaliana*. *Plant Physiology*, **112**, 1023-1033.

Furnari, B., Blasina, A., Boddy, M.N., McGowan, C.H. and Russell, P. (1999) Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. *Molecular Biology of the Cell*, **10**, 833-845.

Furnari, B., Rhind, N. and Russell, P. (1997) Cdc25 mitotic inducer targeted by Chk1 DNA damage checkpoint kinase. *Science*, **277**, 1495-1497.

Gabrielli, B., De Souza, C., Tonks, I., Clark, J., Hayward, N. and Ellem, K. (1996) Cytoplasmic accumulation of Cdc25B phosphatase in mitosis triggers centrosomal microtubule nucleation in HeLa cells. *Journal of Cell Science*, **109**, 1081-1093.

Galaktionov, K. and Beach, D. (1991) Specific activation of Cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, **67**, 1181-1194.

Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient requirements of suspension cultures of soy bean root cells. *Experimental Cell Research*, **50**, 151-158.

Gatz, C. (1996) Chemically inducible promoters in transgenic plants. *Current Opinion in Biotechnology*, **7**, 168-172.

Gatz, C. (1997) Chemical control of gene expression. *Annual Review of Plant Physiology & Plant Molecular Biology*, **48**, 89-108.

Gatz, C. (1998) An intoxicating switch for plant transgene expression. *Nature Biotechnology*, **16**, 140.

Gatz, C., Frohberg, C. and Wendenburg, R. (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants. *Plant Journal*, **2**, 397-404.

Gatz, C., Kaiser, A. and Wendenburg, R. (1991) Regulation of a modified CaMV 35S promoter by the Tn*10*-encoded Tet repressor in transgenic tobacco. *Molecular & General Genetics*, **227**, 229-237.

Gautier, J. (1993) The Cdc25 protein tyrosine phosphatase family. *Adv. Prot. Phosphatases*, **7**, 153-171.

Gautier, J., Norbury, C., Lohka, M., Nurse, P. and Maller, J. (1988) Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle gene *cdc2*. *Cell*, **54**, 433-439.

Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F. and Kirschner, M.W. (1991) Cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. *Cell*, **67**, 197-211.

Geissendörfer, M. and Hillen, W. (1990) Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn*10*-encoded tet regulatory elements. *Appl. Microbio. Biotechnol.*, **33**, 657-663.

Girard, F., Strausfeld, U., Cavadore, J.-C., Russell, P., Fernandez, A. and Lamb, N.J.C. (1992) Cdc25 is a nuclear protein expressed constitutively thoughout the cell cycle in nontransformed mammalian cells. *Journal of Cell Biology*, **118**, 785-794.

Glotzer, M., Murray, A.W. and Kirschner, M.W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*, **349**, 132-138.

Gorst, J.R., John, P.C.L. and Sek, F.J. (1991) Levels of p34cdc2-like protein in dividing differentiating and dedifferentiating cells of carrot. *Planta*, **185**, 304-310.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 5547-5551.

Gossen, M. and Bujard, H. (1993) Anhydrotetracycline, a novel effector for Tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Research*, **21**, 4411-4412.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S. and Nurse, P. (1991) Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. *EMBO Journal*, **10**, 3297-3309.

Gould, K.L., Moreno, S., Tonks, N.K. and Nurse, P. (1990) Complementation of the mitotic activator $p80^{cdc25}$ by human protein tyrosine phosphatase. *Science*, **250**, 1573-1576.

Gould, K.L. and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast Cdc2+ protein kinase regulates entry into mitosis. *Nature*, **342**, 39-45.

Gunning, B.E.S. and Sammut, M. (1990) Rearrangement of microtubules involved in establishing cell division planes start immediately after DNA synthesis are completed just before mitosis. *Plant Cell*, **2**, 1273-1282.

Gutiérrez, C. (1998) The retinoblastoma pathway in plant cell cycle and development. *Current Opinion in Plant Biology*, **1**, 492-497.

Gutiérrez, C. (2000) DNA replication and cell cycle in plants: learning from geminiviruses. *EMBO Journal*, **19**, 792-799.

Gutiérrez, R.A., MacIntosh, G.C. and Green, P.J. (1999) Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. *Trends in Plant Science*, **4**, 429-438.

Hadwiger, J.A., Wittenberg, C., Mendenhall, M.D. and Reed, S.I. (1989) The *Saccharomyces cerevisiae CKS1 gene*, a homolog of the Schizosaccharomyces *pombe suc1*$^+$ gene, encodes a subunit of the Cdc28 protein kinase complex. *Molecular & Cellular Biology*, **9**, 2034-2041.

Hagan, I., Hayles, J. and Nurse, P. (1988) Cloning and sequencing of the cyclin-related *cdc13*$^+$ gene and a cytological study of its role in fission yeast mitosis. *Journal of Cell Science*, **91**, 587-595.

Hamill, J.D. (1993) Alterations in auxin and cytokinin metabolism of higher plants due to expression of specific genes from pathogenic bacteria: A review. *Australian Journal of Plant Physiology*, **20**, 405-423.

Hamill, J.D. and Lidgett, A.J. (1997) Hairy root cultures - opportunities and key protocols for studies in metabolic engineering. In Doran, P. (ed.) *Hairy Roots*. Harwood Academic Publishers Ltd, Reading, UK, pp. 1-30.

Hamill, J.D., Robins, R.J., Parr, A.J., Evans, D.M., Furze, J.M. and Rhodes, J.C. (1990) Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. *Plant Molecular Biology*, **15**, 27-38.

Harbour, J.W., Luo, R.X., Santi, A.D., Postigo, A.A. and Dean, D.C. (1999) CDK phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*, **98**, 859-869.

Hashimoto, J., Hirabayashi, T., Hayano, Y., Hata, S., Ohashi, Y., Suzuka, I., Utsugi, T., Toh-E, A. and Kikuchi, Y. (1992) Isolation and characterization of cDNA clones encoding *cdc2* homologues from *Oryza sativa*: a functional homologue and cognate variants. *Molecular & General Genetics*, **233**, 10-16.

Hata, S. (1991) cDNA cloning of a novel cdc2$^+$/CDC28-related protein kinase from rice. *FEBS Lett.*, **279**, 149-152.

Hata, S., Kouchi, H., Suzuka, I. and Ishii, T. (1991) Isolation and characterization of cDNA clones for plant cyclins. *EMBO Journal*, **10**, 2681-2688.

Hayles, J., Aves, S. and Nurse, P. (1986) *suc1* is an essential gene involved in both the cell cycle and growth in fission yeast. *EMBO Journal*, **5**, 3373-3379.

Hemerly, A., Engler, J.D., Bergounioux, C., Van Montagu, M., Engler, G., Inzé, D. and Ferreira, P. (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. *EMBO Journal*, **14**, 3925-3936.

Hemerly, A.S., Ferreira, P., Engler, J.D., Van Montagu, M., Engler, G. and Inzé, D. (1993) *cdc2a* expression in *Arabidopsis* is linked with competence for cell division. *Plant Cell*, **5**, 1711-1723.

Hemerly, A.S., Ferreira, P.C.G., Van Montagu, M., Engler, G. and Inzé, D. (2000) Cell division events are essential for embryo patterning and morphogenesis: studies on dominant-negative *cdc2aAt* mutants of *Arabidopsis*. *Plant Journal*, **23**, 123-130.

Hepler, P.K., Sek, F.J. and John, P.C.L. (1994) Nuclear concentration and mitotic dispersion of the essential cell cycle protein, $p13^{suc1}$, examined in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 2176-2180.

Hillen, W., Schollmeier, K. and Gatz, C. (1984) Control of expression of the Tn*10*-encoded tetracycline resistance operon. II Interaction of RNA polymerase and Tet repressor with the *tet* operon regulatory region. *Journal of Molecular Biology*, **172**, 185-201.

Hindley, J. and Phear, G.A. (1984) Sequence of the cell division gene *CDC2* from *Schizosaccharomyces pombe*; patterns of splicing and homology to protein kinases. *Gene*, **31**, 129-134.

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.

Hirt, H., Páy, A., Györgyey, J., Bakó, L., Németh, K., Bögre, L., Schweyen, R.J., Heberle-Bors, E. and Dudits, D. (1991) Complementation of a yeast cell cycle mutant by an alfalfa cDNA encoding a protein kinase homologous to $p34^{cdc2}$. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 1636-1640.

Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E. and Draetta, G. (1993) Phosphorylation and activation of human Cdc25-C by Cdc2-cyclin B and its involvement in the self amplification of MPF at mitosis. *EMBO Journal*, **12**, 53-63.

Hoffmann, I. and Karsenti, E. (1994) The role of cdc25 in checkpoints and feedback controls in the eukaryotic cell cycle. *J. Cell Sci. (S)*, **18**, 75-79.

Hunt, T. and Sherr, C.J. (1994) Cell multiplication. Editorial overview. Checks and balances. *Current Opinion in Cell Biology*, **6**, 833-835.

Huntley, R., Healy, S., Freeman, D., Lavender, P., Dejager, S., Greenwood, J., Makker, J., Walker, E., Jackman, M., Xie, Q., Bannister, A.J., Kouzarides, T., Gutiérrez, C., Doonan, J.H. and Murray, J.A.H. (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. *Plant Molecular Biology*, **37**, 155-169.

Huntley, R.P. and Murray, J.A.H. (1999) The plant cell cycle. *Current Opinion in Plant Biology*, **2**, 440-446.

Hush, J., Wu, L.P., John, P.C.L., Hepler, L.H. and Hepler, P.K. (1996) Plant mitosis promoting factor disassembles the microtubule preprophase and accelerates prophase progression in tradescantia. *Cell Biology International*, **20**, 275-287.

Imajuku, Y., Hirayama, T., Endoh, H. and Oka, A. (1992) Exon-intron organization of the *Arabidopsis thaliana* protein kinase genes *CDC2a* and *CDC2b*. *FEBS Letters*, **304**, 73-77.

Ito, M., Iwase, M., Kodama, H., Lavisse, P., Komamine, A., Nishihama, R., Machida, Y. and Watanabe, A. (1998) A novel *cis*-acting element in promoters of plant B-type cyclin genes activates M phase-specific transcription. *Plant Cell*, **10**, 331-341.

Izumi, T. and Maller, J.L. (1995) Phosphorylation and activation of the *Xenopus* Cdc25 phosphatase in the absence of Cdc2 and Cdk2 kinase activity. *Molecular Biology of the Cell*, **6**, 215-226.

Jacobs, T. (1993) Molecular and cell biology of the plant cell cycle - Ormrod,Jc, Francis,D. *Science*, **262**, 1461-1462.

Jacobs, T.W. (1995) Cell cycle control. *Annual Review of Plant Physiology & Plant Molecular Biology*, **46**, 317-339.

Jacqmard, A., De Veylder, L., Segers, G., Engler, J.D., Bernier, G., Van Montagu, M. and Inzé, D. (1999) Expression of *CKS1At* in *Arabidopsis thaliana* indicates a role for the protein in both the mitotic and the endoreduplication cycle. *Planta*, **207**, 496-504.

Jagadish, M.N. and Carter, B.L.A. (1977) Genetic control of cell division in yeast cultured at different growth rates. *Nature*, **269**, 145-147.

Jefferson, R.A., Kavanaugh, T.A. and Bevan, M.W. (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal*, **6**, 3901-3907.

Jeffrey, P.D., Ruso, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N.P. (1995) Mechanism of Cdk activation revealed by the structure of a cyclinA-Cdk2 complex. *Nature*, **376**, 313-320.

Jiminez, J., Alphey, L., Nurse, P. and Glover, D.M. (1990) Complementation of fission yeast *cdc2*^{ts} and *cdc25*^{ts} mutants identifies two cell cycle genes from *Drosophila*: a cdc2 homologue and string. *EMBO Journal*, **9**, 3563-3571.

John, P.C.L. (1996) The plant cell cycle: conserved and unique features in mitotic control. In Meijer, L., Guider, S. and Vogel, L. (eds.), *Progress in cell cycle research*. Plenum Press, New York, Vol. 2, pp. 59-72.

John, P.C.L., Sek, F.J. and Hayles, J. (1991) Association of the plant p34^{cdc2}-like protein with p13^{suc1} implications for control of cell division cycles in plants. *Protoplasma*, **161**, 70-74.

John, P.C.L., Zhang, K. and Dong, C. (1993) A p34cdc2-based cell cycle: its significance in monocotyledonous, dicotyledonous and unicellular plants. In Ormrod, J.C. and Francis, D. (eds.), *Molecular and Cell Biology of the Plant Cell Cycle: proceedings of a meeting held at Lancaster University, 9-10 April 1992*. Kluwer Academic Publishers, Dordrecht, pp. 9-34.

Jones, A.M., Im, K.H., Savka, M.A., Wu, M.J., Dewitt, N.G., Shillito, R. and Binns, A.N. (1998) Auxin-dependent cell expansion mediated by overexpressed auxin-binding protein 1. *Science*, **282**, 1114-1117.

Joubès, J., Chevalier, C., Dudits, D., Heberle-Bors, E., Inzé, D., Umeda, M. and Renaudin, J.P. (2000) CDK-related protein kinases in plants. *Plant Molecular Biology*, **43**, 607-620.

Kakizuka, A., Sebastian, B., Borgmeyer, U., Hermans-Borgmeyer, I., Bolado, J., Hunter, T., Hoekstra, M.F. and Evans, R.M. (1992) A mouse homolog is differentially and developmentally expressed. *Genes Dev.*, **6**, 578-590.

Karaiskou, A., Jessus, C., Brassac, T. and Ozon, R. (1999) Phosphatase 2A and Polo kinase, two antagonistic regulators of Cdc25 activation and MPF auto-amplification. *Journal of Cell Science*, **112**, 3747-3756.

Karlsson, C., Katich, S., Hagting, A., Hoffmann, I. and Pines, J. (1999) Cdc25B and Cdc25C differ markedly in their properties as initiators of mitosis. *Journal of Cell Biology*, **146**, 573-84.

Kong, M., Barnes, E.A., Ollendorff, V. and Donoghue, D.J. (2000) Cyclin F regulates the nuclear localization of cyclin B1 through a cyclin-cyclin interaction. *EMBO Journal*, **19**, 1378-1388.

Krek, W. and Nigg, E.A. (1989) Structure and developmental expression of the chicken CDC2 kinase. *EMBO Journal*, **8**, 3071-3078.

Krek, W. and Nigg, E.A. (1991) Differential phosphorylation of vertebrate $p34^{cdc2}$ kinase at the G1/S and G2/M transitions of the cell cycle: Identification of major phosphorylation sites. *EMBO Journal*, **10**, 305-316.

Kumagai, A. and Dunphy, W.G. (1991) The Cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell*, **64**, 903-914.

Kumagai, A. and Dunphy, W.G. (1992) Regulation of the Cdc25 protein during the cell cycle in *Xenopus* extracts. *Cell*, **70**, 139-151.

Kumagai, A. and Dunphy, W.G. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. *Science*, **273**, 1377-1380.

Kumagai, A. and Dunphy, W.G. (1999) Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes & Development*, **13**, 1067-1072.

Kumagai, A., Yakowec, P.S. and Dunphy, W.G. (1998) 14-3-3 proteins act as negative regulators of the inducer Cdc25 in *Xenopus* egg extracts. *Molecular Biology of the Cell*, **9**, 345-354.

Kumar, A., Taylor, M.A., Arif, S.A.M. and Davies, H.V. (1996) Potato plants expressing antisense and sense S-adenosylmethionine decarboxylase (SAMDC) transgenes show altered levels of polyamines and ethylene - antisense plants display abnormal phenotypes. *Plant Journal*, **9**, 147-158.

Labbé, J.C., Capony, J.-P., Caput, D., Cavadore, J.-C., Derancourt, J., Kaghad, M., Lelias, J.-M., Picard, A. and Dorée, M. (1989) MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO Journal*, **8**, 3052-3058.

Labbé, J.C., Lee, M.G., Nurse, P., Picard, A. and Doree, M. (1988) Activation at M-phase of a protein kinase encoded by a starfish homologue of the cell cycle control gene *cdc2*$^{+}$. *Nature*, **335**, 251-254.

Labbé, J.-C., Martinez, A.M., Fesquet, D., Capony, J.-P., Darbon, J.-M., Derancourt, J., Devault, A., Morin, N., Cavadore, J.-C. and Dorée, M. (1994) p40^{MO15} associates with a p36 subunit and requires both nuclear translocation and Thr176 phosphorylation to generate Cdk-activating kinase activity in *Xenopus* oocytes. *EMBO Journal*, **13**.

Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W. and Hoffmann, I. (1998) The Cdc25b phosphatase is essential for the G2/M phase transition in human cells. *Journal of Cell Science*, **111**, 2445-2453.

Lee, M.G., Norbury, C.J., Spurr, N.K. and Nurse, P. (1988) Regulated expression and phosphorylation of a possible mammalian cell-cycle control protein. *Nature*, **333**, 676-679.

Lee, M.G. and Nurse, P. (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. *Nature*, **327**, 31-35.

Lemcke, K. and Schmülling, T. (1998) Gain of function assays identify non-*rol* genes from *Agrobacterium rhizogenes* Ti-DNA that alter plant morphogenesis or hormone sensitivity. *Plant Journal*, **15**, 423-433.

Lessard, P., Bouly, J.P., Jouannic, S., Kreis, M. and Thomas, M. (1999) Identification of Cdc2cAt: a new cyclin-dependent kinase expressed in *Arabidopsis thaliana* flowers. *Biochimica et Biophysica Acta - Gene Structure & Expression*, **1445**, 351-358.

Lew, D.J., Dulic, V. and Reed, S.I. (1991) Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell*, **66**, 1197-1206.

Lew, D.J. and Reed, S.I. (1993) Morphogenesis in the yeast cell cycle: Regulation by Cdc28 and cyclins. *Journal of Cell Biology*, **120**, 1305-1320.

Lidgett, A.J., Moran, M., Wong, K., Furze, J., Rhodes, M.J. and Hamill, J.D. (1995) Isolation and expression pattern of a cDNA encoding cathepsin B-like protease from *Nicotiana rustica*. *Plant Molecular Biology*, **29**, 379-384.

Lloyd, A.M., Schena, M., Walbot, V. and Davis, R.W. (1994) Epidermal cell fate in *Arabidopsis*: patterns defined by a steroid-inducible regulator. *Science*, **266**, 436-439.

Lopez-Girona, A., Furnari, B., Mondesert, O. and Russell, P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature*, **397**, 172-175.

Love, J., Scott, A.C. and W.F., T. (2000) Stringent control of transgene expression in *Arabidopsis thaliana* using the Top10 promoter system. *Plant Journal*, **21**, 579-588.

Lui, H., Wang, H., DeLong, C., Fowke, L.C., Crosby, W.L. and Fobert, P.R. (2000) The *Arabidopsis* Cdc2a-interacting protein ICK2 is structurally related to ICK1 and is a potent inhibitor of cyclin-dependent kinase activity in vitro. *Plant Journal*, **21**, 379-385.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991) Mik1 and Wee1 cooperate in the tyrosine phosphorylation of Cdc2. *Cell*, **64**, 1111-1122.

MacNeill, S.A. and Nurse, P. (1993) Mutational analysis of the fission yeast $p34^{cdc2}$ protein kinase gene. *Molecular & General Genetics*, **236**, 415-426.

Magyar, Z., Meszaros, T., Miskolczi, P., Deák, M., Fehér, A., Brown, S., Kondorosi, É., Athanasiadis, A., Pongor, S., Bilgin, M., Bakó, L., Koncz, C. and Dudits, D. (1997) Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized Alfalfa cells. *Plant Cell*, **9**, 223-235.

Martín-Castellanos, C., Blanco, M.A., de Prada, J.M. and Moreno, S. (2000) The puc1 cyclin regulates the G1 phase of the fission yeast cell cycle in response to cell size. *Molecular Biology of the Cell*, **11**, 543-554.

Martinez, A., Sparks, C., Hart, C.A., Thompson, J. and Jepson, I. (1999) Ecdysone agonist inducible transcription in transgenic tobacco plants. *Plant Journal*, **19**, 97-106.

Martinez, M.C., Jørgensen, J.-E., Lawton, M.A., Lamb, C.J. and Doerner, P.W. (1992) Spatial pattern of *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 America*, **89**, 7360-7364.

Martinho, R.G., Lindsay, H.D., Flaggs, G., DeMaggio, A.J., Hoekstra, M.F., Carr, A.M. and Bentley, N.J. (1998) Analysis of Rad3 and Chk1 protein kinases defines different checkpoint responses. *EMBO Journal*, **17**, 7239-7249.

Masgrau, C., Altabella, T., Farrás, R., Flores, D., Thompson, A.J., Besford, R.T. and Tiburcio, A.F. (1997) Inducible overexpression of oat arginine decarboxylase in transgenic tobacco plants. *Plant Journal*, **11**, 465-473.

Masui, Y. and Markert, C. (1971) Cytoplasmic control of nuclear behaviour during meiotic maturation of frog oocytes. *J. Exp. Zool.*, **177**, 129-146.

Matsuoka, S., Mingxia, H. and Elledge, S.J. (1998) Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*, **282**, 1893-1897.

McKenzie, M.J., Mett, V., Reynolds, P.H.S. and Jameson, P.E. (1998) Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. *Plant Physiology*, **116**, 969-977.

McKibbin, R.S., Halford, N.G. and Francis, D. (1998) Expression of fission yeast c*dc25* alters the frequency of lateral root formation in transgenic tobacco. *Plant Molecular Biology*, **36**, 601-612.

McNellis, T., Mudgett, M.B., Li, K., Aoyama, T., Horvath, D., Chua, N.-H. and Staskawicz. (1998) Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death. *Plant Journal*, **14**, 247-257.

Meijer, L., Arion, D., Golsteyn, R., Pines, J., Brizuela, L., Hunt, T. and Beach, D. (1989) Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. *EMBO Journal*, **8**, 2275-2282.

Mészáros, T., Miskolczi, P., Ayaydin, F., Pettkó-Szandtner, A., Peres, A., Magyar, Z., Horváth, G.V., Bakó, L., Fehér, A. and Dudits, D. (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. *Plant Molecular Biology*, **43**, 595-605.

Mett, V.L., Lochhead, L.P. and Reynolds, P.H. (1993) Copper-controllable gene expression system for whole plants. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 4567-4571.

Mews, M., Sek, F.J., Moore, R., Volkmann, D., Gunning, B.E.S. and John, P.C.L. (1997) Mitotic cyclin distribution during maize cell division - Implications for the sequence diversity and function of cyclins in plants. *Protoplasma*, **200**, 128-145.

Meyerson, M., Enders, G., Wu, C.-L., Su, L.-K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.-H. (1992) A family of human Cdc2-related protein kinases. *EMBO Journal*, **11**, 2909-2917.

Miao, G.-H., Hong, Z. and Verma, D.P.S. (1993) Two functional soybean genes encoding $p34^{cdc2}$ protein kinases are regulated by different plant developmental pathways. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 943-947.

Millar, J., McGowan, C., Jones, R., Sadhu, K., Bueno, A., Richardson, H. and Russell, P. (1991a) *cdc25* M-phase Inducer. *Cold Spring Harbour Symposia on Quantitative Biology*, **LVI**, 577-584.

Millar, J.B.A., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C. and Russell, P. (1991b) $p55^{cdc25}$ is a nuclear protein required for the initiation of mitosis in human cells. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 10500-10504.

Millar, J.B.A., Lenaers, G. and Russell, P. (1992) *Pyp3* PTPase acts as a mitotic inducer in fission yeast. *EMBO Journal*, **11**, 4933-4941.

Millar, J.B.A. and Russell, P. (1992) The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell*, **68**, 407-410.

Mineyuki, Y., Yamashita, M. and Nagahama, Y. (1991) p34^{cdc2} kinase homologue in the preprophase band. *Protoplasma*, **162**, 182-186.

Minshull, J. (1993) Cyclin synthesis: Who needs it? *Bioessays*, **15**, 149-155.

Minshull, J., Blow, J. and Hunt, T. (1989) Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. *Cell*, **56**, 947-956.

Minshull, J., Golsteyn, R., Hill, C.S. and Hunt, T. (1990) The A- and B-type cyclin associated Cdc2 kinases in *Xenopus* turn on and off at different times in the cell cycle. *EMBO Journal*, **9**, 2865-2875.

Mironov, V., De Veylder, L., Van Montagu, M. and Inzé, D. (1999) Cyclin-dependent kinases and cell division in plants - the nexus. *Plant Cell*, **11**, 509-521.

Mizukami, Y. and Fischer, R.L. (2000) Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 942-947.

Moreno, S., Hayles, J. and Nurse, P. (1989) Regulation of p34^{cdc2} protein kinase during mitosis. *Cell*, **58**, 361-372.

Moreno, S., Nurse, P. and Russell, P. (1990) Regulation of mitosis by cyclic accumulation of p80^{cdc25} mitotic inducer in fission yeast. *Nature*, **344**, 549-552.

Morgan, D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131-134.

Morgan, D.O. and De Bondt, H.L. (1994) Protein kinase regulation - insights from crystal structure analysis. *Current Opinion in Cell Biology*, **6**, 239-246.

Morris, M., Heitz, A., Mery, J., Heitz, F. and Divita, G. (2000) An essential phosphorylation-site domain of human Cdc25C interacts with both 14-3-3 and cyclins. *Journal of Biological Chemistry*, **275**, 28849-28857.

Motyka, V., Faiss, M., Strnad, M., Kaminek, M. and Schmülling, T. (1996) Changes in cytokinin content and cytokinin oxidase activity in response to derepression of *ipt* gene transcription in transgenic tobacco calli and plants. *Plant Physiology*, **112**, 1035-1043.

Mueller, P.R., Coleman, T.R. and Dunphy, W.G. (1995a) Cell cycle regulation of a *Xenopus* Wee1-like kinase. *Molecular Biology of the Cell*, **6**, 119-134.

Mueller, P.R., Coleman, T.R., Kumagai, A. and Dunphy, W.G. (1995b) Myt1 - a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science*, **270**, 86-90.

Müller, R. (1995) Transcriptional regulation during the mammalian cell cycle. *Trends in Genetics*, **11**, 173-178.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassys with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473-479.

Murray, A.M. and Hunt, T. (1993) *The cell cycle: an introduction.* W.H. Freeman and Company, New York.

Murray, A.W. (1989) The cell cycle as a *cdc2* cycle. *Nature*, **342**, 14-15.

Murray, A.W. (1992) Creative blocks: cell-cycle checkpoints and feedback controls. *Nature*, **359**, 599-604.

Murray, E.E., Lotzer, J. and Eberle, M. (1989) Codon usage in plant genes. *Nuclic Acids Research,* **17**, 477-498.

Nagata, A., Igarashi, M., Jinno, S., Suot, K. and Okayama, H. (1991) An additional homolog of the fission yeast *cdc25*$^{+}$ gene occurs in humans and is highly expressed in some cancer cells. *New Biol.*, **3**, 959-968.

Nakagami, H., Sekine, M., Murakami, H. and Shinmyo, A. (1999) Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D in vitro. *Plant Journal*, **18**, 243-252.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y. and Nakayama, K. (1996) Mice lacking p27(Kip1) display increased body size, muliple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell*, **85**, 707-720.

Nakayama, K.-I. and Nakayama, K. (1998) Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. *BioEssays*, **20**, 1020-1029.

Nasmyth, K. (1996) At the heart of the budding yeast cell cycle. *Trends in Genetics*, **12**, 405-412.

Nefsky, B. and Beach, D. (1996) Pub1 acts as an E6-Ap-like protein ubiquitin ligase in the degradation of Cdc25. *EMBO Journal*, **15**, 1301-1312.

Neufield, T.P. and Edgar, B.A. (1998) Connections between growth and the cell cycle. *Current Opinion in Cell Biology*, **10**, 784-790.

Nigg, E.A. (1995) Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays*, **17**, 471-480.

Nigg, E.A., Blangy, A. and Lane, H.A. (1996) Dynamic changes in nuclear architecture during mitosis - on the role of protein phosphorylation in spindle assembly and chromosome segregation. *Experimental Cell Research*, **229**, 174-180.

Nishijima, H., Nishitani, H., Seki, T. and Nishimoto, T. (1997) A dual-specificity phosphatase Cdc25b is an unstable protein and triggers p34cdc2/cyclin B activation in hamster Bhk21 cells arrested with hydroxyurea. *Journal of Cell Biology*, **138**, 1105-1116.

Noble, M., Endicott, J., Brown, N. and Johnson, L. (1997) The cyclin box fold: protein recognition in cell cycle and transcription control. *Trends in Biological Science*, **22**, 482-487.

Norbury, C., Blow, J. and Nurse, P. (1991) Regulatory phosphorylation of the $p34^{cdc2}$ protein kinase in vertebrates. *EMBO Journal*, **10**, 3321-3329.

Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503-508.

Nurse, P. (1999) Cyclin dependent kinases and regulation of the fission yeast cell cycle. *Biological Chemistry*, **380**, 729-733.

Nurse, P. and Bisset, Y. (1981) Gene required for G1 commitment to cell cycle and G2 control of mitosis in fission yeast. *Nature*, **292**, 558-560.

Nurse, P. and Thuriaux, P. (1977) Controls over the timing DNA replication during the cell cycle of fission yeast. *Exp. Cell Res.*, **107**, 365-375.

Nurse, P. and Thuriaux, P. (1980) Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics*, **96**, 627-637.

O'Connell, M.J., Raleigh, J.M., Verkade, H.M. and Nurse, P. (1997) chk1 is a Wee1 kinase in the G2 DNA damage checkpoint inhibiting Cdc2 by Y15 phosphorylation. *EMBO Journal*, **16**, 545-554.

O'Farrell, P., Edgar, B.A., Lakich, D. and Lehner, C.F. (1989) Directing cell division during development. *Science*, **246**, 635-640.

Ohi, R. and Gould, K.L. (1999) Regulating the onset of mitosis. *Current Opinion in Cell Biology*, **11**, 267-273.

Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F. and Rolfe, M. (1995) Role of the ubiquitin-proteosome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science*, **269**, 682-685.

Paris, J., Leplatois, P. and Nurse, P. (1994) Study of the higher eukaryotic gene function CDK2 using fission yeast. *Journal of Cell Science*, **107**, 615-623.

Parker, L.L., Atherton-Fessler, S. and Piwnica-Worms, H. (1992) $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 2917-2921.

Patra, D. and Dunphy, W.G. (1998) Xe-p9, a Xenopus Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase-promoting complexes at mitosis. *Genes & Development*, **12**, 2549-2559.

Patra, D., Wang, S.X., Kumagai, A. and Dunphy, W.G. (1999) The *Xenopus* Suc1/Cks protein promotes the phosphorylation of G(2)/M regulators. *Journal of Biological Chemistry*, **274**, 36839-36842.

Peeper, D.S., Parker, L.L., Ewen, M.E., M., T., Hall, F.L., Xu, M., Zantema, A., A.J., v.d.E. and Piwnica-Worms, H. (1993) A- and B-type cyclins differentially modulate substrate specificity of cyclin-Cdk complexes. *EMBO Journal*, **12**, 1947-1954.

Pelosi, A., Lee, M.C.S., Chandler, S. and Hamill, J.D. (1995) Hormonal control of root primordia differentiation and root formation in cultured explants of *Eucalyptus globulus* seedlings. *Aust. J. Plant Physiol.*, **22**, 409-415.

Peng, C.Y., Graves, P.R., Ogg, S., Thoma, R.S., Byrnes, M.J., Wu, Z.Q., Stephenson, M.T. and Piwnica-Worms, H. (1998) C-tak1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell Growth & Differentiation*, **9**, 197-208.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z.Q., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G2 checkpoint control - regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*, **277**, 1501-1505.

Piggot, J.R., Rai, R. and Carter, B.L. (1982) A bifunctional gene product involved in two phases of the yeast cell cycle. *Nature*, **298**, 391-393.

Pines, J. (1993) Cyclins and cyclin-dependent kinases: take your partners. *Trends In Biochemical Sciences*, **18**, 195-197.

Pines, J. (1995) Cyclins and cyclin-dependent kinases: a biochemical view. *Biochem. J.*, **308**, 697-711.

Pines, J. (1999) Four-dimensional control of the cell cycle. *Nature Cell Biology*, **1**, E73-E79.

Pines, J. and Hunter, T. (1991) Cyclin dependent kinase: a new cell cycle motif? *Trends in Cell Biology*, **1**, 117-121.

Porceddu, A., De Veylder, L., Hayles. J., Van Montagu, M., Inzé, D. and Mironov, V. (1999) Mutational analysis of two *Arabidopsis thaliana* cyclin-dependent kinases fission yeast. *FEBS Letters*, **446**, 182-188.

Raleigh. J.M. and O'Connell, M.J. (2000) The G(2) DNA damage checkpoint targets both Wee1 and Cdc25. *Journal of Cell Science*, **113**, 1727-1736.

Ramirez-Parra, E., Xie, Q., Boniotti, M.B. and Gutiérrez, C. (1999) The cloning of plant E2F, a retinoblastoma-binding protein, reveals unique and conserved features with animal G(1)/S regulators. *Nucleic Acids Research*, **27**, 3527-3533.

Reed, S.I. (1991) G1-specific cyclins: in search of an S-phase-promoting factor. *Trends in genetics*, **7**, 95-99.

Reed, S.I. (1992) The role of p34 kinases in the G1 to S-phase transition. *Annual Review of Cell Biology*, **8**, 529-561.

Reichheld, J.P., Chaubet, N., Shen, W.H., Renaudin, J.P. and Gigot, C. (1996) Multiple A-type cyclins express sequentially during the cell cycle in *Nicotiana tabacum* BY2 Cells. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 13819-13824.

Renaudin, J.P., Savouré, A., Philippe, H., Van Montagu, M., Inzé, D. and Rouzé, P. (1998) Characterization and classification of plant cyclin sequences related to A-

and B-type cyclins. In Francis, D., Dudits, D. and Inzé, D. (eds.), *Plant cell division*. Portland Press, London, pp. 67-98.

Renaudin, J.-P., Doonan, J.H., Freeman, D., Hashimoto, J., Hirt, H., Inzé, D., Jacobs, T., Kouchi, H., Rouzé, P., Sauter, M., Savouré, A., Sorrell, D.A., Sundaresan, V. and Murray, J.A.H. (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organization. *Plant Molecular Biology*, **32**, 1003-1018.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A. and Murray, J.A.H. (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science*, **283**, 1541-1544.

Riou-Khamlichi, C., Menges, M., Healy, J.M.S. and Murray, J.A.H. (2000) Sugar control of the plant cell cycle: Differential regulation of *Arabidopsis* D-type cyclin gene expression. *Molecular & Cellular Biology*, **20**, 4513-4521.

Röder, F.T., Schmülling, T. and Gatz, C. (1994) Efficiency of the tetracycline-dependent gene expression system: complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. *Molecular & General Genetics*, **243**, 32-38.

Rothnie, H.N. (1996) Plant mRNA 3'-end formation. *Plant Molecular Biology*, **32**, 43-61.

Russell, P., Moreno, S. and Reed, S.I. (1989) Conservation of mitotic controls in fission and budding yeasts. *Cell*, **57**, 295-303.

Russell, P. and Nurse, P. (1986) *cdc25*$^{+}$ functions as an inducer in the mitotic control of fission yeast. *Cell*, **45**, 145-153.

Russell, P. and Nurse, P. (1987) Negative regulation of mitosis by *wee1*$^{+}$, a gene encoding a protein kinase homolog. *Cell*, **49**, 559-567.

Russo, A.A., Jeffrey, P.D., Patten, A.K., Massague, J. and Pavletich, N.P. (1996) Crystal structure of the p27(Kip1) cyclin-dependent-kinase inhibitor bound the cyclin A-Cdk2 complex. *Nature*, **382**, 325-331.

Sabelli, P., Burgess, S., Kush, A. and Shewry, P. (1998) DNA replication initiation and mitosis induction in eukaryotes: the role of MCM and Cdc25 proteins. In Francis, D., Dudits, D. and Inzé, D. (eds.), *Plant cell division*. Portland Press, London, pp. 243-268.

Sadhu, K., Reed, S.I., Richardson, H. and Russell, P. (1990) Human homolog of fission yeast *cdc25* mitotic inducer is predominantly expressed in G2. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 5139-5143.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A laboratory manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, R.Q., Piwnica-Worms, H. and Elledge, S.J. (1997) Conservation of the Chk1 checkpoint pathway in mammals -

linkage of DNA damage to Cdk regulation through Cdc25. *Science*, **277**, 1497-1501.

Schena, M., Lloyd, A.M. and Davis, R.W. (1991) A steroid-inducible gene expression system for plant cells. , **88**, 10421-10425.

Schwob, E., Bohm, T., Mendenhall, M. and Nasmyth, K. (1994) The B-Type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1 to S transition in *S. cerevisiae*. *Cell*, **79**, 233-244.

Sebastian, B., Kakizuka, A. and Hunter, T. (1993) Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 3521-3524.

Sekine, M., Ito, M., Uemukai, K., Maeda, Y., Nakagami, H. and Shinmyo, A. (1999) Isolation and characterization of the E2F-like gene in plants. *FEBS Letters*, **460**, 117-122.

Setiady, Y.Y., Sekine, M., Hariguchi, N., Kouchi, H. and Shinmyo, A. (1996) Molecular cloning and characterization of a cDNA clone that encodes a *cdc2* homolog from *Nicotiana tabacum*. *Plant & Cell Physiology*, **37**, 369-376.

Shaul, O., Mironov, V., Van Montagu, M. and Inzé, D. (1999) Tobacco cultures transformed with cyclin-promoter-*gus* constructs reveal a discrepancy between *gus* mRNA levels and GUS protein activity upon leaving the stationary state. *Plant Science*, **141**, 67-71.

Simanis, V. and Nurse, P. (1986) The cell cycle control gene $cdc2^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell*, **45**, 261-268.

Skoog, F. and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Society for Experimental Biology Symposium*.

Slusarenko, A.J. (1990) A rapid miniprep for the isolation of total DNA from *Agrobacterium tumefaciens*. *Plant Molecular Biology Reporter*, **8**, 249-252.

Solomon, M.J., T, L. and Kirschner, M.W. (1992) The role of phosphorylation of $p34^{cdc2}$ activation: Identification of an activating kinase. *Molecular Biology of the Cell*, **3**, 13-27.

Sommer, S., Siebert, M., Bechthold, A. and Heide, L. (1998) Specific induction of secondary product formation in transgenic plant cell cultures using an inducible promoter. *Plant Cell Reports*, **17**, 891-896.

Soni, R., Carmichael, J.P., Shah, Z.H. and Murray, J.A.H. (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. *Plant Cell*, **7**, 85-103.

Sorrell, D.A., Combettes, B., Chaubet-Gigot, N., Gigot, C. and Murray, J.A.H. (1999) Distinct cyclin D genes show mitotic accumulation or constant levels of transcripts in tobacco bright yellow-2 cells. *Plant Physiology*, **119**, 343-351.

Stals, H., Casteels, P., Van Montagu, M. and Inzé, D. (2000) Regulation of cyclin-dependent kinases in *Arabidopsis thaliana*. *Plant Molecular Biology*, **43**, 583-593.

Sudakin, V., Shteinberg, M., Ganoth, D., Hershko, J. and Hershko, A. (1997) Binding of activated cyclosome to $p13^{suc1}$. Use for affinity purification. *Journal of Biological Chemistry*, **272**, 18051-18059.

Sun, Y.J., Dilkes, B.P., Zhang, C.S., Dante, R.A., Carneiro, N.P., Lowe, K.S., Jung, R., Gordon-Kamm, W.J. and Larkins, B.A. (1999) Characterization of maize (*Zea mays* L.) Wee1 and its activity in developing endosperm. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 4180-4185.

Sundaresan, V. and Colasanti, J. (1998) Cyclin-dependent kinases in higher plants: spatial and temporal control of cell division. In Francis, D., Dudits, D. and Inzé, D. (eds.), *Plant cell division*. Portland Press, London, pp. 47-65.

The Arabidopsis Genome Initiative. (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796-815.

Thompson, J.F., Hayes, L.S. and Lloyd, D.B. (1991) Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene*, **103**, 171-177.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, **76**, 4350.

Tréhin, C., Glab, N., Perennes, C., Planchais, S. and Bergounioux, C. (1999) M phase-specific activation of the *Nicotiana sylvestris Cyclin B1* promoter involves multiple regulatory elements. *Plant Journal*, **17**, 263-273.

Uetz, P., Giot, L., Cagney, G., Mansfield, T.A., Judson, R.S., Knight, J.R., Lockshon, D., Narayan, V., Srinivasan, M., Pochart, P., Qureshi-Emili, A., Li, Y., Godwin, B., Conover, D., Kalbfleisch, T., Vijayadamodar, G., Yang, M.J., Johnston, M., Fields, S. and Rothberg, J.M. (2000) A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature*, **403**, 623-627.

Umeda, M., Umeda-Hara, C., Yamaguchi, M., Hashimoto, J. and Uchimiya, H. (1999) Differential expression of genes for cyclin-dependent protein kinases in plants. *Plant Physiology*, **119**, 31-40.

Van Haute, E., Joos, H., Maes, S., Warren, G., Van Montagu, M. and Schell, J. (1983) Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for reversed genetics of the Ti plasmid of *Agrobacterium tumefaciens*. *EMBO Journal*, **2**, 411-418.

Verwoerd, T.C., Dekker, B.M.M. and Hoekma, A. (1989) A small-scale procedure for rapid isolation of plant RNAs. *Nucleic Acids Research*, **27**, 2362.

Vidal, A. and Koff, A. (2000) Cell-cycle inhibitors: three families united by a common cause. *Gene*, **247**, 1-15.

Wang, H., Fowke, L.C. and Crosby, W.L. (1997) A plant cyclin-dependent kinase inhibitor gene. *Nature*, **386**, 451-452.

Wang, H., Qi, Q.G., Schorr, P., Cutler, A.J., Crosby, W.L. and Fowke, L.C. (1998) ICK1, a cyclin-dependent protein kinase inhibitor from *Arabidopsis thaliana* interacts with both Cdc2a and CycD3, and its expression is induced by abscisic acid. *Plant Journal*, **15**, 501-510.

Weinmann, P., Gossen, M., Hillen, W., Bujard, H. and Gatz, C. (1994) A chimeric transactivator allows tetracycline-responsive gene expression in whole plants. *Plant Journal*, **5**, 559-569.

Winkler, K.E., Swenson, K.I., Kornbluth, S. and Means, A.R. (2000) Requirement of the prolyl isomerase Pin1 for the replication checkpoint. *Science*, **287**, 1644-1647.

Woo, E.S., Rice, R.L. and Lazo, J.S. (1999) Cell cycle dependent subcellular distribution of Cdc25B subtypes. *Oncogene*, **18**, 2770-2776.

Wu, L. and Russell, P. (1993) Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature*, **363**, 738-741.

Yamaguchi, M., Fabian, T., Sauter, M., Bhalerao, R.P., Schrader, J., Sandberg, G., Umeda, M. and Uchimiya, H. (2000) Activation of CDK-activating kinase is dependent on interaction with H-type cyclins in plants. *Plant Journal*, **24**, 11-20.

Yang, J. and Kornbluth, S. (1999) All aboard the cyclin train: subcellular trafficking of cyclins and their CDK partners. *Trends in Cell Biology*, **9**, 207-210.

Yang, J., Winkler, K., Yoshida, M. and Kornbluth, S. (1999) Maintenance of G(2) arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO Journal*, **18**, 2174-2183.

Zeng, Y. and Piwnica-Worms, H. (1999) DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. *Molecular & Cellular Biology*, **19**, 7410-7419.

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*, **200**, 2-12.

Zheng, X.-F. and Ruderman, J.V. (1993) Functional analysis of the P-box, a domain in cyclin-B required for the activation of cdc25. *Cell*, **75**, 155-164.

Chemicals and Materials

Unless otherwise stated all biochemicals were supplied by the Sigma-Aldrich PTY LTD, Castle Hill, NSW, Australia. Analytical grade general laboratory chemicals were supplied by AJAX Chemicals, Sydney, Australia or BDH Chemicals, Victoria, Australia.

Applied Biosystems Prism sequencing kit and automated sequencer from PE Applied Biosystems, Scoresby, VIC, Australia.

Chemiluminescent substrate solution was supplied by Amersham International, UK.

Dynabeads® oligo$(dT)_{25}$ beads were purchased from Dynal, Carlton, VIC, Australia.

Express Hyb solution supplied by Clontech, Inc., Palo Alto, CA, USA.

GIGAPRIME Labeling kit from Bresatec, Adelaide, SA, Australia.

Moloney Murine Leukemia Virus(M-MLV H-RT), RNase Inhibitor supplied by Life Technologies GIBCO BRL, Mulgrave, VIC, Australia.

PhosphoImager and ImageQuant software from Molecular Dynamics

Polytron Homogeniser supplied by Selby, Clayton, Victoria, Australia.

Radioactive isotopes ^{32}P and ^{125}I-anti-rabbit-IgG were purchased from Amersham International, UK and Bresatec, Australia.

Restriction endonucleases and enzymes all supplied by Promega, Hawthorn, VIC, Australia – unless otherwise stated.

Restriction endonuclease NdeI was obtained from New England Biolabs *Inc.*

Sephadex G50 from Amrad Pharmacia Biotech, Boronia, VIC, AUST.

Shrimp Alkaline Phosphatase was purchased from United States Biochemical

Sorvall, Ultracentrifuge, opaque quickseal tubes were supplied by Beckman, Mt Waverley, VIC, AUST.