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Optimising delivery of oral plant-made vaccines

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ABSTRACT

Compared to prokaryotic or other eukaryotic expression systems, plants provide many benefits for the production of recombinant proteins. Extensive research has dramatically increased the yield and quality of proteins expressed *in planta*, yet far less is known about the complex physical and immunological characteristics associated with using plants as both expression and oral delivery vehicles for antigenic proteins. The research presented in this thesis investigates the oral delivery and immunological presentation of antigens in the gut.

The type of plant tissue (leaf, hairy roots, and fruit) was shown to alter the yield of the heat-labile enterotoxin B-subunit (LTB) immunogen from the mucosal pathogen enterotoxigenic *Escherichia coli,* and the site of antigen release and the resulting mucosal immune response in mice (Chapter 2). As different expression system and host species have broadly different characteristics, a simpler experimental system was used where the LTB protein was engineered to accumulate in different subcellular locations within the same species and tissue (Chapter 3). Correctly folded LTB was localised to the apoplast, endoplasmic reticulum, non-lytic vacuoles, and protein bodies when expressed in the leaves of *Nicotiana benthamiana*. This study suggests the site of subcellular targeting not only influences the yield and quality of protein accumulation, but also the release characteristics during simulated digestion *in vitro* and the resulting mucosal immunogenicity when delivered orally to mice.

In addition to the physical characteristics of antigen release during digestion, many immunostimulatory agents are endogenous to plants. The concentration of the known adjuvant of *Solanum lycopersicum*, α-tomatine was characterised in fruit expressing Norwalk virus capsid protein virus-like particles (Chapter 5). When freeze-dried transgenic fruit at two ripening stages with different concentrations of endogenous α-tomatine was fed to mice, no variation in the seroconversion or magnitude of humoral or mucosal response was observed. Nor was there any variation in response when fruit was formulated with escalating doses of purified α-tomatine. These data suggest the concentration of α-tomatine alone does not potentiate the mucosal immunogenicity to orally-delivered VLPs.

The primary goal of this research has been to characterise and optimise the delivery and immunogenicity of antigens within plant cells. These experiments highlight the importance of considering the immunological context when using plant cells to deliver vaccine antigens. While the host factors investigated in this thesis are amenable to rational design, they are still only a small part of the highly complex plant-based production and delivery system which requires improved characterisation before progressing beyond proof-of-concept clinical trials.

PUBLICATIONS DURING ENROLMENT

Published research papers

*Pelosi A., ***Shepherd RP**. De Guzman, G., Hamill, J., Meeusen, E., Sanson, G., Walmsley, AM. 2011 *The release of a plant-made and delivered antigen in the mouse gut*. Current Drug Delivery, 8(6), 612-621

* these authors contributed equally to this work

Pelosi, A., Piedrafita, D., De Guzman, G., **Shepherd, RP**., Hamill, JH., Meeusen, E., Walmsley, AM. 2012. *The Effect of Plant Tissue and Vaccine Formulation on the Oral Immunogenicity of a Model Plant-Made Antigen in Sheep.* PLOS One, *7*(12), e52907.

See Appendix 1

Submitted research papers

Shepherd, RP, Walmsley, AM. *Improved oral immunogenicity of the heat-labile enterotoxin B subunit within protein storage vacuoles of Nicotiana benthamiana leaves.* Submitted to Plant Biotechnology Journal.

Shepherd, RP, Walmsley, AM. *The intrinsic saponin of tomato, α-tomatine does not potentiate oral immunogenicity of Norwalk virus-like particles*. Submitted to The Journal of Negative Results in Biomedicine.

Reviews

*Pelosi, A., ***Shepherd, RP**, Walmsley, AM. 2012. *Delivery of plant-made vaccines and therapeutics.* Biotechnology Advances, *30*(2), 440–448.

* these authors contributed equally to this work

See Appendix 2

Book chapters

De Guzman, G., **Shepherd, RP**. Walmsley, AM. 2010. In G Shaw, *Immunoassays in Agricultural Biotechnology*, John Wiley and Sons, Hoboken, USA.

See Appendix 3

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal and two submitted manuscripts. The core theme of the thesis is improving the immune response to orally delivered vaccines made in plants. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Biological Sciences under the supervision of Amanda M Walmsley and David R Smyth.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2, 3 and 4 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	The Release and Induced Immune Responses of a Plant-made and Delivered Antigen in the Mouse Gut	Published	Some experimental design, protein expression in *N. benthamiana*, 33% of animal experiments, all immune response characterisation, and 33% of the manuscript was written by myself
3	Improved oral immunogenicity of the heat-labile enterotoxin B subunit within protein storage vacuoles of *Nicotiana benthamiana* leaves	Submitted	All experiments were designed, performed, analysed, and the manuscript written by myself
4	The intrinsic saponin of tomato, α-tomatine does not potentiate oral immunogenicity of Norwalk virus-like particles	Submitted	All experiments were designed, performed, analysed, and the manuscript written by myself

I have not renumbered sections of published papers in order to generate a consistent presentation within the thesis.

Signed: [redacted] Date: 09 March 2016

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] Date: 10 March 2016

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It has been an honour and privilege to stand on the shoulders of the giants who have advanced the field of molecular pharming. It has been humbling to be involved with this technology during the period where real strides have been taken using plant biotechnology to improve human health, and it is with great hope that the work included in this thesis contributes to the understanding of how plants can be used to deliver vaccine antigens.

I would like to thank the Australian Government for financial support with the Australia Postgraduate Award, and the School of Biological Sciences and Monash University for providing additional funding and research support.

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I would like to acknowledge my family who have facilitated my ‘concoctions’ from the time I could reach a bench top, and have provided love and support for my entire life. Thank you. The limitless love and support of my genuinely long-suffering wife Cathleena really has made all the difference in my life and work.

This thesis is dedicated in loving memory to my family who have passed during my candidature, and our unborn baby who will soon start a new cycle of life for our family.

While there are many people that have helped contribute to this thesis, I am solely responsible for any errors or omissions.

COMMON ABBREVIATIONS

APC	antigen presenting cell
ARR	antigen release ratio
ASC	antibody secreting cell
CaMV	cauliflower mosaic virus 35S regulatory region
CD25	alpha chain of the IL-2 receptor
CFA	colonisation factor antigen
C_{max}	peak plasma concentration
CMIS	common mucosal immune system
CT	cholera toxin
CTA	cholera toxin A subunit
CTB	cholera toxin B subunit
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DPI	days post infiltration
dw	dry weight
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
ETEC	enterotoxigenic *Escherichia coli*
FAE	follicular-associated epithelial
FDA	United States Food and Drug Administration
fw	fresh weight
GALT	gut-associated lymphoid tissue
GFP	green fluorescent protein
GIT	gastrointestinal tract
GM1	monosialotetrahexosylganglioside
GMP	Good Manufacturing Practice
HBGA	histo-blood group antigen
HBsAg	hepatitis B surface antigen
HPLC	high performance liquid chromatography
i.m.	intramuscular
i.n.	intranasal
IFN-γ	interferon-γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgG2a	immunoglobulin G2a
IL	interleukin
ILF	isolated lymphoid follicle
ISCOM	immune-stimulating complex
ISCOMATRIX	immune-stimulating matrix
KDEL	hexapeptide endoplasmic reticulum retention signal
KLH	keyhole limpet hemocyanin
LAV	live attenuated vaccines
LC/MS	liquid chromatograph and mass spectrometry
LMIC	low to middle income countries
LT	enterotoxin
LTA	heat-labile enterotoxin A subunit
LTB	heat-labile enterotoxin B subunit
MALT	mucosa-associated lymphoid tissue

MLN	mesenteric lymph node
NALT	nasopharyngeal-associated lymphoid tissues
NaVCP	Narita virus capsid protein
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NoV	*Norovirus*
NVCP	Norwalk virus capsid protein
NVLPs	*Norovirus* virus-like particles
OD450	optical density at 450 nm
OVA	ovalbumin
PAMP	pathogen associated molecular patterns
PB	protein body
PB-I	protein body type I
PB-II	protein body type II
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PP	Peyer's patch
prGCD	plant-recombinant β-glucocerebrosidase
PSV	protein storage vacuole
QS-21	*Quillaja saponaria* glycoalkaloid fraction 21
RA	retinoic acid
s.c.	subcutaneous
SED	subepithelium dome
SGF	simulated gastric fluid
sIgA	secretory immunoglobulin A
SP	signal peptide
$t_{1/2}$	time to reduce plasma concentration to half the observed maximum
TEM	transmission electron microscopy
TFG-β	transforming growth factor β
T_h1	T helper type 1 response
T_h2	T helper type 2 response
T_{reg}	T regulatory cell
TSLP	thymic stromal lymphopoietin
TSP	total soluble protein
USDA	United States Department of Agriculture
VLP	virus-like particle
WHO	World Health Organisation

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Plant-made recombinant proteins

1.1.1 Ability of plants to express recombinant proteins

In less than 30 years, 'molecular pharming' has moved from proof-of-concept to a viable alternative for the production of high-value recombinant proteins for research, industrial, and pharmaceutical uses. The first report of a recombinant protein expressed *in planta* was published in 1986, where the chimeric plant protein nopaline synthase fused to human growth hormone was expressed in tobacco and sunflower callus.[1] This landmark paper showed that plant cells possess the capacity to transcribe and translate mammalian genes into functional proteins. Soon after, expression of complex, post-translationally modified proteins including monoclonal antibodies [2] and human serum albumin [3] were reported. These first studies were conducted with plant species chosen for their amenability to transformation. However, recent advances in transient and stable transformation technologies have seen a rapid expansion in the number of plant species and expression systems capable of producing recombinant proteins at lab, pilot, and commercial scale.[4,5]

Plants have historically been positioned as a low cost production system for pharmaceutical proteins due to their lower input costs and capacity to be grown at a variety of scales.[6] Yet the production costs associated with established protein expression systems have also decreased rapidly due to technical advancements.[7] In response, developers of plant expression technologies have begun to focus on taking advantage of the specific benefits of plant cells as expression systems. These include the relatively low cost of upstream manufacture, high scalability for commodity proteins, rapid production in weeks as opposed to months, little to no risk of contamination with human or animal pathogens or microbial contaminants such as endotoxin, and the ability to produce complex proteins with designed post-translational modifications including mammalian glycosylation.[4,8-11]

Indeed, the benefits of plant systems have attracted sufficient investment that there are now multiple plant-made products successfully scaled up to pilot and commercial production in Good Manufacturing Practice (GMP)-accredited facilities.[12] For example, high recombinant protein yield and automated plant handling allows Medicago Inc to produce and purify up to 10 million doses of a pharmaceutical grade pandemic influenza vaccine per month, and vaccine candidates from this facility are now entering late stage clinical trials.[13]

Despite these benefits, plant systems have had a slow uptake in the highly regulated pharmaceutical environment due to their relatively slow speed of stable transformation, plant-specific glycosylation (in most species), difficulty in standardisation of downstream

purification, lack of GMP-accredited production systems and facilities, and the general regulatory uncertainty associated with novel expression systems.[14] Although much of the perceived regulatory risk has now been mitigated by the assurance from regulators that plants-based expression is compatible with existing guidelines, development of new products using plants has been slow compared to more commonly used expression systems.[15]

Despite the slower pace of development, continual improvements in the technical characterisation of plant-made recombinant proteins, as well as the ongoing interactions between regulators, public consortia and industrial organisations,[16] has led to the first therapeutic protein, taliglucerase alfa, produced in plants achieving marketing approval for human use in 2012.[17]

1.1.2 Licensed plant-made therapeutic proteins

Plant-made recombinant proteins have repeatedly passed sufficient quality assurance to enter human clinical trials under the Food and Drug Administration (FDA) Investigational New Drug (IND) process.[18,19] However, the most significant recent regulatory event has been the licensure of Protalix Biotherapeutics recombinant form of the human glucocerebrosidase enzyme expressed in carrot cell suspension. Taliglucerase alfa is an excellent case study for the benefits of plant-based expression of recombinant proteins. Unlike mammalian systems, plant cell cultures are not known to harbour human pathogens.[20] This was highlighted when a competing manufacturer of recombinant glucocerebrosidase was forced to stop manufacture due to viral contamination in their Chinese hamster ovary (CHO)-based production system resulting in global rationing of this essential therapeutic protein.[21] Also, glucocerebrosidase produced in CHO cells requires additional *in vitro* N-glycosylation after purification whereas the glucocerebrosidase produced in carrot cells is N-glycosylated during maturation within the plant endomembrane system and requires no further processing.[22] These factors provide a good example of where plants may have intrinsic benefits over other protein production systems by making 'biobetter' rather than 'bioequivalent' or 'biosimilar' proteins.[23]

Despite the recent regulatory acceptance of recombinant proteins produced *in planta*, the cost of producing highly purified and sterile proteins in plants, while potentially less than in other systems, is still prohibitively expensive for use in most therapeutic indications without extensive payer-side reimbursement.[24] For example, despite the upstream benefits of expressing glucocerebrosidase in plant cell culture, taliglucerase alfa still costs payers approximately $150,000 USD per annum,[25] with most of this figure associated with the development, downstream processing, marketing and profit.[26,27]

Plant cells possess the ability to make similar or functionally better recombinant proteins than other expression platforms. However, the capacity for large scale production of plants with lower capital expenditure than other systems has the potential to reduce upstream costs, and may facilitate the development of lower cost therapeutic proteins to treat common diseases in low to middle income countries (LMICs) unable to afford high-cost 'biologics'.[28,29] These 'biosimilars' will likely be predominantly differentiated from their parent drug by their lower price.[30] The pharmacoeconomics of all drugs, and particularly 'biologics' is a challenge to all national health systems regardless of economic status.[31] Indeed, one of the most extensively studied areas of recombinant protein expression in plants is the potential for manufacturing low-cost vaccines against pathogens endemic to LMICs.

1.2 Vaccination with plants

1.2.1 Expression of antigenic proteins in plants

The concept of using plant cells for the expression of vaccine antigens was first suggested in a patent application by Roy Curtis and Guy Cardineau in 1982:

"The invention is directed to transgenic plants expressing colonization and/or virulence antigens specified by genes from pathogenic microorganisms." [32]

The first research paper featuring the use of recombinant vaccine antigens produced in plant cells was 13 years later. In this paper, Haq et al., showed the successful expression, folding, and oral immunogenicity of a vaccine antigen produced in plants.[33] This seminal paper provided evidence that transgenic plants can act as both protein production and oral delivery vehicles for immunogenic proteins, and has inspired a body of basic and applied research towards the goal of using plants for oral vaccination against a variety of diseases.

The authors of this paper noted:

"We anticipate that an increase in recombinant protein concentration in the plant tissue will lead to an increased immune response." [33]

Accordingly, much the focus in the field of 'molecular pharming' has been on improving the yield of heterologous proteins. Great success has been achieved in the production and purification of antigenic proteins for systemic administration of vaccines antigens including the clinical development of several candidate influenza and hepatitis B vaccines.[19,34] Of particular note was the 2006 United States Department of Agriculture (USDA) licensure of the first veterinary vaccine made in plant cells.[35] This product was composed of a liquid culture of transgenic tobacco cells expressing the hemaglutinin neuraminidase protein of Newcastle disease virus (NDV). When administered as a subcutaneous injection of the plant cell lysate, poultry were protected against lethal challenge with NDV.[36] Despite providing sufficient characterisation of manufacturing and efficacy for licensure with the USDA, the product was never commercially manufactured and was instead developed as a proof-of-concept for the licensing of a plant-made veterinary vaccine candidate within the existing USDA regulatory framework.

Alas, the prediction by Haq et al. that an increase in recombinant protein concentration would lead to an increased immune response has not been fully realised. Despite many studies over 30-years investigating the oral delivery of recombinant proteins in plants, no major

advancements in immunogenicity have been discovered nor have projects using this technology advanced into late stage clinical trials.[33]

1.2.2 Orally delivered plant-made vaccines

The concept of plants as both production and delivery vehicles was first raised in the patent application by Curtis & Cardineau:

"… use of such transgenic plants for oral immunization of humans and other animals to elicit a secretory immune response which inhibits colonization of or invasion by such pathogenic microorganisms through a mucosal surface of humans or other animals." [32]

However, the clinical development of plant-made and orally delivered vaccines have stalled due to the inability to reliably induce immunity, and adhere to the stringent characterisation and reproducibility of manufacture required for vaccine production. While these factors have not limited the academic exploration of oral delivery of plant-made vaccines in preclinical and early-stage clinical trials, they pose significant impediments to the licensure of a vaccine for human use.[37]

Many of the regulatory concerns associated with *in planta* production have been substantially de-risked as other plant-made proteins move through the regulatory process,[38] but the limitations associated with the basic understanding of the mechanisms through which plant cells interact with the host mucosal immune response have not been as easy to overcome. It is important to note that these difficulties are not limited to plants. The development of the entire field of orally-delivered vaccines has moved very slowly in comparison to systemically delivered vaccines, predominantly due a lack of understanding of the mucosal immune response to oral vaccination.[39]

The first plant-made vaccine candidates were proposed as low-cost 'edible' vaccines that could be grown and minimally processed in resource poor settings.[40-43] However, the requirements of a controlled dose of active ingredient, and the inherent variability in plant growth and antigen expression makes the control of antigen dose challenging. This has led to a conscious move away from terms such as 'edible vaccine' to a more restrained discussion of using plant cells as part of the production system for oral vaccines.[1]

Plant-made vaccines meet many of the requirements for low-cost, large-scale vaccination programs in lower income regions including low cost of upstream manufacture, ease of oral delivery compared to systematic delivery with needles, and the relative thermal stability of antigenic proteins in plant material compared to vaccines that require cold-chain transport.[3] A

wide variety of antigens from human and animal pathogens have been expressed *in planta*,[4,8] yet despite the advantages of plant systems, the technology has yet to be translated into regions with a high burden of preventable disease for which alternative solutions are either too costly or simply non-existent.[12] Lower cost vaccines could radically reduce the high mortality endemic to LMICs, and there are perhaps no indications more fitting to the characteristics of plant-made oral vaccines than the scourge of diarrhoeal pathogens.

1.2.3 Global burden of diarrhoeal disease

Despite advancements in basic infection and immunity research and increased general health initiatives, diarrhoeal disease remains one of the principal causes of mortality in developing nations and is a significant financial burden in wealthy countries. The United Nations have made great efforts to engage the support of the global vaccine development community with Target 4.A of the Millennium Development Goals "*which aims by 2015 to have reduced the under-five mortality rate of 1990 by two thirds*." Unfortunately, the rate of child mortality, which is highly influenced by diarrhoeal disease, has remained essentially constant over this period.[44] A meta-analysis of epidemiological studies published between 1992 and 2000 suggests that 21% of deaths of children under 5 years of age in developing nations is directly attributable to diarrhoeal disease.[45]

Many effective, non-specific treatments such as oral rehydration therapy and prophylactic administration of anti-motility drugs can address the symptoms and complications of diarrhoeal disease sequelae,[14] yet despite these effective treatments, the high mortality of diarrhoeal disease remains. This is due in part to the large number of etiological agents capable of causing diarrhoea, and the disincentives to diverting limited public health budgets to developing treatments targeted to individual diseases.[15] While many in the global health community perceive vaccines as too expensive to develop and distribute,[18] experience with the global eradication of smallpox suggests that one of the key tools to move away from the prophylactic treatment of symptoms to a significant and long-lasting reduction in the mortality associated with diarrhoeal disease is with preventative vaccination.[19]

The World Health Organisation's (WHOs) list of agents causing the highest burden of diarrhoeal disease (in alphabetical order) includes:

- *Calicivirus* including the *Norovirus* (NoV) genus
- *Campylobacter sp.*
- Enteropathogenic *Escherichia coli*

- Enterotoxigenic *E. coli* (ETEC)
- *Rotavirus sp*.
- *Salmonella typhi*
- Shingella-toxin carrying bacteria
- *Vibrio cholera*

Due to the cost and complexity of research, development, manufacture, registration and distribution of new drugs, successful vaccines have been developed against only a few of these diarrhoeal pathogens. Yet, it is the countries with the highest disease burden that are also the least able to fund basic and translational research into vaccines for these pathogens, and the focus on return on investment for private enterprise in developed economies limits work on low cost vaccines for LMICs.[21] Recently, the economic disparity between development demand and capacity has been targeted by agencies such as the Global Alliance for Vaccine and Immunisation (GAVI). These funds set minimum threshold prices and quantities for vaccines so that manufacturers do not need to rely solely on market forces in each country; thus generating the supply-side certainty required for investment in neglected or low-cost vaccines.[22] This focus on improving the economics of diarrhoeal vaccine development is important because to date all vaccines licenced for diarrhoeal diseases have been researched and initially licenced in developed nations.[46] The potential for plant-made vaccines to change the supply and demand dynamic for vaccine development is already evident, with South Africa showing the feasibility of a dedicated local pandemic influenza vaccine production facility based solely on expression in plant cells.[47]

1.2.4 Licensed diarrhoeal vaccines

Currently marketed vaccines for diarrhoeal disease include:

- Dukoral® (Crucell-Sweden), ORC-Vax® (VaBiotech-Vietnam), Shanchol® (Shanta Biotechniques-India), and Orochol/Mutachol® (Crucell-Switzerland) for the prevention of *V. cholera* associated diarrhoea
- Rotarix® (GlaxoSmithKline-Belgium) and RotaTeq® (Merck-USA) for the treatment of *Rotavirus* associated diarrhoea
- Vivotif Oral® (Crucell-Switzerland), Typhim Vi® (Sanofi Pasteur-France), and Typhrix® (GlaxoSmithKline-Belgium) for the treatment of *Typhoid sp.* associated diarrhoea

These vaccines are based on fundamentally different technologies. The two licenced rotavirus vaccines RotaTeq® and Rotarix® are live attenuated vaccines (LAVs). Vivotif® Oral is also a LAV consisting of an orally delivered, live, attenuated *Salmonella typhi* strain Ty21a. Typhim Vi® and Typhrix are sterile solutions containing the cell surface Vi polysaccharide extracted from *Salmonella enterica* serovar *Typhi*, *S typhi* Ty2 strain and are delivered intra-muscularly (i.m.). The oral cholera vaccines Shanchol® and Dukoral® are inactivated vaccines, and both contain inactivated pathogenic *V. cholera* biotypes. Dukoral® is additionally formulated with the mucosal adjuvant, cholera toxin B-subunit (CTB). While these different technologies have inherent strengths, weaknesses and risks,[48] it is interesting to note that none of these vaccines utilise modern, subunit production.

Subunit-based vaccines are inherently safer than live, attenuated or purified vaccines as the live pathogen is not involved in the manufacturing process. The principal weakness of subunit vaccines is the poor immunogenicity inherent to administering just a few selected components of the pathogen rather than the complete pathogen with its intrinsic immunostimulatory pathogen associated molecular patterns (PAMPs).[49] While not all diarrhoeal vaccines are delivered orally, all diarrhoeal pathogens are present in the gastrointestinal tract (GIT); making the mucosal route of administration highly immunologically relevant and also compatible with oral delivery of plant cells. Recent advancements in mucosal immunology have revealed many of the mechanistic requirements for vaccines to overcome the limitations of oral delivery, including how and where antigens are sensed, and the immunological context required for response rather than tolerance.[50] This understanding of oral immunity now provides a framework to begin rationally investigating whether there are plant-specific factors that may be optimised to improve the oral immunogenicity of antigens both manufactured and delivered in plants and hopefully enable this technology to move beyond proof of concept studies.

1.3 Immunology of diarrhoeal diseases

1.3.1 Mucosal immune geography

To improve the oral immunogenicity of plant-made vaccines, the structure and function of the mucosal immune system must be considered.

The mucosal surface of the GIT is a thin, permeable barrier that is the sole route of macro and micronutrient ingestion It supports the commensal microbiota while defending against a diverse range of pathogens.[51] The continuous surface of the mammalian gut is comprised of a single layers of columnar epithelial cells bound by tight junctions and interspersed with secretory glands and lymphoid tissue.[52] The epithelial surface is protected by non-specific defence mechanisms including a thick mucin-rich glycocalyx containing anti-microbial peptides, as well as a library of secretory immunoglobulin A molecules (sIgAs).[53] The predominantly low-affinity 'natural' or innate sIgAs of the glycocalyx provide 'immune exclusion' where sIgA-bound microbes or toxins are restricted in their movement through the glycocalyx or attachment to the epithelium.[54]

The regions of the epithelium associated with acquired immunity are the mucosa-associated lymphoid tissue (MALT). These regions share common structures between the major mucosal surfaces of the body (gut, nasopharyngeal, bronchial, conjunctival, and urogenital), but also have subtle functional differences. The most practical surfaces for vaccine delivery are the gut-associated and nasopharyngeal-associated lymphoid tissues (GALT and NALT, respectively). These surfaces have a different distribution of antigen sampling sites (GALT lymphoid tissue density varies according to the distribution along the GIT, NALT is more evenly distributed across the nasal epithelium in humans).[55] Other differences include the glycocalyx composition that influences the rate of antigen absorption (GALT is thicker and composed of more highly branched mucin glycoproteins),[56] and lymphatic transport of activated cells that changes the site of the adaptive immune response (GALT drains to the mesenteric lymph nodes (MLNs), NALT to the buccal and retropharangeal lymph nodes).[57]

The GALT can be divided into unorganised effector sites including the lamina propria and intraepithelial lymphocytes, and more organized structures such as MLNs, Peyer's patches (PPs), isolated lymphoid follicles (ILFs), cryptopatches and colon patches.[52] The primary sites for antigen sampling and immune induction in the GALT are the PPs.[58] These regions of organised follicular cells are located along the length of the gastrointestinal tract,[59] and are more densely localised at regions of potential pathogen interaction in the distal ilium,

appendix, colon and rectum.[60] The loose connective tissue of the lamina propria surrounds the epithelium, and is the location of the majority of gut-associated effector cells.[61]

The follicular-associated epithelial (FAE) cells covering the PPs feature a smooth luminal-facing surface. These cells are referred to as M-cells for their microfold or membranous surface.[62] Approximately 1 out of 10 million epithelial cells in the intestinal tract is an M cell (approximately 5% in humans and 10% in mice).[63] The M-cell surface is morphologically distinguished from the surrounding villus epithelium by a reduced brush border and decreased glycocalyx layer.[64] This reduced glycocalyx allows interaction of the luminal contents with the cell surface, and efficient transport of the lumen contents across the epithelium.[65] M-cells are capable of transporting antigens, whole viruses and bacteria from the luminal space via endocytosis, phagocytosis, pinocytosis, macropinocytosis,[66] and receptor-mediated processes.[67] While the M-cells of the FAE are the principal sites of lumen sampling and immune surveillance,[68] epithelial villus M-cells also actively transport antigens from the lumen,[64] and lamina propria-resident dendritic cells (CD103+ DCs) are capable of expanding processes across the epithelial junctions and can directly sample microbes and soluble antigens from the luminal space.[69]

The deep invagination of the basolateral membrane of M-cells referred to as the sub epithelium dome (SED) provides a microenvironment for naïve lymphocytes. Once transported across the epithelium, antigens and processed peptides are presented to the antigen presenting cells (APCs) of the SED including distinct gut lineages of DCs, immunologically naïve B and T cells, and macrophages (see Figure 1-1).[70]

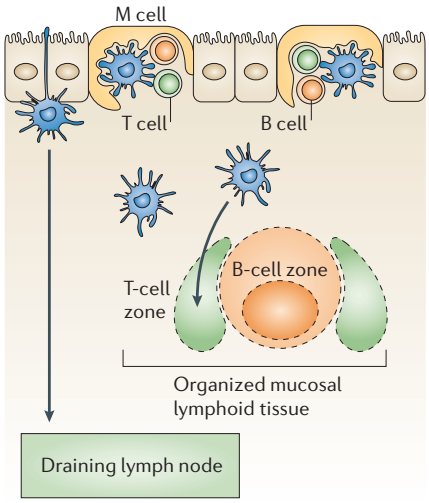

Figure 1-1 Generalised representation of the gut immune geography. The luminal-facing M-cells transport antigens and microbes to the naïve APCs (blue cells). These cells then traffic to the draining lymph node or organised mucosal lymphoid tissue where they present the antigen to naïve lymphocytes. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Neutra, M. R., & Kozlowski, P. A. (2006). Mucosal vaccines: the promise and the challenge. *Nature Reviews Immunology*, *6*(2), 148–158, copyright 2006.

Once activated, APCs in the SED migrate to the local germinal centres or may traffic via the draining lymph nodes to the blood and the distal mucosal sites. Following maturation in the germinal centres of the PPs, MLNs or ILFs, mature, high affinity T- cells and antibody secreting B-cells (ASCs) may also enter the blood and be trafficked to peripheral mucosa.[71]

1.3.2 Mucosal immune response

Systemically administered vaccines are sometimes capable of inducing a protective immune response to some mucosal pathogens including influenza and polio viruses. However, vaccination via the mucosa is the primary mechanism of inducing the gut-specific sIgA and cellular immune responses required for protection from most diarrhoeal pathogens.[72] The goal of all mucosal vaccines is to induce an adaptive response sufficient to provide protection from subsequent pathogenesis, with sIgA essential for generating a neutralising response to subsequent challenge for most mucosal pathogens.[73] Unlike the systemic immune response, the mucosal immune system has the capacity to induce effector cells at the mucosal surfaces. Antigen exposure at an isolated region of a mucosal surface is sufficient to generate a subsequently antigen-specific sIgAs at other mucosal surfaces.[74,75] There is tropism between the compartments of this common mucosal immune system (CMIS): NALT immunisation effectively induces antigen-specific immunity in the respiratory and reproductive tissues, in contrast to GALT immunisation that promotes antigen specific immunity in the GIT.[76]

The difficulty and complexity of inducing a protective mucosal response to oral vaccination is due in part to the fact that unlike the normally sterile environment of the systemic immune response, the mucosal immune system must be tolerant of the barrage of non-pathogenic dietary and microbial antigens while still being able to successful raise an appropriate defence against an invading pathogen or toxin.[77]

When dietary, commensal or pathogenic microbial antigens are presented to the APCs located under the SED (primarily DCs), the combinatorial co-signalling of the microenvironment influences the ‘priming’ of these cells. Priming signals include a diverse range of cytokines, chemokines, PAMPs and metabolites that educate the DC on the local microenvironment and the required characteristics of the immune response.[77,78] The epithelium is involved in many of the priming signals.[70] For example, epithelium-derived thymic stromal lymphopoietin (TSLP) or the vitamin A metabolite retinoic acid (RA), prime DCs into a tolerogenic phenotype and actively suppress inflammatory cytokines.[79,80] Other signals involved in the priming of DCs in the SED include cytokines from B-cells,[81] T-cells,[79] localised tissue damage,[82] systemic inflammatory environment,[83] dietary nutrients,[84] and the bacterial species

present within the GIT lumen.[85] After activation, DCs in the sub-epithelium region migrate away from the epithelial centres and into the proximal T-cell or B-cell-rich germinal centres where they up-regulate maturation markers and begin to present antigen and co-signalling molecules to and from follicular dendritic cells, B- and T- cells (see Figure 1-2).[86,87]

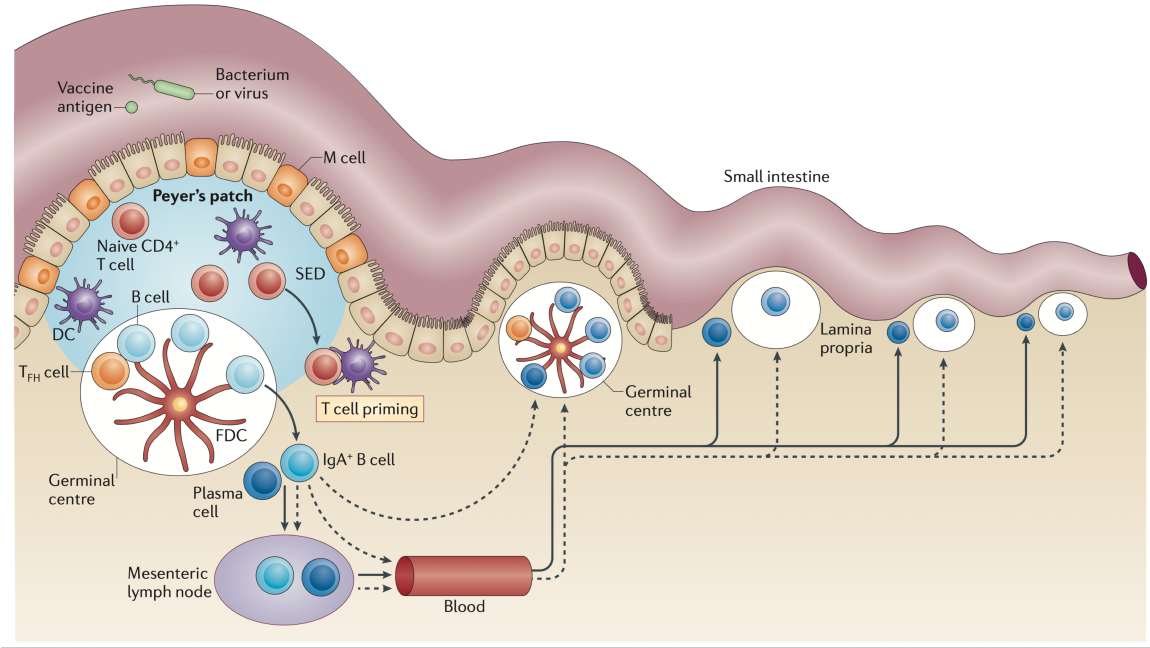

Figure 1-2 **Induction of gut IgA response by mucosal vaccines.** Once antigens are transported across the epithelial barrier and into the SED, antigens and priming signals are processed by naïve DCs that then present antigens to naïve CD4+ T-helper cells and T-follicular helper (T_{fh}) cells. These T_{fh} cells migrate to the edge of the B-cell follicle where the co-localise with naïve B-cells to form a germinal centre in the presence of follicular dendritic cells (FDC). The antigen-specific B-cells in the germinal centre undergo somatic hyper mutation and class switching to IgA, and mature plasma B-cells and memory B-cells leave the PP via the efferent lymph node and move into the circulation. Once in the blood, cells of mucosal origin are attracted back to the lamina propia of the GALT and/or other MALT structures by gut-maturation specific homing molecules. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Lycke, N. (2012) Recent progress in mucosal vaccine development: potential and limitations. Nat Rev Immunol 12, 592–605, copyright 2012.

The co-signalling microenvironment of the PP germinal centre promotes different classes of CD4+ T-helper (T_h) cell maturation. Each class is characterised by distinct cytokines and transcription factor profiles, [88-91] including those predominant in vaccine response:

- T_h1 cells secrete interferon-γ (IFN-γ) and tumour necrosis factor α (TNF-α) to promote CD8+ cytotoxic T-lymphocyte (CTL) response against intracellular pathogens such as viruses.
- T_h2 cells expressing interleukin 4 (IL-4), IL-5 and IL-13 promote B-cell class switching.

- T-follicular helper (T_{fh}) cells promote germinal centre formation, affinity maturation, and high-affinity plasma and memory B–cell maturation.
- Regulatory T-cells (T_{reg}) suppress T_h1 and T_h17 responses by expressing anti-inflammatory cytokines including transforming growth factor β (TGFβ) and IL-10.

As in other lymphoid tissues, inflammatory co-signalling primes naïve T-cells into an antigen-specific CD8+ CTL phenotype,[91] and accordingly the pro-inflammatory cytokines IL-1, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to be effective adjuvants for the induction of antigen-specific CTL in the NALT.[92]

It is clearly beneficial for the majority of mucosal immunity to dietary and commensal bacterial to be mediated by a tolerogenic response rather than an inflammatory T_h1 response. The signalling environment in the SED potentiates this tolerogenic response by maturation of antigen-specific T_{reg}, T_h2, and antigen specific IgA and IgG B-cells.[90] A contrast of this tolerance is the observation that pathogenesis associated with colitis, Crohn's disease and *Helicobacter pylori* infection are characterised by an inflammatory response and dysregulation of the tolerogenic response to commensal and dietary antigens.[93] Indeed, induction of an inflammatory response from the GALT is generally only observed following breakdown of the epithelial barrier or pathogen invasion.[94]

In the healthy GALT microenvironment, naïve B cells undergo class-switching in response to antigen stimulation in the presence of transforming growth factor β (TGF β), IL-10 and the B-cell activating factor secreted by T_h2 and T_{reg} cells.[95] Both T-cell dependent and T-cell independent activation can lead to IgA class switching of B-cells in the GALT,[96] and a T-cell dependent pathway is crucial for vaccine responses, whereas the T-cell independent pathway often generates lower affinity 'natural' sIgAs involved in mucosal homeostasis and pathogen exclusion.[95] Activated IgA-expressing B cells may stay in the PP and undergo hyper somatic mutation to generate higher-affinity IgA plasma cells, or may traffic to other germinal centres in the MLN or lamina propria (see Figure 1-2).[54] The PP are also the location of the B-memory cell formation prior to trafficking to other mucosal sites where they remain functional for several years.[97]

Recently, the key mechanism for the induction of high-affinity sIgA in response to mucosal vaccination has been revealed. Generally, antigen-specific IgA expressing B-cells are induced and undergo affinity maturation at the primary site of activation in the PPs.[68] However, clonally related plasma B-cells are also identified at different germinal centres throughout the lamina propria. Bergqvist et al., observed that the distributed IgA responses to an oral

immunogen was highly synchronized, oligoclonal, affinity matured, and broadly distributed to the inductive and effector sites along the entire small and large intestines.[98] Key to this mucosa-wide seeding of IgA B-cells is the repeated exposure of B-cells to their cognate antigens within the local germinal centres of the PP and ILF.[99] This finding confirms the necessity of repeated exposure to an effective dose of antigen to generate high affinity sIgA for vaccine antigens along the GIT.

The activation and tolerance-inducing properties of T_{reg} cells have constantly been identified as one of the major risks associated with the use of mucosal vaccination with non-living vectors, yet the role of these cells in mucosal vaccine development is far from clear.[100,101]

1.3.3 Mucosal immune tolerance

'Oral tolerance' has traditionally been defined as the suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route.[102] The risk of oral vaccination inducing a tolerogenic response to subsequent immune activation against a live pathogen has been repeatedly suggested as one of the unaddressed risks for the development of safe oral vaccines,[48,94,101,103] and has also been identified as one of the limiting factors in the development of orally-delivered plant-made vaccines.[104-108]

While the tolerance response has been extensively demonstrated in animal models including mice, rats, and guinea pigs; other species including humans generally appear less prone to the development of systemic unresponsiveness.[101] However, humans still possess peripheral tolerance, as exemplified by the success of repeated low dose oral exposure of allergens to re-programme aberrant inflammatory responses at the mucosa.[102,109-111]

Key to the understanding of oral tolerance is the understanding of the function of the peripherally derived T_{reg} cells. These CD4+ T-cells are characterised by their expression of the forkhead box P3 (FoxP3) gene and the alpha chain of the IL-2 receptor (CD25).[100,112] When activated by exposure to their cognate antigens, these cells act to down regulate the inflammatory response of other cells via secretion of the pro-tolerogenic cytokines IL-10 and TGF-β.[113] There appears to be a low risk of tolerance to diarrhoeal antigens as while IL-10 and TGFβ were both originally identified as inhibitors of inflammation, recent evidence suggests both cytokines are also required for promoting of B-cell maturation and IgA expression.[114]

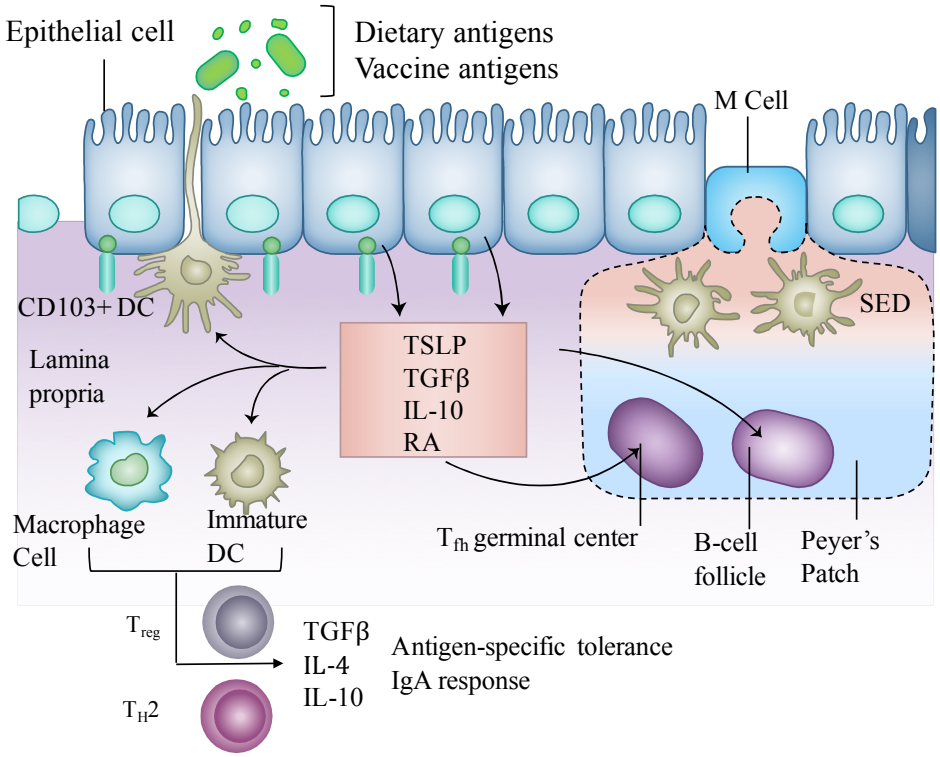

Figure 1-3 Molecular signalling associated with the induction of mucosal tolerance. Dietary and vaccine antigens sampled by epithelial cells and transepithelial CD103+ DCs are translocated to the SED and lamina propria. In the strong tolerising environment of TSLP, TGFβ, IL-10 and RA, antigen specific T_{reg} and T_h2 cells are trafficked into the periphery where they produce a pro-IgA and anti-inflammatory signalling cascade of TFGβ, IL-4 and IL-10 in response to their cognate antigen.

Tolerance in humans may also favour an antibody-dominant response to subsequent challenge with antigen after mucosal vaccination. When soluble keyhole limpet hemocyanin (KLH) was used to immunise naïve volunteers by oral or intranasal (i.n.) routes, subsequent boosting with systemic administration of KLH resulted in increased antibody titres but not T-cell responses.[115] A similar response was observed in the *ex vivo* stimulation of human peripheral blood mononuclear cells with common dietary antigens bovine gamma globulin, ovalbumin (OVA), and soybean protein. Most volunteers had detectable dietary antigen-specific salivary sIgAs, but low to no proliferation of peripheral T-cells when stimulated with the dietary antigens.[116] This suggests that the induction of T_{reg} cells at the mucosa may in fact assist in the generation of a mucosal IgA response rather than limit it. However, it is important to note that T_{reg} cells may reduce CTL response, and may still be a limiting risk for pathogens such as *Mycobacterium tuberculosis* that require CTL response for clearance.[117]

Despite the complexity of the signalling pathways that need to be considered in the development of mucosal vaccines, the very fact that several licenced orally-delivered vaccines

provide protective immunogenicity to diarrhoeal diseases indicates that oral vaccination is an achievable goal. Indeed, many of the major areas of investigation for mucosal vaccination focus on improving the delivery and presentation of antigens to the GALT.

1.4 Immunological hurdles of oral vaccination

The lack of vaccines for most diarrhoeal diseases is evidence of the complexity of developing a safe and effective formulation.[118] Specific to the context of oral vaccines, much of this complexity is related to the need for antigens to traverse the microbiota, mucosa, and highly proteolytic environment of the GIT while providing the correct immunomodulatory signalling to ensure the generation of a long lasting and protective response.

Unfortunately, the study of natural infections has yet to reveal the immune correlates of protection to most diarrhoeal pathogens, and this makes it harder to optimise vaccines to induce a specific response.[118] Known correlates of protection include antigen-specific sIgA, humoral IgA and IgG, antigen-specific CTL, memory B and T cell responses. However, it is also likely that many of the correlates of protection may not yet have been identified.[119]

While correlates may not be well understood (particularly on a population level),[120] there is a prerequisite for oral vaccines to effectively deliver antigens to the GALT in a sufficient dose to induce an appropriate response. Accordingly, many technologies of oral vaccination have focused on improving both the effective delivery and immunological context of vaccine antigens.

1.4.1 Improving formulation and oral delivery of vaccine antigens

The gastrointestinal tract is a highly heterogeneous environment and the physiological conditions and resident microbiota vary dramatically between individuals and geographic regions.[121-124] Individuals also respond differently to dietary and commensal antigens depending on their prior exposure, nutritional status, genetics, co-infections, and overall microbial burden.[125] It is therefore unsurprising that there is a huge variation in immune response between individuals to vaccination with the same formulation.[118] This variation is observed following administration of most oral vaccines, with up to 50% of individuals not responding to a licenced whole-cell killed oral Cholera vaccine when delivered to individuals in Cholera-endemic regions of India, despite the same vaccine being over 90% effective when administered to individuals in Switzerland.[126]

Generally, LAVs fare better during transit in the GIT due to the capacity of the cell or virus to maintain integrity during digestion. This highlights the need for improved delivery of antigens

in non-living oral vaccines, and protective antigen carriers are one of the keys to minimising the heterogeneity of response between individuals.[127] Many delivery systems encapsulate antigenic proteins or epitopes, or add protective epitopes to more robust macromolecules.

This rapidly expanding list includes:

- Live carrier systems in non-pathogenic bacteria e.g. *Salmonella sp.*[128]
- Bacterial 'ghosts' [129]
- Virus-like particles [130]
- Virosomes [103]
- Phage surface display [131]
- Starch micro-particles [132,133]
- Latex micro-spheres [134]
- Water-in-oil-in-water and oil-in-water-in-oil emulsions [135]
- Chitosan nanoparticles [136]
- β-glucan micro particles [137]
- Bilosomes [137]
- Functionalised liposomes [138]
- Proteasomes and polymeric nano particles [139]
- Immuno-stimulating complexes (ISCOMs) [140-143]

There have been many highly effective vaccination regimes using these technologies in small-animal studies, but translation of these results to larger animals such as livestock or humans has often been limited due to the dramatically different kinetics and mucosal environments between model species.[39] For example, strong humoral and mucosal immune responses to oral delivery of a starch micro-particle formulated diphtheria sub-unit vaccine was observed when administered to mice,[132] but no immune response was observed when the dose was scaled for use in humans.[133]

In addition to improving protection from proteolysis, many carrier systems have been designed to optimise delivery of antigens to the GALT and particularly the M-cells via receptor-mediated pathways.[65,144] Targeting M-cells for antigen delivery has repeatedly shown improvement in the consistency and magnitude of the immune response.[138,145-151]

The most intensely studied carrier molecule used to increase delivery of antigens across the epithelium is CTB (see 1.6.3), which binds the monosialotetrahexosylganglioside (GM1) ganglioside of mucosal epithelial cells and translocates across the epithelium.[152,153] Conjugation of antigens to CTB has induced both tolerance and immunogenicity when used as a carrier, and CTB is also a moderately potent adjuvant when delivered alone.[154]

The plant lectin UAE-1 from *Ulex Europaeu–I* has also been well characterised for binding the apical surfaces of epithelial and endothelial cells, and is one of the classical histological markers of epithelial cells.[155] UAE-1 and other plant lectins are effective at increasing the consistency and magnitude of immune response when conjugated to various antigen delivery systems including liposomes and latex micro particles.[156]

Recently, an M-cell specific antibody, NKM 16-2-6 has been shown to be specific for α1,2-fucosylated M-cells.[151] When conjugated to recombinant tetanus toxoid, botulinum toxoid, or OVA, mice generated a faster and stronger IgA and IgG immune response than to either antigen conjugated to a control antibody, and mice administered the NKM16-2-6/BT conjugate were completely protected from challenge with live *Clostridium botulinum* whereas control mice were not.[151]

The ability of so many different carrier systems to improve the magnitude and consistency of immune response highlights the immunological importance of delivering antigens to the GALT. It is must be noted that none of the epithelial targeting systems have been sufficiently efficacious in clinical trials to reach licensure. In addition to the delivery of antigens to these cells, the co-signalling context provided with the antigen is equally important in generating a protective immune response.

1.4.2 Increasing the immunological context of antigen presentation

Without additional signalling, prolonged binding or transcytosis at the cellular surface, the default response to soluble antigens is generally ignorance.[94] However, increased delivery of antigens without altering the immunological context also triggers the default T_{reg}/T_h2 pathway of the GALT.[157]

Orally delivered soluble antigens require formulation with additional pathogen-like characteristics to induce an antigen-specific cellular (effector T cells) or humoral (serum antibody) immune response.[39] Sansonetti, 2011 proposed that the most likely way to perturb the immunological ignorance or tolerogenic response is to mimic the processes of mucosal pathogens.[94] This may include the mucosal immune effectors cells sensing microbial components within tissues, direct access of microbes to the host cellular surfaces, introduction

of PAMPs into the cell cytoplasm, entry of pathogens into cells, or alteration of host membranes.[94] The importance of the immunological context of antigen presentation for the development of oral vaccines is confirmed by the fact that all currently licenced diarrhoeal vaccines utilise one or more of these 'microbial-like' characteristics.

The most extensively investigated mucosal adjuvants are the AB_5 group of bacterial enterotoxins. These holotoxins belong to the AB_5 group of toxins so named as they comprise a single catalytic A subunit, and a pentameric glycan-binding B subunit. AB_5 toxins are common to many pathogens including *Bordetella pertussis*, *Shingella dysenteriae*, *V. cholera*, and ETEC.[158] The canonical member of this class of adjuvants is the cholera toxin (CT).

CT is both a potent immunogen and a mucosal adjuvant for conjugated, fused, or co-administered antigens and was first characterised as an adjuvant following the observation that mice co-administered CT did not develop oral tolerance to the otherwise tolerogenic protein KLH.[159] Several studies have shown an improved T_h2-mediated immune response to antigens co-delivered with CT,[160] and it has recently been shown that CTs adjuvant effect requires direct interaction with gut-resident DCs.[161] Despite being used in livestock vaccines, the intrinsic oral toxicity of CT and safety concerns related to induction of Bell's Palsy following i.n. administration has prevented licensure for human use.[162,163]

The non-toxic B subunits of CT (CTB) and the heat-labile toxin (LT) from ETEC (LTB) are also effective, if less potent, adjuvants than the complete holotoxin. The LTB protein shares 83% sequence identity with CTB and shares similar but distinct ligand-binding properties;[164] LTB is a more promiscuous receptor of non-GM1 ligands including bacterial LPS.[165] These proteins effectively bind epithelial cell surface receptors and translocate fused, conjugated or co-delivered antigens across the epithelium and into the SED where they promote the induction of a T_{reg}/T_h2-mediated responses.[154,166-168]

Mutant forms of enterotoxins have been investigated for their ability to retain adjuvant effects while minimising intrinsic toxicity.[169] Of particular note is the CTA1-DD adjuvant derived from the genetic fusion of the ADP-ribosyltransferase subunit from *V. cholera* (CTA1) to a B-cell binding synthetic analogue of protein A of *Staphylococcus aureus* (DD).[166] This adjuvant has been shown to be an efficient mucosal adjuvant for the induction of T_h1, T_h17 and memory and plasma B-cells when delivered orally or i.n. with a variety of antigens and has been used successfully in several clinical trials.[170,171]

The triterpene glycosides (saponins) from *Quillaja saponaria* and other species have been characterised as adjuvants (see section 1.7.1). While highly labile to the proteolysis of the GIT, many cytokines including IL-1, IL-6, IL-12, IFN-γ, the chemoattractants chemokine C

motif ligand 1 and CCL5 have been shown to be effective as mucosal adjuvants when combined with encapsulation in carrier formulations.[172]

Another class of well characterised mucosal adjuvants are based on PAMPs formulated in oil and water emulsions that stimulate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) inflammatory response. These adjuvants include CpG oligonucleotide formulations that activate toll-like receptor (TLR) 9,[173,174] monophosphoryl lipid A formulations that act via TLR 4, bacterial flagellin that acts via TLR5,[175] synthetic triacylated lipopeptide Pam3CSK4 that acts via TLR2,[176] and muramyldipeptide that acts via nucleotide-binding oligomerisation domain containing 2 (NOD2).[177] Oral co-delivery of many of these PAMPs with antigens have shown strong DC-mediated IgA production.[175]

In addition to the enterotoxins and PAMP-like adjuvants, other mucosal adjuvants include oil and water emulsions. The specific mechanism of action of emulsions in the GALT are not clear, but cationic liposomes, squalene (MF59) emulsions, α-galactosylceramide formulations and the mineral oil-based Montanide ISA-51 and Montanide ISA-720 VG all enhance immune response.[138]

Modern expression technologies now provide high levels of heterologous protein accumulation in plants, allowing a refocusing from the plant cell as an antigen expression system to the plant cell as a vaccine delivery vehicle. It is essential to understand and take advantage of these recent advancements in the understanding of mucosal immunology and adjuvants for the development of plant cells as effective delivery systems for diarrhoeal disease vaccines.

1.5 Improving oral delivery of plant-made vaccines

From the very first *in planta* expression of recombinant proteins, plant cells have been investigated as oral delivery vehicles for the production and delivery of antigenic proteins from diarrhoeal diseases.[178] Many single or multimeric proteins and virus-like particles (VLPs) from diarrhoeal diseases have been shown to be immunogenic in preclinical trials when delivered orally in plant cells,[107] and there are at least five Phase I clinical studies that have shown immunogenicity to three diarrhoeal antigens delivered in plant cells:

- LTB from ETEC [179,180]
- Norwalk capsid protein (NVCP) from *Norovirus* [181]
- G protein from rabies [182]

While there has been recent progress towards a clinical candidate of CTB expressed in rice,[183,184] not a single new clinical study using plant cells to deliver vaccine antigens has been published in the past decade. Many of the limiting factors related to the development of plant-made vaccines are similar to those faced by oral vaccines in general.

"Certainly, while the technical immunological difficulties of oral vaccination remain unsolved for conventional vaccines, it is unlikely that the vaccines produced in plants will make the breakthrough" [185]

The use of the highly complex plant cell to deliver vaccine antigens requires a far better understanding of the mechanistic processes associated with how plant cells act as delivery vehicles before such a vaccine could be licenced for use in humans. There is no real consensus on the the key mechanistic processes of how and where plant cell release antigens during transit in the GIT, or on the role (if any) plant cells play in influencing the mucosal immune response. As such, it is unlikely that plant-made vaccines will be able to answer the mechanistic questions required to advance the field of oral vaccines. However, by utilising the recent breakthroughs in mucosal immunology and the extensive literature of pre-clinical and clinical studies of plant-made vaccines, it may be possible to untangle some of the factors associated with using plant cells to deliver vaccine antigens.

1.6 Bioencapsulation of antigens during oral delivery of plant cells

Live, attenuated oral vaccine formulations have the advantage of using the bioencapsulation of the pathogen to protect the immunodominant antigens against the low pH and highly proteolytic environment of the GIT.[172] Due to the proteolytic attack on all macromolecules in this environment, unformulated proteins not evolved to withstand this environment are generally rapidly degraded.[172]

Members of the research community have repeatedly surmised that the cellular matrix of the plant cell may protect antigens from proteolysis during transit in the GIT,[19,40,185-196] and there is extensive evidence of antigens delivered in fresh or minimally processed plant cells that induce a mucosal immune response.[40-43,107,194,196-202] Also evident is the ability of plants to orally deliver proteins into the circulation that are normally labile in the GIT, including green-fluorescent protein (GFP),[107,199] extendin-4,[203] proinsulin,[196,204] and coagulation factor IX.[201] Most recently, Protalix Biotherapeutics have completed pre-clinical and Phase I clinical studies indicating successful, safe and tolerable oral delivery of two therapeutic proteins; the binding domain of the human TNF receptor fused to the Fc component of a human antibody domain, and the glucocerebrosidase enzyme (prGCD) expressed in transgenic carrot cell culture. The recent publication by Shaaltiel et al., provides strong evidence that lyophilised carrot cells are able to protect prGCD from degradation in simulated gastric and intestinal fluids (SGF and SIF, respectively), and cells are capable of delivering bioactive prGCD into the blood stream of both rats and pigs.[205] These studies suggest that regardless of any immunological impact of plant cells when delivering antigenic proteins, oral delivery of functional proteins to the intestinal epithelium is certainly possible.

Despite these observations, there is no consensus as to what role the plant cells play in any proposed ‘bioencapsulation’ of antigenic proteins, including how and where the plant cells release antigen during digestion. There is no evidence as to whether this process can be optimised using the plant-specific tools available.

There are two primary mechanisms that have been postulated by which encapsulation by plant cells may be able to increase the bioavailability of recombinant antigens. The first involves cellular encapsulation of antigens within the cell wall or endomembrane system, that act as a physical barrier to prevent or limit access to low pH and high concentration of GIT-resident proteases.[205,206] The second mechanism involves a ‘bystander’ effect by which other plant proteins and cellular components act as decoy substrates for the GIT-resident proteases, leaving the target antigen intact.[207]

Several studies have suggested that the cellular encapsulation within plant cells is responsible for the improved mucosal immune response observed for orally delivered antigens in transgenic plant cells compared to purified recombinant proteins delivered in aqueous formulations. One study compared the immunogenicity of orally delivered soluble yeast-derived HBsAg and potato-derived HBsAg in mice.[208] In this study, two 150 μg doses of yeast-derived HBsAg with bicarbonate buffer and 10 μg of CT adjuvant did not stimulate a serum or mucosal response. Whereas, three 142 μg doses of HBsAg delivered in 5 g of potatoes with 10 μg of CT resulted in a high anti-HBsAg serum response. The authors state "*the unique features of bioencapsulation of the antigen within plant cells may be the actual reason why plant-based HBsAg is effective for oral immunisation*".[208] However, despite the strong antibody response when HBsAg was delivered in plants, no control was made for the potential bystander effect protecting antigens administered in the complex formulation of the plant cells compared to purified HBsAg formulated without decoy substrates.

In another study, LTB was expressed in transgenic maize, and the processed maize meal pelletised and administered orally to mice. Mice were immunised with a control diet of 50 μg soluble recombinant LTB mixed with "mouse chow", or formulations of 5 or 50 μg of LTB in pelletised transgenic maize meal. Mice administered both the 5 or 50 μg LTB dose within the transgenic maize pellet produced a higher systemic anti-LT IgG and mucosal anti-LT sIgA antibody response compared to mice administered the 50 μg purified LTB in "mouse chow".[197] However, it is unclear from this study whether the improved immunogenicity was due to encapsulation within the plant cells, the stability of the recombinant LTB in the 'mouse chow,' or the different components of the 'mouse chow' and the pelletised transgenic maize meal.

The plant biomatrix may also contribute to a 'bystander' effect by which the components of the plant cells provide non-specific 'decoy' substrates for the GIT proteases. This effect has been shown to protect the common mucosal adjuvants CT, LT and the muco-adhesive plant lectin UAE-1 during incubation with natural and simulated rat intestinal fluid *in vitro*.[207] In this study, CT, LT and UAE-1 were degraded in natural and simulated rat intestinal fluid, but could be rescued by the addition of exogenous 'bystander' protein substrates in the form of bovine serum albumin or ovalbumin. Further studies have shown the addition of casein as a 'bystander' substrate was effective in protecting insulin-like growth factor from proteolysis in pig, rat and dog intestinal fluid.[209] Casein has also proved an effective substrate decoy against proteolytic degradation of a therapeutic peptide incubated in rat small intestine fluid.[210] While these studies suggest that decoy substrates may rescue antigens *in vitro*, the influence of

additional proteolytic substrates *in vivo* is less clear as there are generally no shortage of dietary and microbial proteins in the GIT lumen that may act as protease substrates.

To date, two studies involving the expression of LTB in maize endosperm have provided the strongest evidence for the enhancement of mucosal immunogenicity when antigens are encapsulated within plant cells. In these studies, the LTB protein was expressed in maize endosperm where it was shown to localise to novel starch granules not present in non-transgenic endosperm.[211] In the first study, soluble recombinant LTB added to non-transgenic maize meal was completely digested in SGF within 5 minutes, whereas LTB encapsulated in transgenic maize meal resisted digestion for up to 15 minutes.[211] In the second study, mice were fed pellets made from transgenic maize meal expressing 10 μg of LTB, or pellets made from non-transgenic maize meal spiked with 10 μg of purified bacterial LTB.[212] The use of this 'spiked' control accounts for the potential 'bystander' effect of protection. Mice fed the transgenic maize pellets developed significantly higher anti-LTB serum and faecal antibodies compared to the meal spiked with the same dose of LTB. Despite the difference in immunogenicity, both groups were protected from subsequent challenge with heat-labile toxin (LT) challenge.[212]

While these two experiments provide evidence that expression within plant cells improves the oral immune response to the same dose of antigen delivered outside the cell *in vivo*, the interpretation of the result in this context is made difficult by the complexities of processing the maize meal into pellets and the stability of LTB in the maize pellet. The authors noted that the improved *in vivo* immunogenicity of mice fed transgenic LTB compared to those fed pellets spiked with purified bacterial LTB could possibly be explained by the spiked LTB being "*more vulnerable to proteolytic degradation during the process of pellet preparation and feeding*" or that "*that there was more LTB in transgenic maize than the ELISA assay indicate*".[212]

The most fundamental choice for protein expression *in planta* is the selection of the expression system and host species, yet this choice has not received particular attention related to antigen encapsulation or the potential for improving the mucosal immune response.

1.6.1 Selection of expression system for oral administration of plant cells

The plant species used for orally-delivered vaccines has historically been chosen based on the amenability for transformation, antigen yield, palatability, and capacity for processing (e.g. milling).[4,186] Given the huge variation in the physical and chemical properties between plants and the unknown immunological impact of different expression systems and host species, the

choice of the species and tissue type has frequently been made without an understanding of the influence on the resultant oral immunogenicity.

Many palatable and non-palatable plant tissues been used to successfully induce an oral immune response after oral priming and/or boosting, but to date there has been no comparisons as to whether the specific tissue used as the vaccine delivery vehicle directly influences the oral immunogenicity of an encapsulated antigen. Also, modern, high-yielding plant expression systems now regularly allow the expression of >1% total soluble protein (TSP) of antigen accumulation in the vegetative leaf tissue (particularly the deconstructed viral expression systems),[213,214] but these systems have not been frequently investigated as expression systems for orally delivered vaccines.

The following list, while not exhaustive, provides an example of the diversity of species and tissues types that have been used to deliver antigenic proteins as formulations of whole plant cells or minimally purified plant extracts:

- *Arabidopsis thaliana* leaves [215]
- *Chlamydomonas reinhardtii* cells [216,217]
- *Daucus carota* root [192,218]
- *Glycine max* endosperm [219,220]
- *Lactuca sativa* leaf [220-222]
- *Lupinus luteus* callus [221]
- *Medicago sativa* leaf [182,204,223,224]
- *Nicotiana benthamiana* leaf [225,226]
- *Nicotiana tabaccum* leaf,[105,199,200,227-232] NT-1 cells,[190,204] and hairy root cell culture [226,232]
- *Orviz sativa* endosperm,[106,110,233-243] and callus [244]
- *Solanum lycopersicum* fruit [191,245-248]
- *Solanum tuberosum* tuber [33,42,208,249-254]
- *Spinacia oleracea* leaf [182]
- *Vigna unguiculata* leaf [185,255]
- *Zea maize* endosperm [180,212,256-259]

Different species accumulate proteins at different concentrations within the vegetative and storage organs, and a wide variety of different tissue types have been investigated as locations for recombinant protein accumulation.[197] Given the diversity of these tissue types, it is likely that the highly varied plant biomatrix may influence the oral bioavailability or immune presentation of antigens.[260]

Plant tissues have radically different cell wall structures, compositions, thicknesses and ease of digestion.[261,262] These factors have been studied extensively in the agricultural literature as ease of access to the macronutrients within plant cells is one of the important factors in the ability for feedlot animals to convert feedstock into biomass.[263] The cell wall integrity during transit in the gut lumen is one of the key factors optimised in agricultural feeding studies, and the rate of macromolecule release during digestion is essential in determining the feeding requirements of livestock.[264] Because of this, the digestibility of the cell walls of forage grasses have been well characterised, including the pre-treatment with cellulosic enzymes to increase the rate of protein and carbohydrate release during digestion.[263] The ability of plant cell walls to resist chemical and enzymatic degradation during digestion is multifactorial and involves the composition and assembly of lignin and polysaccharide modifications that cross-link the cell wall structure and generate cell wall architectures.[265] This cross-linking has been shown to alter the resistance to cellular fracture during mastication and digestion.[266] In addition to the cell wall, the ability of different expression systems such as corn or rice endosperm to be milled to specific particle sizes is known to affect the kinetics of macronutrient release during digestion.[267] While the recent *in vitro* and *in vivo* characterisation of the therapeutic protein prGCD indicates that the carrot cell retains integrity in SGF,[205] there are no reported investigations of the cell wall structure on the oral bioavailability of recombinant vaccine antigens.

Once released from the plant cell during transit in the GIT, the composition of the co-delivered plant biomatrix may also indirectly influence the bioavailability of the recombinant antigens. For example, commonly used plant expression systems including corn, potato, soybean, tobacco and rice have dramatically different compositions of macromolecules known to influence the uptake and digestion of proteins via stimulation of the host digestive enzymes and microbiome.[268,269] Between common species, protein content ranges from 2% w/w in potato to 37% in soybean, carbohydrates from 18% w/w in potato to 78% in rice, lipids from 0.1% w/w in potato to 20% in soybean, fibre between 0.2% w/w in rice to 15% in tobacco leaf, and phenolics from 0.09 mg/g dry weight in rice to 30 mg/g in tobacco leaf.[27] This variation of the macromolecule content of the plant biomass provides a radically different digestive environment and substrates for proteolytic enzymes.

The study of protein release and digestion *in vivo* is complicated by the many separate factors associated with digestion including the physiological, behavioural, and biochemical differences between species.[270] For this reason, *in vitro* models are the primary source of assessing digestion of bioactive molecules. While many modern *in vitro* systems include physiologically relevant complexities such as microbial and physical digestion,[271,272] there is

no consensus as to the mechanistic process of how plant cells release their cellular contents during digestion nor a standard assay for comparing release between different formulations. Indeed, the processes and host-mechanisms involved in protein and peptide uptake in the gut have been contentiously debated in the nutritional literature since the 1970s.[273] Early studies on the bioavailability of plant and animal proteins identified a small but nutritionally significant influence on their dietary source,[274,275] with plant proteins exhibiting altered digestibility depending on the host species.[276] However, it is unclear as to whether the intrinsic resistance to proteolysis is in any way involved in the sampling and receptor mediated translocation of immunogens across the GALT or the resultant immune response.[277]

In addition to the influence of the macromolecules on the host physiology, the tolerogenic immunity to dietary proteins is an important factor in oral delivery of immunogens. Mice previously exposed to soy proteins in their diet did not raise soy-specific humoral or cellular antibodies when they were co-administered CT mixed with extracted soy proteins.[278] Importantly, the authors noted that the anti-CT humoral and mucosal responses were similar for CT delivered with soy proteins or phosphate buffered saline (PBS).[279] This suggests that the existing tolerogenic response to dietary antigens was not broken by co-administration of the strong mucosal adjuvant CT and was not able to sensitise the animals to the host proteins to which they were already tolerised. The same effect has been observed where mice immunised with transgenic rice containing CTB did not generate measurable antibodies against rice storage proteins.[233] While these studies indicate that administration of the potent immunogen and adjuvants CT and CTB is insufficient to break immunological tolerance to the pre-exposed antigens, there is evidence that mice naïve to soy proteins could be sensitised to soy proteins after 3 doses of soy protein extract mixed with CT.[280] It is unclear as to whether the choice of plant species, homology with other dietary proteins, or mucosal adjuvant influences the immunological tolerance or response to dietary antigens co-delivered with mucosal adjuvants.

Significant differences exist between the European Medicines Agency (EMA) and the United States Food and Drug Administration regarding the choice of specific plant transformation technologies used for GMP production of biologically active substances. The EMA restricts production to stable lines of transgenic plants while the FDA is more permissive of the specific plant technology used for the upstream production of GMP products.[37] Despite 'palatability' being one of the early considerations for 'edible vaccines,'[189] any plant-made vaccine licenced for human use will involve at least minimal processing and homogenisation of the plant material to ensure consistency of dose. Therefore, the choice of 'palatability' when deciding on the host expression species may not be an essential consideration.[15]

However, expression in plants with known toxins such as the alkaloids of *Nicotiana sp*. leaf or the solanine-containing skin of potato tubers may still be of concern when choosing a host species.[281,282]

In addition to the choice of expression host, protein accumulation in different subcellular organelles of the plant cell may also influence antigen release and immune response.

1.6.2 Subcellular localisation of proteins in plants

Like the pragmatic selection of plant species and tissue type for the development of oral vaccine candidates, the use of signalling sequences to manipulate recombinant protein trafficking and accumulation in subcellular locations within the plant cell has often been optimised to enhance yield or post-translational modifications, rather than considering the impact on oral immunogenicity.

Manipulation of the subcellular location of recombinant proteins within prokaryotic and eukaryotic cells has been explored in most protein production systems as a means to improve yield, ease downstream harvesting of proteins, reduce proteolysis, or alter the biochemical or structural properties of the post-translational product.[283] In addition to nuclear encoded genes, many recombinant proteins have been expressed following transgene insertion into the chloroplast genome, where transplastomically expressed proteins are correctly folded and can undergo some post-translational modification [107,284] while remaining encapsulated within the chloroplast.[285]

The complex internal protein synthesis, trafficking, and degradation pathways present in plant cells often makes the optimal location for subcellular accumulation of recombinant proteins a predominantly pragmatic choice based only on the few locations where proteins will accumulate to a practical yield.[286] Other reasons for manipulating the subcellular localisation of recombinant proteins include increased protein folding efficiency and reduced degradation,[287,288] reduced cellular cytotoxicity of protein accumulation,[289] post-translational modification including lipid modification, phosphorylation and disulfyl bridge formation,[4] and ease of purification and downstream formulation.[290]

The understanding of the plant trafficking system has provided a molecular toolbox of signal peptides that can direct the accumulation of recombinant proteins at different subcellular locations.[4,290,291]

Recombinant proteins have been successfully targeted to a wide range of subcellular locations in plants including (see Figure 1-4):

- Cytoplasm [292]
- Endoplasmic reticulum (ER) [293-295]
- Protein storage bodies (PBs) [296-299]
- Lytic and non-lytic vacuoles [300-302]
- Mitochondria [303]
- Oil bodies [304]
- Maize starch granules [211]
- Apoplastic space [305-307]
- Chloroplast [305,308-310]

In addition to rational trafficking using fusions of signal sequences, the endogenous signal peptides from many bacterial and viral heterologous proteins are trafficked to different subcellular locations in plants than when expressed in their native host. For example, proteins are trafficked to different regions of the plant cell despite not containing any known plant-specific trafficking signals.[11,211,311] Trafficking has also been shown to depend on assembly of multimeric protein structures such the short and long chains of monoclonal antibodies.[312] The accumulation of the same protein in different subcellular organelles can also depend on the host plant tissue or development stage.[313,314]

In addition to the intrinsic plant organelles, several systems exist for the use of novel or adapted plant signal sequences to induce PB formation in tissues that do not commonly accumulate them such as vegetative leaf cells.[206,315]

These protein body-inducing sequences include:

- Zeins (including the Zera™ tag) [298]
- Elastin-like polypeptides [299,316,317]
- Hydrophobins [318,319]

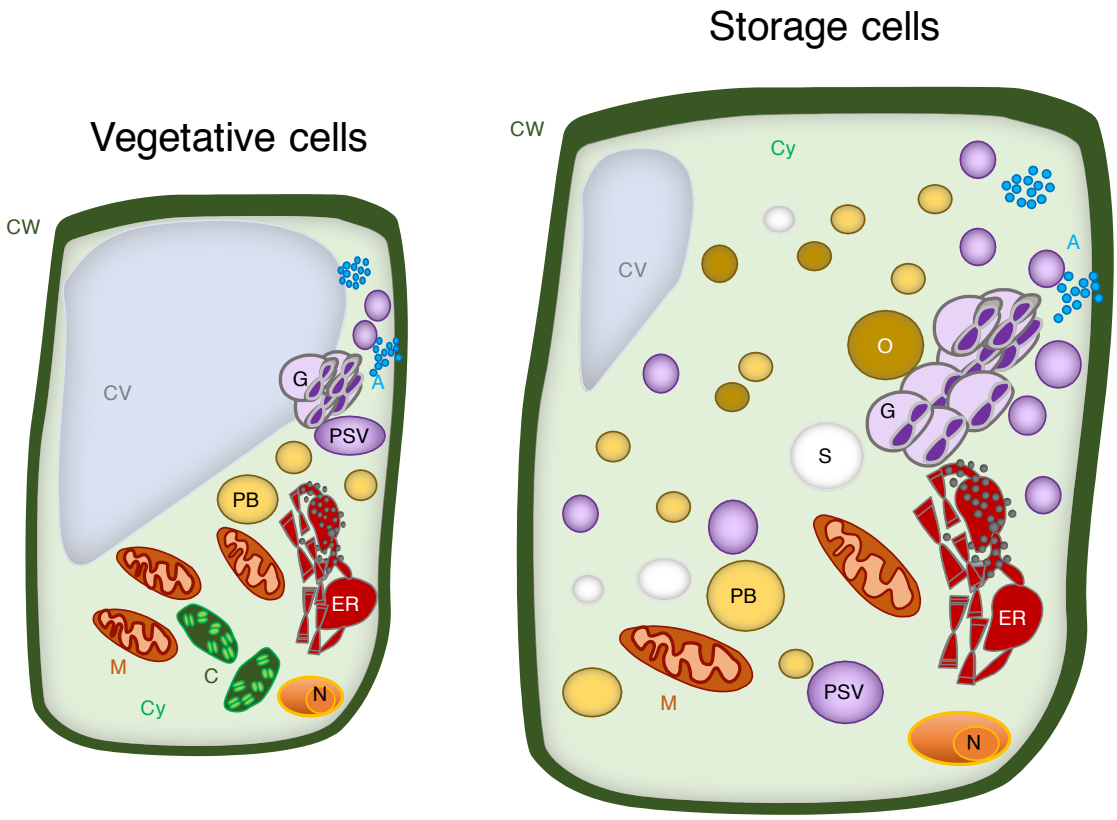

Figure 1-4 Potential sites and pathways of recombinant protein accumulation in vegetative and storage plant cells. Genes transcribed in the nucleus (N) are translated by ribosomes in the cytoplasm (Cy), and proteins with a chloroplast transit peptide or mitochondrion signal sequence are trafficked to the chloroplast (Ch) or mitochondrion (M) membranes, respectively. Proteins carrying a signal peptide are translocated into the endoplasmic reticulum (ER). Once within the secretary system, the default path of protein trafficking is through the Golgi (G) to the apoplast in post-Golgi vesicles (A) where they accumulate between the cell membrane and cell wall (CW). Alternate protein transport is directed by trafficking specific motifs that may shuttle proteins to proteins storage vesicles (PSV), protein bodies (particularly in endosperm cells) (PB), starch granules in endosperm cells (S), or oil bodies (O).

In addition to the biochemical and purification benefits of subcellular localisation,[320] limited evidence suggests that the localisation of recombinant antigens within the plant cell may influence the *in vitro* and *in vivo* characteristics of plant cells as delivery vehicles for antigens.

The main storage proteins in rice endosperm are the alcohol-soluble prolamins that accumulate in protein body I (PB-I) organelles, and the water-soluble glutelins that accumulate in protein body II (PB-II) organelles. Because PB-IIs are water soluble, they are more vulnerable to digestion in the gastrointestinal tract than are prolamins.[233] In one study, a synthetic tolerogen designated 3Crp was targeted to PB-I or PB-IIs of rice endosperm.[239] When compared to purified chemically synthesised 3Crp peptide, 3Crp localised in PB-I was highly resistant to pepsin-based digestion *in vitro*. When exposed to pancreatin-based digestion *in-vitro*, purified chemically synthesised 3Crp peptides were completely digested within 2 minutes, whereas PB-II and PB-I localised 3Crp remained detectable at 30 min and

18 h, respectively. Importantly, mice fed PB-II or PB-I localised 3Crp required 10 and 20-fold less 3Crp respectively, than mice fed purified 3Crp to confer IgE suppression.[239] These data suggest the choice of protein localisation in PB-I or PB-II could be optimised to tailor the release characteristics of the tolerogens.

In another study, correctly folded CTB protein localised within the PB-I structures of rice endosperm. When tested *in vitro*, CTB in PB-Is were resistant to digestion with pepsin.[233] Importantly, when delivered orally to mice, CTB was observed binding directly to the M cells of the GALT indicating that CTB was bioavailable and did not remain within the PB-I organelles.[233] However, a GFP-CTB fusion protein delivered in minimally processed transplastomic leaf cells also showed direct binding of CTB with M-cells.[199] The affect of subcellular encapsulation on this process is unclear. Despite both of these studies showing that encapsulated proteins are released from the plant cell during digestion, there is no indication whether this release or resulting immunogenicity can be modified by altering the subcellular accumulation.

Chikwamba et al., have shown that the LTB protein localises to induced starch granules within the parenchyma of the maize endosperm.[211] LTB localised within these starch granules is highly thermostable and resistant to proteolysis in SGF. The authors suggest that the strong immunogenicity observed when mice were orally immunised with LTB within the starch granules compared to the LTB admixed with non-transgenic maize was a result of encapsulation with the starch granules.[212] However, it is not clear if it was the encapsulation or the processing of the food pellet that influenced the response.

Although these studies hint at the role the plant species, tissue type, and subcellular localisation play in the oral immunogenicity of antigens delivered in whole cells, there has been no systematic studies conducted to investigate the effect of these variables. Due to its extensive characterisation as a mucosal immunogen, the LTB protein from ETEC was selected for the rational investigation of plant tissue type and subcellular localisation affects on oral immunisation.

1.6.3 Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) is a diarrhoea-causing strain of *E. coli* that generates a high disease burden in LMICs.[321] ETEC is transmitted by the faecal-oral route and is the major cause of 'travellers' diarrhoea' in nations where the pathogen is endemic.[322,323] In LMICs, approximately 10-20% of diarrhoeal events in children are caused by ETEC, leading to 280 -

400 million diarrhoeal cases and an estimated mortality of 300,000 to 500,000 deaths due to ETEC-related acute dehydration per year in children under the age 5.[324]

Pathogenesis is caused by the action of the heat-labile enterotoxin (LT) and the heat-stabile enterotoxin (ST). The multimeric LT toxin is a canonical member of the AB_5 family of holotoxins common amongst many lethal pathogens including *B. pertussis*, *S. dysenteriae*, *V. cholera*, and ETEC,[158] and shares 83% sequence identity with CT[158]

In *E. coli*, LTB subunit is transcribed and exported to the periplasm via the general secretory pathway. Once the signal peptide is cleaved in the periplasm, the protein assembles into a functional pentamer and associates with the A subunit (LT-A) to form the LT holotoxin before being secreted from the cell via a type II protein secretion pathway.[325] However, unlike CT that is secreted into the gut lumen by the host pathogen, LT is actively delivered to the cell surface in vesicles after ETEC contact with the epithelium.[326,327] The AB_5 complex irreversibly binds to the surface of the intestinal epithelium by attaching to cell surface receptors including monosialotetrahexosylganglioside (GM1)-containing lipid rafts, and is rapidly translocated via retrograde transport to the basolateral surfaces of the epithelium.[328] The A subunit is translocated into the endomembrane system where it activates adenylate cyclase, resulting in an increase in cyclic adenosine monophosphate (cAMP) causing chloride and water efflux from the epithelium.[158] The ST toxin is a non-antigenic peptide consisting of 18 to 19 amino acids that binds reversibly to guanylate cyclase, resulting in increased levels of cyclic guanosine monophosphate (cGMP).[329] The cAMP and cGMP-mediated fluid efflux leads to watery diarrhoea, and if untreated, acute dehydration.[329]

Both toxins are involved in the pathogenesis, but it is the strong immunogenicity of the LT toxin and the immunogenic but non-toxic B subunit that has led to further investigation in traditional and plant-expression vaccine candidates for the treatment of ETEC.

1.6.4 Vaccines for ETEC

At present, there is no licenced vaccine for the prevention or treatment of diarrhoea caused by any strain of ETEC.[321]

Early studies suggested that protective immunity to ETEC could be conferred by oral immunisation with inactivated ETEC prototype strain H10407.[330] Human subjects receiving this candidate vaccine developed strong anti-colonisation factor antigen (CFA)/I adhesion and anti-LT antibodies. However, it was subsequently shown that the immune response was only effective for homologous strains of ETEC carrying the same CFA antigen.[331] As CFA/I is

only prevalent in 15-25% of ETEC strains, modern vaccines have focused on inducing an immune response to multiple CFAs and LT.[332,333]

In an effort to avoid the oral toxicity of LT, pre-clinical development of a transcutaneous patch containing LT and a single CFA (CF6) showed protective immunity in mice that developed both anti-LT and anti-CF6 antibodies.[334] Further clinical development of the patch containing CF6 and purified LT indicated that CF6 was only minimally immunogenic despite almost complete seroconversion of subjects to LT.[335] Larger Phase 2 studies showed that transdermal LT without CFAs led to patients experiencing reduced illness when challenged with a homologous strain of ETEC.[336] Similar results were obtained in a double-blind field trial of patients vaccinated with LT or placebo prior to travel into ETEC-endemic countries.[337] However, in a recent Phase 3 field study, despite transcutaneous LT retaining strong immunogenicity, the formulation provided only 60% protection against LT-related ETEC diarrhoea and no protection against the heterologous ST-related ETEC.[338] Due to the lack of efficacy in the field setting, further development of this LT-only formulation has ceased.

Despite candidate ETEC vaccines inducing strain-specific protective responses in trials conducted in wealthy nations, most ETEC vaccines are still limited by their short periods of protection, heterogeneous immune response and difficulty overcoming the environmental enteropathy when delivered in LMICs where ETEC is endemic.[321,339,340] The lack of efficacy in LMICs has been attributed to highly varied CFAs between endemic ETEC strains, and an inability to induce neutralising anti-CFA responses to the immunodominant CFAs.[323,338]

To provide protection against a broader range of ETEC strains, more recent vaccine candidates include multiple CFAs. The rCT_B-CF vaccine is a killed vaccine formulation containing strains expressing six distinct CFAs supplemented with recombinant CTB.[341] The ACE527 vaccine is a live attenuated formulation of three strains of attenuated *E. coli* expressing five CFA adhesions and recombinant LTB.[342] The multivalent ETEC vaccine uses an inactivated recombinant *E. coli* strain expressing the four most prevalent CFAs and a non-toxic double-mutant form of LT (dmLT).[343] All candidates are under active clinical development.

Due to the homology between the AB_5 holotoxins, several trials have investigated the efficacy of licenced oral cholera vaccines for the prevention of diarrhoea caused by ETEC. An early clinical study indicated that there was partial cross protection against ETEC-caused diarrhoea for up to 3 months in individuals vaccinated with a killed whole cell cholera vaccine containing additional purified CTB.[344] However, larger more recent studies with the licenced

cholera vaccine Dukoral® have not shown cross protection against ETEC-related pathogenesis.[321]

The strong immunogenicity and amenability to expression *in planta* makes LTB an excellent model protein to investigate the plant-intrinsic factors associated with oral delivery of whole plant cells.

1.6.5 Plant-based expression of LTB

The LTB protein has been expressed in a wide variety of prokaryotic and eukaryotic expression systems.[345-349] Indeed, LTB was not only one of the first antigens expressed *in planta* but the first transgenic plant-based vaccine formulation orally administered to human volunteers.[42,179] The LTB protein has been subsequently shown to correctly fold and form stable, GM1-binding pentamers when expressed using stable transgenic, transplastomic and transient expression systems in a wide range of palatable and non-palatable plant species including *N. tabacum, Solenacae tuberosum, Zea mays, Solanaum lycopersicum, N. benthamiana, Eleutherococcus senticosus, Lactuca sativa, Glycine max, Daucus carota, Orzya sativa, Peperomia pellucida, Nasturtium officinale, and Petunia parodii* (see Table 1).

The LTB subunit has also been expressed in plants as a carrier protein for transmucosal delivery of a wide range of biomolecules including:

LTB-ZP3,[350] LTB-ESAT6,[351] LTB-PEDV,[352] LTB-COE,[352] LTB-ST,[231] LTB-MOMP,[235] LTB-GP5-T,[230] LTB-MUC1,[353] and LTB-LTA-K63.[354]

The LTB protein accumulates at a variety of concentrations in different plant tissues (see Table 1). Due to the different units of measurement between research groups, it is hard to make a confident assessment of the highest level of accumulation, but it is likely to be the report of 12 % TSP in vacuolar-targeted LTB in maize endosperm.[286] One of the consistent trends observed across studies is the improved accumulation of LTB after entry into the plant endomembrane system. When the signal peptide of bacterial LTB is truncated, the accumulation of LTB in maize seeds is dramatically lower (3000-fold).[278] In both protein storage and vegetative tissues, the use of the endoplasmic reticulum retention signal (KDEL) generally leads to an increased accumulation of functional protein.[33] However, entry into the ER is not implicitly required as high concentrations (up to 2.5% TSP) of LTB have been shown to accumulate when LTB was expressed transplastomically without ER-entry or retention signals.[355] The native LTB protein has been localised to subcellular organelles in maize and soy. In maize, LTB with the native bacterial N-terminal signal peptide or with the maize γ-zein signal peptide localises within proteolytic-resistant starch granules of the

parenchymal cells.[211] Plant signal peptides have been fused to direct LTB to different regions of the maize endosperm cell, but assessment of where LTB actually accumulated has not been reported (see Table 1). [286] When expressed in soy with a KDEL C-terminal sequence, the protein localises to novel electron dense protein bodies that are absent in non-transgenic seed.[219]

LTB has also been successfully tagged with purification tags such as hexahistidyl and FLAG while maintaining high protein accumulation (up to 0.75 % TSP LTB-KDEL-His in *N. benthamiana,*[356] and up to 3.5% TSP LTB-KDEL-FLAG in soy endosperm).[219] Plant expression of LTB influences the biochemical properties of the resulting protein. LTB produced in bacterial systems dissociates from the immunogenic pentameric form between 66 and 78°C.[357] However, in corn endosperm LTB remains associated as pentamers during extrusion processing with temperatures up to 170°C,[278] potentially due to the encapsulation of LTB within starch granules of the endosperm.[211]

Many of the studies that have investigated the expression of LTB in plants have gone on to use the plant material in a variety of fresh, air-dried and lyophilised formulations for oral immunisation.

Table 1 Summary of literature reporting LTB expression *in planta*

PLANT SPECIES	EXPRESSION SYSTEM	YIELD	SUMMARY	REF.
N. tabacum cv. Samsun *S. tuberosum* cv. "Frito-Lay 1607"	Stable nuclear transformation 35S promoter	Leaf: LTB up to 5 μg/g TSP (0.00005% TSP) Leaf: LTBK up to 14 μg/g TSP (0.00014% TSP) Tuber: LTB up to 30 μg/g TSP (0.0003% TSP) Tuber: LTBK up to 120 μg/g TSP (0.0012% TSP)	First paper to show *in planta* expression of LTB using bacterial delivered sequence of LTB. Increased accumulation when retained in the ER with SEKDEL C-terminal signal sequence.	33
S. tuberosum cv. "Frito-Lay 1607"	Stable nuclear transformation 35S promoter	Leaf: LTB 0.2 ng/g TSP Leaf: sLTB up to 1.9% TSP Tuber: sLTB 4.3-17.2 μg/g (up to 0.19% TSP)	Yield of LTB protein increased 5-40 fold by increasing codon bias towards potato and maize usage. LTB concentration stable for up to 3 months in tubers stored at 4°C.	42
Z. mays cv. Hi-II	Stable nuclear transformation "maize expression cassette"	Endosperm: LTB yield not noted, but sufficient to immunise mice with up to 50 μg.	Synthetic gene optimised for highly expressed maize codons and using an N-terminal barley α-amylase signal sequence.	197
S. tuberosum cv. Désirée	Stable nuclear transformation, Class I Patatin promoter	Tuber: LTBK up to 17 μg/g of fresh weight tuber, with 20% identified as pentameric form.	Synthetic gene optimised to remove putative polyadenylation stop and mRNA instability motifs, and codon usage altered in favour of use in solanaceous crops. SEKDEL tag used.	358
Z. mays cv. Hi-II	Stable nuclear transformation Ubiquitin promoter	Endosperm: LTB up to approximately 9% TSP with α amylase SP	Synthetic gene optimised for highly expressed maize codons, with "over 3000-fold higher level" expression when LTB gene included α-amylase SP. It is unclear if the native "only Lt-B" sequence included the bacterial SP. Expression of LTB increased "approximately 5-fold through plant breeding".	278
Z. mays cv. Hi-II	Stable nuclear transformation γ-zein promoter	Endosperm: LTB 6 transformation events 0.004–0.19% TSP pentameric LTB in R_2 generation	Synthetic gene optimised as [42]. The LTB expression level varied not only between independent events, but also between ears within the same transformation event.	212
Z. mays cv. Hi-II	Stable nuclear transformation 35S promoter	Callus: 35S LTB 3 transformants 0.01-0.04% TSP Callus: 35S LTBK 5 transformants 0.01-0.04% TSP Endosperm: 35S LTB up to 0.28% TSP in R_3 Endosperm: γ-zein LTB up to 3.7% TSP in R_3, up to 350 μg/g dry ground kernel Endosperm: γ-zein LTBK up to 0.9% TSP for in R_1	Synthetic gene optimised as [42]. High accumulation of LTB in maize kernels, enhanced by using the seed-specific γ-zein promoter and ER retention	359
Z. mays cv. Hi-II	Stable nuclear transformation Ubiquitin promoter	Endosperm: same line as [278]	Study showed that processing endosperm into defatted germ enriched the LTB concentration from approximately 0.8 mg/g in kernels to 5.5 mg/g in processed defatted germ. LTB was also stable in kernels stored at 4 or 23°C for 400 days	256

PLANT SPECIES	EXPRESSION SYSTEM	YIELD	SUMMARY	REF.
Z. mays cv. Hi-II	Stable nuclear transformation Ubiquitin promoter	Endosperm: Native LTB (cell surface) up to 1.8 % TSP Endosperm: LTB (vacuole) up to 12% TSP Endosperm: LTB (ER) up to ~0.9% TSP Endosperm: LTB (nucleus) up to ~0.01% TSP Endosperm: LTB (plastid) up to ~0.007% TSP Endosperm: LTB (cytoplasm) up to 0.0008% TSP Defatted corn germ: Native LTB (cell surface) ~0.27 mg/g	First study that investigated the affect of subcellular localisation on the accumulation of LTB in planta. Plant signal sequences were fused to LTB, but actual location within the endosperm was not confirmed.	286
S. lycopersicum, cv. Tanksley TA234TM2R	Stable nuclear transformation Synthetic constitutive promoter	Fruit: LTB 37.8 μg/g dry weight	First study reporting LTB expression in tomato fruit. Fruit was harvested upon reaching the firm, orange-red stage.	360
Z. mays cv. Hi-II	Stable nuclear transformation γ-zein promoter	Endosperm: LTB up to 2 μg/g in maize meal Starch granules: LTB up to 1.3 μg/g in starch fraction.	Synthetic gene optimised as [42]. LTB localised within starch granules of maize endosperm. Native signal peptide of LTB was not necessary for starch localisation.	211
N. tabacum cv. TI560	Stable plastid transformation	Leaf: LTB 2.5% TSP	Native bacterial SP truncated. First expression of LTB in plastid.	355
N. benthamiana	TMV-mediated infection	Leaf: LTBK-His up to 0.75 % TSP	First expression of LTB with SEKDEL and hexahistidyl tag, via transient viral expression in *N. benthamiana*. Bacterial SP cleaved. LTB forms GM1-binding pentamers.	356
N. tabacum cv. TI560	Stable nuclear transformation 35S promoter	Leaf: LTBK 2.2 % TSP of pentameric form	Synthetic gene sequence optimised for expression in *Tobacco*	361
E. senticosus	Stable nuclear transformation Ubiquitin promoter	Embryogenic cells: LTBK 0.36% TSP	First study to show LTB expression in non-crop species, Siberian ginseng. Pentameric form identified not quantified.	362
D. carota cv. Nantes	Stable nuclear transformation 35S promoter	Taproot: pentameric LTBK up to 0.3% TSP	First expression in carrot. Synthetic gene based on carrot codon usage and removal of mRNA destabilizing sequences.	363
L. sativa	Stable nuclear transformation 35S promoter	Leaf: LTBK 1.0-2.0% TSP	First paper to express LTB in lettuce. Gene optimized as per [361]	364
G. max Merrill cv. Jack	Stable nuclear transformation soybean glycinin promoter	Endosperm: LTBK-FLAG up to 3.5% TSP	First expression in soy. LTBK-FLAG localised to electron dense protein bodies in endosperm parenchyma cells absent in control (non-transgenic) endosperm.	219
D. carota cv. Nantes	Stable nuclear transformation 35S promoter	Taproot: pentameric LTBK 3 μg/g fresh weight.	Sample preparation for oral delivery.	363
A. thaliana *Z. mays*	Transient transformation of *A. thaliana* protoplasts Stable nuclear transformation of *Z. mays*	Protoplasts: yield not determined, but LTB localised into endomembrane system and trafficked to cell surface Endosperm: LTB localised to starch and fibre cell fractions	This is the first study to visualise the subcellular localisation of LTB with fluorescent markers, and showed that that the native SP of LTB or plant-derived SP both direct LTB into the endomembrane system where it is trafficked to the cell surface.	365
O. sativa cv. Dongin	Stable nuclear transformation 35S promoter	Callus: pentameric LTBK 0.12% TSP, 86 μg/g dry weight	First study showing transformation of rice with LTB. Synthetic gene optimised as [42].	244
P. pellucida	Stable nuclear transformation 35S promoter	Leaf: LTBK up to 0.75% TSP	First expression in a drought tolerant species. Synthetic gene optimised as [42]. Pentameric form identified not quantified.	366

PLANT SPECIES	EXPRESSION SYSTEM	YIELD	SUMMARY	REF.
N. officinale, synonym: N. micro-phylum	Stable nuclear transformation 35S promoter	Leaf: LTBK 0.85-1.3 % TSP	Synthetic gene optimised as [42]. Pentameric form identified not quantified.	[367]
L. sativa cv. Potinosa and Green Wave	Stable nuclear transformation 35S promoter	Leaf (cv. Potinosa): LTBK 0.01-0.07% TSP pentameric form, no seed set. Leaf (cv. Green Wave): LTBK 0.005–0.07% TSP pentameric form in T0 Leaf (cv. Green Wave): LTBK up to 0.05% TSP pentameric form in T1, 30 μg/g dry weight.	Synthetic gene optimised as [363]. Highest expression in Potinosa cultivar, but no seed set so lower expressing cultivar used for subsequent immunogenicity testing.	[222]
N. tabacum, *S. lycopersicum* and *P. parodii* hairy root cultures	Stable nuclear transformation 35S promoter	*N. tabacum*: LTB 65-70 μg/g fresh weight *S. lycopersicum*: LTB 10 μg/g fresh weight *P. parodii*: LTB 65-70 μg/g fresh weight	First study to express LTB in hairy root cell culture. Synthetic gene optimised as [42]. Quantification of LTB as GM1-binding pentameric form.	[368]
N. tabacum, *S. lycopersicum* and *P. parodii* hairy root cultures	Stable nuclear transformation 35S and wound-inducible NtQPT2 promoters	*N. tabacum*: 35S- 115 μg/g fresh weight, NtQPT2- 60 μg/g, NtQPT2(wounded)- ~330 μg/g fresh weight. *S. lycopersicum*: 35S- 10 μg/g fresh weight, NtQPT2- 10 μg/g fresh weight *P. parodii* root: 35S- 100 μg/g fresh weight, NtQPT2- 40 μg/g fresh weight	First expression using an inducible promoter system. Synthetic gene optimised as [42]. Quantification of sLTB as GM1-binding pentameric form. Despite lower yield per gram in NtQPT2 lines, increased overall biomass allowed increased total accumulation per culture.	[369]
P. parodii hairy root culture, *N. benthamiana*	Stable nuclear transformation 35S promoter MagnICON® TMV-based system	Root: LTB 300 μg/g dry weight Leaf: LTB 300 μg/g dry weight	Synthetic gene optimised as [42]	[226]
N. tabacum cv. Xanthi	Stable nuclear transformation 35S promoter	Leaf: LTBK 0.35– 0.76% TSP	Optimisation of coding sequence not reported.	[370]
S. lycopersicum	Stable nuclear transformation 35S promoter	Fruit: LTBK 1.04-1.19 % TSP, 14-16 μg/g fresh weight	Synthetic gene optimised as [363]. Pentameric form identified not quantified.	[371]
O. sativa	Stable nuclear transformation constitutive globulin promoter	Endosperm: LTB 3.4 ng/μg TSP	The authors co-expressed both CTB and LTB. The LTB gene was not codon optimized, and the CTB was codon optimised. CTB was expressed at 21.3 ng/μg TSP.	[243]

cv. cultivar, TSP- total soluble protein, LTB- full length LTB protein, LTBK- full length LTB protein with SEKDEL endomembrane retention signal, 35S- Cauliflower mosaic virus 35S, ER– endoplasmic reticulum, sLTB- synthetic plant-codon optimised protein (only used when directly compared to non-optimised coding sequence), 'pentameric LTB' refers to quantification using the GM1 binding assay, SP- ER-entry signal peptide, γ-zein- maize 27 kDa gamma zein promoter, LTBK-His- full length LTB protein with SEKDEL endomembrane retention signal with hexahistidyl tag, sLTBK-FLAG- LTB protein N-terminal signal peptide from bacterial chintinase and C-terminal with SEKDEL endomembrane retention signal and FLAG tag, TMV- tobacco mosaic virus.

1.6.6 Oral immunogenicity of plant-made LTB

The immune response to LTB is one of the best studied systems for oral vaccine development, and is one of the few examples of a plant-made vaccine reaching clinical trials where it was shown to be safe and well tolerated when delivered in potato and corn.[179,180]

The oral immune response to LTB is predominantly a T_h2 dominated response, with a generally dose-dependent increase in antigen-specific IgG1 humoral and sIgA mucosal response with an increased dose of LTB.[33,42] This response is common to oral delivery of LTB regardless of the host cell used to manufacture the protein.[340]

The LTB protein generates an antigen-specific IgG1 humoral and sIgA mucosal response when delivered in plant cells to BALB/c, Swiss brown, and C57Blk/6 mice, and outcrossed sheep (see Table 2). Two separate clinical trials have shown a similar T_h2-mediated response in healthy human volunteers following oral administration of LTB in potato tuber and corn (see Table 2). Other pre-clinical oral immunisation trials have included delivery of LTB in *S. tuberosum* tuber, *Z. mays* kernels, meal and de-fatted meal, *S. lycopersicum* fruit, *G. max* soluble protein, *D. carota* powder, *O. sativa* callus and endosperm powder, *L. sativa* leaf, *P. parodii* hairy root culture, and *N. benthamiana* leaf (see Table 2). Doses of LTB in these studies have ranged from 0.33 to 65 μg of LTB per dose in mice, 5 mg LTB in sheep, and 0.75-1.0 mg LTB in humans (see Table 2). A dose-dependent serum IgG and mucosal IgA response was observed in mice administered 0.33, 3.3 and 33 μg LTB in defatted germ from transgenic *Z. mays*, consistent with the dose response of increased serum and mucosal antibody titre observed following increasing doses of LTB in non-plant studies.[256,347,372]

Due to the widely varied immunisation schedules employed in different studies, it is impossible to directly compare the magnitude or kinetics between studies. There is a trend that plant-delivered LTB is orally immunogenic after one dose if animals have been previously exposed to the antigen, or two administrations if naïve. Heterologous prime boost strategies with subcutaneous priming and oral boosting are highly effective at inducing mucosal immune responses, and many studies have shown that the mucosal sIgA is partially or wholly protective against challenge with LT or CT toxins in the 'patent mouse' assay. However, this assay does not use live ETEC and is not representative of protection against live ETEC due to the complex interaction between live ETEC and the host epithelium,[372] and lack of immunity to ETEC CFA serotypes.[373]

In addition to the wide variety of plant species and tissue types used in pre-clinical models, the processing of the plant mass prior to oral administration has been equally as varied. Some

formulations have used raw plant tissue, but the majority of formulations have been composed of freeze dried formulations.

The LTB protein has been delivered in (see Table 2):

- Crude soluble extract of *N. tabacum* leaf,
- Crude soluble extract or raw cubes of diced *S. tuberosum* tuber,
- Whole kernels, meal, endosperm meal, or de-fatted meal of *Z. mays*,
- Lyophilised and powered *S. lycopersicum* fruit,
- Soluble protein extract of *G. max*,
- Lyophilised and powered *D. carota*,
- Lyophilised *O. sativa* callus or endosperm powder,
- Lyophilised *L. sativa* leaf,
- Lyophilised *P. parodii* hairy root culture,
- Lyophilised *N. benthamiana* leaf formulated with oil.

Despite the wide variety of formulations used to deliver the LTB protein, there has been no direct comparisons between the species, tissue types, or subcellular localisation of LTB with regard to immunogenicity.[226] Comparison between LTB administered within plant cells compared to soluble LTB spiked into the same, non-transgenic plant material has shown that encapsulation within the plant cell leads to improved seroconversion and magnitude of the mucosal immune response. Encapsulation of LTB in potato,[42] whole maize kernels,[197] and processed maize pellets,[212] increased the IgA immune response of mice relative to the same dose of LTB mixed with non-transgenic plant material.

In one of the first human trials of plant-made vaccines, fourteen healthy volunteers ingested 50 or 100 g of raw transgenic potato tuber containing 0.4 to 1.1 mg of recombinant LTB, or wild-type tubers on days 0, 7 and 21.[179] Volunteers who ingested the transgenic tubers had an increase in peripheral IgA-secreting cells from an undetectable pre-vaccination level to a peak of 19.1 IgA anti-LT ASC per 10^6 peripheral blood mononuclear cells (PBMCs) at 1-week after the third dose. Ten volunteers had a 4-fold increase in serum anti-LT IgG antibodies, and 6 had a 4-fold rise in serum anti-LT IgA antibodies. The authors note that while not directly compared in this study, the immune response to LTB in potato tubers was similar to a previous study where volunteers were inoculated 10^9 colony forming units (cfu) of live ETEC.[179]

In a second clinical trial, nine healthy volunteers were administered a more concentrated source of LTB consisting of 2.1 g of transgenic de-fatted corn germ meal containing 1 mg LTB, or the same mass of wild-type de-fatted corn germ meal on days 0, 7 and 21.[180] Seven out of 9 volunteers developed at least a 4-fold rise in serum IgG anti-LT after vaccination, and 4 out of 9 developed at least a 4-fold rise in serum IgA anti-LT antibodies. Seven of 9 volunteers developed anti-LT IgA ASC and the same 7 volunteers developed anti-LT IgG ASCs. Four out of 9 volunteers developed at least a 4-fold rises in stool anti-LT sIgA concentration after vaccination. Interestingly, two responses occurred after the first dose of vaccine suggesting that the plant-derived LTB boosted a pre-existing immunological memory to the antigen.[180]

Despite these promising clinical results with non-optimised plant formulations, there have been no further reports of clinical studies of LTB administered to humans. Given the large number of reports of oral immunogenicity to LTB in plant systems, and the suggestions that encapsulation within the plant cell may improve the immune response compared to un-encapsulated antigen, the LTB protein is used in this work to further investigate the impact of expression system and subcellular localisation on oral delivery of antigenic proteins.

Table 2 Summary of studies involving oral delivery of plant-derived LTB

HOST PLANT	DOSE AND FORMULATION	IMMUNOGENICITY	SUMMARY	REF.
N. tabacum leaf extract and *S. tuberosum* raw tuber delivered to BALB/c mice	12.5 μg LTBK in crude soluble extract by gavage on days 0, 4, 21, 25 15-20 μg LTBK in 5 g of raw tuber on days 04, 4, 14, 18	Leaf extract: Similar antigen-specific serum IgG and neutralising antibodies, but reduced mucosal IgA compared to administration of 20 μg rLTB by gavage. Tuber: Similar mucosal IgA and reduced serum IgG compared to administration of 20 μg rLTB by gavage.	First study to show oral immunogenicity of a recombinant immunogen expressed *in planta*. Immunogenicity of leaf versus tuber not directly compared.	[33]
S. tuberosum tuber, raw cubes delivered to healthy human volunteers	50 or 100 g of transgenic raw tuber with 0.75 ± 0.35 mg LTB on days 0, 7, 14, 21	10/11 volunteers seroconverted (4-fold rise) for anti-LT IgG. 6/11 seroconverted (4-fold rise) for anti-LT IgA. 8/11 volunteers developed LT neutralizing serum titres. 5/10 volunteers had a 4-fold increase in sIgA in stool samples.	First report of human immune response to orally delivered plant-made antigen. The mass (50 or 100 g) of potato was used to investigate volunteer response to raw potato, not to influence dose of LTB. Anti-LT IgA antibody secreting cells were observed for all volunteers given transgenic potato, but not all volunteers seroconverted.	[179]
S. tuberosum tuber delivered to 'mice'	~20 or 50 μg LTB in 5 g of raw tuber fed to fasted mice on days 0, 7, 14	Increase serum and mucosal Abs compared to 5 μg rLTB via gavage. Both doses of LTB in plant cells induced lower toxin neutralisation capacity than 5 μg rLTB.	LTB delivered in potato tubers was immunogenic, but the mucosal immune response was less effective at neutralising LT toxin challenge compared to a 4-fold lower dose of LTB.	[42]
Z. mays kernels delivered to BALB/c mice	5 or 50 μg LTB in an unknown mass of transgenic corn on days 0, 7 and 21	Serum IgG and mucosal IgA higher in both groups fed transgenic corn than in mice administered regular mouse chow with 50 μg purified rLTB.	This study provides evidence that LTB delivered orally within plant cells may be more immunogenic than that delivered in soluble form. However, the specific feeding regime and volume of chow is not noted.	[197]
S. tuberosum tuber TSP as gavage, and tuber as raw cubes delivered to female Swiss mice.	s.c. prime/oral boost: prime with 45 μg rLTB or tuber extract LTBK, both with butyl16-p(AA) adjuvant. Boost 5 g raw tuber Oral immunisation: ~65 μg LTBK in 5 g raw tuber or gavage of ~2 μg LTBK in 0.4 mL of tuber extract on days 0, 2 and 4 and boost on days 21, 23 and 25	Mice administered LTBK via oral feeding or gavage priming and oral boosting did not develop detectable anti-LT serum IgA or faecal IgA. Mice primed via s.c. with rLTB or tuber-deriver LTBK and then boosted by feeding with raw tuber or gavage did develop an antigen-specific immune response.	The authors note that the dose of antigen used to fed or gavage mice was similar to previously studies. However, the 'triple' dose regime was unable to induce a mucosal response without s.c. priming, after which serum IgG and faecal IgA were detected. The authors suggest that effective priming may be needed before oral delivery of plant-made LTB.	[358]

HOST PLANT	DOSE AND FORMULATION	IMMUNOGENICITY	SUMMARY	REF.
Z. mays meal formed into 1 g pellets delivered to 5-week old female BALB/c mice	10 μg LTB in transgenic maize meal pellet, or 10 μg rLTB spiked into wild type maize meal pellet on days 0, 3, 7, and 21	Mice administered LTB in transgenic maize induced higher serum IgG and faecal IgA antibodies by day 27 compared to mice fed wild type meal spiked with rLTB. Mice fed transgenic maize and wild type spike maize pellet were partially protected from LT and CT challenge.	This study showed an improved (increased IgG and IgA) response to the same antigen when encapsulated inside plant cells, and indicates possible reactivity of IgA with LT and CT following administration of LTB only.	[212]
Z. mays defatted corn germ delivered to BALB/c mice	0.33, 3.3, or 33 μg LTB in defatted corn germ on days 0, 7, and 21	0.33 μg LTB: 8/10 detectable serum IgG, faecal IgA 3.3 μg LTB: 10/10 detectable serum IgG, faecal IgA 33 μg LTB: 10/10 detectable serum IgG, no faecal IgA.	Study indicated that LTB could be enriched from maize kernels via standard agricultural processing into defatted corn meal, and that there is an dose response with increased dose leading to increased seroconversion and antibody titres.	[256]
S. lycopersicum powdered lyophilised fruit delivered to adolescent BALB/c mice	12.6-50.4 μg LTB in 4g of freeze dried tomato powder formulated with or without saponin adjuvant and apple cider on days 0, 3, 14, 17, 28, 31, 42, 45, 56, 59, 70, 73, 84, 87	LTB: 4/4 adult mice developed serum anti-LTB IgG LTB with adjuvant: 2/2 adult mice developed serum anti-LTB IgG. LTB:17/17 pups developed serum anti-LTB IgG LTB with saponin: 13/13 pups developed anti-LTB serum IgG.	Showed that oral immunisation of mice with transgenic tomato fruit is able to passively immunise offspring through transplacental transfer or ingestion of colostrum. Study used food-grade saponins added extracellularly with no change response observed.	[360]
Z. mays defatted corn germ meal delivered to 9 healthy human volunteers	1 mg LTB in 2.1 g defatted transgenic corn meal on days 0, 7, and 21	7/9 volunteers developed at least 4-fold rise in serum IgG anti-LT, and 4/9 developed at least a 4-fold rise in serum IgA anti-LT antibodies. 7/9 volunteers developed specific IgA ASC and 7/9 developed IgG ASC. 4/9 volunteers developed at least 4-fold rises in stool sIgA anti-LT concentrations after vaccination; two responses occurred after the first dose of vaccine.	This study is the second clinical trial of plant-derived LTB to show seroconversion and mucosal IgA response following vaccination. Improved seroconversion compared to similar dose delivered in raw potato. Processing of corn meal unlikely to retain antigen in cell, but LTB may be encapsulated in starch granules as per [212]	[180]
G. max soluble protein extracts delivered to female C57BL/6j mice	25 μg LTBK-FLAG in 150 μL soluble protein extracts following s.c. prime and gavage boost, or gavage on days 0, 7 and 14	Strong humoral IgG response in s.c. prime, gavage boost. Similar anti-LTB serum IgA and reduced faecal IgA when gavaged compared to prime boost. Gavaged mice challenged with 25 μg LT were partially protected.	First study to show immunogenicity of LTB expressed in soy. Gavage was with TSP and LTB was not encapsulated in plant cells. Soy expressed LTB was also shown to be an effective adjuvant for a co-delivered bacterial antigen.	[219]
D. carota lyophilised powdered root delivered to 12-14 week old BALB/c mice	10 μg LTB in 430 mg powdered carrot and formulated to 2.0 mL with water for gavage on days 1, 7, 14	Carrot powder suspension induced serum IgG and mucosal IgG and IgA, but lower titres than 10 μg of rLTB delivered via gavage. Mice challenged with LT were similarly protected by carrot LTB and rLTB.	First study to show oral immunogenicity in carrot root. Despite the lower serum and antibody titres to carrot-derived LTB, mice were equally protected from LT challenge as mice administered rLTB.	[192]
O. sativa lyophilised callus delivered to BALB/c mice	~1.7 μg LTBK in 20 mg lyophilised transgenic rice callus powder on days 0, 7, 14 and 21	Mice immunised with LTBK induced serum IgG and faecal IgA.	First study showing oral immunogenicity in rice callus. Predominant IgG1 serum isotype indicative of a T_h2-mediated response. Competitive binding assay showed serum anti-LTB antibodies were successful in competing for LT in a GM1 binding assay.	[244]

HOST PLANT	DOSE AND FORMULATION	IMMUNOGENICITY	SUMMARY	REF.
L. sativa freeze-dried and ground leaf delivered to 12-14 week old BALB/c mice.	~8 μg of LTBK in 260 mg powdered lettuce leaf resuspended in water via gavage on days 0, 7 and 14	Increased serum IgG response, but low mucosal IgA response compared to control group administered 10 μg rLTB formulated in water.	First study showing oral immunogenicity in lettuce. Despite low mucosal IgA response compared to mice administered rLTB, mice gavaged with lettuce leaf showed complete protection following challenge with CT.	[222]
Z. mays seed ground and prepared as per [212] delivered to 4-week old BALB/c mice	5 μg LTB, 5 μg LTB and 5 μg CTB, 5 μg CTB, or 10 μg CTB, in maize meal pellets on days 0, 7, 21 and 49	All mice fed LTB or pellets containing CTB raised serum IgG and faecal IgA anti-CT antibodies. There was no significant difference in faecal anti-LTB IgA response between groups administered 5 μg LTB or 5 μg LTB and 5 μg CTB. However, an increase anti-CTB antibody response was observed between mice fed 5 μg CTB and those fed 5 μg CTB and LTB.	LTB expressing maize was from [212]. Mice administered plant-derived LTB had higher humoral and mucosal response to those fed the same or higher dose of CTB. The authors suggested that the reduced immunogenicity may be in part due to reduced signal peptide cleavage from CTB. Also, LTB acted synergistically as a cis-adjuvant to increase LTB response, but not *vice versa*.	[259]
P. Parodii hairy root culture and *N. benthamiana* leaf, lyophilised and delivered to outbred Merion/Merino sheep	5 mg LTB in 19 g freeze dried transgenic hairy root or transgenic leaf material formulated in oil on days 0, 14, 28 and 38	Hairy root: 0/5 sheep seroconverted with anti-LTB IgG, 0/5 mucosal IgA response. Leaf: 1/5 seroconversion with anti-LTB IgG, and 3/5 sheep responded with a mucosal anti-LTB IgA response.	Intact hairy root or leaf tissue 0.5-1.0 mm^2 used to immunise animals. Increased detection of mucosal IgG and IgA response in sheep immunised with transgenic leaf material compared to root material. No LTB was recovered from faecal samples.	[226]
N. tabacum leaf protein extract delivered via gavage to female BALB/c mice	0.75 μg LTBK, or 0.75 μg LTBK and 0.38 μγ HPV16L1 VLPs on days 0-2, 8-10, 29-31 and 50-52	Mice immunised with LTBK or LTBK and HPV16L1 generated serum IgG and mucosal IgA response to LTB after vaccination. Mice immunised with LTBK were partially protected from challenge with 75 μg LT and ST.	Mice immunised with LTBK and HPV16L1 VLPs generated strong humoral and mucosal immune responses to the VLPs than mice administered VLPs alone. This indicates LTBs cis-adjuvant properties when co-delivered with another antigen.	[370]
O. sativa ground and suspended in PBS delivered to 5-week old female BALB/c mice	s.c. or oral 50 μg rLTB or ~90 μg LTB and 110 μg CTB on days 1, 4, 7, 10, 13, 16, 19, 22, 25	The seroconversion and titre of IgG and IgA in serum, and IgA in faeces was similar in mice immunised with rLTB and LTB produced in rice.	Despite the potential for synergistic cis-adjuvant capacity of LTB and CTB, the immune response to LTB was similar to that of rLTB alone. This is similar to the observations of [259]	[243]

Abs- antibodies, rLTB- bacterial-derived recombinant LTB, rCT- purified bacterial-derived cholera toxin, LTBK- plant produced LTB protein with a c-terminal KDEL ER-retention signal, s.c.- subcutaneous, LTBK-FLAG- LTBK with a c-terminal FLAG protein purification tag, LT- heat-labile toxin from ETEC, CT- cholera toxin , CTB- B-subunit from heat-labile cholera toxin

1.6.7 Experimental strategy for determining the impact of bioencapsulation

The primary considerations for choosing the LTB protein to investigate the influence of plant species and subcellular localisation on oral immunogenicity was the record of LTB experimentation in plant systems, the molecular reagents available to investigate the subcellular localisation within the plant cell, and the well characterised immune response to oral administration.

The first experiments directly compared three different plant expression systems: vegetative leaf of *N. benthamiana*, fruit of *S. lycopersicum*, and hairy root cell culture of *P. parodii*. These species and tissues were chosen because of their amenability for transformation, significantly varied physiology and cell wall architectures.[261] If the choice of expression host does indeed influence oral immunogenicity, it was hypothesised that the different characteristics of these species and tissues would be revealed. The identity and quality of the denatured and native protein as well as the capacity of each plant to produce pentameric LTB was determined by western blot. Plant material was harvested, lyophilised, and processed in an identical manner to minimise processing variation. Each plant and tissue type was roughly chopped and sieved to a known particle size. An animal study investigated the ratio of LTB released into the lumen versus that retained in the plant cells at different locations within the mouse GIT. It was hypothesised that this would provide an understanding of where the plant tissues released the antigen during digestion. Another animal study immunised mice with the different plant species and measured their humoral and serum antibody responses after 28 days to investigate the influence of the expression system on oral immunogenicity

To investigate if subcellular encapsulation improves the seroconversion or magnitude of the mucosal immune response, LTB was expressed in *N. benthamiana* leaves using the TMV-based MagnICON expression system.[214] This system was chosen as high yields of heterologous protein have previously been expressed in *N. benthamiana* leaves, and the tripartite expression system is amenable to inclusion of additional protein signalling sequences.[213,214] The yield of LTB, and the identity and quality of the denatured and native protein was determined by western blot. The subcellular localisation of LTB within the leaf cells was confirmed using immuno-gold labelling and transmission electron microscopy. The primary alkaloid metabolites of the *N. benthamiana* leaves were quantified to establish if there was a difference in the plant host response to the differential sites of protein accumulation. Leaf material was lyophilised and processed to generate a consistent particle size. The delivery kinetics of different subcellular localisations of LTB was investigated *in vitro* in an SGF assay. Control leaf material formulated with purified recombinant LTB was

used to ascertain if encapsulation within the leaf cell influences the immune response in mice. By delivering the same antigen in the same species and only varying the subcellular localisation, it was hoped that the impact of antigen encapsulation in different subcellular locations within the *N. benthamiana* leaf cell would be revealed.

1.7 Influence of plant-metabolites on oral vaccination

In addition to the encapsulation of antigens in plant cells, the plant matrix itself may include compounds that improve the immune response to co-delivered antigens.

Mucosal adjuvants tend to act in one of three (known) mechanisms: protecting the antigen from degradation, targeting and improving transport of antigens across the epithelium, or direct modulation of the immunological signalling at the epithelium. While the encapsulation or 'decoy' action of the plant matrix may improve the oral delivery of antigens or therapeutic proteins in plant cells, phytometabolites may also play a role in potentiating the immune response. Indeed, many of the known systemic and mucosal adjuvants are plant components such as glycoside saponins, lectins, and pectins.[374] Several studies suggest that the mucosal immune response to plant-made vaccines may be potentiated by using an exogenous formulation with saponins from the soapbark tree *Quillaja saponaria*,[215,228,375] or by delivering antigens in plant cells with endogenous metabolites such as alkaloids, lectins or saponins.[191,220,376]

One of the earliest plant-made vaccine patents provided evidence of this adjuvant affect when they showed that food-grade saponins formulated with transgenic *S. tuberosum* tuber expressing Norwalk virus-like particles (NVLPs) resulted in higher humoral and mucosal immune responses than mice fed the same formulation without saponins.[375] The same patent showed that mice fed transgenic *S. lycopersicum* fruit expressing a human respiratory syncytial virus fusion protein delivered with food-grade saponins or CT resulted in a higher humoral antigen-specific antibody titre, compared to the same fruit without the adjuvant.[375]

A later study showed that powdered tobacco leaves containing the measles virus H protein (MV-H) formulated with 2.5 mg crude saponins from *Q. saponaria* was significantly more orally immunogenic than the same leaf material formulated with CT or the mutant LT adjuvant LT(R192G).[228]

Most recently, purified saponins from *Q. saponaria* have been shown to provide protective immunity following oral administration of lyophilised *A. thaliana* leaf expressing haemagglutinin H5.[215] Mice administered powdered leaf formulated with 2.5 mg of food-grade saponins raised a much higher antigen-specific serum IgG and mucosal IgA response

than mice administered H5 alone or formulated with other adjuvants including 10 μg CT or 50 ng of recombinant flagellin. Furthermore, 90% of mice administered the saponin formulation were protected from challenge with live influenza that killed all mice administered H5 alone.[215]

However, the efficacy of formulating plant-made vaccines with exogenous saponins is questioned by another study that observed no significant antigen-specific humoral response in mice fed transgenic *S. lycopersicum* fruit expressing LTB when formulated with 10 mg of food-grade saponins compared to mice fed the same fruit without exogenous saponins.[360]

In addition to the exogenous application of plant metabolites, it is conceivable that plants producing endogenous immunomodulatory metabolites may be able to act as self-adjuvanting delivery vehicles.[374,377]

One study that suggested this 'self-adjuvanting' concept noted the unexpectedly high mucosal immune response to a very low dose (100 ng) of HBsAg VLPs delivered orally in powdered *L. sativa* leaf.[220] The authors note that "*it might be carefully assumed that raw edible plants can be used at the same time as an oral vaccine producer and, at least partially, as a source of endogenous adjuvants too.*"[220]

One of the most compelling observations of plant cells providing an endogenous adjuvant effect was seen following NVLP delivery in *S. lycopersicum* fruit.[191] Oral administration of two doses of 100 μg purified insect-derived VLPs (irVLPs) by gavage induced a Norovirus (NoV) specific humoral IgG response in 40% of mice, similar to other studies of orally delivered NoV VLPs in plants.[181,249] However, lyophilised tomato powder containing ~64 μg NVCP without any adjuvant induced NoV-specific serum IgG in 80% and mucosal IgA in 100% of mice, and all mice fed NVCP in the fruit raise higher, longer-lived titres than mice fed the purified VLPs alone. The authors suggest that the improved immunogenicity observed in mice fed tomato powder containing NVCP may be due in part to *"α-tomatine, an alkaloid glycoside of tomato may serve as a natural adjuvant potentiating immune responses in mice."* [191] A separate study also observed an increased humoral and mucosal immune response to CTB expressed and delivered orally in *S. lycopersicum* fruit compared to purified CTB.[378] The authors of this study suggest that the improved immune response was likely due to the protection against the "*degradation by protease in the digestive tract of mice*" offered by the "*fibrous tissue of fruit*."[378]

Interestingly, plant metabolites may also negatively influence the oral immunogenicity of some co-delivered antigens. The best example of this effect is the reduced immunogenicity to

LTB and NVCP when delivered in cultivars of potato tubers with high concentrations of phenolic compounds.[379] A lower immune response to LTB delivered in the high-phenol Désirée cultivar was observed compared to the same dose of LTB delivered in the lower-phenol Frito-Lay 1607 cultivar.[42,358] Similarly, a significantly reduced immune response was observed to NVCP delivered in Désirée potatoes compared to tomato fruit, with the authors suggesting that the Désirée cultivar *"might contain some immuno-inhibiting substance"*.[191] It is unclear if the reduced immunogenicity to these antigens delivered in Désirée potatoes is due to increased degradation of the antigen during digestion, or an inherent 'immuno-inhibiting' compound of the host expression system.

Despite these reports, the impact of endogenous plant metabolites on oral immunogenicity of plant-made vaccines has not been investigated. This is surprising since an extensive body of literature exists on the influence of plant metabolites on non-plant based oral vaccines, lending credence to the potential of self-adjuvanting vaccines produced in plants.

1.7.1 Plant metabolites as oral adjuvants

Many mucosal adjuvants are derived from plants. These compounds cover the gamut of mucosal adjuvant mechanisms; carrier adjuvants that decrease proteolysis and/or protect antigens during transit in the GIT such as polysaccharides and gelling agents, targeting adjuvants to increase translocation across the host epithelium such as lectins, and direct immunomodulating compounds such as saponins and inulin-type fructans.

Pectin and other complex plant polysaccharide gelling agents protect antigens from proteolysis during digestion by encapsulating proteolytic labile regions or the protein within the gel matrix as well as increasing the retention time of antigen transit during digestion.[380] A wide variety of plant-made polysaccharides have been investigated as oral adjuvants including plant-intrinsic starch granules,[211,381] and polysaccharide-antigen formulations. [381-384]

Plant lectins are carbohydrate-binding glycoproteins found in a variety of plant species.[156] Lectins bind carbohydrate moieties on cell surfaces, particularly M cells.[385] This mechanism of targeting has been used extensively as a mucosal carrier to target antigens or therapeutic proteins to the epithelium surface.[138,145,156,386-388]

Saponins are a highly diverse group of glycosides occurring predominantly in plants, and play important roles in plant defence against pathogens.[389] The principal source of commercial and pharmaceutical saponins is the bark and wood of *Q. saponaria*,[390] and saponins of *Q. saponaria* are used as systemic and oral adjuvants in many veterinary and clinical trials of human vaccines.[391]

Plant-derived saponins are natural glycosides of steroid or triterpene origin that exhibit many biological and pharmacological actions including immunomodulatory,[392] oncolytic,[393] antiviral,[391] antifungal,[394] antibiotic,[395] hypoglycemic,[396] and hypocholesterolemic properties.[397] The canonical saponin structure comprises a lipophilic chain (sapogenin) formed by a steroid or other triterpene aglycon decorated with one or more hydrophilic glycoside chains.[389] Aglycon derivatives can also incorporate nitrogen, and these nitrogen-containing saponins often exhibit the chemical and pharmacologic characteristics of alkaloids.[389] The surfactant qualities of saponins are derived from the amphypatic structure of the molecule, and saponins are used extensively in commercial food and beverage production as foaming agents.[390]

It is the capacity of saponins to modulate the mammalian immune response following oral and systemic delivery that has led to significant interest in their potential as vaccine adjuvants.[397,398] Crudely purified (often referred to as 'food-grade') saponin extracts are too toxic for systemic delivery in humans due to the haemolysis and disruption of cell membranes caused by cholesterol scavenging.[399] However, saponins can be delivered orally with far lower toxicity. Crudely purified extracts may be further refined into semi-purified fractions including the well characterized Quil-A sub-fraction used in veterinary vaccines since the 1950s, or more highly purified into the QS-21 fraction used in some veterinary and human vaccine formulations.[400-402]

When used as a systemic or oral adjuvant, crude, semi-purified and highly purified saponins potentiate humoral and mucosal antibody responses and the production of Class I-restricted CTLs.[403,404] These properties are ideal for subunit vaccines against intracellular pathogens such as viruses where protection is conferred by multiple arms of the immune response.

Crude or purified saponins may be formulated with antigens as powders or liquids, but are also used as one of the principal components of immune-stimulating complexes (ISCOMs): Particulate structures composed of a saponin, cholesterol, and lipid**.**[405] When formulated with an amphipathic protein or peptide, the immunogen is incorporated into the particulate structure.[406] Without antigen, the ISCOM is referred to as an ISCOM matrix and is the basis of the proprietary ISCOMATRIX™ adjuvant used in veterinary vaccines. However, ISCOMATRIX has also been studied in 8 separate human clinical trials where it was effective at inducing long-lived CD4+ and CD8+ T-cell and humoral responses to antigens including hepatitis B virus, human papilloma virus and influenza virus.[407,408]

Most importantly for the development of vaccines for diarrhoeal pathogens, exogenously formulated 'food-grade' saponins enhance the humoral and mucosal immune response to a

variety of orally delivered antigens. When BSA was formulated with food-grade *Q. saponaria* saponins, the resulting anti-BSA mucosal and humoral response was improved compared to other adjuvants including lithium or taurine.[409] Food-grade saponins are effective oral adjuvants for orally delivered proteins including HBsAg, haemagglutinin from influenza, a mucin epitope conjugated to KLH, chimeric plant viruses,[375] and rabies virus antigen.[410]

The mechanism of action for the immune potentiation of orally-delivered food-grade saponins is not understood, but saponins have been shown to facilitate the increased rate of antigen sampling and basolateral M-cell mediated transport of a co-delivered rabies virus,[411] and to directly interact with APCs in the SED.[412,413] The surfactant qualities of saponins have also been suggested as a mechanism for the increased antigen translocation across the GIT epithelium.[399,414,415] Intragastric administration of 99mTc-radio-labeled human serum albumin together with quinoa saponins revealed an increased presence of the radio labelled protein in blood, liver, spleen and lungs of mice compared to mice administered labelled serum albumin without saponins.[416] However, the physicochemical properties required for the increased epithelium transport has not been determined, and at least *in vitro*, the surfactant qualities of saponins are not explicitly required for their adjuvant effect,[417] so long as they retain an amphipathic structure.[418]

More highly purified fractions of *Q. saponaria* saponins also promote immunogenicity when formulated with protein antigens. One important study characterised the immune response to varying doses of QS-21 formulated with a constant dose of tetanus toxin (TT) orally administered to mice.[419] Low doses of QS-21 induced anti-TT IgG1 and IgG2b antibodies characteristic of a T_{reg}/T_h2 immune response, whereas higher doses resulted in anti-TT IgG1, IgG2a, IgG2b, and IgG3 antibodies and TT-responsive IFN-γ secreting splenocytes characteristic of a T_h1 response. Most interestingly, mucosal anti-TT IgA antibodies were only observed in groups of mice administered the lower doses of QS-21 suggesting that the increased T_h1 response abolished the default T_{reg}/T_h2 mucosal immune response. Moreover, while TT delivered without QS-21 induced moderate serum anti-TT IgG titres, mice were only protected from systematic challenge with live *Clostridium tetani* when vaccinated with a formulation containing QS-21. Importantly, the authors observed no architectural changes or histopathological damage to the GIT epithelium.[419] Together, these results suggest oral QS-21 is capable of influencing the magnitude and polarisation of the effector cell subsets via modulation of co-signalling, and does not involve gross damage to the epithelium.

The adjuvant effect of orally delivered saponins is also observed when they are formulated into particulate ISCOMs. The first evidence of oral immunogenicity involving ISCOMs

showed an OVA-specific humoral response of mice given a single dose of OVA formulated as an ISCOM.[420] Mice repeatedly administered OVA-formulated ISCOMs induced a T_h1/T_h2 balanced immune response represented by high titres of serum IgG and IFN-γ expressing OVA-specific splenocytes, in addition to increased mucosal IgA titres.[406] Orally delivered ISCOMs more rapidly undergo transcytosis by APCs compared to antigen alone (potentially due to their particulate nature), and are more rapidly transported to the draining lymph nodes than soluble protein.[141] *In vitro* assessment of ISCOM transcytosis shows that ISCOMs are dissociated and processed into antigenic peptides in the SED.[421] However, there have been multiple reports of ISCOM-based oral formulations showing no or low immunogenicity, and as such, the factors associated with generating reliable immune response to orally-delivered ISCOMs are not well understood.[141]

The adjuvant properties of saponins are not restricted to those from *Q. saponaria*.[400,422] Saponins from *Chenopodium quinoa* potentiate IgG and IgA responses in serum, intestinal and lung secretions,[416] and saponins from the stem and leaf of *P. ginseng* have also been shown to generate a balanced T_h1/T_h2 immune response to orally delivered foot-and-mouth disease virus antigens.[423]

Interestingly, mice fed food-grade saponins without formulation with other antigens show 'pre-conditioning' of the immune system with antigen-independent clonal expansion and lymphocyte proliferation.[424] Mice orally administered food-grade saponins prior to being immunised i.m. with inactivated rabies vaccine raise significantly higher humoral antigen-specific antibodies compared to mice not 'pre-conditioned' with saponins.[424] Similar 'pre-conditioning' has been observed in chickens orally delivered *Panax ginseng* saponins and subsequently administered a Newcastle disease virus vaccine i.m.[425] These data suggest that oral delivery of saponins may act to globally potentiate the immune response.

While the potent and well characterised saponin fractions from *Q. saponaria* are clearly capable of improving the oral immunogenicity of co-delivered antigens, it would be ideal for the development of a low-cost plant-made oral vaccine if the plant host cell also produced an immunostimulatory compound. Fortuitously, *S. lycopersicum* fruit is not only one of the model species used to express and deliver antigenic proteins, but also possesses a naturally occurring saponin glycoalkaloid.

1.7.2 Tomato as a model of metabolite influence on immunology

The fruit of *S. lycopersicum* was one of the earliest expression systems used for the production of antigenic proteins.[426] Tomato is amenable to stable nuclear and chromoplastic

transformation and there are a wide variety of expression plasmids available using constitutive and fruit-specific promoters.[427]

Orally immunogenic vaccine antigens produced in *S. lycopersicum* fruit include:

- Respiratory syncytial virus fusion protein [428]
- LTB protein [360]
- Hepatitis E virus E2 protein [429]
- Human immunodeficiency virus (HIV) envelope and group-specific antigen proteins, and HBsAg [247,430-432]
- NVCP [191,245]
- Severe acute respiratory syndrome (SARS)-coronavirus spike protein [433]
- *Rotavirus* viral-coat protein 2 and 6 [434]
- *Yersinia pestis* F1-V protein [246]
- CTB protein [378]
- HIV trans-activator of transcription protein [435,436]
- *Corynebacterium diphtheriae*, *B. pertussis* and *C. tetani* exotoxin epitopes [437]

Tomato has also one of the most highly characterised metabolomes of edible crop species, and is frequently used as a model of crop ripening.[438,439] The principal metabolite in leaves and unripe fruit of *S. lycopersicum* is the saponin glycoside, α-tomatine. As expected given its saponin structure, α-tomatine possesses antibiotic,[395] antifungal,[394] anti-malarial,[440] cholesterolemic,[396] and oncolytic properties.[393,441-443] α-tomatine has also been characterised as a systemic adjuvant,[444] and tomatine (an alkaloid fraction including α-tomatine and dehydrotomatine) increases permeability of epithelial cells without observable histopathology similar to saponins from *Q. saponaria*.[445]

Immature commercial tomato cultivars contain up to 500 mg per kg fresh weight of α-tomatine and ripe fruit of commercial cultivars typically contain approximately 5 mg of α-tomatine per g of fresh fruit.[446] Far higher concentrations have been observed in rare tomato cultivars including the Andean cerasiform cultivar which can accumulate between 500-5000 mg per g fresh weight in immature fruit.[447] Accumulation of α-tomatine (and other glycosides common to the *Solanaceae* family) in the vegetative and reproductive tissues is up-regulated by the alkaloid biosynthesis pathways in response to stress and herbivory.[448] As such, the

glycosides of *Solanaceae* present in the leaves and unripe fruit are a key defence mechanism against herbivory.[440,449] As the fruit ripens, most of the α-tomatine and its secondary metabolite dehydrotomatine are actively degraded.[450,451] Despite being a potent defence mechanism against insects, the low oral toxicity of α-tomatine in mammals (LD_{50}: 500mg/kg in mice) is similar to saponins from other species, and is relatively low compared to other *Solanaceae* glycoalkaloids such as α-chaconine and α-solanine.[282]

As observed with saponins from other species, tomatine formulated with OVA dramatically increases the anti-OVA CD8+ IFN-γ cells in mice compared to OVA alone.[452] It is superior to other systemic adjuvants including aluminium hydroxide or incomplete Freund's adjuvant at inducing strong CTL responses.[453] Formulation of tomatine with a pre erthrocytic malaria vaccine candidate antigen sufficiently increased the CTL immune response to provide a level of protection unachievable with other adjuvants such as oil-based emulsions and TLR agonists.[454]

The mechanism of action for α-tomatine's adjuvant affect is not well understood. It has been shown to induce apoptosis and inhibit NFκb activation in adenocarcinoma and breast cancer cell lines.[443,455] Tomatine formulated with antigen may result in an antigen 'depot' slowing release of antigen to the APCs,[452] but it has also been shown to stimulate loading of antigen onto MHC I molecules.[444] Most recently, α-tomatine has been shown to increase phagocytosis in complex antigens, and the adjuvant effect of tomatine is abrogated when the phagocytic process is blocked in mature APCs.[413]

To date, there is no evidence of α-tomatine potentiating the immune response to orally delivered antigen either as an exogenous component of a vaccine formulation, or co-delivered in a plant formulation. Indeed, limited evidence suggests that α-tomatine may increase epithelial permeability to a similar extent to saponins from other species.[456] This raises the possibility that *S. lycopersicum* fruit may be able to act as an antigen expression and delivery system, with an intrinsic T_h1 adjuvant property, making it an ideal system for investigation as a candidate system for a diarrhoeal virus such as NoV.

1.7.3 Norovirus

Norovirus is a member of the *Caliciviridae* genus, and noroviruses are now recognised as the leading cause of gastroenteritis epidemics and a major cause of sporadic gastroenteritis in children and adults.[457] NoV infection accounts for up to 20% of severe gastroenteritis cases among children less than 5-years of age and 12% of all mild and moderate diarrhoea.[458]

Several meta-analyses suggest that the acute diarrhoeal disease caused by NoV infection is responsible for up to 200,000 deaths annually in children less than 5-years of age in LMICs, and up to 800,000 annual deaths across all ages groups.[458-461] In the United States, the direct and indirect costs of food-borne NoV infection is estimated at over $2 billion annually.[462]

Transmission of NoV between individuals is by the faecal-oral route, and is enhanced by the long period of asymptomatic shedding (with a mean period of viral shedding in stool of 28-days),[463] the stability of the virus capsid, the difficulty in removing infectious particles from work surfaces,[464] and the low number of particles required for infection.[463] Between 15-35% of humans infected with NoV are asymptomatic despite shedding virus.[463] Although acute gastroenteritis associated with NoV infection clears within 2-4 days, viral shedding continues for approximately 30-days,[463] and immunocompromised individuals may become chronically infected and continuously shed infectious virus.[465]

Norwalk virus (NV) was first identified as the etiological agent of acute gastroenteritis in the town of Norwalk, USA in 1972.[466] The 'Norwalk virus' was first identified as 27 nm particles in stool samples of patients experiencing acute gastroenteritis,[466,467] and was subsequently cloned and shown to possess a single positive-sense 7.7 kb RNA genome enclosed in a capsid.[468] Norwalk virus is the sole species in the *Norovirus* genus and is divided into six genogroups.[469] The GI, GII and GIV strains are known to infect humans, and the other genogroups infect cattle (GIII), mice and rats (GV), and dogs (GVI).[470] The GII genogroup is most prevalent in humans causing approximately 95% of human infections.[471] The majority of recent NoV outbreaks are caused by the GII.4 genotypes, and in the mid 1990s the global identification of GII.4 genotypes was classified as a pandemic.[472]

The NoV capsid binds histo-blood group antigens (HBGA) expressed on epithelial cells in a genogroup and strain-dependent manner, with certain strains of virus only binding to specific surface antigens of the host epithelium.[473] The mechanism of norovirus pathology and replication *in vivo* is not well understood, but changes to cells of the epithelium and lamina propia following infection include increased enterocyte apoptosis, flattened villi, crypt hypertrophy, mucosal inflammation, and disruption of the epithelial barrier function.[457,474] The acute diarrhoea associated with infection is likely caused by epithelial barrier damage and increased anion transport,[474] while the histological changes to the epithelium likely indicate the epithelium as the site of viral replication.[457] In addition to the acute effects of gastroenteritis, there is growing recognition that NoV infection may be associated with long-term inflammatory-related sequelae including post-infection irritable bowel syndrome,[475] and exacerbation of inflammatory bowel disease.[476]

1.7.4 Vaccines for *noroviruses*

Despite the identification and characterisation of NoV as the causative agent of acute gastroenteritis, the lack of understanding of the correlates of immunity and the difficulty studying the virus *in vitro* [477,478] has resulted in a lack of treatment options for this acute and debilitating disease. There is no vaccine licensed for the prevention of NoV-related gastroenteritis and the only treatment is oral rehydration therapy. Error-prone RNA replication also leads to a high rate of antigenic drift further complicating the development of a vaccine.[479]

Protection from NoV infection involves both genetic and immunologic requisites. Genetic protection requires the absence of strain-specific receptors for viral binding in the loci that control human ABO, Lewis and HBGAs.[473] The correlates to acquired immunity are less clear. Capsid-specific serum IgG titre,[480] memory IgG and memory IgA cells,[481,482] as well as HBGA-blocking antibodies,[480] have all been associated with reduced viral load and reduction in disease symptoms in infected patients.

Despite difficulty studying live virus, highly immunogenic NVLPs are amenable for expression and self-assembly in cell-free, prokaryotic and eukaryotic expression systems including alphavirus, baculovirus, yeast, Venezuelan encephalitis virus replicons and plants.[483,484] NVLPs fold into 27 or 38 nm icosahedral particles consisting of 90 dimers of the 58 kDa capsid protein VP1.[466,485] In many heterologous expression systems, recombinant VLPs range is size from 27 to 38 nm and both size particles are immunologically identical to live virus.[485,486] Recombinant NVLPs are also stable in the low pH of the GIT.[485,487] The ease of manufacture and the strong immunogenicity of NVLPs makes them the most likely candidate for a prophylactic NoV vaccine.

Several clinical studies have investigated the immune response to NVLPs when delivered orally. The first pre-clinical study of NVLPs using the VP1 protein expressed in a baculovirus–insect cell expression system was immunogenic in mice when delivered by oral gavage.[488] The same system was used to express VLPs for a clinical study of healthy adult volunteers already seropositive for NoV.[489] In this study, 24 volunteers were orally administered 2 doses of 100 or 250 μg of NVLPs in water, 3-weeks apart. The resultant serum anti-NVLP IgG responses was dose-dependent, and all volunteers that received the highest dose responded with more than a 4-fold increase in serum IgG titres. Fifteen of the 24 volunteers responded after the first dose, indicating that NVLPs are not only immunogenic, but are capable of boosting the existing immunological memory.[489]

Several candidate VLP vaccines delivered i.n. have entered clinical trials. The first study used NVLPs (GI.1 genotype) produced in a baculovirus–insect cell expression system and formulated with the TLR4 agonist monophosphoryl lipid A (MPL) and the mucoadhesive chitosan.[490] Healthy adult volunteers were randomized to receive placebo or 5, 15, 50, and 100 μg NVLPs in 2 doses administered 21 days apart. Norwalk VLP-specific serum IgG and IgA increased 4.8 and 9.1-fold, respectively at the 100 μg dosage level, and all subjects that received the 50 or 100 μg vaccine dose developed IgA ASCs that expressed mucosal homing markers.[490] In a follow-up Phase 2 study with the same formulation,[491] 98 volunteers were randomised to receive two doses of placebo or 100 μg Norwalk VLPs and later challenged with a homologous GI.1 strain of NoV.[492] An NVLP–specific IgA response was detected in 70% of vaccine recipients and vaccination significantly reduced the frequencies of NV gastroenteritis. The same vaccine formulation has also been shown to significantly increase IgA or IgG B memory responses in seronegative subjects.[493]

Most recently, i.m. administration of a NoV vaccine candidate comprised of GI.1 and GII.4 NVLPs with MPL and alum has been shown to induce high seroconversion and anti-NVLP IgG titres to both genotypes and to mucosal-homing ASCs,[494] but resulted in only partial protection from infection and symptoms when challenged with a heterologous GII.4 substrain of live virus.[495]

Together, these results suggest that NVLPs are a likely candidate for a NoV vaccine. To date, all NoV clinical candidate vaccines have been expressed in baculovirus-insect cell systems. While purified VLPs from baculovirus-insect cells are safe and tolerable in early stage clinical studies, plant cells are also a valid alternative to existing expression systems and GMP-grade NVLPs are being produced in plants for future clinical trials.[496]

1.7.5 Plant-based expression of Norovirus VLPs

Following the cloning of the Norwalk genome [468] and the observation that recombinant capsid protein spontaneously forms NVLPs when expressed in a heterologous system,[483] NVCP became one of the first antigens to be expressed *in planta* (see Table 3).[249]

The 58 KDa NVCP protein expressed in stable, nuclear transformants of *N. tabacum* and *S. tuberosum* forms 38 nm NVLPs.[249] This first study reported relatively high levels of NVCP expression with 0.23% and 0.37% TSP observed in *N. tabacum* leaf and *S. tuberosum* tuber, respectively. Like other NVLP expression systems, VLPs made in plants have been observed in both ~38 and ~27 nm particles.[191,245,249,497-501]

To date, NVLPs have been expressed in *N. tabacum* leaf, *S. tuberosum* tuber, *S. lycopersicum* fruit, *N. benthamiana* leaf, and *L. sativa* leaf using a variety of expression systems including stable nuclear transformation with constitutive promoters, the MagnICON™ TMV-based system, transient expression with non-replicating and replicating DNA plasmids, and a geminivirus based expression system (see Table 3). Due to the different units of measurement between studies reporting NVCP or NVLP yield in units of fresh weight or TSP, it is difficult to directly compare the yields of un-associated NVCP and authentically folded VLPs. However, the highest yield of *in planta* accumulation of NVLPs is likely 1 mg/g fresh weight of NVLPs expressed in *N. benthamiana* leaves.[500] High yields of NVCP have also been observed in *S. lycopersicum* fruit with accumulation of up to 8% TSP.[191] Due to the lack of a known plant signalling sequence on the NVCP amino acid sequence,[468] NVCP and NVLPs are assumed to accumulate in the cytoplasm. While the sub-cellular localisation of NVCP and NVLP accumulation has not yet been confirmed within the plant cell, NVCP fused to a SP for entry into the ER or a chloroplast transit peptide resulted in approximately 5-fold lower yield than when untargeted.[497]

Interestingly, while both the native coding sequence from GI.1 Norwalk virus [181,245,249] and a plant-codon optimised version of the same gene [191] have been shown to be highly expressed in multiple plant systems, expression of the GII Narita 104 coding sequence required modification to increase the yield from 0.001 to 0.04% TSP.[502]

Unlike other expression systems where VLPs are purified prior to administration, plant systems have been used for both expression and delivery of NVLPs in pre-clinical and clinical studies.

Table 3 Summary of literature reporting NVLP expression *in planta*

PLANT SPECIES	EXPRESSION SYSTEM	YIELD	SUMMARY	REF.
N. tabacum cv. "Samsun" and *S. tuberosum* cv. "Frito-Lay 1607"	Stable nuclear transformation 35S and Patatin promoter	*N. tabacum* leaf: NVCP up to 0.23% TSP *S. tuberosum* tuber: NVCP up to 0.37%, ~10-20 μg/g fresh weight	First study to show expression of NVCP *in planta*. NVCP self assembles into 38 nm VLPs. Purified tobacco NVCP contained 'mostly' VLPs, while approximately half of NVCP extracted from tubers was as VLPs.	[249]
S. tuberosum cv. "Frito-Lay 1607"	Stable nuclear transformation patalin promoter	Tuber: NVCP 1.4-5 μg/g fresh weight	High variation in NVCP expression and VLP formation observed between individual tubers. VLP size not determined.	[181]
S. lycopersicum cv. TA234TM2R	Stable nuclear transformation 35S promoter	Fruit: NVCP 30 μg/g dry weight	First expression of NVCP in tomato. Only 23 nm VLPs produced in fresh and dried fruit compared to other species producing 23 and 38 nm VLPs.	[245]
S. tuberosum cv. Désirée and *L. esculentum* (lycopersicum) cv. TA234	Stable nuclear transformation Dual-enhancer 35S promoter	*S. tuberosum* tuber: NVCP up to 0.4% TSP, ~24% as 23 nm VLPs and 40% as 38 nm VLPs *S. lycopersicum* fruit: NVCP up to 8% TSP, ~41% as 23 nm VLPs and 22% as 38 nm VLPs.	Gene sequence optimised to substitute dicotyledonous plant-favoured codons and remove 'CG' dinucleotides, 'CNG' methylation sites and polyadenylation motifs. Both 23 and 38 nm VLPs observed in contrast to [245].	[191]
N. benthamiana	MagnICON™ TMV-based system	Leaf: NVCP 800 μg/g fresh weight	First expression of NVCP in *N. benthamiana*, with the highest reported expression in any plant system. Only 38 nm VLPs observed.	[497]
N. benthamiana	Agroinfiltration of a DNA replicon system 35S promoter	Leaf: NVCP 340 μg/g fresh weight	Highest expression of NVLPs when co-expressed with p19 inhibitor of gene silencing. Approximately 30 nm VLPs observed.	[498]
S. tuberosum cv. Désirée and *N. benthamiana*	Stable nuclear transformation Agroinfiltration of non-replicating plasmids Agroinfiltration of a DNA replicon All 35S promoter	*S. tuberosum* tuber: NaVCP 0.001-0.002% TSP *N. benthamiana* leaf, non-replicating: NaVCP ~0.002% TSP Leaf, non-replicating: NaVCP mutant no polyadenylation ~0.006% TSP Leaf, DNA replicon: NaVCP 0.02% TSP Leaf, DNA replicon: NaVCP mutant no polyadenylation 0.04% TSP	Study showed low yield of NaVCP compared to NVCP expressed in other studies. The authors identified several premature polyadenylation sites within the NaVCP coding sequence that substantially limited NaVCP expression *in plants*, and mutation of these sites increased expression ~400-fold.	[502]
N. benthamiana	MagnICON™ TMV-based system	Leaf: ~400 μg/g fresh weight	Study details scale-up of upstream and downstream processes required to manufacture NVCP VLPs to GMP-grade for pre-clinical and clinical studies.	[496]
L. sativa cv. red leaf	Gemnivirus expression system with p19 silencing repressor	Leaf: NVCP ~200 μg/g fresh weight	First expression of NVCP in lettuce. All NVLPs 38 nm. Co-expression of p19 had no observable impact on yield.	[499]
N. benthamiana	Agroinfiltration of non-replicating plasmids 35S promoter	Leaf, non-optimised: low yield Leaf, codon optimised: low yield Leaf, co-expression of vp1, vp2 and 3'UTR from genotype 4 isolate: ~1 mg/g fresh weight	Study examined expression of different inhibitors of post-translational gene silencing and coding variants of NVCP. Highest expression when vp1 and vp2 genes co-expressed. 23 and 38 nm VLPs observed.	[500]
N. benthamiana	MagnICON™ TMV-based system	Leaf: 285 μg/g fresh weight	High yield of "plant optimised NaVCP gene" based on [502]. Both 33 nm VLP and ~20 nm VLPs were observed.	[501]

cv.- cultivar, NVCP- Norwalk virus capsid protein, 35S- cauliflower mosaic virus 35S constitutive promoter, VLPs- virus-like particles, TSP- total soluble protein, NVLP- Norwalk virus virus-like particle, p19- p19 protein of tomato bushy stunt virus, NaVCP- Narita 104 virus capsid protein, GMP- good manufacturing practice, *vp1*- viral coat protein 1, *vp2*- viral coat protein 2, 3'UTR- 3' untranslated region

1.7.6 Mucosal immunogenicity of plant-made NVLPs

The first study of NVLP expression *in planta* also used the plant cell to deliver the antigen orally.[249] To date, there have been eight published studies that investigated the oral or i.n. administration of plant-made NVLPs including a Phase 1 clinical study (see Table 4).

NVLPs expressed in plants and administered orally appear to provide an immunologically similar response to NVLPs expressed in other systems, characterised by seroconversion to humoral anti-NoV IgG1 antibodies, and in most cases, increased faecal and/or mucosal surface anti-NoV IgA antibodies.[497,503-505] No appreciable difference in the resultant immune response has been observed when compared to an equivalent dose of NVLPs manufactured using a baculovirus-insect cells expression host.[497,501,504,505]

Plant-derived VLPs have been delivered orally in a variety of plant-matrix and purified formulations including *S. tuberosum* as fresh [181,249] and lyophilised tuber,[191] *N. tabacum* leaf extract,[249] and *S. lycopersicum* lyophilised [191,245] and air-dried fruit.[191] Pre-clinical studies have used doses from 10 μg of NVCP (representing ~2.5-5 μg NVLPs) in tuber, up to 352 μg NVCP (containing ~110 μg NVLPs) in lyophilised fruit powder (see Table 4). Purified plant-derived NVLPs have also been delivered i.n. in several pre-clinical studies with doses between 5 and 250 μg NVLPs (see Table 4). Human volunteers have received doses ranging from 215-751 μg NVCP in potato tubers.[181]

Orally delivered NVLPs elicit varied seroconversion rates in preclinical and clinical studies, with the response rate influenced by source, dose and formulation with mucosal adjuvants. For example, mice fed NVCP in *S. tuberosum* tuber without adjuvant had a 40% seroconversion rate, whereas mice administered the same dose with 10 μg CT had a seroconversion rate of 70%.[249] In the same study, mice administered only 50 μg of NVCP as crude lysate from *N. tabacum* leaf induced a higher seroconversion with or without CT (89% and 90% seroconversion, respectively).[249] A subsequent study observed lower immunogenicity when mice were fed 340 μg NVCP in *S. tuberosum* tuber compared to 100 μg of insect-derived VLPs via gavage. The reduced immune response to NVLPs delivered in tuber may be due to the lower concentration of assembled VLPs or the affect of the plant matrix composition in potato tubers compared to other expression hosts.[249,497] However, several studies have suggested that immune-suppressing metabolites in potato may also negatively influence the oral response to antigens in whole-cell formulations (see 1.7).[181,249]

Low seroconversion and low IgG and IgA titres were observed in the first and only clinical study of NVLPs administered in whole plant cells.[181] Twenty-four healthy adult volunteers

were randomised to receive two doses of transgenic tuber on days 0 and 7, or three doses of transgenic potato on days 0, 7, and 21, with 20 volunteers completing the study per protocol.[180] Each dose contained approximately 500 μg of NVCP, approximately half of which was assembled into VLPs. Nineteen of 20 (95%) volunteers who ingested two or three doses of transgenic potatoes developed significant rises in the numbers of anti-NoV IgA ASCs. Four of 20 (20%)volunteers that received transgenic potatoes developed serum IgG anti-NVCP titres (mean 12-fold rise), and four of 20 (20%) volunteers (three of whom did not develop IgG responses) developed serum IgM anti-NVCP titres (mean 7-fold rise). Stool IgA anti-NVCP was detected in six volunteers (30%) who ingested transgenic potatoes (mean 17-fold rise). This response to plant-expressed NVLPs was relatively low compared to a previous clinical study by the same authors where 100% of 15 volunteers who ingested 2 doses of purified NVLPs (250 mg/dose) developed increases in serum IgG antibodies.[489]

Despite the relatively low immunogenicity of NVLPs delivered in *S. tuberosum* tubers in preclinical and clinical studies, higher seroconversion and antibody titres have been observed when NVLPs are expressed and delivered in *S. lycopersicum* fruit. When mice were fed 80 μg of NVCP in 3 g of dry tomato powder with or without food-grade saponins, only 3 of 5 mice seroconverted with increased humoral anti-NoV IgG.[245] However, all mice showed a NoV faecal IgA response.[245] A subsequent study administered lyophilised tomato fruit powder from an early stage of ripening ("harvested at the pink stage of ripening" compared to the earlier study that used 'red' fruit) and showed a robust systemic and mucosal antibody response.[191] Mice were administered 4 doses of fruit powder containing 192, 240 and 352 μg of NVCP, equivalent to 60, 75 and 110 μg NVLPs. Unlike baculovirus-insect cell derived NVLPs, tomato fruit NVLPs include 23 and 38 nm variants. All mice administered 192 μg of NVCP in the fruit powder consistently showed rapid increase of both serum and intestinal NV-specific antibodies after the first two feedings, with little difference between the groups fed low or high doses of fruit powder. In contrast, a control group administered 100 μg of purified NVLPs showed consistently lower rates of seroconversion and reduced humoral and mucosal antibody titres compared to the mice fed tomato fruit. Interestingly, there was no dose response observed between groups fed fruit powder containing 192, 240 and 352 μg of NVCP, suggesting the maximum immune response to the formulation was reached with the lowest dose. The authors investigated lower doses of NVCP in lyophilised or air-dried fruit powder, and observed dose-dependent humoral and mucosal immunity. Surprisingly, mice fed powdered, air-dried tomato fruit responded with consistently higher humoral and mucosal immune responses. The authors of this paper suggest that the robust response to tomato-

delivered NVLPs could be a result of increased immunogenicity to a smaller 23 nm VLP produced in the tomato fruit, bioencapsulation of the antigen within the plant matrix, or the presence and possible adjuvant activity of α-tomatine within the fruit.[191] However, the lack of any adjuvant property of food-grade saponins in a separate study where NVCP were administered in tomato fruit,[360] suggests the affect of saponins on the oral immunogenicity of NVCP delivered in tomato fruit is not completely understood.

Given the robust immunogenicity of NVLPs observed when delivered in earlier 'pink' tomato fruit, [245] and the known change in α-tomatine concentration observed during fruit ripening,[506] it may be possible to harvest tomato fruit with different concentrations of α-tomatine and determine if the concentration of this known adjuvant does indeed influence the mucosal immune response to NVLPs.

Table 4 Summary of studies involving oral or intranasal delivery of plant-derived norovirus VLPs

HOST PLANT	DOSE AND FORMULATION	IMMUNOGENICITY	SUMMARY	REF.
N. tabacum leaf extract and *S. tuberosum* raw tuber delivered to CD1 mice	10, 50 or 80 μg of NVCP purified from tobacco leaf extract with or without 10 μg CT by gavage, or 40-80 μg NVCP in 4 g raw tuber on days 1, 2, 11, and 28.	Leaf extract: 10 μg NVCP with 10 μg CT, 4/4 seropositive (GMT 800), 1/4 positive faecal IgA. Leaf extract: 50 μg NVCP with 10 μg CT, 9/10 seropositive (GMT 3200), 5/9 positive faecal IgA. Leaf extract: 50 μg NVCP no CT, 8/9 seropositive (GMT 800), 5/8 positive faecal IgA Leaf extract: 80 μg NVCP with 10 μg CT, 3/3 seropositive (GMT 25,600), faecal IgA ND Tuber: 40-80 μg NVCP with 10 μg CT, 7/10 seropositive (GMT 200), 0/10 positive faecal IgA Tuber: 40-80 μg NVCP no CT, 4/10 seropositive (GMT 200), 1/10 positive faecal IgA.	First study to show expression and oral immunogenicity of NVLPs *in planta*. All VLPs 38 nm. Low seroconversion and faecal IgA response in groups administered NVLPs in tuber compared to those administered leaf extract. Lower immune response in tuber may occur due to reduced percentage of NVCP in VLPs in tuber compared to leaf.	[249]
S. tuberosum raw tuber delivered to healthy human volunteers	~215-751 μg of NVCP in raw potato tuber in 2 doses on days 0 and 7, or 3 doses on days 0, 7 and 21.	2 doses: 10/10 IgA ASCs, 4/10 IgG ASCs, 1/10 serum IgG (8-fold mean rise), 0/10 serum IgM, 2/10 stool IgA response (16.6-fold mean rise) 3 doses: 9/10 IgA ASCs, 2/10 IgG ASCs, 3/10 serum IgG (13.3-fold mean rise), 4/10 serum IgM (7-fold mean rise), 4/10 stool IgA response (17.8 fold mean rise).	First and only clinical study to deliver NVLPs to humans in plant cells. VLP size not determined. Relatively modest immune response, well below purified insect-derived VLPs (i-rVLPs) or infection with wild type NV.	[181]
S. lycopersicum lyophilised fruit powder delivered to female CD1 mice	~80 μg NVCP in 3 g dry tomato powder with and without 10 mg food-grade saponin on days 0, 4, 17, 21.	Without saponin: 3/5 seroconversion, serum IgG 10,456 GMT, faecal IgA 3,066 GMT With saponin: 3/5 seroconversion, serum IgG 13,395 GMT, faecal IgA 3,915 GMT	First study to express and deliver NVCP in tomato fruit. Only moderate seroconversion. No significant increase in seroconversion or immunogenicity in response to co-administration of exogenous saponins.	[245]
S. tuberosum lyophilised tuber and *S. lycopersicum* lyophilised or air-dried fruit powder delivered to 'mice'	~120, 144, and 240 μg NVCP containing ~63, 18 and 30 μg VLPs in 1, 1.2 and 2 g of lyophilised tuber on days 1, 4, 17, and 20. ~192, 240 and 352 μg NVCP containing 60, 75 and 110 μg VLPs in 1.2, 1.5 and 2.2 g of tomato powder on days 1, 4, 17, and 20. 0.1, 0.4, and 0.8 g of lyophilised or air-dried fruit powder fed on days 1, 4, 17 and 20.	All mice seroconverted to NVCP in tuber, serum IgG peak GMT at 201, mucosal IgA GMT 1,293. Only 20-40% of mice seroconverted at low levels in groups fed 1.2 and 2 g tuber. The serum IgG responses to all doses of tuber was lower than mice gavaged 100 μg i-rNV VLPs, but similar faecal IgA responses. All mice seroconverted to NVCP in fruit, serum IgG peak GMT 192 and mucosal IgA GMT 2465. No significant difference between groups fed different doses. The serum and mucosal response higher in all groups fed transgenic tomato compared to 100 μg i-rNV. Dose dependent response to serum IgG and mucosal IgA for mice administered lower doses of 0.1, 0.4, and 0.8 g lyophilised or air-dried fruit. Significantly higher IgG and IgA GMTs in all mice administered air-dried fruit compared to lyophilised fruit.	Tomato harvested at the 'pink' stage of ripening. Both 23 and 38 nm VLPs observed. The authors note reduced immunogenicity in the two groups fed more tuber as the powder was mixed with water, decreasing the dose of VLPs. Potato dose of VLPs was less than i-rNVs and accordingly lower immune response. However, ~60 μg VLPs in tomato fruit provided higher immune response than 100 μg i-rNVs. Lower dose of NVLPs showed dose dependent response. Air-dried fruit significantly more immunogenic than lyophilised fruit. All mice could be boosted by subsequent oral gavage of i-rNV even if no detectable response at first administration.	[191]
N. benthamiana produced VLPs, purified and delivered to CD1 mice.	100 and 250 μg NVCP with or without 10 μg CT by gavage on days 0, 21 and 42.	Dose dependent response of increasing serum IgG and vaginal IgA when mice administered 100 μg NVCP without CT, 100 μg NVCP with CT, 250 μg NVCP without CT, and 250 μg NVCP with CT. Peak serum IgG GMT, vaginal and intestinal IgA ~ 6-weeks after 3rd dose. Balanced IgG1/IgG2a ratio.	First study to investigate immunogenicity of highly purified NV VLPs produced *in planta*. VLPs 38 nm. Immunogenicity enhanced in response to CT co-administration.	[497]

N. benthamiana produced VLPs, purified and delivered to female 5-week old BALB/c mice.	5 and 25 μg NVCP VLPs alone, or co-delivered with 1 μg CT, 10 μg gardiquimod or 10 and 25 μg resiquimod via i.n.. on days 0 and 21.	Intranasal immunisation with NV VLPs co-delivered with CT or gardiquimod is superior to resiquimod in the induction of a robust NV-specific systemic (serum IgG1>IgG2a) and mucosal (salivary, intestinal, nasal and bronchoalvelolar IgA) immune response.	First study to investigate the influence of co-delivered adjuvants with purified plant-derived VLPs. The authors suggest that the results indicate that the TLR7 agonist gardiquimod induces antigen-specific systemic and mucosal immune response superior to those induced by the TLR7/8 agonist resiquimod and comparable to those induced by CT.	[505]
N. benthamiana produced VLPs, purified and delivered to female 5-week old BALB/c mice.	10 and 25 μg NVCP VLPs alone, or with gardiquimod, and with or without 0.25% w/w GelVac mucoadhesive i.n. on days 0 and 21.	GelVac powder formulation elicits a superior systemic (serum IgG1>IgG2a) and mucosal (salivary, intestinal, nasal and bronchoalvelolar IgA) immune response compared to a PBS liquid formulation and synergistic adjuvant effect of gardiquimod retained. GelSite liquid formulation elicits an equivalent mucosal immune response compared to a PBS liquid formulation.	Study shows improved systemic and mucosal immune response when NV VLPs are formulated with a powdered mucoadhesive to delay mucociliary clearance.	[504]
N. benthamiana produced VLPs, purified and delivered to female 5-week old BALB/c mice.	25 μg NaVLPs via i.n. administration on days 0 and 21.	All mice administered NaVLPs developed specific serum IgG, and nasal IgA antibodies. IgG1>IgG2a ratio.	Purified Narita 104 VLPs were highly immunogenic with a T_h2-mediated IgG1-dominant response when delivered via i.n. route.	[501]

NVCP- Norwalk virus capsid protein, VLPs- virus-like particles, CT-cholera toxin, GMT- geometric mean titre, ND- not determined, ASCs- antibody secreting cells, NV-Norwalk virus, i-rVLP- insect-derived recombinant norovirus, NaVLPs-Narita 104 VLPs

1.7.7 Experimental strategy for determining the impact of intrinsic adjuvants

To investigate whether an intrinsic plant metabolite influenced the oral immunogenicity of an antigen expressed and delivered *in planta*, previously transformed lines of *S. lycopersicum* expressing NVCP were used. The concentration of α-tomatine is known to decrease during fruit ripening,[446] allowing a natural variation in the α-tomatine concentration within the vaccine formulations by harvesting earlier or later during the fruiting period.

The concentration of α-tomatine and other metabolites was characterised in batches of transgenic and control fruit at two ripening stages. These fruit were taken from stable, nuclear transgenic lines of *S. lycopersicum* cv. TA234.[245] Wild type, isogenic plants were grown alongside the T4 generation of NVCP-expressing seed in environmentally controlled conditions, and fruit from different stages of ripening characterised for accumulation of NVCP and α-tomatine. Fruit was harvested from an immature stage, 3-4 weeks after pollination and less than 35 mm in diameter, as well as at a riper stage 3 as the colour began to change from green to yellow. The ability of the tomato fruit to correctly assemble NVLPs at both stages was determined by enzyme-linked immunosorbant assay (ELISA), western blot, sucrose ultracentrifugation and TEM.

Finally, to investigate the influence of α-tomatine on the oral immunogenicity of NVLPs within tomato fruit, feeding studies with formulations of lyophilised fruit were conducted. Mice were administered a relatively low dose of NVCP to ensure any dose response was not obscured by high titres.[191] As the influence of exogenous adjuvants on the immune response to NVCP in tomato fruit is unclear,[245] mice were also administered lyophilised fruit formulated with different concentrations of purified (exogenous) α-tomatine.

The influence of α-tomatine on the oral immunogenicity of NVLPs, may provide an understanding of whether plant cells can be rationally optimised to improve their capacity as both antigen production and delivery vehicles.

1.8 Summary of literature and rationale for experimental hypothesis

Plant-based production of recombinant proteins has developed from an academic curiosity to a viable commercial manufacturing platform within the past two decades.[10] Most advancements have occurred due to a better understanding and optimisation of expression and purification technologies. Due to the complexities associated with reliable dose delivery and the complex immune response to oral vaccination, less progress has been made on advancing the basic understanding of plant cells as delivery vehicles for vaccine antigens.

Plant-made vaccines have been promoted as a mechanism to cheaply express and deliver antigens from enteric pathogens,[28] in the hope of combatting the high mortality associated with diarrhoeal disease in LMICs.[118] However, the understanding of how orally-delivered plant cells release protein antigens during digestion and induce the mucosal immune response is not sufficiently advanced for the progression of plant-based vaccine candidates into clinical development. This thesis elucidated two plant-specific factors for the optimisation of immunogenicity to vaccine antigens; bioencapsulation and plant-intrinsic adjuvants.

Bioencapsulation or decoy substrate mechanisms have been suggested in the literature as mechanisms to prevent proteolysis of antigens during transit in the GIT. The non-toxic model protein immunogen LTB has been expressed in at least 13 different plant species (see 1.6.5), but there has been no direct comparison as to whether the intrinsic differences in these expression systems influenced the bioencapsulation or oral immunogenicity. Therefore, the LTB protein was produced in three different plant expression systems, and the *in vivo* release and oral immunogenicity characterised in mice.

The subcellular location of antigen accumulation within the plant cell has been suggested in the literature as a mechanism to increase bioencapsulation during digestion, and the LTB protein has been rationally targeted to a range of different subcellular locations within plant vegetative and storage tissues (see 1.6.2). However, there has been no direct comparisons of the release or immunogenicity of LTB when localised to different regions of the plant cell. To test this, LTB was targeted to different subcellular locations of the leaf cell and the *in vitro* release and *in vivo* immunogenicity characterised.

Lastly, plant metabolites including saponins are used extensively as systemic and mucosal adjuvants for vaccine antigens, and it has been suggested that the most abundant glycoalkaloid of tomato fruit, α-tomatine, may influence the mucosal immune response to co-delivered vaccine antigens. Despite these assertions, there are no known reports of the quantification or characterisation of α-tomatine in a vaccine formulation (see 1.7.2). To

determine if α-tomatine does potentiate the immune response to a co-delivered antigen, virus-like particles from the enteric pathogen NoV were expressed in tomato fruit and the concentration of α-tomatine measured by HPLC/MS. The immunogenicity of NVLPs in response to varying concentrations of endogenous and exogenous α-tomatine was determined.

Taken together, this body of work reveals some of the plant-specific factors that can be optimised to improve oral immunogenicity of vaccine antigens delivered in plant cells..

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2. THE RELEASE AND INDUCED IMMUNE RESPONSES OF A PLANT-MADE AND DELIVERED ANTIGEN IN THE MOUSE GUT

2.1 Introduction

Modern systems for *in planta* expression of recombinant proteins differ dramatically in the yield and quality of the proteins produced, as well as the optimal growth conditions, speed and scale of production. Many systems, particularly those using transient expression, are capable of rapidly producing recombinant proteins. However, transient systems are still not as easily scaled as field-grown stable transgenic plants nor do they have the precise environmental control offered by tissue culture based systems. Despite the wide variety of plant expression systems used to produce recombinant vaccine antigens, there has been little comparative evidence of the intrinsic capacity of expression systems to act as a vehicle for the oral delivery of enteric antigens.

To investigate whether the choice of expression system influences the release or immune response of vaccine antigens, we expressed the heat-labile B subunit (LTB) protein from the mucosal pathogen enterotoxigenic *Escherichia coli* (ETEC) using three different systems: transient viral expression in *Nicotiana benthamiana* leaves, stable expression in *Solanum lycopersicum* fruit and *Petunia parodii.* hairy root tissue culture. These systems were chosen based on their different cell types (leaves, fruit, and roots) and expression systems (transient viral expression, stable transformation of whole plants, and stable transformation of hairy root cell cultures). To minimise other variables associated with delivery, careful consideration was given to standardising the formulation used to administer the plant material, the particle size of the plant material, and the dose of antigen. Unless indicated otherwise, immunoassays for antigen detection in plant material and the resulting immune response were developed and optimised as per Appendix 3.

This study showed that the choice of expression system and formulation (aqueous or lipid-based) influenced the *in vivo* release and oral immune response to LTB. The different expression systems and formulations released LTB at different regions of the mouse gut, and the highest immunogenicity was observed when LTB was expressed and delivered in *N. benthamiana* leaves in an aqueous solution. However, due to the complex differences

between these systems, the mechanism of action for these effects was not able to be determined and each system had relative merits that warranted further investigation.

2.2 Statement of contribution

The nine authors of this paper are Assunta Pelosi (AP), Robert P Shepherd (RPS), Giorgio De Guzman (GDG), John Hamill (JH), Els Meeusen (EM), Gordon Sanson (GS), and Amanda M Walmsley (AMW).

In the case of Chapter 2: "The Release and Induced Immune Responses of a Plant-Made and Delivered Antigen in the Mouse Gut", the nature and extent of contribution to the work was the following:

- Some experimental design, LTB expression in *N. benthamiana*, 33% of animal experiments, all immune response characterisation and analysis, and 33% of the manuscript was written by RPS. The extent of this work was 40%.
- Some experimental design, LTB expression in cell culture, antigen release experiments, 33% of animal experiments, and 33% of the manuscript was written by AP. The extent of this work was 40%.
- Some experimental design, LTB expression in hairy roots, 33% of animal experiments, and 33% of the manuscript was written by GDG. The extent of this work was 10%.
- Experimental design and review, and manuscript review by JH. The extent of this work was 1%.
- Experimental design, planning and review, and manuscript review by AMW. The extent of this work was 7%.
- Experimental design and review, and manuscript review by EM. The extent of this work was 1%.
- Experimental design by GS. The extent of this work was 1%.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

09 March 2016

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Signature *Date*

Robert P Shepherd (candidate)

10 March 2016

.. ……

Si *Date*

David R Smyth (supervisor)

2.3 Publication

The Release and Induced Immune Responses of a Plant-Made and Delivered Antigen in the Mouse Gut

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Abstract: This study investigated the site of release of a model vaccine antigen from plant cells and the corresponding induced immune response. Three plant tissues (leaf, fruit and hairy root) and two formulations (aqueous and lipid) were compared in two mouse trials. A developed technique that enabled detection of antigen release by plant cells determined antigen release occurred at early sites of the gastrointestinal tract when delivered in leaf material and at later sites when delivered in hairy roots. Lipid formulations delayed antigen release from all plant materials tested. While encapsulation in the plant cell provided some protection of the antigen in the gastrointestinal tract and influenced antigen release, formulation media was also an important consideration with regards to vaccine delivery and immunogenicity. Systemic immune responses induced from the orally delivered vaccine benefited from late release of antigen in the mouse gastrointestinal tract. The influences to the mucosal immune response induced by these vaccines were too complex to be determined by studies performed here with no clear trend regarding plant tissue site of release or formulation media. Expression and delivery of the model antigen in plant material prepared in an aqueous formulation provided the optimal systemic and mucosal, antigen-specific immune responses.

Keywords: Gastrointestinal delivery, plant-made vaccine, antigen release, vaccine formulation.

INTRODUCTION

Oral delivery is a low cost, labor efficient way to vaccinate animals and humans. It eliminates needles and syringes, eliminates the need for the vaccine to be sterile, increases ease of vaccine delivery, increases patient compliance and enables a simultaneous induction of antigen-specific mucosal, humoral, cell mediated and systemic immune responses. However the complex and dynamic environment of the gastrointestinal tract (GIT) may result in inconsistent patient response to an orally delivered vaccine.

It is well documented that plant-made and delivered vaccines can induce antigen-specific immune responses when orally administered [1-6]. However the recent trend for plant-made vaccines has been to use plants only as the production system and the vaccine antigen(s) purified and highly characterised before parenteral vaccination [7-11]. Previous studies of oral delivery of plant-made vaccines have not standardized formulation. The priority was consumption of the greatest amount of the test diet therefore plant-made vaccines were fed fresh to previously fasted animals [1,2,12] or formulated to entice consumption in for example apple cider [5, 6], or complex formulations made from apple, nuts and honey [13].

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The model antigen used in this study is the B subunit of the heat labile toxin (LT) of enterotoxigenic *Escherichia coli* (LTB). LTB was chosen as it is a well characterised, strong mucosal antigen/adjuvant from an enteric pathogen. LTB has been extensively studied for its ability to target subunit vaccines to the mucosal immune system. It was also one of the first antigens expressed in plants [1,3,5,12,14,15]. Plant-made LTB has been tested in mouse feed trials [1] and in the first human clinical trial with a transgenic, plant-made, antigen [3]. Different plant systems have since been investigated for the expression of LTB. Transgenic corn [14, 16], soybean [17] and rice [18] were shown to express recombinant LTB that retained its native pentameric form. The corn-made material was later used successfully in human clinical trials [19].

In an attempt to improve oral delivery of plant-made vaccines with regards to increasing strength of the induced immune response, this study makes the first comparison of oral delivery of a model antigen in different formulation media and plant tissues. We also perform the first investigation into the site of release of plant-made antigen in the mouse GIT. We believe a deeper understanding of antigen release and delivery in the intestinal tract is necessary to optimise the plant-made vaccine technology for consistent oral vaccination. The transgenic plant lines that we developed use plasmids Supplementary Fig. (**1**) with LTB expression under the control of strong constitutive promoters and co-translationally inserted into the endoplasmic reticulum. The goal of this study was to investigate the site of release of a model vaccine antigen from plant cells and the correspond-

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ing induced immune response in attempt to improve oral delivery of plant-made and delivered vaccines. We also aimed to establish if recombinant proteins are protected in the gut by containment within plant cells or whether it is merely the presence of plant cell matrices/contents that provide protection.

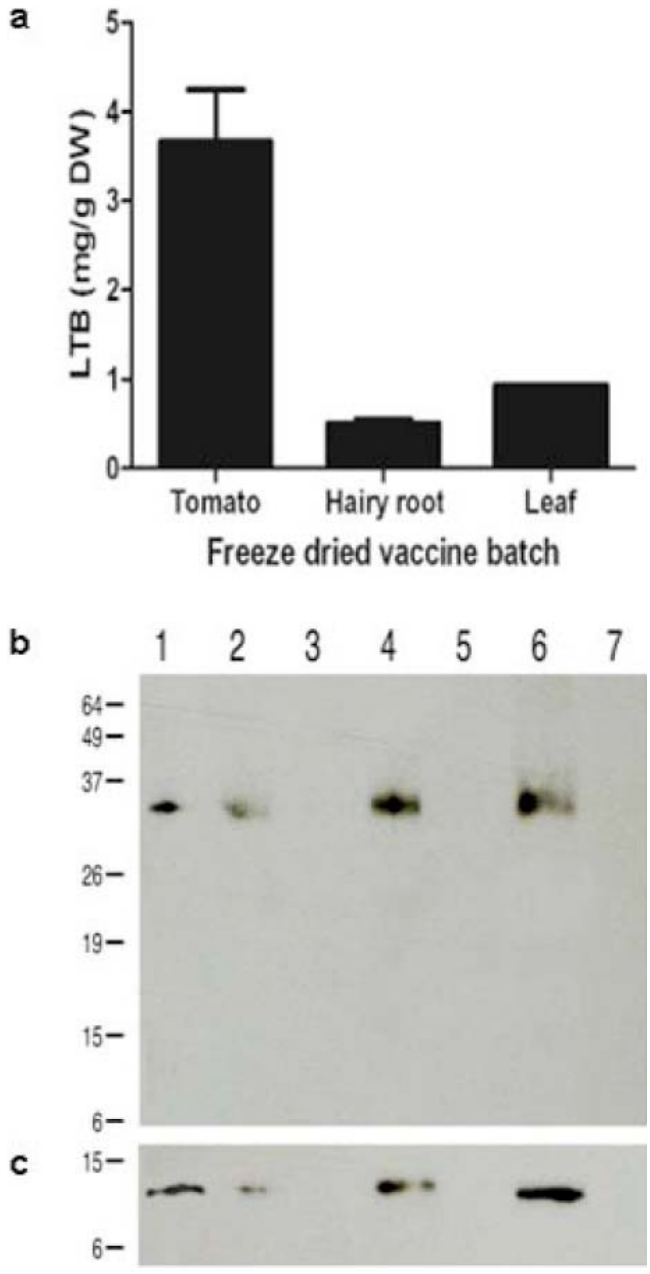

Fig. (1). Expression of LTB in plant materials **a** LTB content of freeze-dried tomato fruit, petunia hairy root and *N. benthamiana* leaf batches, where the bars indicate the mean of two repeated ELISAs taken from the same batch and the error bars the standard error of the mean. Western blot analysis of **b** native and **c** denatured LTB in freeze dried vaccine batches. Lane 1, 3 ng purified recombinant bacterial-made LTB positive control (rLTB); Lanes 2-7, crude protein extracts from LTB transformed *N. benthamiana*, control *N. benthamiana*, LTB-transformed tomato, Control tomato, LTB-transformed hairy root and Control hairy root respectively.

MATERIALS AND METHODS

Plant Materials

Plant Cell Culture Production

Liquid cell cultures of *Nicotiana tabacum* (NT1 cell cultures) were gown in 250 ml conical flasks containing 40 ml MS medium [20], 2.21 mg/L 2,4-D at 22-25°C with orbital shaking at 110 rpm. Cells were subcultured weekly by transferring 2 ml cell suspension to 40 ml fresh media. 14 days after subculture, cells were harvested under vacuum in a Bucher funnel lined with a Whatman No.1 filter disc. NT1 cells were freeze-dried for a minimum of 48 h with a maximum shelf temperature of 20°C.

CLTB24b Tomato Line

The plasmid pCLTB is a proprietary plant expression vector belonging to Dow AgroSciences Pty., and is described in the supplementary figures Supplementary Fig. (**1a**). The pCLTB plasmid was prepared from cultures of *E. coli* DH5a and electroporated into *Agrobacterium tumefaciens* EHA105 [21]. *Agrobacterium*-mediated transformation of *Solanum lycopersicum* cotyledons (variety Tanksley TA234TM2R) was performed according to Van Eck et al [22].

Plantlets were regenerated on MS medium [20] containing 10 mg/ml ammonium glufosinate. Individual lines were screened by GM1 ganglioside-dependent ELISA for LTB expression in leaves and fruit [1]. Lines with greatest LTB fruit expression were self-pollinated and the resulting seeds germinated on MS medium supplemented with 7.5 mg/ml ammonium glufosinate. Surviving seedlings were transferred to soil in the glasshouse and self-pollinated. Fresh fruit from each T1 plant were taken and freeze-dried for 48 h.

Hairy Root Cultures

Leaf discs were obtained from *Petunia parodii* plants grown in a greenhouse under 8 h photoperiod. A 20 ml *Agrobacterium rhizogenes* (strain LBA9402) culture containing pBin+35SLTB Supplementary Fig. (**1b**) was grown in YMB medium (AustraTechnology Laboratories, Australia) with 25 mg/L of kanamycin, for 48 h at 25°C and used to infect surface sterilised leaf segments (1 cm^2) by submersion for 1 min and blotted dry. The disks were then placed onto solid MS plates [20] containing 0.8% agar and incubated in the dark for 48 h. The leaf disks were transferred onto solid MS agar plates containing 500 mg/l cefotaxime and left 7 days for root formation. Single roots were cultured in conical flasks containing 50 ml of MS medium containing 25 mg/l kanamycin and 500 mg/l cefotaxime with constant orbital shaking. The surviving roots were screened by PCR for the presence of pBin+35sLTB using primers **FWD** 5' GCCATGGTGAAGGTGAAGTGCTA 3' **REV** 5' CCATGGTGAAGGTGAAGTGCTA 3'. For batch processing, cultures were grown in conical flasks containing 50 ml MS medium and harvested 22 days after subculture. The harvested material was snap frozen in liquid N_2 then freeze-dried for 48 h.

Transient Expression of LTB in *Nicotiana benthamiana* Leaves

N. benthamiana leaves transiently expressing LTB were produced using the MagnICON system (Icon Genetics

GmbH, Germany). The LTB gene (including the signal peptide responsible for co-translational insertion into the ER) was amplified using primers with flanking *Nco*I and *Bam*HI sites and ligated into pICH11599 to give pICH-LTB Supplementary Fig. (**1c**). pICH-LTB was transformed into *A. tumefaciens* strain GV3101, and co-transformed with required MagnICON modules into the leaves of 6-8 week old greenhouse-grown *N. benthamiana* leaves as per Marillonnet *et al.* [23]. Plants were harvested at 7 days post infiltration, and freeze-dried. Mice administered a control leaf diet were fed *N. benthamiana* leaves expressing cytoplasmic GFP using the MagniCON system as per Marillonnet *et al.* [23] and processed identically to LTB expressing leaves.

Crude Protein Extracts

Crude protein was extracted by homogenising freeze-dried plant material in 1:6 (w/v) extraction buffer [PBS supplemented with 0.1% Tween 20 (PBST) and 2x Roche Complete Protease Inhibitor Cocktail tablets] with two 3 mm tungsten carbide beads for 1 min at a frequency of 28/s in a Qiagen Mixer Mill. Insoluble material was removed by centrifugation at 13,000 rpm at 4°C for 5 minutes.

Direct Bind LTB-Specific ELISA

The concentration of LTB in all plant materials was determined in this study by direct bind LTB ELISA. This relatively inexpensive method was preferred for gross quantification over a GM1 capture given the costly nature of monosialoganglioside GM1. All vaccine batches were quantified by the same method.

Direct bind LTB-specific ELISA analysis was performed using Costar 9018 96-well, microtitre plates (Corning Life Sciences) coated with 50 ml of diluted (1:50 in PBS) crude protein extract per well. The plates were sealed and incubated overnight at 4^{o}C. All subsequent incubations were performed at 37°C for 1 h with three washes with PBST performed between each. Plates were blocked with 5% dry skim milk powder (DM) in PBST, followed by incubation with 1:2,000 rabbit anti-LTB (Benchmark Biolabs), then 1:15,000 goat anti-rabbit IgG HRP conjugate (Sigma). In each case antibodies were diluted in 1% DM in PBST. Bound antibodies were visualised using TMB-peroxidase substrate (Bio-Rad Laboratories) according to manufacturer's instructions. The amount of LTB in the freeze-dried plant materials was calculated against a recombinant, bacterial-made rLTB (Benchmark Biolabs) standard.

Western Blot Analysis

Crude protein was loaded (1.95 µg total soluble protein for *N. benthamiana*, 2.3 µg for tomato and 68.2 µg for hairy roots) onto 5x SDS gel loading buffer [24] and separated on a 12% Ready Gel Tris-HCl Precast Gel (Bio-Rad Laboratories) with (denatured) or without (native) boiling for 10 min. The separated proteins were transferred onto PVDF membrane (GE Healthcare) and immunodetection performed with 1:4000 rabbit anti-LTB (Benchmark Biolabs) and 1:20,000 goat anti-rabbit IgG HRP conjugate (Sigma) using the SNAP i.d. Protein Detection System (Millipore) according to manufacturer's instructions. Bound antibody was detected using ECL Plus (GE Healthcare).

Preparation of Test Diet

With the exception of the NT1 cells, the particle size of all freeze-dried plant material was ground in a commercial coffee grinder and standardised to between 0.5 and 1.2 mm^2 by seiving. All mice were fed a total of 0.2 g freeze-dried plant material. Mice receiving test treatments were fed enough plant material to deliver an antigen dose of 50 µg LTB and the weight increased to 0.2 g with control material. The plant material was then mixed into a paste in either a lipid (peanut butter) or aqueous (apple juice and honey) media immediately before administration. The total mass (plant material + formulation media) of treatment provided to the mice was 1.0 g of tomato or NT1 cells, 2.0 g of hairy roots and 0.4 g of *N. benthamiana*. The variation in mass delivered was due to the mixability or solubility of the different plant materials. The pH of the peanut bitter (PB) media (1 part peanut butter: 3 parts peanut oil) was 6.7 while the pH of the apple juice and honey (AH) media (3 parts apple juice: 1 part honey) was 3.6. The PB media comprised 6.2% protein, 80.2% fat and 6.6% carbohydrate (including 2.7% sugars) and the AH media 0.25% protein, 0.03% fat, 64.9% carbohydrate (including 66.7% sugars). A vaccine diet formulated with control plant material or wild type NT1 with the addition of 50 µg bacterial-made rLTB served as negative and free LTB controls respectively. The undifferentiated NT1 plant cell line was chosen to represent a generic plant cell.

Animal Trials

Female, 8 week old, C57BL/6 were maintained in a controlled environment in the School of Biological Sciences Animal Holding Facility at Monash University under conditions amenable to the Animal Ethics Committee of Monash University. Mice were housed in individual cages provisioned with water and standard food pellets except when fed the test vaccine diet. Mice were monitored daily for health and condition. Mice were acclimatised for 1 week during which they were randomly assigned to 18 groups of 3-5 mice each (Table **1**) and introduced to a mock vaccine diet consisting of control plant material mixed with either PB or AH at days -6 and -4 Fig. (**2**).

Trial 1: Antigen Fate in the Mouse GIT

At day 0, mice were fasted for 4 h before receiving the test diet which remained with the animals for 16 h. At trial termination (day 1), mice were humanely killed by CO_2 inhalation and their stomach, duodenum (2 cm length from the stomach), ileum (4 cm length from the large intestine) and large intestine harvested, contents removed and the flushed with 100 ml of PBST supplemented with 2x Roche Complete Protease Inhibitor Cocktail tablets. The ingesta and gut washes were combined, weighed and analysed for LTB content at each site. Faecal pellets were collected at day -1 and 1. All samples were stored at -20°C.

Trial 2: Oral Immunisation

Mice were fed the test diet on day 0, 7 and 14. On day 28 (trial termination) all mice were humanely killed by CO_2 inhalation and their GIT sampled and flushed with PBST as described in Trial 1. Faecal samples were collected at day -1 and 28, gut washes performed on day 28 from small intestine

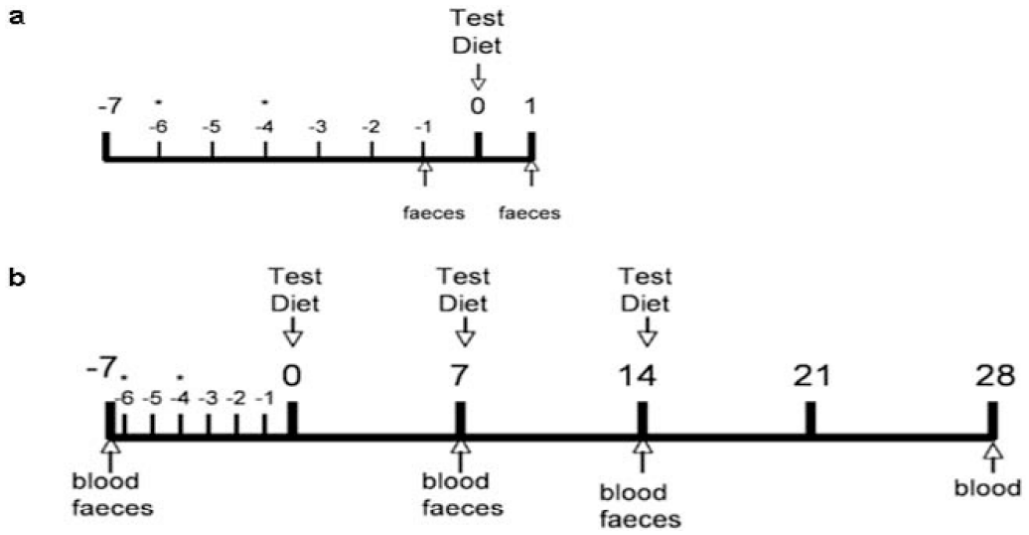

Fig. (2). Timelines for oral administration of vaccines in **a** Trial 1 and **b** Trial 2. Acclimatisation feed with control material is indicated by *.

as described for trial 1, and blood samples taken from tail bleeds at day -1, 7, 14 and cardiac puncture at day 28. Serum was separated from whole blood by clotting at room temperature before centrifugation at 8,000 rpm for 10 min. All samples were stored at -20°C.

Release of Plant-Made Antigens in the Gut

The intact and disrupted gut ingesta samples were analysed for antigen content using GM1 ganglioside-direct ELISA as per Haq *et al*. [1]. The detectable antigen content of intact and disrupted ingesta samples were compared to estimate the proportions of antigen released and still residing in plant cells. When comparison of detectable antigen was performed in our investigations, only GM1 ganglioside-direct ELISA was used. Our goal was not to account for all LTB ingested, but to deliver the same relative dose from each vaccine batch and determine the site of its release. The combined intact ingesta and gut wash samples were mixed by brief vortex, centrifuged at 13,000 rpm for 10 min at 4°C and then the supernatant diluted 1:5 in 1% DM in PBST and incubated on the coated plate for 1 h at 37°C. Homogenised ingesta samples were disrupted in 2 volumes of extraction buffer using a Qiagen Mixer Mill for 1 min at a frequency of 28/s with two 3 mm tungsten carbide beads. Antigen content was enumerated as ng LTB/g ingesta.

Measurement of Ingesta Particle Size

Ingesta particle size was compared between treatment groups using MIX MFC Version 1.0.0.1 software. The collected ingesta was smeared onto the surface of a glass slide and scanned after air drying using a CanoScan 9950F scanner (Canon). Images were analysed to determine the area of each particle identified.

Antigen Surviving Passage Through the GIT

Faecal samples were extracted in 6 ml/g extraction buffer using two 3 mm tungsten carbide beads and a Qiagen Mixer Mill for 1 min at a frequency of 28/s. The homogenised samples were then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant analysed for LTB content by GM1 ganglioside-direct ELISA as described above. Three replicate readings were performed for each sample.

Immunogenicity of Plant-Made LTB

Serum and faecal samples was analysed by anti-LTB-antibody-specific ELISA to assess systemic and mucosal immune responses. Briefly, LTB was adsorbed to the plate by incubating 50 µl of 5 mg/ml bacterial rLTB at 37°C overnight. The plates were washed with PBST then blocked with 10% foetal calf serum in PBS for 1 h at 37°C. The plates are then washed with PBST before serial dilutions of the serum samples were made down the plate. A polyclonal antibody against LTB was used as the standard and serially diluted down the plate starting at 1:100 dilution. The plates were incubated 1 h at 37°C before washing three times with PBST. Goat anti-mouse IgG1 or IgG2a (Sigma) diluted 1:2000 in 5% DM in PBST was then added to the plate and incubated for 1 h at 37°C. Plates were washed three times with PBST before detection using TMB according to manufacture's directions (Bio Rad). Serum samples were also analysed for LTB-specific IgG1 and IgG2a using Zymed MonoAb ID kit (Invitrogen, Carlsbad USA) as per the manufacturers instructions. Three replicate readings were performed for each sample.

Gut wash samples were assayed for LTB-specific IgA using a GM1 ganglioside capture ELISA. Costar 9018 96-well plates (Corning Life Sciences) were coated with 50 ul of 10 µg/mL GM1 monoganglioside (Sigma) in carbonate buffer (0.1 Na_2CO_3, 0.1M $NaHCO_3$, pH 7.4) and incubated overnight. All subsequent incubations were conducted at room temperature on a shaking platform at 100 rpm, and three washes with PBST were conducted between steps. Plates were blocked with 1% DM for 2 h, then each well coated with 25 ng LTB (Sigma) diluted in PBS for 1 h. Gut wash samples were added to each plate at a starting dilution of 1 in 5, and serially diluted in PBS. Samples were incubated for 1 h followed by incubation with 1:2,000 anti-mouse IgA-HRP diluted in PBS. Plates were developed and

read with TMB (Biorad) as per manufacturer's instructions. Three replicate readings were performed for each sample.

Statistical Analyses

GraphPad Prism 5 was used for all statistical analyses performed in this study. With regards to antigen content, one-way ANOVA was used to determine statistical significance between means or medians using probabilities of $P<0.05$ as the limit for observed differences to be considered significant. Since the titre data resulting serum and wash titres were not normally distributed, statistics designed to describe normal distributions could not be used. Instead geometric means, confidence intervals of 95% and fold increase over day 0 titres were used to compare induced immune responses.

RESULTS

LTB Content and Integrity in Transgenic Plant Material

Batches of freeze-dried plant material were prepared from stably expressing transgenic lines of *S. lycopersicum* (tomato) fruit (TrTom), *P. parodii* (petunia) hairy root culture (TrHR) and transiently expressing leaves of *N. benthamiana* (TrLeaf). All plant lines demonstrated LTB content ranging from 0.5 to 3.7 mg/g dry weight (DW) Fig. (**1a**). The accumulation of pentameric LTB, the functional form required for GM1-ganglioside binding to the mucosal surface of the GIT epithelium, was confirmed by western blot Fig. (**1b** and **1c**). In its native state, LTB pentamer was observed at approximately 35 kDa Fig. (**1b**). Upon denaturation, the LTB pentamer was reduced to its monomeric form at approximately 12 kDa Fig. (**1c**) in the purified, bacterial-made, recombinant LTB (rLTB) positive control as well as the transgenic plant lines. Non-specific bands were not seen in the correlating control lanes. Western analysis displayed no qualitative difference between purified, recombinant, bacterial-made LTB, tomato-, leaf- and hairy root-derived LTB as in native or denatured conditions there was no evidence of difference in band sizes introduced by differences in post-translational modifications. The four different LTB vaccine batches (purified, bacterial-made; fruit-made; leaf-made; or hairy root-made) were therefore tested for immunogenicity in mouse feed trials.

Mouse Feed Trials

The first of two mouse feeding trials was designed to investigate protection and release of plant-made antigen by plant cells in the mouse gut and determine if this was influenced by the type of plant cell and/or the formulation media used, i.e. peanut butter (PB) or apple juice and honey (AH). A summary of the feed treatments (Table **1**) and the timing of immunization and sampling Fig. (**2**) are given. All mice were fed 0.2 g of freeze-dried plant material; those receiving test vaccine treatments were fed enough transgenic material to deliver a dose of 50 µg LTB.

Formulated vaccine materials were weighed before and after feeding. Comparisons of the mass of uneaten vaccine revealed no significant difference in the amount of vaccine material consumed between the treatments groups Supplementary Fig. (**3**) (One way ANOVA, $P>0.05$) with an average of 48.3 and 48.6 µg LTB consumed in trials 1 and 2 respectively. The particle size of the ingesta recovered from the stomach was determined to assess the possible effect that particle size may have on antigen release. The median particle size ranged from 0.001-0.007 mm^2 across all treatments but was not statistically different (1 way ANOVA, $P>0.05$) between the different plant materials or formulation media used Supplementary Fig. (**4**). This suggested antigen release was not influenced by variation in ingesta particle size. The results we observed were therefore due to the treatments given, not differences in antigen dosage or ingesta particle size.

Table 1. Treatments and Number of Mice Employed in Trials 1 and 2

Treatment	Mice
Normal diet, pellets	3
Wt plant cells + rLTB delivered in peanut butter (WtNTPB+LTB)	3
Wt plant cells + rLTB delivered in apple juice and honey (WtNTAH+LTB)	3
Wt plant cells delivered in peanut butter (WtNTPB)	3
Wt plant cells delivered in apple juice and honey (WtNTAH)	3
Control tomato fruit delivered in peanut butter (ContTomPB)	3
Control tomato fruit delivered in apple juice and honey (ContTomAH)	3
Control hairy roots delivered in peanut butter (ContHRPB)	3
Control hairy roots delivered in apple juice and honey (ContHRAH)	3
Control leaves delivered in peanut butter (ContLeafPB)	3
Control leaves delivered in apple juice and honey (ContLeafAH)	3
Test tomato fruit delivered in peanut butter (TestTomPB)	5
Test tomato fruit delivered in apple juice and honey (TestTomAH)	5
Test hairy roots delivered in peanut butter (TestHRPB)	5
Test hairy roots delivered in apple juice and honey (TestHRAH)	5
Test leaves delivered in peanut butter (TestLeafPB)	5

Ingesta was collected from four sites along the mouse GIT, stomach, duodenum, ileum and large intestine and subjected to LTB-specific ELISA analysis. LTB content was determined for both intact and homogenised (plant cells broken open) ingesta samples (Supplementary Table **1**). The antigen release ration (ARR) was calculated Fig. (**3a**) by dividing the amount of antigen found in the intact ingesta samples (amount of antigen released into the intestinal tract)

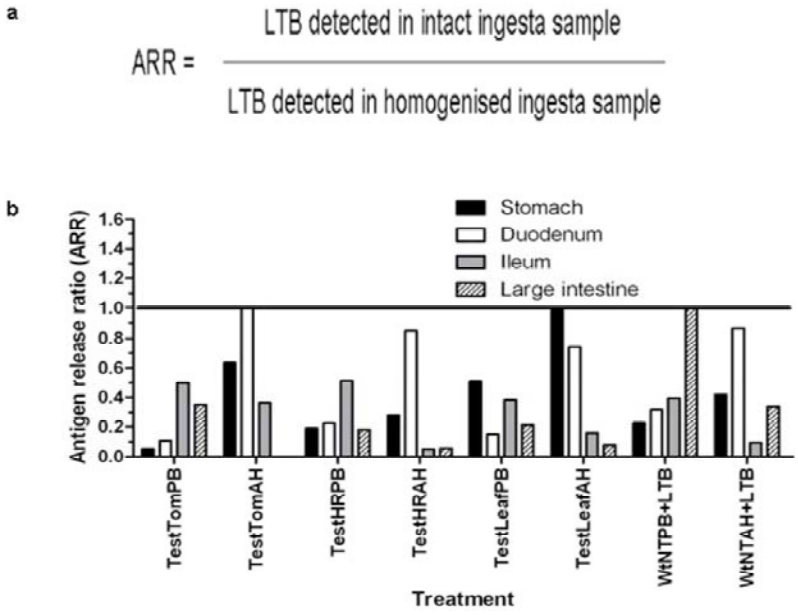

Fig. (3). Antigen release from plant materials. **a** The formula used to calculate the Antigen Release Ratio (ARR). To calculate the ARR, LTB content of intact and homogenised ingesta was determined from at least two replicates. **b** Graphic representation of the ARR and corresponding site in the mouse gut. The bars represent the ratios of the ingesta samples of 5 mice.

by the amount of antigen found in the homogenised ingesta samples (total antigen present in tract). Therefore when the ARR equaled 1, heavy line in Fig. (**3b**), the detectable antigen in intact ingesta was equal to the total detectable antigen (when plant cells were homogenised), meaning all antigen had been released by the plant cells. The site in the mouse GIT where the majority of plant cells had broken open and the contained antigen released was taken as being the point where ARR approached 1.

Irrespective of plant material, vaccine resuspended in AH released the majority of the LTB antigen in the first half of the GIT (stomach or duodenum) Fig. (**3b**). Three out of four vaccines resuspended in PB released the maximum amount of antigen in the last half of the GIT (either ileum or large intestine) Fig. (**3b**). To evaluate the protective effect of the delivery media, the percentage of LTB antigen released at the four tested sites of the mouse GIT was also calculated and compared Supplementary Fig. (**5**). No significant difference was found between the degrees of LTB release/ protection for the different formulation media. An investigation into the presence of LTB pentamer in mouse faecal pellets also revealed no significant difference between control and test treatments or the formulation media used Supplementary Fig. (**6**).

The second mouse feeding trial was designed to investigate the LTB-specific immune response induced by the different plant-made vaccine formulations. Since the titre data resulting from our study was not normally distributed, geometric means, confidence intervals of 95% and fold increase over day 0 titres were used to compare induced immune responses. Very little difference was displayed with LTB-specific IgG titres between repetitions of control plant materials in the same formulation media (AH or PB) with the three plant materials (fruit, root, leaf) merely displaying background titres. Therefore all control materials in the same formulation media were pooled into formulation media groups Fig. (**4**). Serum samples were taken on days 0, 7, 14 and 28 with gut washes also taken on day 28. Serum samples of responders became positive for LTB-specific antibodies by day 14 with titres increasing by day 28 (data not shown). The greatest LTB-specific systemic (IgG) immune response was shown by mice fed test leaf in AH (TestLeafAH), which by day 28 saw a 67.5-fold increase over day 0 titres (Table **2**), followed by mice fed purified LTB delivered with plant cells in PB (WtNTPB+LTB) (33.5 fold increase over day 0 titres), test leaf in PB (TestLeafPB, 17-fold increase over day 0 titres), mice fed test hairy roots in PB (TestHRPB, 4.9-fold increase), test hairy root delivered in AH (TestHRAH, 4.6-fold increase), test tomato delivered in PB (TestTomPB, 3.4 fold increase), free purified LTB delivered with plant cells in AH (WtNTAH+LTB, 2.7-fold increase) and test tomato delivered in AH (TestTomAH, 1.8-fold).

As found with the LTB-specific IgG titres, very little difference was displayed with LTB-specific IgA titres between repetitions of control plant materials in the same formulation media (AH or PB) with the three plant materials (fruit, root, leaf) merely displaying background titres. Therefore all control materials in the same formulation media were pooled into formulation media groups Fig. (**5**). The greatest LTB-specific, mucosal immune response (IgA) was found in gut washes from mice fed TestLeafAH, which saw a 31.8-fold increase over the same mice at day 0 (Table **2**). The next highest LTB-specific IgA titres were found in mice fed purified rLTB delivered in PB (13.7-fold increase over day 0 titres), TestHRPB (5.8-fold increase over day 0 titres), WtNTAH+LTB (3.4-fold increase), TestHRAH (2.4-fold increase) and TestLeafPB (2-fold increase). Test tomato treatments showed a weak IgA response in 1 from 5 mice (1.8-fold increase) only when delivered in PB. This weak response consisted of an IgG1:IgG2a ratio close to 1. The

Table 2. Day 28 LTB-Specific Serum IgG and Gut Wash IgA Titres

Treatment	Final IgG Titre	Fold Increase	IgG1:IgG2a	Final IgA Titre	Fold Increase
Pellets	10	-	-	5	-
WtNTPB	29.3	-	-	10	-
WtNTAH	103.2	-	-	10	-
WtNTPB+LTB	983	33.5	7.8b	137	13.7
WtNTAH+LTB	278	2.7	9.1	34	3.4
ContTomPB	23	-	-	4	-
ContTomAH	33	-	-	5	-
TestTomPB	76	3.37	1.1c	7	1.75
TestTomAH	184	5.6	1.1c	5	-
ContHRPB	38	-	-	6	-
ContHRAH	40	-	-	10	-
TestHRPB	186	4.9	2.9b	35	5.8
TestHRAH	184	4.6	4.1	24	2.4
ContLeafPB	21	-	-	8	-
ContLeafAH	54	-	-	4	-
TestLeafPB	357	17	8.6b	16	2
TestLeafAH	3647	67.5	20.8a	127	31.8

Titres are the geometric mean of 3 (control treatments) to 5 (test treatments) repetitions. Fold Increase represents fold increase over corresponding control treatments. Titre represents the average day 0 titre subtracted from the corresponding treatment's average day 28 titre. Different superscript letters in the main section of the IgG_1:IgG_{2a} column indicate significant difference (ANOVA, $P<0.01$).

test leaf and test hairy root treatments resulted in high IgG1:IgG2a ratios, (Table **2**). The TestLeafAH treatment ratio was significantly higher (ANOVA, $P = 0.0058$) and the test tomato significantly lower (ANOVA, $P = 0.0377$) than the other treatments.

DISCUSSION

Reaching the full potential of pharmaceuticals relies on effective delivery systems. While the most advanced plant-made vaccine are purified and only use plants as a production system, oral and nasal routes of administration of pharmaceuticals are considered the easiest means of delivery with increased patient compliance. Much progress has been made in the field of plant-made vaccines but to our knowledge, no studies have investigated how immunogenicity may vary after oral delivery depending on the delivering plant material; the site of antigen release from plant cells in the gut, or how antigen delivery may differ for different plant tissue types or formulation media used. We used the enteric antigen LTB as a reporter antigen in our novel approach to study immunogenicity of plant-made and delivered LTB in relation to plant cell degradation and antigen release in the mouse GIT. LTB was chosen as our model antigen because it is stable in the gut environment [25], at acidic pH as low as 2.0 [26], can be produced in numerous plant systems and has been shown to induce immune responses following oral delivery to mice and humans. The authors note that while the stability of LTB in the GIT environment was beneficial in this study, in particular to enable the detection of the antigen once it had been released from the plant cell, the performance of LTB is not indicative of all antigens. For example, the site of release would be extremely important to the activity of a less pH robust protein such as the haemagglutinin protein (HA) of H5 and H7 strains of influenza. This homotrimer loses its conformation at low pH [27] so delivery of HA by the leaf expression system and the consequent release of the protein in the stomach (pH 3 when fed and 4 when fasted [28]) would result in the denaturing of the protein and most likely its degradation before its uptake and recognition by the immune system.

Although an LTB coding region of the same nucleotide sequence was used to transform the different plant materials, different genetic constructs (and hence regulatory elements) were used to drive LTB expression in the different plant-made vaccine batches. A direct comparison of antigen production was therefore not made. However since the same coding region was used, the same amount of antigen was delivered to each animal Supplementary Fig. (**3**), and the antigen was shown to be qualitatively similar Fig. (**1b** and **1c**), we compared the immunogenicity of the different plant-made vaccine batches within mouse trials.

The first mouse trial studied the effect of plant cell tissue and formulation media on antigen release. By determining the LTB content of intact and disrupted ingesta samples, we were able to determine the site of antigen release from plant cells in the mouse GIT Fig. (**3b**). The particle size of ingesta and the amount of antigen released into the mouse GIT did not differ between plant materials or formulations Supplementary Fig. (**4** and **5**) and can therefore be discounted as a contributing factor for site of antigen release and induced immune response in this mouse model. Also, there was no significant difference between LTB found in the faecal pellets of mice fed control materials as opposed to test vaccines Supplementary Fig. (**6**). This suggests that either all available LTB was bound to GM1-ganglioside receptors along the GIT or was ultimately degraded. Taking these aspects into consideration it is probable that the differences we observed in the timing of antigen release from the plant materials tested were due to variations in formulation or structure and composition of the respective plant cell walls. The fibrous nature of the hairy root material for example, may have contributed to its increased tolerance to mechanical and chemical digestion in the stomach. Similarly, the composition of the delivery media used for vaccine formulation influenced antigen protection, such that the lipid based PB formulation (80% fat, 6% protein, 3% sugar) may have required more prolonged enzymatic degradation to breakdown its constituents to their simplest form. This notion is supported by a recent study reporting that the addition of irrelevant bystander protein inhibited proteolysis of LTB in intestinal fluid for at least 3 hours [25]. In contrast, the aqueous based AH formulation (0.3% protein, 0.03% fat, 64% sugar) comprising mostly simple sugars would have required relatively less intensive digestion. We contend therefore, that while the plant cell wall acts as a protective barrier that encapsulates the expressed antigen, PB serves as a defensive coating, rich in proteins and lipids that act as a decoy for proteases and digestive enzymes in the GIT. While the inclusion of groups consisting of plant materials delivered without formulation may have offered a clearer understanding of the effect plant matrices themselves had on antigen release, this was not possible. In our experience, the dehydrated (freeze-dried) nature of our vaccine batches is such that it is harmful, sometimes lethal if delivered unformulated to the mouse, having killed two of three mice in a previous, unreported trial. In this case, autopsy suggested the cause of death to be dehydration.

The defensive coating ability of PB was demonstrated in the second trial by comparing the induced immune responses to purified LTB formulated in PB and AH with the PB formulation inducing more robust, antigen specific immune responses Table **2** and Figs. (**4** and **5**). Also, in two out of three plant-made LTB vaccines, PB treatments resulted in larger LTB-specific, systemic immune responses than the corresponding tissue delivered in AH and in three out of four cases the PB treatments resulted in antigen release in the later sections of the mouse GIT sampled Fig. (**3**). The divergent treatment however was that which induced the highest fold increase in LTB-specific IgG and IgA over day 0 titres, the leaf material-made LTB delivered in AH. This high immune response may have been due to an adjuvanting effect by the infiltrated bacterium, in the form of *Agrobacterium*-derived lipoplysaccharide endotoxin or perhaps plant-made alkaloids. Although bacterial endotoxins are often encountered by the GIT the quantity involved in this experiment may have adjuvanted an antigen-specific immune response. In addition, the strong immune response induced by the TestLeafAH treatment may have resulted due to release of plant cell contents including possible adjuvanting alkaloids occurring more readily from the AH rather than the PB formulation.

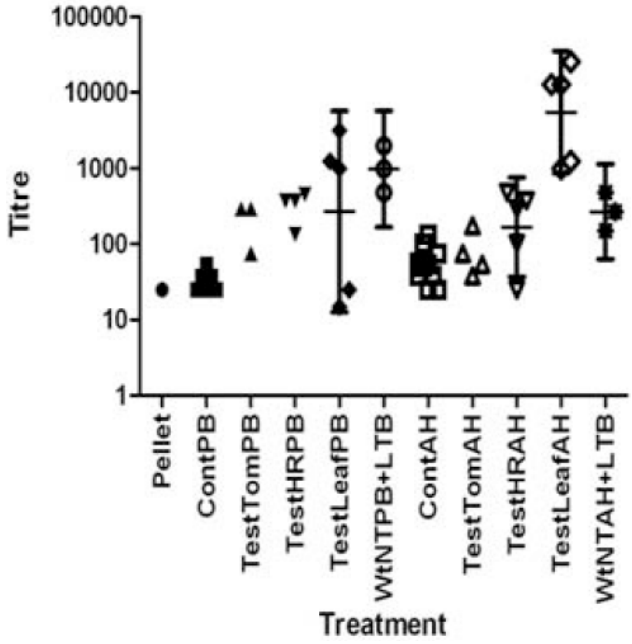

Fig. (4). LTB-specific IgG response. Data points represent individual responder mouse titres where the large horizontal line represents the geometric mean and the outlier horizontal lines the 95% confidence interval.

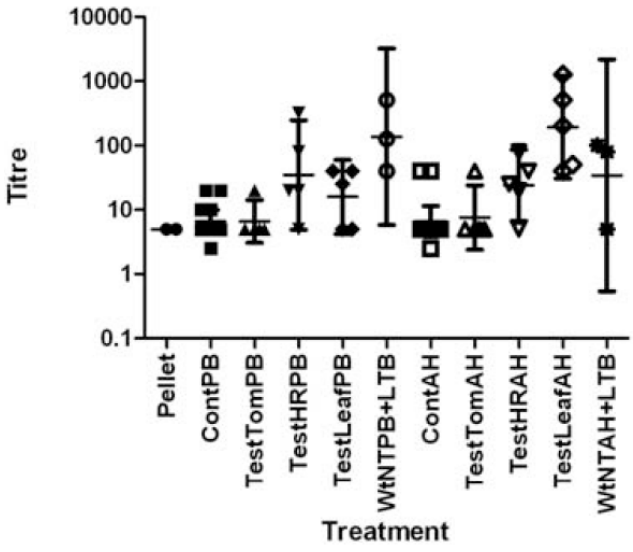

Fig. (5). LTB-specific IgA response in gut washes. Data points represent individual responder mouse titres where the large horizontal line represents the geometric mean and the outlier horizontal lines the 95% confidence interval.

The presence of LTB-specific antibodies (IgG and IgA), particularly in transgenic leaf and hairy root treatments indicated that LTB released from the orally delivered plant material persisted long enough to stimulate both systemic and mucosal immune responses. The TestTom treatments in this study were in agreement with previous studies with regards to IgG_1:IgG_{2a} ratios induced by the LT antigen, as they were close to 1 and therefore indicated a balanced Th1 and Th2 response [29]. However the test leaf and hairy root treatments induced immune responses biased towards a Th2 response, being significantly higher than the test tomato treatments. The TestLeafAH treatment in particular induced a response strongly biased towards Th2 with an IgG_1:IgG_{2a} ratio significantly higher (ANOVA, $P = 0.0058$) than the other treatments. It is important to note however that the route of immunization, oral or nasal, is important with regards to the adjuvanticity or immune modulation of LT [30]. For example in agreement with our transgenic leaf and hairy root results, a recent publication found orally delivered, rice-made LTB to induce a predominant Th2 response [18].

It appears that systemic immune responses induced from orally delivered plant-made vaccines benefit from delayed release of antigen in the mouse GIT since three out of four PB vaccine formulations induced higher LTB-specific IgG titres compared to their AH counterparts. However the formulation media and site of antigen release did not have an apparent effect on mucosal immunogenicity with the PB and AH treatment displaying no trend with regards to induced LTB-specific IgG and IgA responses. Not surprisingly, the influences to the mucosal immune response induced by plant-made vaccines and their formulations were too complex to be determined by studies performed here and further work perhaps involving more complex formulations needs to be performed.

Although a difference was seen with production of LTB-specific IgG production, transgenic tomato fruit did not induce robust systemic or mucosal immune responses. This was surprising since there is evidence in the literature and in our own studies [5, 13] that tomato fruit–made vaccines successfully induce specific immune responses not only to LTB [5] but other antigens including Norwalk virus capsid protein [31], *Yersinia pestis* F1 and V [13]. Since LTB-specific ELISA and western analysis demonstrated antigen expression of similar quality Fig. (**1**) by test tomato fruit, and systemic and mucosal immune responses were observed for all other plant encapsulated LTB or free LTB groups, it may be possible that an unknown component of the tomato plant cell contents may be acting to suppress antigen presentation to relevant immunocompetent cells. Perhaps previously reported success of tomato-fruit delivered vaccines would be improved through expression by a different plant system (leaf or hairy root).

Expression and delivery of LTB in *Nicotiana* leaf material induced the highest increase in serum tires over day 0 samples. We propose that while LTB encapsulated in *N. benthamiana* leaves formulated in AH stimulated strong mucosal and systemic immune response in mice, less pH resistant antigens or target animals with a longer or more complicated GIT, such as ruminants, may require increased protective properties offered by either hairy roots and/or lipid (PB) formulations to encapsulate the antigen of interest.

This study reports for the first time, the ability to determine where plant cells break open in the mouse GIT. Using this technique we were able to determine that while encapsulation in the plant cell provides some protection of the antigen from the hostile GIT and affects the site of antigen release, formulation media is also an important consideration with regards to antigen delivery/release and immunogenicity, particularly with induction of systemic immune responses after oral delivery. The choice of plant material and formulation therefore require careful consideration to ensure targeted delivery to immune responsive sites and to ensure safe passage of less stable antigens to specific sites of the intestinal tract. This study represents the first step in elucidating the optimal conditions for oral delivery of plant-made vaccines with the final goal being increased titres.

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3. IMPROVED ORAL IMMUNOGENICITY OF THE HEAT-LABILE ENTEROTOXIN B SUBUNIT WITHIN PROTEIN STORAGE VACUOLES OF *NICOTIANA BENTHAMIANA* LEAVES

3.1 Introduction

In Chapter 2, we provided evidence that the choice of plant expression system effects the mucosal immune response following oral administration of plant material containing the heat-labile enterotoxin B subunit (LTB). The strongest immune response was induced when LTB was delivered in the leaves of *Nicotiana benthamiana*. While purposely using expression systems based on very different expression platforms and species, this diversity made it difficult to determine which plant-specific factors were responsible for the variation in immunogenicity. To minimise the confounding variables, the effect of a single characteristic; subcellular localisation was chosen for further investigation. This property was chosen due to the amenability of the MagnICON tobacco mosaic virus system to test the effectiveness of different cellular signals such as N- and C-terminal signalling peptides, and the known ability of LTB to accumulate in different cellular organelles (see 1.5.1.5).

In Chapter 2, the full-length plant-codon optimised LTB coding sequence was cloned into a construct amenable for use in the MagnICON system. This genetic sequence included the native bacterial signal peptide allowing entry of the nascent poly-peptide chain into the endoplasmic reticulum (ER), where it is predicted to traffic through the plant endomembrane system and into the apoplastic space. In this study, the default site of accumulation of LTB within a plant cell was altered using a series of DNA constructs compatible with the MagnICON system. The DNA constructs were produced with plant-specific protein signal sequences fused to the LTB coding sequence. These signal sequences included native, synthetic and plant-derived N-terminal signal peptides, chloroplast transit peptides, gamma zein and phaseolin-based fusion tags, vacuole targeting sequences, and the Zera® protein-body tag. When LTB was expressed with these signal sequences, four different constructs were found to yield sufficient protein for further investigation (nominally > 500 μg/g dry

weight): LTB with the native signal peptide, LTB with a KDEL ER-retention signal, LTB with a vacuole targeting sequence, and LTB fused with the Zera protein-body tag.

The yield of LTB was characterised in *N. benthamiana* plants for each construct to determine the optimum age and period between infiltration and harvest (Figure 3-1).

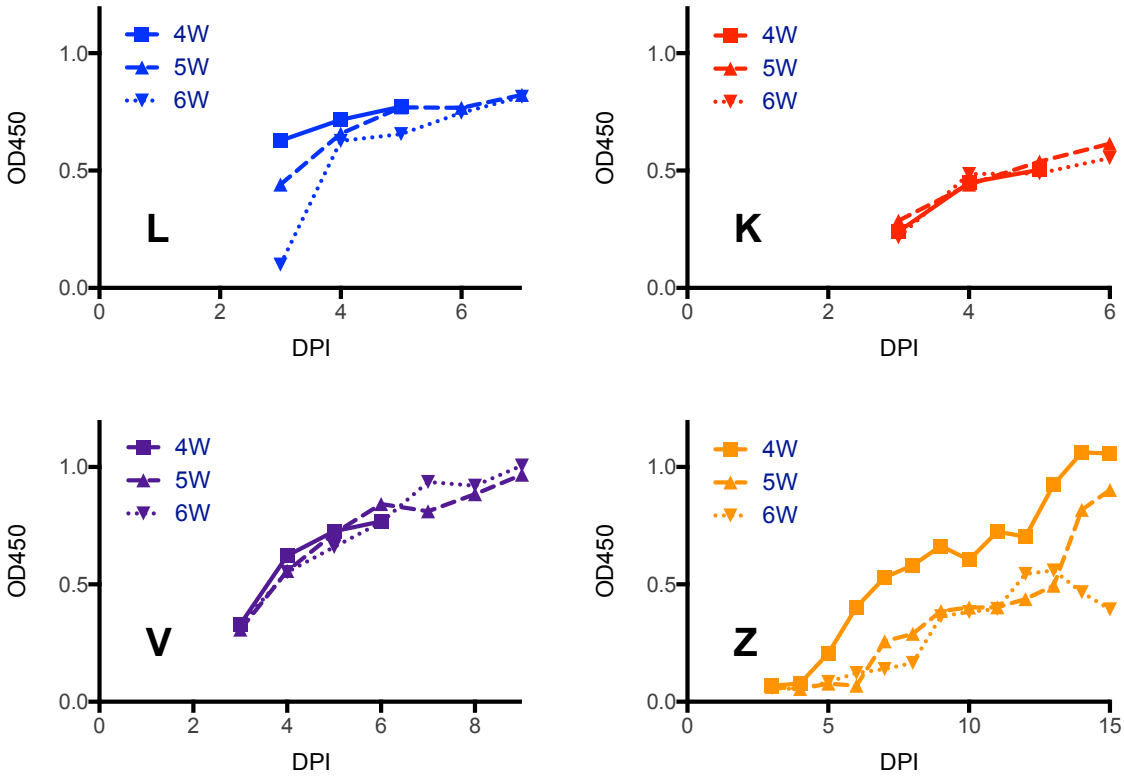

Figure 3-1 Accumulation of GM1-binding LTB in *N. benthamiana* leaves infiltrated with MagnICON vectors targeting LTB to different subcellular locations. Ten leaf discs (each 10 mm^2) at each time point were randomly sampled from 10 plants transiently infiltrated with the full-length LTB coding sequencing containing the native bacterial signal sequence (L), the same protein with a C-terminal SEKDEL (K), the vacuolar targeting sequence (V), or the LTB protein truncated to remove the native signal peptide and replaced with the Zera protein body tag (Z). Plants were 4, 5 or 6-weeks old (4W, 5W, 6W). Sampling was stopped when leaves became necrotic. DPI- days post infiltration, OD450- optical density 450 nm. Experimental methods as per 3.3.

The yield of GM1-binding LTB was highest for all constructs in 4-week old plants. Expression of L, K and V constructs led to gross necrosis of the plant leaf tissue by 7, 6 and 9 days post infiltration, respectively. Interestingly, expression of LTB targeted to accumulate in ER-derived protein bodies (Z) did not cause necrosis at any time point. A separate batch of plants were grown for the subsequent analysis and immunogenicity testing (detailed in 3.3). Despite the lower immunogenicity of *N. benthamiana* leaf formulations mixed with lipid observed in Chapter 2, an oil-based palatability enhancer was chosen for this study to minimise rehydration of plant-endogenous proteases. The results of these experiments showed that localisation of LTB within the protein storage vacuoles of the *N. benthamiana* leaf cell

improved the LTB-specific immune response compared to responses induced through oral delivery of plant tissues that had LTB targeted to other subcellular locations.

3.2 Statement of contribution

The two authors of this paper are Robert P Shepherd (RPS) and Amanda M Walmsley (AMW).

In the case of Chapter 3: "Improved oral immunogenicity of the heat-labile enterotoxin B subunit within protein storage vacuoles of *Nicotiana benthamiana* leaves", the nature and extent of contribution to the work was the following:

- All experiments were designed, performed, analysed, and the manuscript written by RPS. The extent of this work was 80%.
- Experimental plan and review, and manuscript review by AMW. The extent of this work was 20%.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

…… ……. 09 March 2016

Signature Date

Robert P Shepherd (candidate)

… ……. 10 Mar 2016

Sig ate

David R Smyth

3.3 Manuscript

This manuscript is formatted for the Plant Biotechnology Journal

Improved oral immunogenicity of the heat-labile enterotoxin B subunit within protein storage vacuoles of *Nicotiana benthamiana* leaves

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KEYWORDS

Oral vaccine, subunit vaccine, delivery, kinetics, subunit vaccine, subcellular localisation

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SUMMARY

Oral delivery of antigenic proteins in transgenic plant material was heralded as a safe, rapid and economical platform for the development of vaccines. However, as with conventionally produced oral vaccines, degradation of the vaccine antigens during digestion leads to inconsistent dosing and immunogenicity. Improving the protection of recombinant antigens within the plant cell may improve the dose consistency and mucosal immune response. Here, we used plant signal sequences to localize the B subunit of the heat-labile toxin (LTB) from enterotoxigenic *Escherichia coli* (ETEC) within discrete subcellular compartments of transiently transformed *Nicotiana benthamiana* leaf cells including the apoplastic space, endoplasmic membrane system, protein storage vacuoles, and protein bodies. LTB localised to these regions formed ganglioside-binding pentamers characteristic of bacterial LTB, and *in vitro* treatment of leaf cells with simulated gastric fluid showed differential release and degradation of LTB depending on the subcellular encapsulation. Feeding trials in mice revealed that localisation of LTB to the protein storage vacuoles improved the magnitude and consistency of an antigen-specific IgG1-dominant humoral and mucosal sIgA response compared to LTB localised to other compartments. The optimisation of protein release from plant cells during digestion may facilitate improved oral delivery of antigenic or therapeutic proteins by inducing more robust and consistent immune responses.

INTRODUCTION

Vaccination remains one of the key tools in the fight against infectious disease, yet with a few exceptions, vaccines are delivered by systemic injection. While systemic administration is efficacious for many diseases, there is demand for oral vaccines as the antigen-specific secretory antibody response at the mucosa is a key determinate of protection against a variety of pathogens (Pasetti et al. 2011; Pabst 2012). The concept of expressing antigenic proteins in transgenic plant cells as a combined production and delivery system is now over three decades old, with new expression systems and host modifications dramatically improving the capacity of plants to produce high yields of complex, well characterised therapeutic proteins (Curtiss and Cardineau 1997; Rybicki 2014; Daniell et al. 2015). Recent studies have shown that plant cells are effective delivery vehicles for small therapeutic proteins (Kwon and Daniell 2015; Shaaltiel et al. 2015) yet the concept of whole plant cells as a delivery system for oral vaccines is marred by the inability to reliably induce immunity in the complex environment of the gastrointestinal tract (GIT).

Our previous work has shown that there are significant differences in the systemic and mucosal immune response when the same antigen is delivered in whole cells from different plant tissues (Pelosi et al. 2011; 2012). In comparison to tomato fruit and *Petunia parodii* hairy root cell culture, the leaf tissue of *N. benthamiana* was shown to rapidly release antigen in the murine stomach. These release kinetics correlated with a strong secretory immunoglobulin A (sIgA) response in 60% of mice, but did not translate as effectively in a larger sheep model of oral immunity. This observation raises the question as to whether the release of antigenic proteins from *N. benthamiana* leaf material can be optimised to increase immunogenicity?

We investigated the effect of subcellular localisation on the release kinetics and oral immunogenicity of an antigen from the mucosal pathogen enterotoxigenic *Escherichia coli* (ETEC). We utilized the MagnICON tobacco mosaic virus system to express the B-subunit of

the heat-labile enterotoxin (LTB) as this antigen has been extensively characterised and used in previous pre-clinical and clinical trials of orally-delivered plant-made vaccines (Gleba et al. 2005; Tacket 2009). The LTB protein is closely related to the B-subunit of the cholera toxin (CTB), a component of the licensed human oral cholera vaccine Dukoral® (Sanchez and Holmgren 2011; Svennerholm 2011). The strong mucosal immunogenicity of both LTB and CTB derives from the tertiary pentameric structure common to AB_5 holotoxins (Beddoe et al. 2010), where monomers undergo disulphide bond formation to assemble pentamers during protein maturation in the endomembrane system. These complexes bind with high specificity to GM1-receptors on the surface of epithelial and intra-epithelial cells in the GIT (Goins and Freire 1988; Gustafsson et al. 2013). If delivered at a sufficient dose, CTB and LTB are capable of inducing a T_h2-like adaptive response characterised by an IgG1-dominant humoral and sIgA mucosal response (Svennerholm 2011), but immunity to the heat-labile toxin (LT) alone has been shown to be ineffective in preventing ETEC-related diarrhoeal in larger field trials (Zhang and Sack 2015).

The LTB protein is immunogenic when delivered orally in potato tubers (Haq et al. 1995; Mason et al. 1998; Lauterslager et al. 2001), maize meal (Streatfield et al. 2001; Chikwamba et al. 2002; Lamphear et al. 2002; Karaman et al. 2012), tomato fruit (Walmsley et al. 2003; Pelosi et al. 2011), soy beans (Moravec et al. 2007), carrot cells (Rosales-Mendoza et al. 2007), rice callus (Kim et al. 2010), rice endosperm (Soh et al. 2015), hairy root cell culture, and tobacco leaves (Pelosi et al. 2011; 2012). LTB expression and delivery in plant cells has been one of the few plant system to progress to early-stage clinical studies where LTB in potato tubers (Tacket et al. 1998) and defatted corn meal (Tacket et al. 2004) were moderately immunogenic when delivered to healthy volunteers.

Previous studies have shown that LTB localised to the starch granules of maize improved thermal stability during processing (Chikwamba et al. 2002), and was highly immunogenic when orally administered to mice (Chikwamba et al. 2003). In comparison, the immune

response to the same concentration of LTB formulated outside of the plant cell was barely detected (Chikwamba et al. 2003). Sub-cellular accumulation of LTB in maize cells leads to over 1000-fold variation in antigen yield (Streatfield et al. 2003), but the influence of subcellular localisation on the resulting immunogenicity has not been investigated.

In this study, we show that LTB can be targeted for accumulation in the subcellular compartments of *N. benthamiana* leaf cells, and that localisation influences the rate of LTB release and degradation *in vitro*. Additionally, accumulation of LTB in the protein storage vacuoles increased the magnitude and rate of antigen-specific mucosal response when administered orally to C57Blk-6 mice.

RESULTS

Genetic fusions of LTB with trafficking signals facilitate high-level expression in *N. benthamiana* leaf cells

Pentameric LTB has been observed in plant cells when expressed using the native bacterial signal sequence (Haq et al. 1995; Mason et al. 1998; Tacket et al. 1998; Lauterslager et al. 2001; Lamphear et al. 2002; Streatfield et al. 2003; Rosales-Mendoza et al. 2008; Pelosi et al. 2011; 2012), a KDEL ER-retention sequence (Pimpl and Denecke 2000; Chikwamba et al. 2002; Streatfield et al. 2003; Moravec et al. 2007; Kim et al. 2010; Martínez-González et al. 2011), vacuolar transit peptide, nuclear transit signal sequence, and chloroplast transit peptides (Streatfield et al. 2003). We show that LTB can also be rationally engineered to traffic and accumulate at high concentration in the protein storage vacuoles and induced protein bodies of *N. benthamiana* leaves (Figure 2A). Plants infiltrated with the full-length LTB (L) yielded 773 ± 250 µg of GM1-binding, pentameric LTB per gram of dry weight (DW) leaf material. This yield is equivalent to 0.66% of the total soluble protein (TSP). When the KDEL tag was fused C-terminal to the same sequence, LTBK (K) accumulated to 523 ± 36 µg/g DW (0.33% TSP). By fusing the vacuolar transit peptide of tobacco chitinase C-terminal of LTB (V), the yield was dramatically increased to 3346 ± 1829 µg/g DW (2.29% TSP), and when Zera™ (containing its own signal peptide) was fused N-terminal to truncated LTB without the native signal peptide (Z), LTB accumulated at 703 ± 110 µg/g DW (0.43% TSP).

Fusion proteins are the expected molecular weight

To determine if there were qualitative differences in LTB recovered from the different subcellular locations, TSP was separated by SDS-PAGE and visualized by western blot. No banding was detected in leaves infiltrated with a GFP-expressing construct (not shown). When samples of TSP were heated to 95°C for 1-minute, all constructs showed distinct banding patterns representative of multimer formation (Figure 2B). The purified LTB

standard forms monomer (11 kDa) and dimers (20 kDa; Figure 2B S). Protein extracts from plants expressing the L construct displayed monomer, dimer, trimer (~28 kDa), tetramer (~39KDa), and pentamer (~50 kDa) formation. Extracts from tissues expressing the K and V constructs show evidence of the monomer and dimer only. Extracts expressing the Z construct show the predicted ~18 kDa band of the Zera™ΔLTB construct as well as bands corresponding to LTB monomer, trimer, tetramer, and pentamers (see Figure 2B Z10, Z5, Z2).

LTB fusion proteins traffic to discrete regions of the cell

To ensure the LTB fusion proteins were localised to the predicted subcellular locations, immune-gold labelling was performed on cryopreserved leaf sections. Gold labelling shows native LTB (L) protein accumulation proximal to the leaf cell margin (see Figure 3L). All gold particles in LTB samples were observed within close proximity to the cell wall, and often in small clusters suggestive of transport vacuoles moving toward the outer cell membrane. In LTBK samples (K), labelling is observed at the inner folded surface of the ER-membrane, but is not observed in any of the other ER-derived concomitant structures such as transport vesicles or outer cell membrane. Samples of LTBV (V) show gold labelling in morphologically-distinct, dark-staining vacuolar compartments within the plant cytoplasm (see Figure 3V). These structures did not co-locate with the central leaf vacuole in any samples (see insert Figure 3V). We suggest that the protein is localised to post-ER vesicles, as there is no clear accumulation on the folded ER surface as observed in LTBK samples. Using the Zera™ fusion tag (Z), gold labelling is observed within and around the edge of electron dense bodies ~0.5-2 μm in diameter (see Figure 3Z). Despite cleavage of the ΔLTB fragment *in vitro* (Figure 2B, Z), gold labelling and all staining of Z was associated with dark staining punctate organelles only.

Subcellular localisation influences the release of antigen *in vitro*

To determine if the subcellular location alters the kinetics of antigen release in conditions similar to those in the murine stomach, we performed release and degradation assays using simulated gastric fluid (SGF). In this assay, the total quantity of GM1-binding LTB in the SGF supernatant was determined at time points physiologically relevant to murine gastric clearing (Schwarz et al. 2002). No degradation was observed when purified recombinant LTB was incubated in PBS alone (see Figure 4A, rL PBS). However, when purified LTB was incubated in SGF, a rapid and complete destruction of the protein was observed within 30 minutes (Figure 4A, rL). To account for bystander plant proteins acting as decoy substrates (Reuter et al. 2009), purified LTB was mixed with control leaves before digestion in SGF. Protection from proteolysis was extended in this experiment (Figure 4, rL GFP). Samples of LTBK and Zera™ΔLTB leaf material did not provide additional protection compared to the rL GFP control group. In contrast, both LTB and LTBV groups provided extended protection of pentameric LTB during SGF digestion. Repetition of this experiment showed similar trends, as did additional experiments with LTBV-expressing leaf material diluted with control leaf (60-40% and 20-80% of LTBV-control leaf, respectively; S2). To quantify this trend and determine if LTB remained within the leaf cell after 30 minutes of incubation with SGF, free LTB in the supernatant at 30 minutes was compared to LTB released after mechanical rupture of the plant cell. The same trend of release kinetics was observed as in the time course experiment, with GM1-binding LTB significantly increased in the supernatant of LTB (L) and LTBV (V) samples, compared to rL GFP, LTBK (K) or Zera™ΔLTB (Z; Figure 4B). There was no significant difference between LTB concentrations in the supernatant before (Figure 4B, open boxes) or after the plant cells were lysed by mechanical disruption (closed boxes) for L, K or V constructs. This confirms that by 30 minutes, the plant cell has released all of the cellular contents into the supernatant. However, in Zera™ΔLTB samples, a decrease in pentameric LTB was observed following disruption of the cells. This suggests reduced

stability of the Zera™ΔLTB protein following lysis, potentially due to degradation of the integrity of the PB.

Mice fed LTB in PSVs showed increased serum immune response compared to other subcellular locations

The next step was to determine if the prolonged protection of LTB in the apoplast and PSVs influenced oral immunogenicity. To do this, we conducted a feeding trial of C57BL/6J mice. Mice were fed instead of gavaged to ensure intact cells were delivered. Mice were fed three doses of dried leaf material containing 75 µg of LTB one week apart. Doses were formulated to contain LTB-expressing samples and control material (leaves infiltrated with a GFP-expressing MagnICON construct) to give a final concentration of 500 µg/g so that each mouse received 150 mg of leaf material per dose. We assayed for the toxic pyrimidine alkaloids of *N. benthamiana* including nicotine, nornicotine, anatabine and anabasine (Supplemental Data, Figure S1), but only low levels of nicotine were observed (11.47 to 10.23 µg/g). Mice ate on average 94% of the dose within the 16- hours it was offered, and no appreciable degradation of LTB was observed within the sample over this period (data not shown). Pre-immune serum was taken prior to trial start, at day 14 (D14) and day 28 of the trial (D28). No significant LTB-specific humoral antibody response was observed prior to D28 (see Supplemental Data, Figure S3). As previously reported (Wagner et al. 2004; Pelosi et al. 2011), orally administered LTB in *N. benthamiana* cells induced an IgG1-dominant humoral response. All groups show a higher IgG1:IgG2a ratio: 2.284 ± 1.090 for mice in the LTB group (L), 1.691 ± 0.0804 for LTBK (K), 2.497 ± 0.08793 for LTBV (V), and 1.262 ± 0.3688 for Zera™ΔLTB (Z; Figure 5A). The consistent IgG1>IgG2a ratio suggests that the humoral response is similar to the IgG1-dominant response to pathogenic ETEC (Martin et al. 2000). There was no significant difference in the IgG1 response between control groups fed purified LTB with (Figure 5, GL) or without bystander control leaves (Figure 5, rL) or when compared to the control group not administered LTB (Figure 5, G). This implies that delivery

of purified LTB not contained within the cell, regardless of the presence of 'decoy' substrates, is insufficient to generate a consistent IgG1 humoral immune response when fed in this manner and dose. In comparison, all mice groups administered LTB within leaf cells (LTB, LTBK, LTBV, Zera™ΔLTB) had a significant humoral antibody response compared to the control (G). Of groups administered LTB encapsulated with the plant cell, only LTBV (Figure 5, V) had a significantly increased IgG1 response at D28 when compared to the other groups when adjusted for multiple comparisons, and 100% (10/10) mice responding to the vaccination. A neutralisation assay was conducted with LTB and CT to determine if there was cross-reactivity between LTB and CT (Figure 5B & C). Mice fed LTB (L) and LTBV (V) leaf induced neutralising antibodies to both LTB and CT when compared to controls, but only mice fed LTB encapsulated in the vacuole raised an significantly improved neutralising titre compared to other groups.

Mice fed LTB in PSVs show higher amplitude and consistency of mucosal sIgA response

One of the key correlates of protective immunity against ETEC and cholera-caused disease is the presence of toxin-specific sIgA at the mucosal surface (Tokuhara et al. 2010; Harro et al. 2011). To determine if subcellular encapsulation also affected mucosal sIgA production, we sampled the small intestines for LTB and CT-binding sIgA. Unlike previous studies where not all responders that raised a humoral IgG1 response converted to a sIgA production (Tacket et al. 1998), in this study all mice that responded with a humoral IgG titre also responded with an concomitant sIgA response. Figure 6 shows the LTB (A) and CT (B) reactive sIgA recovered from the intestinal surface. The sIgA of individual animals is highly correlated with the total antigen-specific humoral IgG1 isotype (Figure 5). There is a significantly improved magnitude and response rate (90%, 9/10) of cross-reactive sIgA at the mucosa in mice fed LTB contained in the PSVs compared to all other groups.

DISCUSSION

In this study, we report the influence of subcellular antigen accumulation on yield, release kinetics and immunogenicity of a mucosal vaccine delivered orally in whole plant cells. Previous reports have indicated that LTB sequestered to the ER of potato tubers, and to the apoplastic space of maize endosperm is safe and moderately immunogenic when delivered to human volunteers (Tacket et al. 1998; Streatfield et al. 2001). Our data suggest that encapsulation with the PSVs of leaf cells may increase the oral immunogenicity of this antigen delivered in *N. benthamiana* leaf cells by reducing the dose of antigen lost to proteolysis during digestion.

Targeting recombinant proteins to subcellular compartments of the plant cell has been used extensively to optimise yield, post-translational modifications, and downstream processing of proteins (Hood et al. 2007; Maclean et al. 2007; Streatfield 2007; Hassan et al. 2008; Hofbauer and Stoger 2013). Despite an increased understanding of the plant cell as an expression system (Benchabane et al. 2008; Hehle et al. 2011; Hassan et al. 2012; Hehle et al. 2015), there is only limited ability to predict which organelle will yield the highest concentration of recombinant protein (Meyers et al. 2008). In this study, we see a dramatic variation in the accumulation between the same antigen localised to different subcellular compartments. The variation in accumulation, despite identical viral transcriptional expression machinery, suggests protein stability is altered by the transit within the plant endomembrane system or final cellular location. Whether accumulation is limited by increased stability or proteolytic mechanisms is not clear (Doran 2006). In addition to the yield of correctly-folded proteins, subcellular localisation also led to a difference in biochemical functionality of LTB. Trafficking to the apoplast or retention within PBs produced LTB multimers that were highly resistant to thermal dissociation compared to other cellular locations. This functional difference may occur due to variation in chaperone and

folding mechanisms, or post-translational modification within the various trafficking pathways.

It is interesting then, that LTB localised to the PSVs and the apoplast showed a improved resistance to proteolysis *in vitro* when compared to other subcellular locations. Whether it is the biochemical or physical protection of LTB that is associated with this proteolytic resistance is currently under investigation. What is apparent is the correlation between improved protection from proteolysis observed *in vitro*, and the immune response observed *in vivo*.

Mice fed purified LTB alone had a detectable, but low magnitude of humoral and mucosal antibody response. Mice fed LTBK or Zera™ΔLTB responded with a statistically similar (ANOVA, $p>0.05$) seroconversion or sIgA than mice fed LTB mixed with control leaves, closely correlating with the lower resistance of these formulations to proteolysis *in vitro.* Mucosal sIgA response was detected in 70% of mice administered LTB localised to the apoplast, similar to what we have observed previously (Pelosi et al. 2011). However, when mice were fed LTB in the PSVs, all had an IgG1 titre above background, and 90% of mice generated detectable sIgA to both LTB and CT.

Previous studies have shown that encapsulation of antigens within subcellular organelles protects antigens from proteolysis during digestion (Chikwamba et al. 2003; Takagi et al. 2010), and several studies have shown that bioactive molecules delivered in plant cells are capable of successfully reaching the blood stream (Kwon et al. 2013; Shaaltiel et al. 2015). While these studies suggest that plant cells may indeed encapsulate the antigens and protect them from proteolysis, it is unclear if the localisation of the antigen within the plant cell was explicitly involved in protecting these proteins from proteolysis. In this study, we show that targeting protein accumulation within the protein storage vesicles of *N. benthamiana* improves the oral immunogenicity compared to targeting to other cellular locations. While encapsulation within the PSVs also improved protection from proteolysis *in vitro*, it is not

known if the improved immune response to LTB in PSVs was due to the stability of the antigen during digestion of the other biochemical variations between proteins localised to the different locations. Previous studies have shown M-cells are directly responsible for translocation of LTB and CTB across the epithelium (Limaye et al. 2006; Nochi et al. 2007; Yuki et al. 2013), and it is unclear if antigens still encapsulated within storage organelles also can be translocated across the epithelium. It would be interesting to determine if the immunogenicity of LTB in PSVs is observed in larger animal species with longer intestinal transit times, as apoplasticly located LTB in *N. benthamiana* leaf many not be strongly immunogenic in other species such as sheep (Pelosi et al. 2012).

In summary, we have shown that the localisation of antigen within discrete compartments of the *N. benthamiana* leaf cell is sufficient to influence the release and immunogenicity of LTB orally administered in leaf material. While it is unlikely that plant material expressing LTB alone will provide sufficient protective immunity to be used as a vaccine for ETEC, (Zhang and Sack 2015) by manipulating host cell factors such as subcellular accumulation we can begin to optimise the interactions of plant cells as both expression and delivery systems.

EXPERIMENTAL PROCEDURES

DNA constructs

The coding region of the plant-codon optimised LTB protein was amplified using PCR from pTH110 (Mason et al. 1998) using primers with flanking restriction sites *NcoI* and *BamHI*, and inserted into the 3' module vector pICH11599 of the MagnICON expression system (Icon Genetics GmbH, Germany) to create pICH-LTB (Figure 1; LTB, L). PCR with primers containing the SEKDEL sequence, or the vacuolar transit peptide GNGLLVDTM (Neuhaus et al. 1991; Di Sansebastiano et al. 1998) including flanking restriction sites *NcoI* and *BamHI* were used to create amplicons and ligated into pICH11599 to create pICH-LTBK and pICH-LTBV respectively (Figure 1). These vectors are hereafter abbreviated as LTBK (K) and LTBV (V). The Zera™ storage protein fusion sequence RX3 (MD et al. 2009) was synthesized (GeneArt GmbH, Germany) with a flexible GGN^3 linker, LEVLFQ/GP-Precission Plus™ cleavage sequence (GE Healthcare Life Sciences, USA) and flanking *NcoI* restriction sites. The LTB sequence without the 5' leading signal peptide sequence (Mason et al. 1998) was cloned into pICH11599 with the Zera-GGN3-cleavage fragment to give pICH-Zera™ΔLTB. This construct is referred to as Zera™ΔLTB (Z). All plasmids were confirmed by sequencing, and constructs electroporated into *Agrobacterium tumafaciens* strain GV3101. All constructs were co-infiltrated with the MagnICON 5' cytosol module pCIH15879 and the integrase module pICH14011T. Control leaf material was produced using the 3' GFP-expressing construct pICH7410.

Production of plant material

N. benthamiana plants were propagated in a temperature-controlled greenhouse set to 28ºC and supplemented with 16-hour light (Phillips Son-T, Germany). Seeds were germinated on Jiffy-7 pellets (Jiffy International AS, Sweden) and supplemented with a 10:10:10 liquid fertilizer (Hortico, Australia) weekly starting 14 days after germination. Plantlets were inoculated at 28 days old using combinations of the integrase, 5' cytosol, and 3' construct

LTB, LTBK, LTBV, Zera™ΔLTB or GFP as per (Marillonnet et al. 2005). Leaf material was harvested 5 days post infiltration (DPI) for LTB, 5 DPI for LTBK, 6 DPI for LTBV, 13 DPI for Zera™ΔLTB, and 7 DPI for GFP. Leaves were snap frozen in liquid N_2, and freeze dried (Dynavac Model FD12, Australia) for 72-112 h with a maximum shelf temperature of 20°C. Material was milled in a bladed coffee grinder and sieved three times to standardize the particle size to 0.5-1.0 mm^2. Plant material was stored in airtight containers containing desiccant at -20ºC prior to analysis.

Capture ELISA and Western blot

TSP was extracted from 10 mg of dried leaf material by homogenization in 1 mL ice-cold extraction buffer PBST2IE (PBS containing 0.05% v/v Tween-20, 2 x Complete Protease Inhibitor Cocktail (Roche, Australia) and 100 mM ethylenediaminetetraacetic acid) with 2 x 2 mm tungsten carbide beads in a Mixer Mill (Qiagen, Australia) set at 28 Hz for 2 minutes. Samples were stored on wet ice for immediate use. GM1-binding LTB was quantified using Costar 9018 96-well microtitre plates (Corning Life Sciences, USA) coated with GM1-monoganglioside (Sigma-Aldrich, Australia) overnight in carbonate buffer (15 mM Na_2CO_3, 10.5 mM $NaHCO_3$) at 4ºC (note: unless otherwise noted, all ELISA incubations were performed at room temperature). Plates were washed and coated with 5% w/v skim milk powder (Sigma-Aldrich, Australia) in PBS. Subsequent steps including TSP determination via Bradfords Reagent (Bio-Rad, Australia) were performed as per (Pelosi et al. 2012). All quantifications were performed on triplicate TSP extractions. Partially denatured TSP samples were heated to 95ºC for 1 minute and Western blot conducted as per (Pelosi et al. 2011).

Immunohistochemistry and transmission electron microscopy

Agrobacterium cultures were syringe infiltrated into six-week old *N. benthamiana* plantlets and leaves harvested 4 DPI. Leaves were re-infiltrated with PBS, and 5 mm diameter leaf discs punched and let soak in PBS for 1 h. Samples were snap frozen using a high pressure freezing apparatus (Leica, Australia), transferred to liquid nitrogen, then substituted with

white resin in an automatic cryo-substitution system (Leica, Australia) for 7 days. Thin sections were prepared on gold grids, and labelled with chicken anti-cholera toxin antibody (Sigma-Aldrich, Australia) and donkey anti-chicken antibody conjugated to 24 nM gold particles (Fitzgerald, USA). Samples were positively stained with 5% uranyl acetate before analysis on a Philips CM120 BioTwin transmission electron microscope.

Simulated gastric fluid (SGF) assay

Leaf material from each of the LTB, LTBK, LTBV and Zera™ΔLTB samples was formulated with GFP-expressing control leaf material to a concentration of 500 μg/g. Dried leaf material was subjected to the SGF assay as per (Takagi et al. 2003). Briefly, 100 mg of plant material was placed in a 5 mL flat bottom glass scintillation vial. Control samples containing 50 μg of purified LTB in PBS, and 50 μg purified LTB mixed with GFP leaf material were also prepared immediately prior to assay. Fifty mL of SGF was made with 100 mg NaCl and 30 mg pancreatic pepsin from porcine intestine (Sigma-Aldrich, Australia) and heated to 37ºC. Warmed SGF was added to the leaf samples, and aliquots of the supernatant taken at 0, 1, 2, 5, 10, 30, 60 and 120 minutes following mixing. Samples were kept at 37ºC in an incubated orbital shaker set to 100 RPM. Aliquots of the supernatant were stopped for ELISA analysis by adding 50 μL of stop solution (0.2 M $NaHCO_3$, 10 mM EDTA, and 10 x Complete Protease Inhibitor Cocktail) and snap frozen in liquid nitrogen. Functional LTB was assayed by GM1-specific ELISA as previously described. LTB remaining in leaf cells was determined by preparing leaf material as described, and incubating with SGF for 30 minutes (Schwarz et al. 2002). A sample of the supernatant was removed, the leaf material resuspended in Stop Solution, and the remaining solution homogenized in a Mixer Mill as previously described. Equivalent volumes of supernatant and homogenate were assayed for pentameric LTB via GM1 ELISA.

Plant material formulation and mouse feeding trial

Dried leaf material from LTB, LTBK, LTBV and Zera™ΔLTB samples was mixed with control material expressing GFP to yield a final concentration of 500 µg/g. Each experimental group of 10 mice received 75 µg LTB per dose per construct, consisting of 150 mg of leaf material mixed with 1 mL of oil-based emulsion to aid palatability (Pelosi et al. 2011). Control groups (rL, n=6 mice) were fed 75 µg of purified LTB mixed with the oil-based emulsion immediately prior to feeding, or 150 mg of GFP leaf formulated with 75 µg of purified LTB (GrL, n=8 mice) and the oil emulsion, also mixed immediately prior to feeding. All procedures involving mice were performed in accordance with the Monash University School of Biological Sciences Animal Ethics Committee. Sixty-five female C57BL/6J mice were purchased from the Monash Animal Research Platform at 4 weeks of age, and housed in individual cages with water *ad libitum* and a standard diet of grain-based rodent pellets. Mice were fed the leaf formulations on day 0, 7 and 14. Prior to feeding, mice were fasted (water remained) for 16 hours overnight, and the dose administered 30 minutes prior to darkness and left overnight. Any remaining dose was removed the following morning and normal feed returned. Blood samples were taken on day 0, 14 via submandibular puncture during the trial, and via cardiac puncture at trial end. Blood samples were allowed to clot, and sera collected following centrifugation at 5,000 g. At trial end, mice were killed via CO_2 asphyxiation, and immediately dissected with the first 20 cm of the small intestine proximal to the stomach excised, sliced open-lengthways, and incubated shaking at 4ºC for 4-hours in 400 µL ice-cold extraction buffer (PBS, 0.1% Tween-20, 200mM EDTA, 10 x Complete Protease Inhibitor Cocktail). Gut samples were centrifuged at 4ºC to pellet remaining tissue and the supernatant retained for analysis. All biological samples were stored at -20ºC until analysis.

Serum and gut wash ELISA

The presence of LTB-specific IgG1 and IgG2a antibodies were assessed using a direct-bind ELISA antibody isotype kit (Sigma-Aldrich, Australia). Purified recombinant LTB was bound to Costar 9018 96-well microtitre plates overnight in PBS at 4ºC. Plates were blocked with

5% w/v skim milk powder, washed, and 1:100 dilution of serum in PBS applied to wells. All subsequent steps were performed as per manufacturers instructions, and detection was performed using an anti-goat IgG(fc)-HRP secondary antibody (Sigma-Aldrich, Australia). Samples were assayed in duplicate, and OD450 values reported as the mean value of separate plates. Gut wash samples were assayed for LTB-specific sIgA within the gut lumen as per (Pelosi et al. 2011). The LTB and CT neutralising assay was conducted as per (Nochi et al. 2007). Briefly, 5 ng of LTB or CT (Sigma-Aldrich, Australia) in 90 μL PBS was incubated with 10 μl mouse serum from day 28 of trial for 2-hours at 37°C before and applied to GM1-coated 96-well plates and processed as previous described for capture ELISA.

Statistical analyses

All analysis and graphing was performed using GraphPad Prism 6.0. Variation quoted in the text is for 1 standard deviation. Mean values of endpoint titre are the geometric mean of triplicate samples. All immunological data compared using 1-way ANOVA and corrected for multiple comparisons with Tukey-Kramer post-hoc test. A difference between treatment groups was considered significant if $p < 0.05$ following correction. Mice were considered to respond to vaccination if the OD450 value in gut wash samples was greater than the mean + 3 x SD of the control group G.

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FIGURES & FIGURE LEGENDS

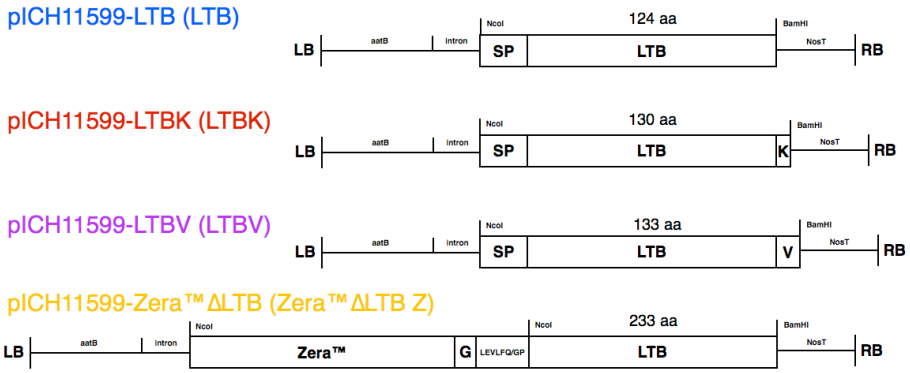

Figure 1 Diagram of DNA constructs used for subcellular LTB accumulation. The plant-optimised coding region for the native bacterial signal peptide (SP) was included in the native LTB construct (LTB), KDEL construct (LTBK) and vacuolar construct (LTBV). The SP was truncated in the Zera™ (Zera™ΔLTB) construct as the Zera™ fusion tag includes a functional plant signal peptide. LTB- plant optimised coding region of the B-subunit of the heat-labile toxin of enterotoxigenic *E. coli*, K- endoplasmic retention signal sequence SEKDEL, V- vacuolar transit peptide GNGLLVDTM, Zera™- protein body fusion tag, G-GGN[3] flexible linker, LEVLFQ/GP- Precission Plus™ cleavage tag, attB- *in planta* recombination site for MagnICON expression system, NosT- nopaline synthase terminator sequence, LB- left border of T-DNA, RB- right border of T-DNA, endonuclease sites *NcoI* and *BamHI* used in construction. Coding regions are to scale.

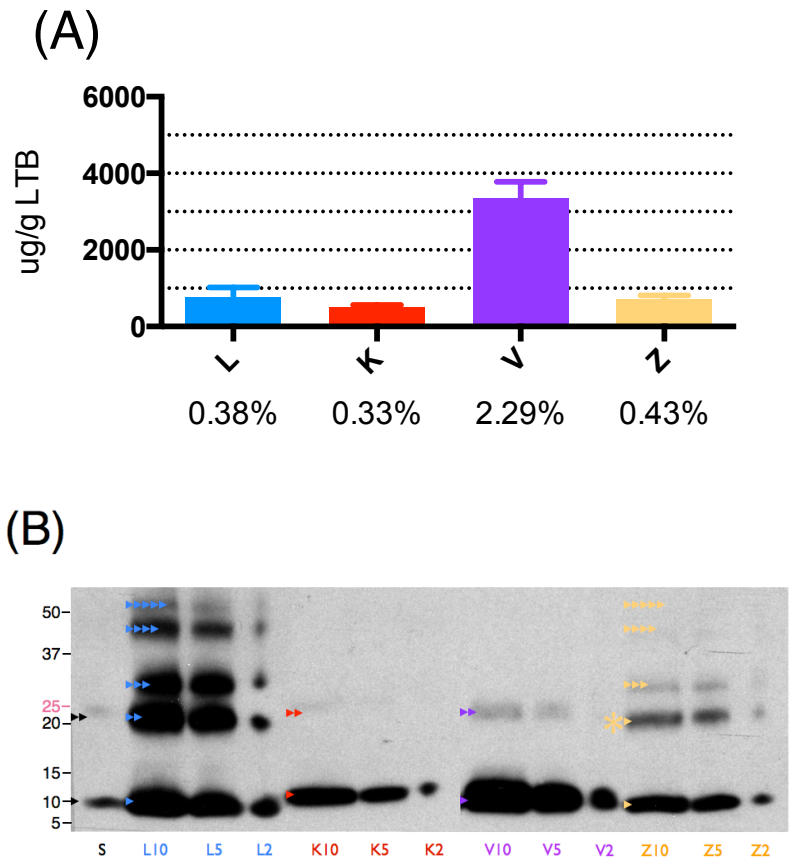

Figure 2 Characterization of LTB expressed in leaf cells. (A) GM1-based quantification of pentameric LTB in dry weight leaf material, with the percent of LTB in TSP indicated below chart. L- LTB, K- LTBK, V- LTBV, Z- Zera™ΔLTB. (B) Western blot of TSP probed with LTB-specific polyclonal antibody. S- 5 ng recombinant LTB standard. Lanes L10, L5 and L2, contain TSP from leaves expressing the LTB construct with 10, 5, and 2 µg of TSP, respectively. K10-2, V10-2, and Z10-2 are loaded in the same arrangement. Despite heat treatment, multiple bands are consistently observed for LTB and Zera™ΔLTB constructs, and to a lesser extent LTBK and LTBV samples. Putative multimers are indicated by ▶ monomer (11 KDa), ▶▶ dimer (20 KDa), ▶▶▶ trimer (28 KDa), ▶▶▶▶ tetramer (34 KDa), ▶▶▶▶▶ pentamer (50 KDa). * indicates the predicted molecular weight of the the full length Zera(TM)-ΔSPLTB fusion (18 KDa). No banding was observed on blots of TSP from control (GFP expressing) leaves across multiple experiments (not shown).

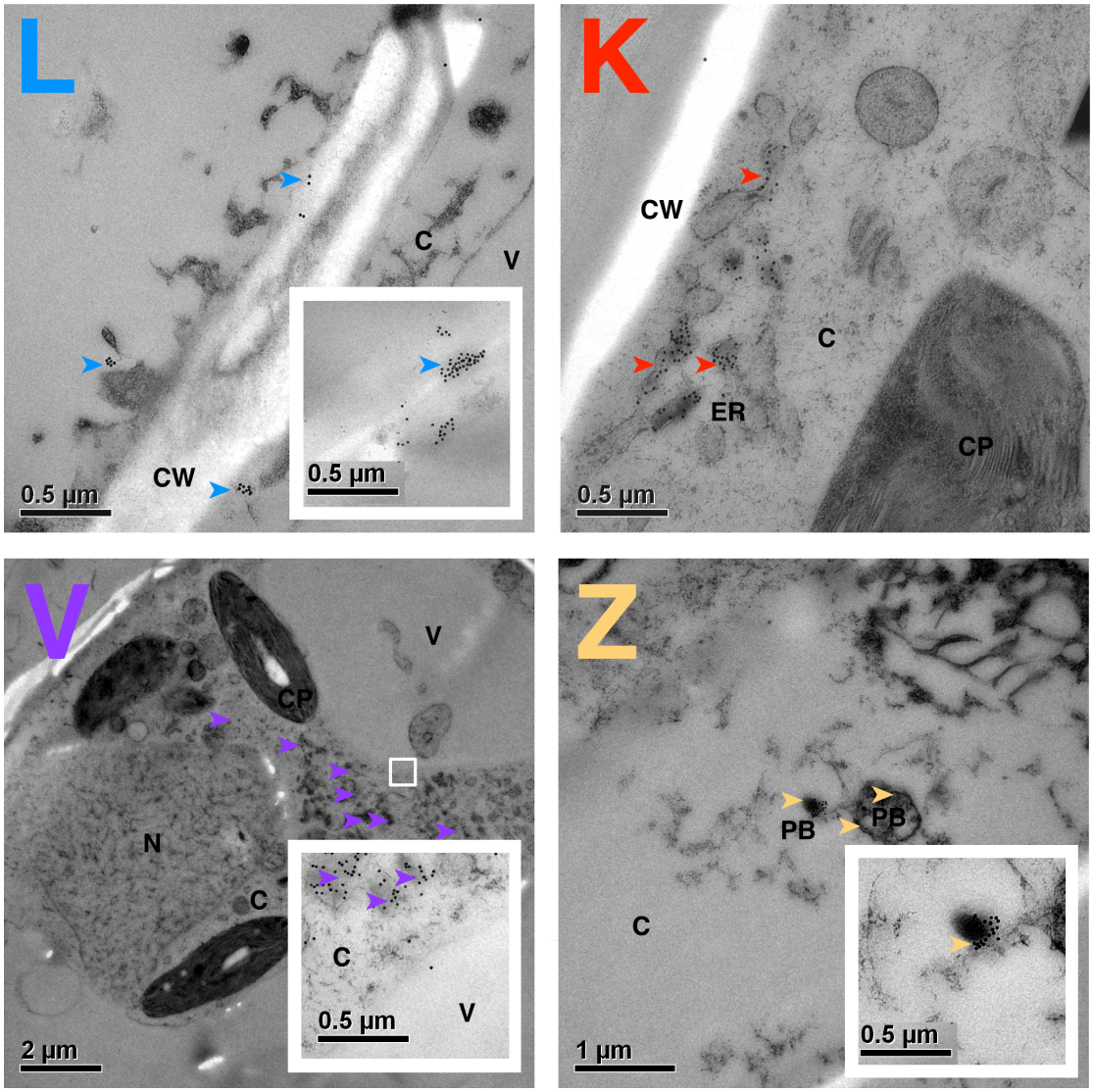

Figure 3 Immuno-gold labelling of LTB in leaf cells. LTB is labelled with chicken anti-cholera toxin primary antibody and secondary antibody conjugated to 24 nm gold particles. L- LTB, K- LTBK, V- LTBV, Z- Zera™ΔLTB. C-cytoplasm, CW- cell wall, ER-endoplasmic membrane associated structures, CP- chloroplast, V- central vacuole, N- nucleus, PB- protein body. No regular labelling pattern was observed in control leaf sections expressing GFP (not shown).

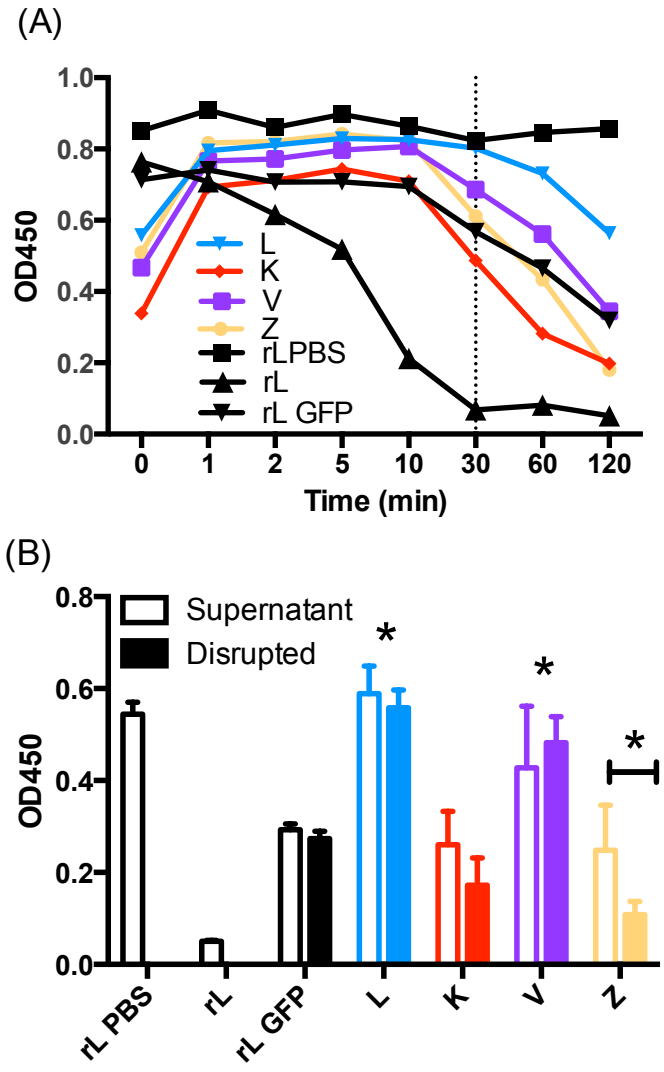

Figure 4 *in vitro* digestion assay (A) Time course of GM1-binding LTB recovered from the supernatant during *in vitro* SGF assay. Leaf samples with equal LTB content were suspended in SGF at 0 minutes, and the supernatant sampled over time. L- LTB, K- LTBK, V- LTBV, Z- Zera™ΔLTB. rL- purified recombinant LTB with no plant material, rL PBS- purified recombinant LTB in PBS rather than SGF, rL GFP- purified LTB mixed with control leaves. Figure representative of 2 separate experiments. (B) Quantification of pentameric LTB in supernatant (closed boxes) and released from cells following homogenization (open boxes) of samples incubated in SGF for 30 minutes. Data are for 3 separate experiments, error bars indicate SD.* indicates $p < 0.05$ where GM1-binding LTB was higher than the control rL GFP, and between supernatant and disrupted cell lysate for Z sample.

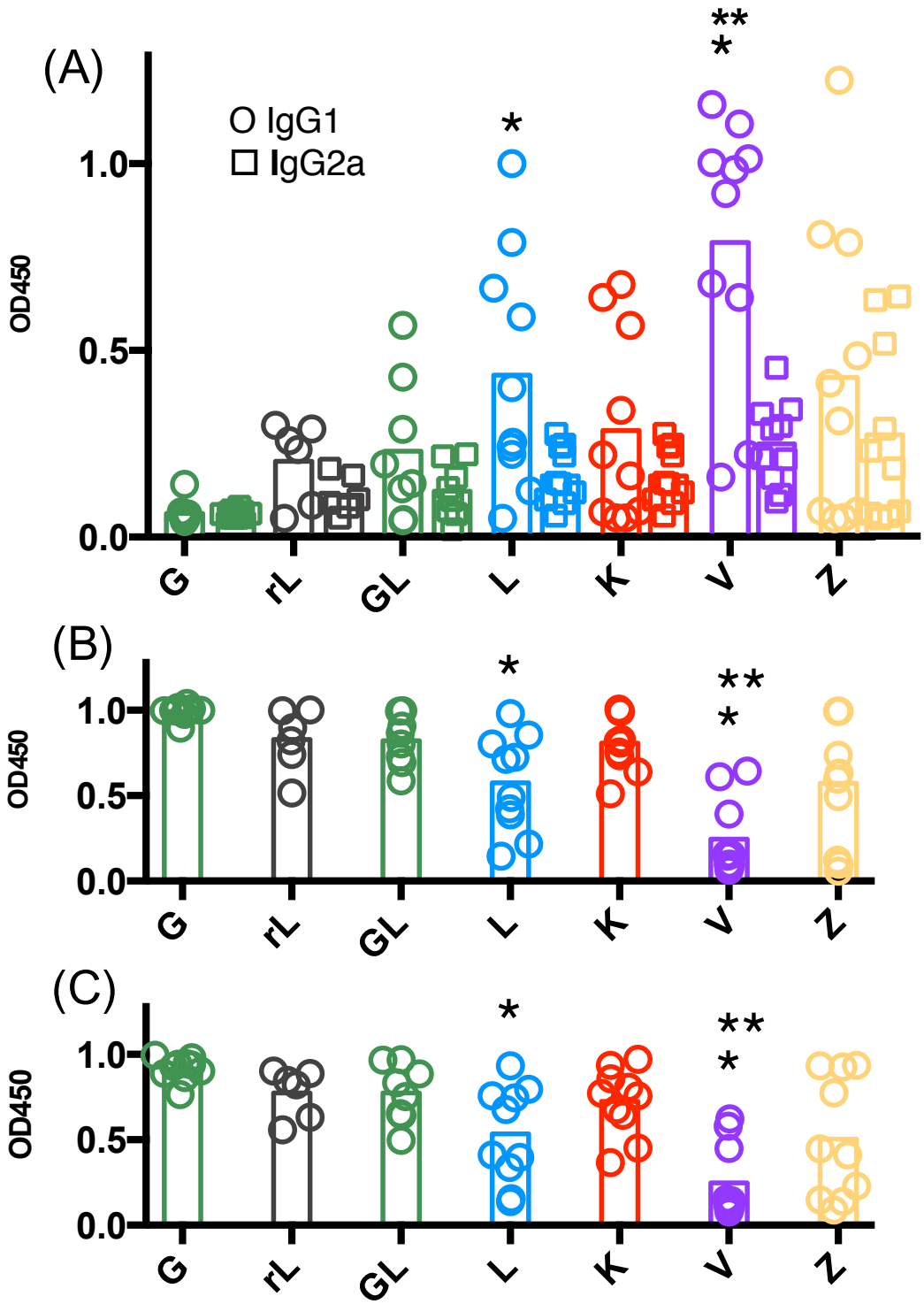

Figure 5 Humoral immune response (A) LTB-specific IgG1 and IgG2a in serum at day 28 of trial. (B) Neutralising capacity of humoral antibodies against (B) LTB and (C) CT. G- mice fed control leaves formulated with peanut mixture, rL- mice fed purified recombinant LTB formulated with peanut mixture, GL- mice fed purified LTB formulated with GFP-expressing leaf material and peanut mixture, L, K, V, and Z- mice fed plant material expressing LTB,

LTBK, LTBV, Zera™ΔLTB constructs, respectively. Each shape represents an individual animal, bars represent group mean. * indicates $p < 0.05$ compared to GrL or rL groups, ** indicates $p < 0.05$ compared to other groups fed LTB in leaf samples (1-way ANOVA adjusted for multiple comparisons).

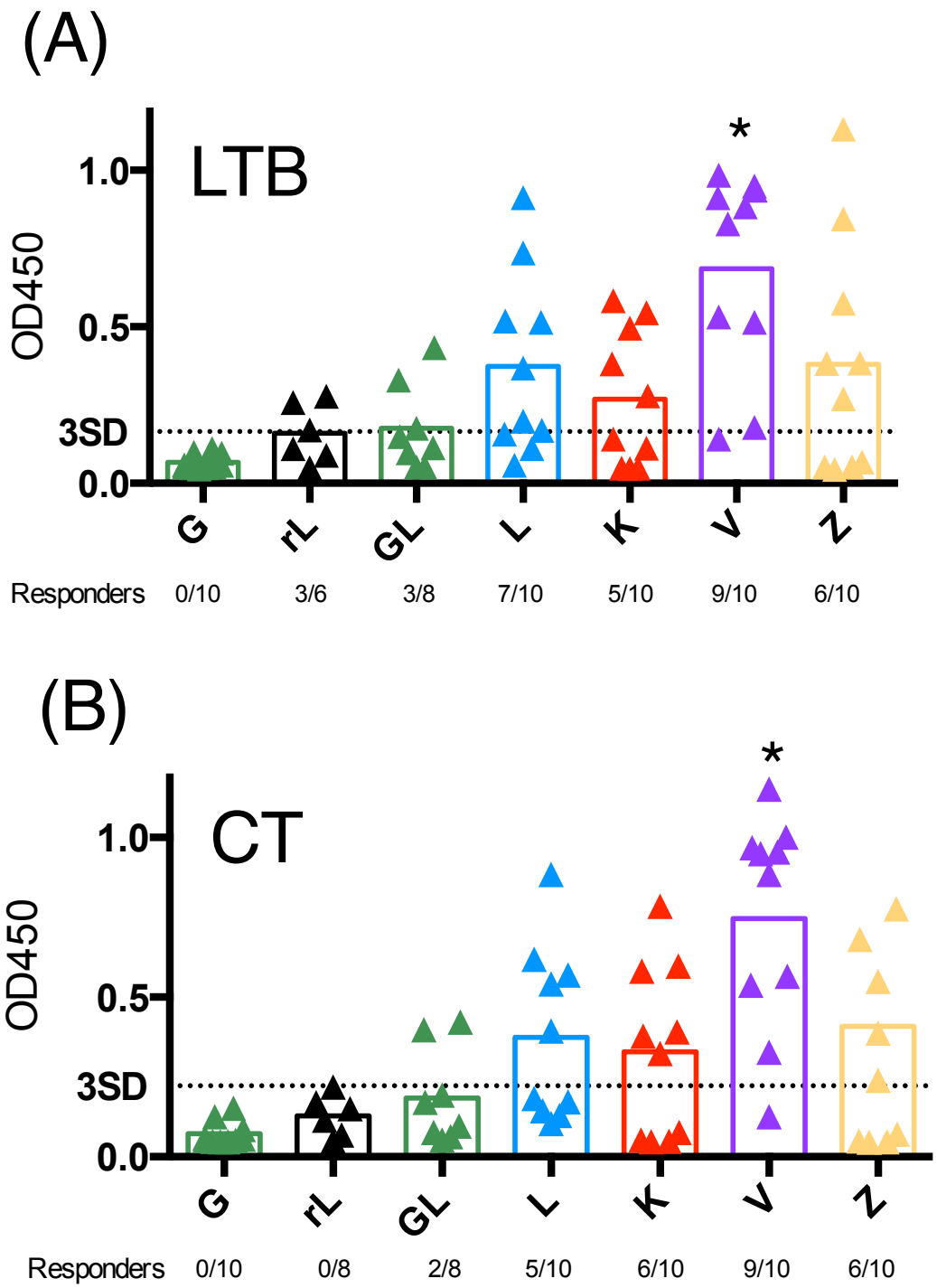

Figure 6 Mucosal immune response (A) LTB and (B) CT-capture IgA ELISA of intestinal mucosa wash samples at trial end. G- mice fed only leaf material expressing GFP formulated with peanut mixture, rL- mice fed purified recombinant LTB formulated with peanut mixture, GL- mice fed purified LTB formulated with control leaf material and peanut mixture, L, K, V, and Z- mice fed plant material expressing LTB, LTBK, LTBV, Zera™ΔLTB constructs,

respectively. Each data point represents assay of gut wash from an individual animal, bars represent group mean. * indicates $p<0.05$ compared to other trial groups (1-way ANOVA adjusted for multiple comparisons). Dotted line representing responder threshold is the mean of the negative control group (G) plus 3 SD.

SUPPLEMENTAL FIGURES & FIGURE LEGENDS

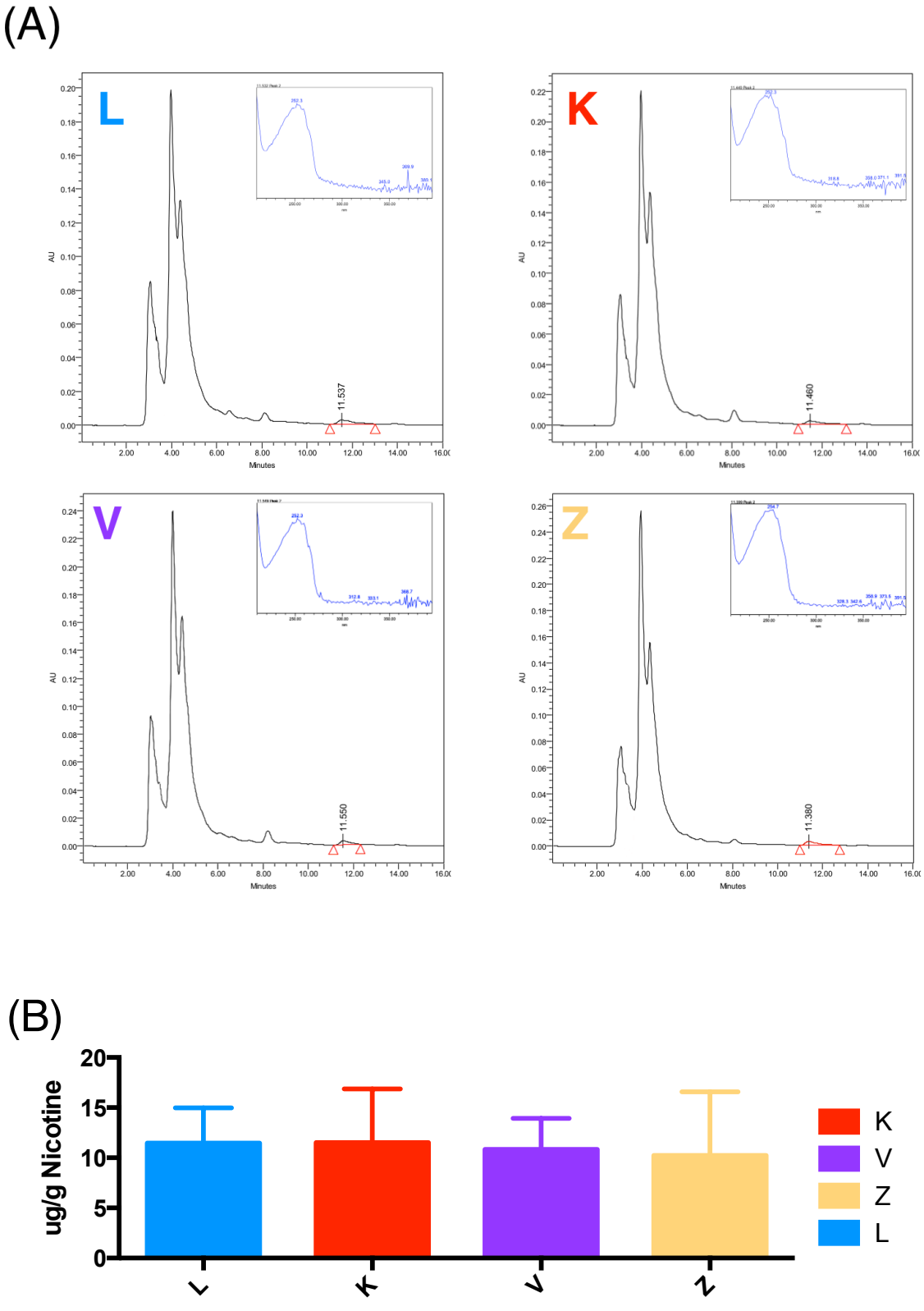

Figure S1. Pyrimidine alkaloid absorbance profile of *N. benthamiana* leaves expressing LTB, LTBK, LTBV, Zera™ΔLTB constructs. Plants were grown 4-6 weeks post germination and 20-70 plants per construct were infiltrated. Leaves were harvested and processed as

previously described. Pyradine alkaloids were extracted from dried leaf (DW) material via homogenization with a Polytron 1200 handheld homogenizer (Kinematica AG, Germany) in acidic 40% methanol, clarified by centrifugation, syringe filtered through a 0.45 μm membrane, and analysed on uBondPak C18 reverse phase column (Waters, Australia). 20 μL was injected onto the column into acidified 40% methanol mobile phase running buffer (pH 7.25) using a Waters 717 Auto Sampler (Waters Australia), and samples run at 1 ml/min using a Water 600 controller (Waters, Australia). Absorbance and spectral analysis was performed using a Waters 2996 Photodiode Array (Waters, Australia) and peak detection was automatically determined and manually confirmed using Water Empower Software (version 2.0, Waters, Australia). Nicotine was identified by the distinctive 252.2 nm absorbance (see insert) and was quantified against a known standard (Sigma-Aldrich, Australia). Additional pyrimidine alkaloids including nornicotine, anatabine and anabasine were tested, but not detected in any samples. Each sample was quantified in duplicate. (A) Representative sample absorbance versus elution time for metabolites from leaves expressing L- LTB, K- LTBK, V-LTBV, Z- Zera™ΔLTB constructs. (B) Nicotine levels were 11.47 μg/g DW in L-expressing leaves, 11.27 μg/g DW in leaves expressing the K construct, 10.80 μg/g DW in V-expressing leaves, and 10.23 μg/g in Zera™ expressing leaves. There was no significant difference between constructs ($p < 0.05$, students t-test). n=6, error bars indicate SD.

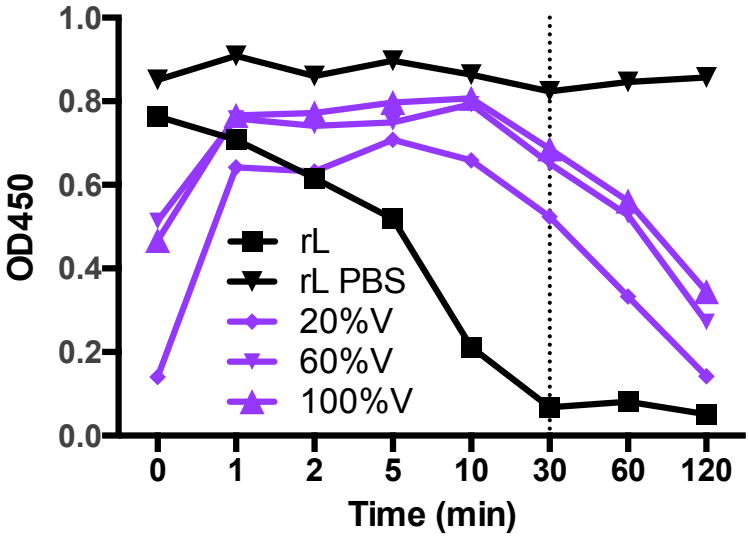

Figure S2. Release kinetics of different concentrations of LTBV leaf diluted with leaf material not containing LTB. LTBV leaf material was mixed with control leaf material expressing the unrelated GFP protein in a ratio of 100%-0% LTBV:GFP (100% LTBV) , 60% LTBV:GFP (60% LTBV), and 20% LTBV:GFP (20% LTBV) and the SGF digestion assay performed as described. Control samples of purified LTB in PBS (rl PBS) and in SGF (rL) are shown as controls.

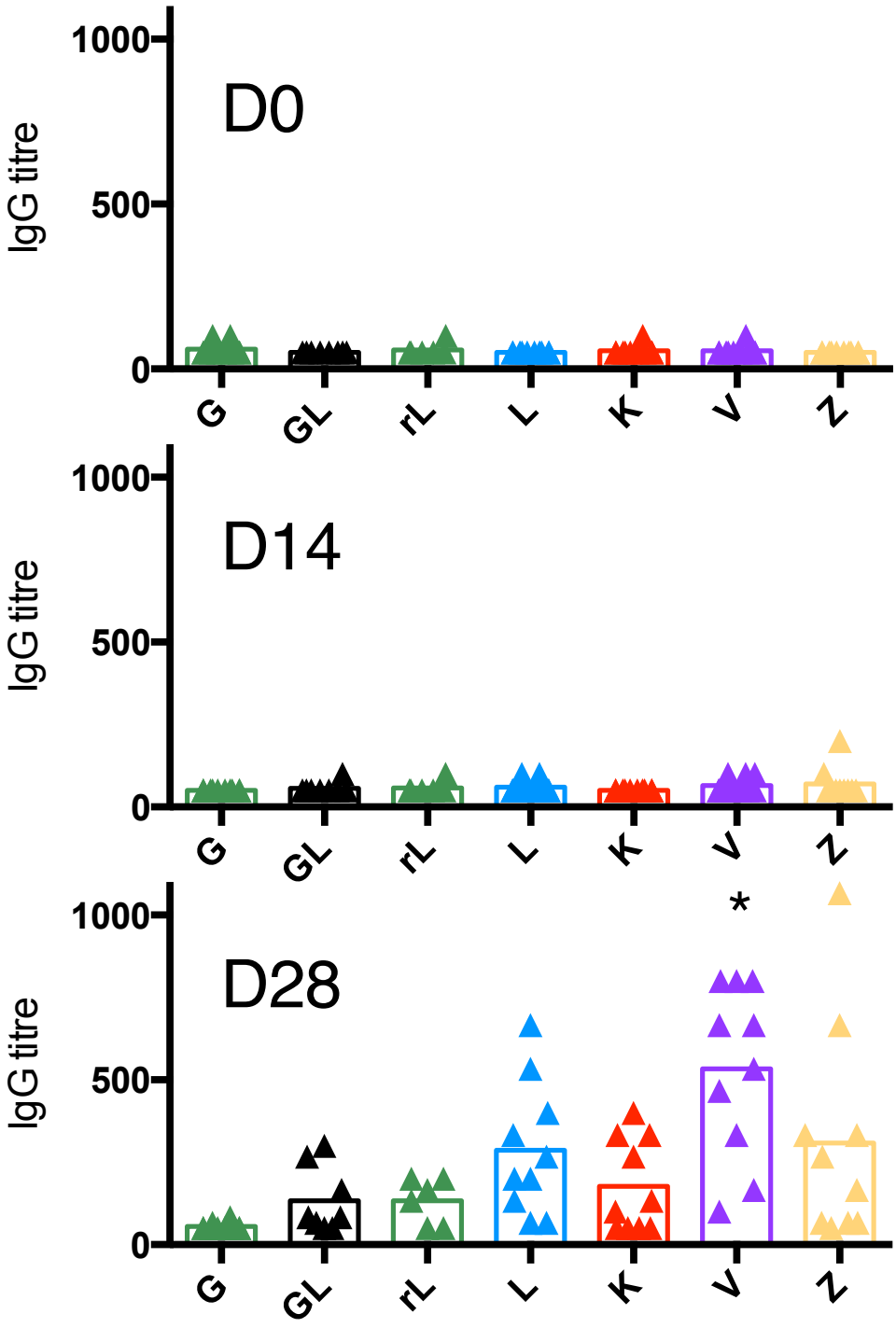

Figure S3. Humoral LTB-specific IgG endpoint titre at baseline (D0), day 14 (D14) and end of trial (D28). Endpoint titre was determined by ELISA. Initial serum dilution was 1:100 in PBS. G- mice fed only leaf material expressing GFP formulated with peanut mixture, rL- mice fed purified recombinant LTB formulated with peanut mixture, GL- mice fed purified LTB formulated with control leaf material and peanut mixture, L, K, V, and Z- mice fed plant

material expressing LTB, LTBK, LTBV, Zera™ΔLTB constructs, respectively. Each data point represents an individual animal where the reciprocal endpoint dilution was greater than OD450 0.1. If the starting dilution OD450 was less than 0.1, a titre of 50 was assigned. Day 28 samples repeated in triplicate, and the geometric mean shown. * indicates a humoral IgG titre where $p < 0.05$ compared to all other groups (adjusted 1-way ANOVA).

4. THE INTRINSIC SAPONIN OF TOMATO, α-TOMATINE DOES NOT POTENTIATE ORAL IMMUNOGENICITY OF NORWALK VIRUS-LIKE PARTICLES

4.1 Introduction

As many mucosal adjuvants are plant derived, it is possible that the intrinsic compounds of the plant cell may enhance the immunogenicity of co-delivered antigens. The data presented in Chapter 2 and 3 shows that the expression system and subcellular localisation of a bacterial antigen delivered in plant cells affects mucosal immunogenicity. However, it is likely that a huge range of other plant-specific factors might alter the mucosal immunogenicity of plant-made antigens. In order to focus on a single plant-specific factor amenable to optimisation, we investigated whether the endogenous metabolite α-tomatine of *Solanum lycopersicum* fruit influenced the mucosal immunogenicity of co-delivered Norwalk virus capsid protein (NVCP).

Many characteristics of tomato fruit change during ripening including cell wall biosynthesis, proteolytic enzymatic activity, and expression of recombinant transgenes (see 1.7.2). In addition to the physiological and transcriptional changes during ripening, there is a shift in the metabolome of the fruit throughout the ripening process that changes many of the bioactive metabolites including the concentration of saponin-like adjuvant α-tomatine. α-tomatine has been shown to potentiate the cellular immune response to systemic administration of antigens, but has not been characterised for its ability to potentiate the mucosal immune response.

To begin this investigation, a series of experiments were designed to orally vaccinate mice with transgenic fruit containing different concentrations of α-tomatine. Before beginning this work, we formed a collaboration with the Victorian Department of Primary Industries to develop a high-performance liquid chromatography and mass spectrometry (HPLC-MS) analytical method to isolate and quantify α-tomatine in freeze dried tomato fruit (Figure 4-1).

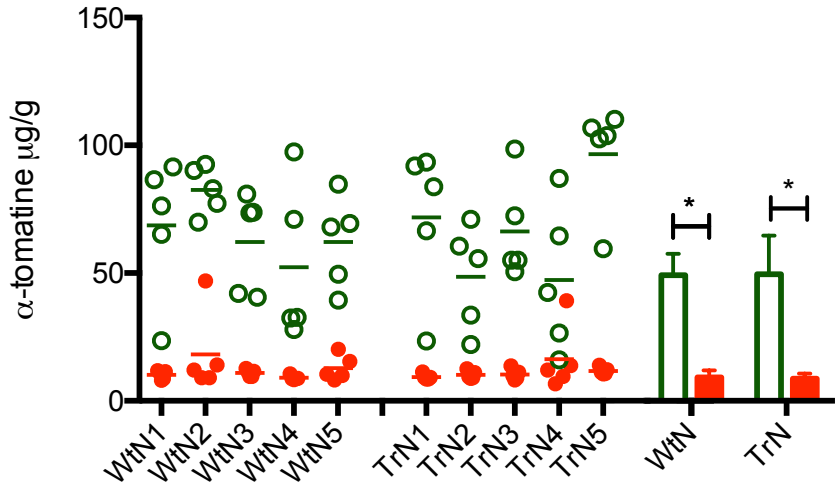

Figure 4-1 α-tomatine concentration in fruit from wild type plants (Wt1-5) or transgenic plants (Tr1-5). Each data point represents the α-tomatine concentration of a single unripe (green) or Stage 3 (S3; red) fruit. Horizontal bars represent the mean α-tomatine concentration from either stage of ripening. The four final columns summarise the α-tomatine concentration for all wild type (wtN) and transgenic (TrN) fruit represented to the left. Errors bars are SD, and significance shown with * ($P < 0.05$, unmatched t-test) between ripening stages. Experimental methods are included in the following manuscript (4.3).

Interestingly, several studies have shown that α-tomatine concentration is influenced by the presence of agronomic and antigenic transgenes (see 1.7.2), yet in this study we observed similar concentrations in wild type isogenic plants and those expressing the NVCP antigen and selectable marker (*Kan*). We observed a significant decrease in α-tomatine during ripening in both wild type and transgenic fruit (**Figure 4-1**). To investigate the relationship between α-tomatine and transgene accumulation in individual fruit, the concentrations of both analytes were quantified in five fruit from five separate plants. No significant correlation between transgene and α-tomatine accumulation was observed in fruit at either stage of ripening (Figure 4-2), effectively uncoupling the concentrations of α-tomatine and the NVCP antigen. Indeed, the concentration of α-tomatine in individual unripe fruit was highly varied while the concentration of α-tomatine in S3 fruit was generally low across the S3 fruit sampled (Figure 4-2).

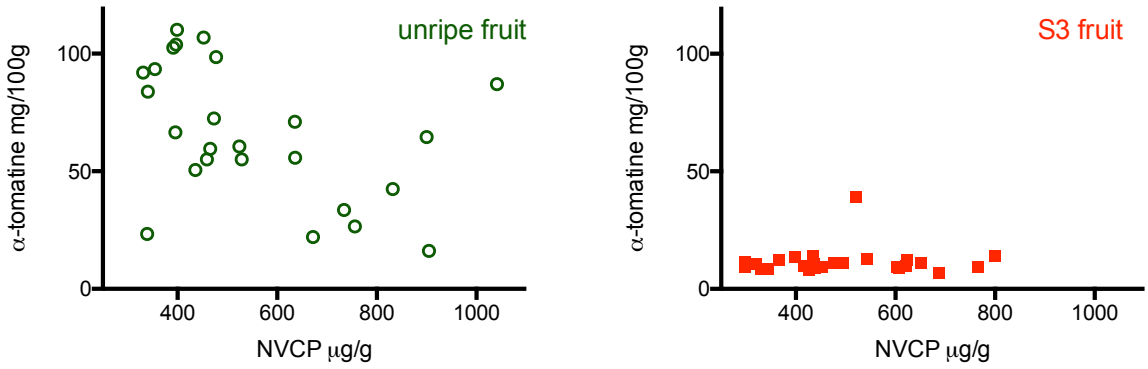

Figure 4-2 The relationship between NVCP protein and α-tomatine accumulation in individual fruit determined by ELISA and HPLC-MS. Each mark represents the concentrations of α-tomatine and NVCP in a single unripe (open green circles) or S3 fruit (red squares). Experimental methods are included in the following manuscript (4.3).

The accumulation, stability, post-translational modification, and ability of the NVCP protein to form virus-like particles (VLPs) was also investigated. The ripening stages 'unripe' and S3 were chosen as these stages show a significant separation in α-tomatine concentration (high when unripe, and low at S3, **Figure 4-1**) while maintaining a similar concentration of NVCP. To determine the impact of ripening stage and intrinsic α-tomatine concentration on the mucosal immune response, two studies in C57BL/6J mice were conducted. Lyophilised fruit from each ripening stage was formulated with or without exogenous purified α-tomatine, and three doses fed to mice in weekly intervals. These studies measured the antigen-specific humoral (serum IgG titre and IgG1/IgG2a ratio) and mucosal sIgA response to oral vaccination with both stages of ripening. Due to the surfactant quality of α-tomatine, additional groups of mice in the second study were administered fruit formulated with the surfactant Tween-20 to approximate the critical micelle concentration of exogenous α-tomatine in the formulations. The concentration of α-tomatine or surfactant did not change the immunogenicity to Norwalk VLPs (NVLPs) and these data were not included in the manuscript.

The evidence generated from these experiments suggests that despite the characterisation of α-tomatine as a systemic adjuvant, at least in this vaccination model of NVLPs delivered in tomato fruit, α-tomatine does not influence the mucosal immune response.

4.2 Statement of contribution

The two authors of this paper are Robert P Shepherd (RPS), and Amanda M Walmlsey (AMW).

In the case of Chapter 5: "The intrinsic saponin of tomato, α-tomatine does not potentiate oral immunogenicity of Norwalk virus-like particles", the nature and extent of contribution to the work was the following:

- All planning, execution and analysis of experiments and writing of the manuscript by RPS. The extent of this work was 80%.
- Research conceived and manuscript reviewed by AMW. The extent of this work was 20%.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

09 March 2016

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Signature Date

Robert P Shepherd (candidate)

Mar 2016

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David R Symth

4.3 Manuscript

This manuscript is formatted for the Journal of Negative Results in Biomedicine

The intrinsic saponin of tomato, α-tomatine does not potentiate oral immunogenicity of Norwalk virus-like particles

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ABSTRACT

Background

Plants are capable of producing recombinant antigenic proteins from a variety of human and animal pathogens, yet no system using plants for both expression and oral delivery has moved beyond early-stage clinical trials. The difficulty of inducing a consistent and robust immune response to the co-delivered antigen is one one of the key impediments to further clinical development. In this study, we investigate whether the adjuvant glycoalkaloid of *S. lycopersicum* fruit, α-tomatine, alters the immunogenicity lyophilised transgenic fruit containing recombinant Norwalk virus-like particles (NVLPs) fed to C57BL/6J mice.

Results

Wild type and transgenic plants expressing the Norwalk virus capsid protein (NVCP), and plants were grown under controlled greenhouse conditions and fruit harvested at two stages of ripening. The NVCP spontaneously assembled into ~38 nm NVLPs in fruit, and we observed no significant difference in NVCP accumulation between ripening stages (~200 μg/g dry weight). Unripe fruit accumulated approximately 5-fold higher α-tomatine concentrations than stage 3 fruit (~650 mg/100 g versus 120 mg/100g) in both transgenic and wild type lines. Mice were fed three, weekly doses of 50 μg NVCP in a total of 300 mg lyophilised tomato fruit. An antigen-specific serum IgG and mucosal IgA response was observed in 20-50% of mice, but no significant variation in the rate of seroconversion or magnitude of response was observed between mice fed fruit from different ripening stages. Nor was there a difference in immunogenicity when fruit was formulated with exogenous, purified α tomatine.

Conclusion

These results indicate that the concentration of intrinsic or exogenous α-tomatine does not change the oral immune response to a co-delivered antigen (NVCP) in tomato fruit, and is an unlikely solution to the challenge of improving immunogenicity to plant-made vaccines.

KEYWORDS

Plant-made vaccine, metabolite, mucosal adjuvant, *Norovirus*

BACKGROUND

Advancements in protein expression technology using plant cells has led to the recent licensure of the first human therapeutic protein manufactured *in planta* [1] and several purified therapeutic proteins produced in plant cells have entered clinical testing [2]. Despite these advancements, arguably the most significant advantage plant cells have over alterative expression systems is their intrinsic capacity as an expression host and delivery system [3, 4]. Indeed, evidence has shown that lyophilised plant cells can effectively deliver low-molecular weight therapeutic proteins into the bloodstream following oral administration [5-9]. Plant cells have also been suggested as effective oral delivery vehicles for vaccine antigens [10-12], and eight early stage clinical trials have shown plant made and delivered antigens are safe and well tolerated [13]. However, initiation of new clinical studies have stalled due to the many unknown and uncontrolled factors associated with using plants as vaccine delivery vehicles, including heterogenetic seroconversion, low magnitude of response, risk of aberrant immune polarisation or tolerance, and the need to manufacture plants under Good Manufacturing Practice (GMP)-based quality systems [13, 14]. In an effort to better understand the immunological aspects of *in planta* antigen delivery, we have investigated the influence of the endogenous saponin-like adjuvant glycoalkaloid of tomato fruit, α-tomatine, on the oral immunogenicity of recombinant NVLPs expressed and delivered in transgenic lyophilised tomato fruit.

Norwalk virus is a member of the Norovirus (NoV) genus in the *Caliciviridae* family, and causes acute gastroenteritis [15, 16]. In the United States, NoV is the leading cause of medically attended acute gastroenteritis in children under 5 years [17]. Moreover, NoV infection is responsible for up to 800,000 deaths annually in low and middle income countries [18-21]. At present, there is no vaccine for the prevention of NoV-related diseases [22]. Most vaccine candidates have utilised the capacity of NoV capsid proteins to self-assemble into VLPs [23].

To date, NVLPs have been successfully expressed in plant systems including stable lines of *Nicotiana tabacum* leaves [24], *Solanum tuberosum* tubers [24-26], and *S. lycopersicum* fruit, [27, 28], as well as transient viral-based expression in *Nicotiana benthamiana* leaves [26, 29-33] and *Lactuca sativa* leaves [34]. NVLPs delivered in plant cells are immunogenic, although NVLPs in potato tuber induce only low humoral and mucosal seroconversion in mice [24]. Human volunteers administered NVLPs expressed in potato tuber also show a low but detectable serum anti-NoV IgG and IgM response, but this immunogenicity was far lower than infection with live NoV [25]. A later study showed that NVLPs delivered in tomato fruit increased immunogenicity, and the authors suggest the improved response may involve the intrinsic adjuvant in tomato fruit, α-tomatine [28]. Saponins are a broad group of plant-derived glycoside or triterpene immunomodulating adjuvants, characterised for their ability to induce balanced T_h1/T_h2 immune responses following systemic or mucosal administration in animals and humans [35-38]. Moreover, α-tomatine in the leaves and unripe fruit of *S. lycopersicum* [39, 40] is a systemic adjuvant with immunological properties similar to other saponins [38, 41-45].

The aim of this study was to determine if α-tomatine in tomato fruit potentiates the oral immunogenicity of a plant-made vaccine candidate. To do this, we expressed and characterised NVLPs in fruit from different ripening stages with different concentrations of α-tomatine, and fed the formulations to mice with varying levels of exogenous purified α-tomatine. We report no variation in the NVCP-specific immune response to differing levels of α-tomatine.

RESULTS

The NVCP protein accumulates at high concentration in unripe and S3 tomato fruit.

The capsid of NoV has previously been expressed in stable, transgenic lines of *S. lycopersicum* and shown to form orally immunogenic VLPs. [27, 28] In this study, fruit from wild type and transgenic plants were harvested when unripe (mean mass 15.1±1.9 g and diameter 33.8±1.2 mm) or at the S3 stage of development (mean mass 80.4±18.9 g and diameter 58.5±5.2 mm). No NVCP was detected in fruit from wild type plants (Figure 1A), and no significant difference in NVCP accumulation was observed in unripe fruit (189.4±22.4 μg/g dry weight) and S3 fruit (219.3±27.6 μg/g dry weight) from transgenic plants (Figure 1A). Western blot analysis of the total soluble protein (TSP) extract from wild type fruit showed no signal (Figure 1B, wt unripe and wt S3), but both unripe and S3 fruit samples (Figure 1B, NV unripe and NV S3) showed accumulation of a 58 kDa protein comparable with the reference NVCP protein produced in insect cells (Figure 1B STD). Transgenic fruit from both ripening stages also showed 28 and 65 kDa bands not present in the purified standard. To investigate if the larger band was a glycosylation-variant of the 58 kDa protein, TSP from unripe fruit was treated with peptide-N-Glycosidase F to cleave N-terminal glycans but no decrease in the intensity of band-shift was observed (Figure 1B, NV unripe PNGase F).

NVCP in unripe and ripe fruit are the same density as insect-derived NVLPs and form 38 nm VLPs

To ensure capsid proteins correctly folded and formed bona fide VLPs, analytical centrifugation and electron microscopy were used to confirm the presence of NVLPs. TSP was extracted from unripe and S3 fruit and prepared on a 10-60% discontinuous sucrose gradient. Transgenic fruit at both stages of ripening showed similar sedimentation to insect-derived NVLPs (iNVLPs), with a single peak observed corresponding to the reference VLPs produced in insect cells (Figure 2). To further confirm that NVCP in tomato fruit forms NVLPs, the peak fraction (Figure 2, F13) was concentrated and negatively stained before

being visualised by electron microscopy. Distinct, ~38 nm VLPs were observed in the peak sucrose gradient fraction from both ripening stages, whereas these structures were not observed in other fractions (fraction 4, not shown).

α-tomatine accumulation is higher in unripe fruit than S3 fruit, but similar between untransformed and transgenic fruit of the same ripening stage

To determine the concentration of α-tomatine in unripe and S3 fruit, alkaloids were extracted and separated on a C18 column. The α-tomatine fraction was the largest glycoalkaloid peak identified in all samples (Figure 3B), and was unambiguously identified by its MS spectrum (not shown). While NVCP concentration was similar between ripening stages, the α-tomatine concentration differed significantly. Wild type isogenic plants accumulated 655.9±111.6 mg/100 g dry weight α-tomatine in unripe fruit, and 122.2 mg/100g dry weight in S3 fruit. Similarly, 661.2±201.4 mg/100g dry weight α-tomatine accumulated in unripe transgenic fruit, and 115.7±27.56 mg/100g in S3 transgenic fruit. There was no significant difference between α-tomatine concentration in wild type or transgenic fruit at the same ripening stage (t-test, unripe $p=0.96$, S3 $p=0.76$).

Endogenous α-tomatine of tomato fruit does not potentiate the immune response to oral NVLPs

To determine if α-tomatine influenced the oral immunogenicity to NVCPs, wild type and transgenic fruit material with known concentrations of α-tomatine were mixed to a final concentration of 166 μg/g NVCP to provide a consistent dose of 50 μg NVCP in 300 mg total plant mass (Table 1). Mice ate greater than 90% of all doses with no significant difference in the mass of test diet consumed between groups or in stability of NVCP during the feeding period (not shown). No anti-NoV response was observed at day -7 or day 14 (not shown) in either trial, and all data presented is for samples taken at trial end on day 28. No anti-NoV serum or mucosal antibodies were observed in mice administered standard feed pellets or wild

type fruit (Figure 4A, 4B, and 4C, pellet, wt unripe, wt S3). Two mice (2/10) administered unripe transgenic fruit showed anti-NoV humoral IgG and mucosal IgA response, and three mice (3/10) administered S3 fruit responded (Figure 4A, 4C, NV unripe, NV S3). To determine if formulation with exogenous α-tomatine affected the immune response, 1.63 mg of purified α-tomatine was added to a formulation of S3 fruit so as to provide the same total concentration of α-tomatine in the unripe fruit. Six mice (6/10) in this group showed humoral and mucosal response to vaccination when mixed with purified α-tomatine. Despite the different number of responders, no significant differences were observed between the mean serum IgG titer or mucosal IgA response between groups administered transgenic fruit (one-way ANOVA, IgG $p=0.84$ and IgA $p=0.34$). To determine the T_h response, serum IgG1 and IgG2a antibodies were quantified by ELISA. All groups responded with higher IgG1 than IgG2a antibodies.

Exogenously formulated α-tomatine does not potentiate the immune response to NVLPs

While not statistically significant, there was higher seroconversion in the group formulated with exogenous α-tomatine compared to other groups. To determine if the exogenous α-tomatine influenced this increased seroconversion, a second study was conducted with the same formulations of fruit material mixed with escalating concentrations of purified α-tomatine. In this second study, control groups administered wild type fruit showed no NoV-specific humoral or mucosal response (not shown). Three mice (3/10) fed unripe transgenic fruit and five (5/10) mice fed S3 transgenic fruit seroconverted with anti-NoV specific humoral IgG antibodies and mucosal IgA antibodies. Increasing the concentration of purified α-tomatine to 3 times (5.94 mg) and 5 times (9.90 mg) that present in unripe fruit failed to alter the seroconversion, magnitude or dominant isotype. Four (4/10) mice administered both 3 and 5-fold α-tomatine concentrations showed seroconversion and a mucosal IgA response. As observed in the first study, IgG1 was the dominant humoral antibody isotype in all mice that responded to vaccination. Also similarly to the first study, no significant difference in the

humoral IgG titer or mucosal IgA response was observed between any of the treatment groups (one-way ANOVA, IgG $p=0.67$ and IgA $p=0.93$).

DISCUSSION

Plant-based protein expression platforms have many advantages compared to other systems, including the ability to produce complex post-transcriptionally modified proteins, relatively low expenditure for capital and operational costs, and ease of scale-up [46]. However, the use of plants solely as production platforms ignores the intrinsic advantage of plants cells to also deliver antigens. One unknown aspect of using plant cells as delivery vehicles, is whether the adjuvant compounds intrinsic to the expression system influence immunogenicity. Indeed, many well-characterised oral adjuvants such as lectins, saponins, and polysaccharides are intrinsic to plants [47], and several pre-clinical examples show that formulation with exogenous saponins improves the immunogenicity of plant-expressed and delivered vaccines [48-50]. Despite being an effective systemic adjuvant [38], we report no significant effect of α-tomatine on the heterogeneity or magnitude of the immune response to NVLPs when delivered as part of the endogenous metabolome of tomato fruit, or when purified α-tomatine is added to the formulation.

To ensure NVLPs in fruit at different ripening stages were similar, we first characterised the accumulation of NVCP during ripening. The NVCP yield was not significantly different in the two stages of ripening chosen for this study, but NVCP decreased in fruit harvested at later stages of ripening (not shown) consistent with the known decrease in transcription of genes driven by the 35S viral promoter during ripening [51]. One of the practical limitations of feeding fruit material to mice is the limit of the total biomass mice will eat overnight [28]. To ensure the dose of plant-material was small enough for mice to consume in a 12-hour period, relatively early stages of fruit ripening were chosen so that the expression was sufficiently high that at least 50 μg of NVCP could be delivered in 300 mg of fruit material. Western blot analysis revealed a similar concentration of the putative 58 kDa form of NVCP in both stages of ripening, yet additional ~65 kDa and ~25 kDa bands were present in the transgenic plant lines that were not visible in the purified 58 KDa insect-derived standard.

The identity of these bands is unknown, and they are not present in western blots of NVCP expressed in other plant species such as potato, tobacco and lettuce [24, 29-32, 34]. Interestingly, neither of the previous reports of NVCP expression in tomato presented a western blot analysis of the TSP [27, 28], and therefore it is difficult to determine if this banding pattern is related to expression in tomato or to this transgenic line. While post-translational modification of the NVCP sequence is not likely due to the lack of signal peptide required for entry into the endoplasmic reticulum, there are 4 putative N-glycosylation sites along the NVCP polypeptide. To ensure that the bands were not related to aberrant N-glycosylation, an attempt was made to remove any N-linked glycans with PNGase F. This did not decrease the intensity or shift the additional bands, indicating that N-glycosylation was not involved in the ~65 kDa band or the smaller ~25 kDa band. N-terminal sequencing is planned to reveal the sequence of these unexpected fragments.

Regardless of the presence of these aberrant bands on the western blot, the NVCP protein formed VLPs of a similar density to the reference iNVLPs when passed through a 10-60% sucrose gradient. These VLPs were ~38 nM, similar to other reported expression of Norwalk VLPs expressed *in planta* [24, 27, 29, 30, 32, 34]. A previous report identified 23 nm and 38 nm NVLPs in tomato fruit [28], but the smaller 23 nm VLPs were not observed at either stage of ripening in this study via sedimentation or TEM (Figure 2). Repetition of the sedimentation analysis (not shown) indicated that the concentration of NVCPs as VLPs was similar at both stages of ripening, and as such there does not appear to be a yield or quality variation in the NVLPs at either stage or ripening using the analytical methods presented in this study.

While the NVCP yield and NVLP quality did not differ significantly between ripening stages, the α-tomatine concentration decreased rapidly as fruit ripened. The decrease has been observed in other wild type cultivars and transgenic lines [52], where synthesis of α-tomatine is highest during early fruit development, and decreased synthesis and increased degradation of α-tomatine reduces the total concentration during the ripening process [53]. The

concentration of α-tomatine does not appear to differ between the pNV210-2 line and the isogenic wild type line at the same stage of fruit maturation. (Figure 1A). This is in contrast to other reports that α-tomatine is decreased in field-grown transgenic lines expressing aminoglycoside 3'-phosphotransferase II [52], or increased in greenhouse-grown transgenic lines expressing a tuberculosis antigen complex (C.A. Penney, unpublished). The cause of the variation in α-tomatine between different transgenic lines is not clear, but α-tomatine also varies in response to environmental and crop-management practices [54], and there are clearly pleotropic influences on both the accumulation and degradation of this metabolite.

In contrast to previous studies that have shown an improved immune response to orally-delivered plant formulations containing exogenously added saponins [48-50], we did not observe any significant effect on the humoral or mucosal immune response to varying concentrations of α-tomatine. As the dose response to oral delivery of NVCP in tomato may be masked at higher doses (192 μg NVCP [28]), a relatively low dose of 50 μg was chosen to investigate the influence of α-tomatine. Accordingly, we observed a similar seroconversion and magnitude of the humoral and mucosal response to 50 μg NVCP as previously when mice were fed 9-45.6 μg NVLPs in tomato fruit [28]. In our study, between 2 and 6 mice seroconverted in each group, but no trend or significant difference was observed in the humoral IgG titer or mucosal IgA response between groups. We observed concordance between humoral and mucosal response, with an anti-NoV mucosal IgA titer only observed in mice that also showed a humoral response (Figure 4). However, as observed in previous studies [24, 25, 28], there were mice in several groups that seroconverted to IgG but did not have a detectable mucosal IgA response. The signalling pathways leading to induction of humoral but not mucosal response to NVLPs are unclear.

Systemic and oral delivery of saponins from *Quillaja saponaria* induce a T_h1 inflammatory response in a dose-dependent manner, with increased IgG2a class switching and cytotoxic T lymphocyte (CTL) activation with increasing dose of saponins [55]. A similar dose-dependent

pro-T_h1 activation is also observed when α-tomatine is administered systematically [37]. However, in this study we noted no change to the ratio of serum IgG1/IgG2a isotypes at any dose of α-tomatine. This is despite mice in the "NV unripe 5tom" group being fed a total of 9.9 mg of α-tomatine per dose, approaching the acute oral LD50 (500 mg/kg), and approximately half the sub-acute LD50 (800-1000 mg/kg) [43]. These data together suggest that orally-delivered, extrinsic α-tomatine does not influence the de facto tollerogenic signalling environment in the gut mucosa [56].

While unlikely to mask the effect of the α-tomatine on the resultant immune response, the influence of the oil-based formulation used to make the tomato fruit palatable cannot be ruled out. The oil-based emulsion was chosen to limit proteolytic degradation of the NVLPs by reactivation of the endogenous proteases of the fruit mixed in an aqueous solution. Previously, we have shown humoral and mucosal immune response in mice and sheep to a lipid-based formulation including a mucosal immunogen expressed in leaf, tomato fruit and root biomass [57]. However, it is unlikely the choice of an oil-based formulation solely masked any impact of α-tomatine, as even when formulated in an aqueous solution, exogenously saponins do not always improve immunogenicity [58]. At present, the specific factors required for consistent mucosal immunogenicity with saponin adjuvants are not known, but may involve poor bioavailability due to interaction with dietary cholesterol [59].

Utilising plant-cells to their full potential as production and delivery vehicles is clearly far more challenging than was first envisioned for 'edible vaccines' [60], both from a production and immunological perspective. The slow development and propagation of stable lines of *S. lycopersicum* is unlikely to be an effective vaccine for rapidly evolving pathogens such as Norovirus [61]. However, rapid transient plant-expression systems show promise in the ability to rapidly produce large quantities of well characterised and immunogenic purified NVLPs in response to changing strains [29, 31, 62, 63]. Stable transgenic, plant-made vaccines may have a niche expressing protective antigens of pathogens with low mutational

rates where low-cost and ease of oral delivery are key factors in vaccine success. Indeed, significant effort has been made in the production, characterisation and understanding of the mucosal immunogenicity of stable lines of transgenic rice expressing cholera toxins [9]. This investigation into the ability of α-tomatine to potentiate oral immunogenicity of NVCP highlights one of the many unknown factors in how plant-based formulations present antigen to the mucosal immune system, and the safety and reproducibility of these formulations. Until the characteristics of plant cells as delivery vehicles are better characterised, this technology will be underutilised in the effort to fight infectious disease.

METHODS

Stable transformation and plant growth

Lines of transgenic *S. lyopersicum* cv. Tanksley TA234TM2R were generated as previously published [64], using a binary plasmid containing a codon-optimized NVCP coding sequence (GenBank: AY360474.1) driven by repeat cauliflower mosaic virus 35S promoters [28]. The resulting pNV110 transgenic lines were self-pollinated over 3 generations, and the pNV110-2 line chosen for high transgene expression and simple pattern of inheritance (data not shown). Seeds from the T_4 generation were germinated on Jiffy7 propagation pellets (Jiffy Products, Norway) and transgene expression confirmed at the 4-leaf stage by spray application of 400mg/l kanamycin in water. Control seedlings of the near isogenic line TA234TM2R were germinated at the same time and treated identically excluding selection. Following selection and at the 8-leaf stage, both pNV110-2 and wild type plants were transferred into 8L pots with commercial potting mix and grown in temperature controlled greenhouse conditions (22.3±2.4°C). Plants were drip-irrigated with Peters General Purpose solution (20-8.7-16.6 N-P-K; Everiss International B. V. Netherlands) and provided with supplemental full-spectrum light to simulate 16-hours of daylight. Plants self-pollinated, and fruit was harvested randomly throughout the fruiting season. Unripe fruit were harvested 3-4 weeks after pollination when fruit were less than 35mm diameter, and S3 fruit were harvested when fully grown and exhibiting 10-30% colour change from green [65]. Harvested fruit were halved, and the pulp and seed removed before being snap frozen in liquid nitrogen and freeze dried (FD12 Dynavac, Australia) at -45°C for a minimum of 120 h. Dried fruit were chopped in a blade grinder, and the resulting batch of material repeatedly passed through 500-1000 μm^2 test sieves to standardise particle size. Prior to analysis, particulate fruit material was stored at room temperature in dark, airtight containers with a silicon-based desiccant (Sigma-Aldrich, Australia).

Analysis of NVCP in fruit

Purified, insect-derived 58 kDa NVLPs were used as the reference standard for all analysis [24]. TSP was extracted from lyophilised fruit material in PBST2IE (phosphate buffered saline with v/v 0.05% Tween-20, 2x recommended concentration Roche Complete Protease Inhibitor Cocktail tablets, and 10 mM EDTA) with stainless steel beads in a Qiagen Mixermill (Netherlands) at 28 Hz for 1 min. NVCP-specific ELISA was performed as published [28], modified so that polyclonal rabbit anti-NVCP capture antibody (courtesy M.K. Estes) was diluted 1:10,000 in PBS prior to binding to the Corning Costar 1096 polystyrene plate (Corning Inc, USA) overnight at 4°C, with primary and secondary antibody samples diluted in PBST. Western blot was conducted using 10 μl of TSP extract or iNVLPs mixed with 2x Laemmli buffer containing 2% β-mercaptoethanol prior to denaturing at 95°C for 1 minute. Denatured proteins were separated on a 12% Tris-HCl Ready Gel (Bio-Rad, USA). Proteins were transferred onto PVDF membrane (GE Healthcare) and detected with polyclonal rabbit anti-NV diluted 1:7,500 in PBSM (PSB with w/v 0.05% skim-milk powder) and monoclonal goat anti-rabbit IgG HRP conjugate (Sigma, Australia) diluted 1:10,000 in PBSM using the SNAP i.d. Protein Detection System (Millipore) according to manufacturer's instructions. Blots were visualised by chemiluminescence with ECL Plus (GE Healthcare) following the manufacturers instructions. Deglycosylation reaction was conducted with a PNGase F kit (Sigma-Aldrich, Australia) with TSP extract following buffer exchange from PBST2IE to PBS as per manufacturers instructions. Sucrose gradient sedimentation was performed with 1 mL TSP or iNVLPs in PBST2IE layered on a discontinuous sucrose gradient made by layering 1.6 mL volumes of 60, 50, 40, 30, 20, and 10% sucrose in a 10 mL polycarbonate centrifuge tube (Beckman, USA). The tubes were centrifuged at 150,000 g for 22 h at 4°C in a TH-641 rotor (Beckman, USA). Sucrose fractions were analysed by ELISA as described. Sucrose fractions with detectable NVCP by ELISA were pooled before being applied to a Quick Spin Protein Column (Roche, USA) for concentration and buffer exchange to PBS. Concentrated samples were used to float film-coated 200-mesh copper grids for 15

minutes at room temperature. Dried grids were floated on 2% uranyl acetate for 30 seconds and dried prior to analysis on a Philips CM120 BioTwin transmission electron microscope.

Quantification of α-tomatine

Five aliquots of batched fruit material (50 mg ± 0.2) were extracted in 800 µl of 80% methanol via sonication for 10 minutes. Samples were centrifuged for 10 min at 20,000 g in a bench-top centrifuge, the pellet re-extracted in another 800 µl of 80% methanol, and the supernatants combined in amber HPLC glass vials. Extracts were kept at 4°C, and 2 µl injected onto the column for analysis. Analyses of extracts was performed on Agilient (Germany) 1290 series HPLC equipped with binary gradient pumps, autosampler with sample cooler (maintained at 4°C), and diode array detector coupled with a Thermo Scientific (USA) LTQ Velos orbitrap mass spectrometer. Two standard brackets of α-tomatine standards (Sequoia Research Products Ltd, UK) were run alongside samples. Optimal separation of plant metabolites was achieved using an Agilent Rapid Resolution C18 column (1.8 µm 150 x 20 mm) with a gradient of 97% H_2O/3% ACN (0.1% FA) to 70% H_2O/30% ACN (0.1% FA) over 15 minutes with a flow of 300 µl/min. The average retention time for α-tomatine was 11.60 ± 0.08 min. LC-MS data was acquired in profile mode with ESI positive-negative switching mode with a mass range of 80 to 2000 amu. A HESI ion source was used with the source heater at 350°C, the heated capillary was maintained at 300°C, and the sheath, auxiliary and sweep gases were at 40, 16 and 8 units respectively. Source voltage was set to 3.3kV for negative mode and 3.6kV for positive mode. MS tune conditions were optimized using a 100 ppm α-tomatine standard infusion via T-piece into the HPLC flow.

Animal feeding study

All procedures involving animals were approved by the School of Biological Sciences Monash University Animal Ethics Committee. Two separate studies were conducted using 4-6 week-old female C57BL/6J mice (Monash Animal Services, Australia) housed in individual cages and fed a standard rodent diet containing a nutritionally balanced, grain-based

formulation with water *ad libitum*. All transgenic formulations were mixtures of transgenic and wild type fruit of the same ripening stage to produce a final NVCP concentration of 166 μg/g, providing 50 μg of NVCP in 300 mg of plant material (Table 1). For groups administered exogenous α-tomatine, purified α-tomatine powder (Sequoia Research Products Ltd, UK) was mixed with fruit material prior to delivery. Each dose of fruit material was mixed with 700 μl of a 1:3 formulation of commercial peanut butter and peanut oil immediately prior to administration [66]. Mice were fasted for 3-4 hours prior to provision of formulations at midday on days 0, 7 and 14, and the test diet replaced with normal feeding pellets 24-hours later. The test diet formulation was weighed before and after administration. Blood was collected by submandibular bleed at day -7 and day 14, and mice were killed by CO_2 asphyxiation on day 28. Whole blood samples were taken by cardiac puncture immediately after death, and the first 20 cm of the small intestine proximal to the stomach was excised, sliced open-lengthways, and incubated rocking at 4ºC for 4-hours in 400 μL ice-cold extraction buffer (PBS, 0.1% Tween-20, 200mM EDTA, 10 x Complete Protease Inhibitor Cocktail). Anti-NoV serum IgG and mucosal IgA were analysed as published [62] using the same primary and secondary antibodies as noted for western blot, with serum samples diluted 1/75 and gut wash samples diluted 1/2. Serum endpoint IgG titres were determined from triplicate plates, the geometric mean titer is shown (Figure 4A & B). NoV-specific IgG1 and IgG2a antibodies in serum were assessed using a direct-bind ELISA antibody isotype kit (Sigma-Aldrich, Australia).

Statistical and analysis

All analysis and graphing was performed using GraphPad Prism 6.0. Error bars show one standard deviation (SD). All comparisons of NVCP and α-tomatine concentrations between plant lines and ripening stages used unpaired t-tests, and immunological data compared using 1-way ANOVA and corrected for multiple comparisons with Tukey-Kramer post-hoc test. A difference between groups was considered significant if $p < 0.05$ following correction. The

cut-off for endpoint titer was an OD450 < 0.1. Mice were considered to respond to vaccination if serum IgG GMT value was greater than the geometric mean + 3 x SD of the groups administered wild type fruit.

LIST OF ABBREVIATIONS

ELISA: enzyme-linked immunosorbant assay, GIT: gastrointestinal tract, GMP: Good Manufacturing Practice, IgA: immunoglobulin A, IgG: immunoglobulin G, IgM: immunoglobulin M, iNVLP: insect-derived Norwalk virus-like particle, LD50: lethal dose for 50% of the population, LTB: heat-labile enterotoxin B-subunit, NoV: norovirus, NVCP: Norwalk virus capsid protein, NVLP: Norovirus virus-like particle, QC: quality control, T_h1: T-helper cell type 1, T_h2: T-helper cell type 2, T_{reg}: T-regulatory cell, TSP: total soluble protein, VLP: virus-like particle.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTION

AMW conceived the study and helped to draft the manuscript. RPS carried out all experiments, collected data, performed data analysis, and wrote the manuscript. Both authors read and approved the final manuscript.

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ILLUSTRATIONS AND FIGURES

Figure 1 Characterisation of NVCP expression in tomato fruit. A) Concentration of NVCP in pooled batch of lyophilised unripe and stage 3 (S3) fruit from wild type isogenic lines of plants (wt), and T_4 plants transformed with pNV210 (NV) as determined by ELISA, and **B)** western blot of TSP from wild type and transgenic unripe and S3 fruit compared to the 58 kDa iNVLPs standard (STD). No shift in banding was observed when TSP from unripe fruit was treated with peptide-N-glycosidase F (PNGase F). n.d.- none detected, TSP- total soluble protein.

Figure 2 Characterisation of NVLPs produced in unripe and S3 transgenic fruit. A) Sedimentation of NVLPs produced in insect cells (iNVLPs) and unripe (NV unripe) and S3 fruit (NV S3) expressing NVCP following centrifugation through a 10-60% discontinuous sucrose gradient (top of the gradient is at the left) and assayed with ELISA, and **B)** electron microscopy of VLPs recovered from fraction 13 (F13) of the sucrose gradients. All VLPs in sucrose fraction were approximately 38 nm in diameter. Scale bar 100 nm.

Figure 3 Concentration of α-tomatine in unripe and S3 fruit from wild type and transgenic tomato. A) α-tomatine concentration in a pooled batch of isogenic wild type (wt) and NVCP-expressing (NV) unripe and S3 lyophilised fruit grown in a greenhouse with controlled temperature and light conditions. Mean value of 5 separate extractions from pooled material analysed by LC-MS against α-tomatine standards, and **B)** representative LC traces of unripe (upper panel) and S3 fruit (lower panel) with α-tomatine identified by coloured peak. Significant difference in unripe and S3 fruit indicated by * ($p<0.05$).

Figure 4 Immune response of mice administered wild type or transgenic tomato fruit containing different concentrations of endogenous or exogenous α–tomatine. Geometric mean serum anti-NV IgG titer (GMT) at day 28 for **A)** first animal trial and **B)** second trial. No anti-NV antibodies were detected in mice administered regular pellet diet, wild type fruit (wt) unripe or S3 fruit in either trial (data not shown for second trial). In the first trial (Figure

4A), no difference in response was observed when mice were fed transgenic fruit from unripe (NV unripe) and S3 fruit (NV S3), or when S3 fruit was formulated with purified α–tomatine to the same total concentration as the unripe fruit (NV S3 tom). In the second trial (Figure 4B), no difference in response was observed when mice were again administered NV unripe or NV S3 fruit, or when mice were administered unripe transgenic fruit formulated with purified α-tomatine at 3 (NV unripe 3tom) and 5 (NV unripe 5tom) times the concentration in unripe fruit, respectively. The ratio of serum IgG1/IgG2a was greater than 1 for all mice administered transgenic fruit in the first **C)** and second **D)** trial. The mucosal anti-NV observed in gut wash samples from the first **E)** and second trial **F)** were correlated with the humoral response, mice having a higher humoral response also had a higher mucosal response, with no significant difference in mucosal response observed between treatment groups administered transgenic fruit. Significant differences between anti-NV IgG1 and IgG2a serum antibodies represented with * (t-test, $p < 0.05$).

TABLES

Table 1 Treatment groups and test diets

Group	No. mice	NVCP (μg)	Mass wt fruit (mg)	Mass transgenic fruit (mg)	Total mass fruit (mg)	Endogenous α-tomatine (mg)	Exogenous α-tomatine (mg)
Trial 1							
wt unripe	10	-	300	-	300	2.00	-
wt S3	10	-	300	-	300	0.37	-
NV unripe	10	50	36	264	300	1.98	-
NV S3	10	50	72	228	300	0.35	-
NV S3 tom	10	50	72	228	300	0.35	1.63
Trial 2							
NV unripe	10	50	36	264	300	1.98	-
NV S3	10	50	72	228	300	0.35	-
NV unripe 3tom	10	50	36	264	300	1.98	3.96
NV unripe 5tom	10	50	36	264	300	1.98	7.92

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Figure 1

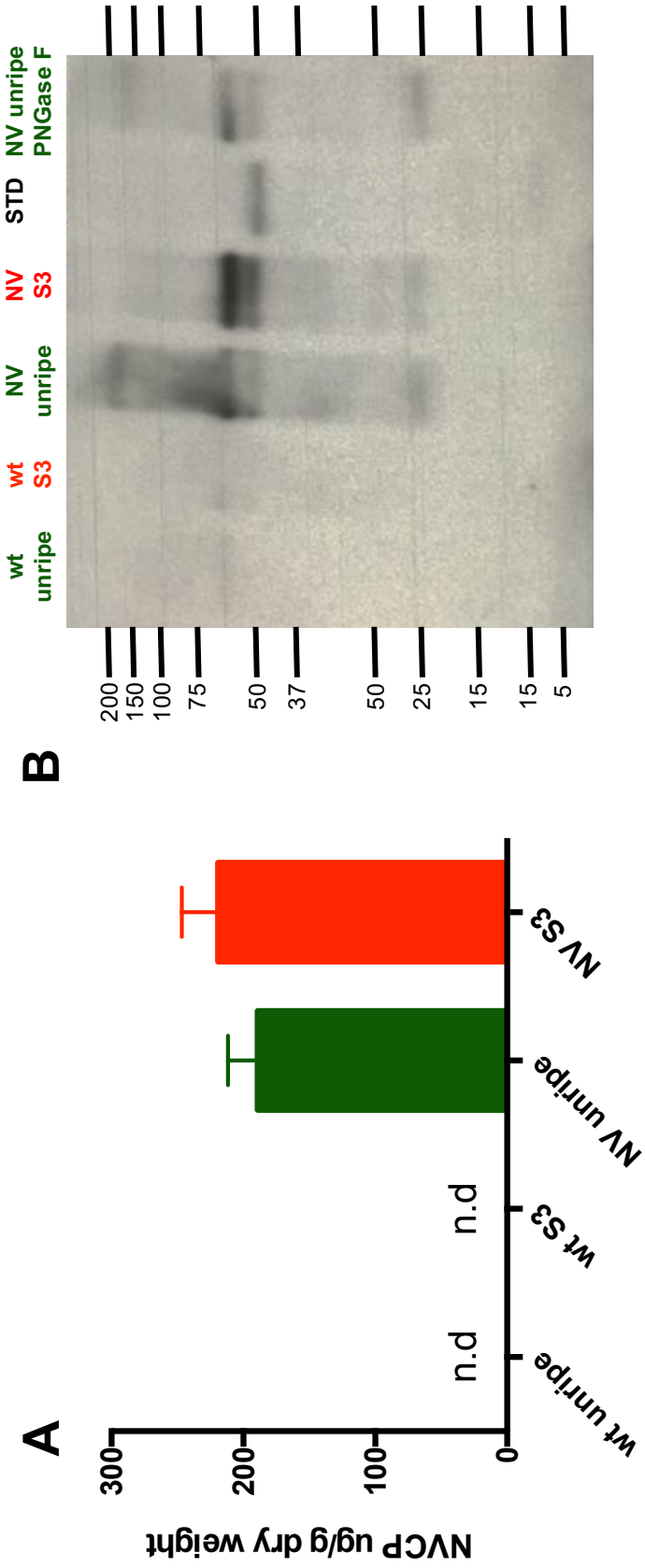
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Figure 2

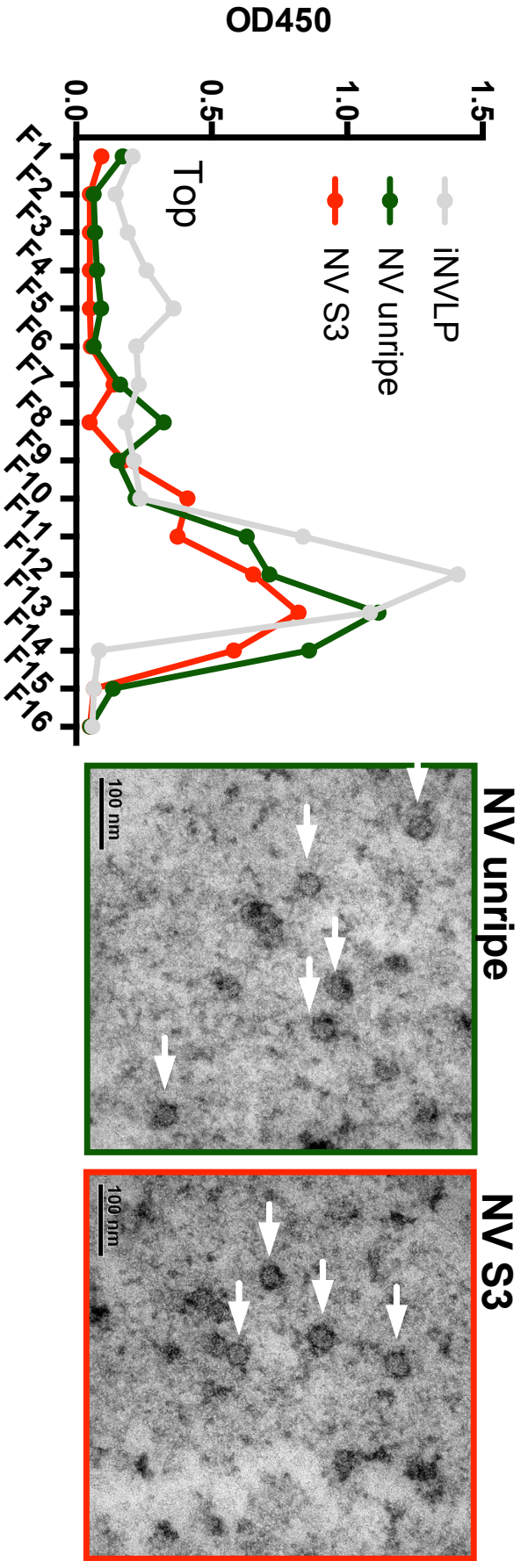
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Figure 3

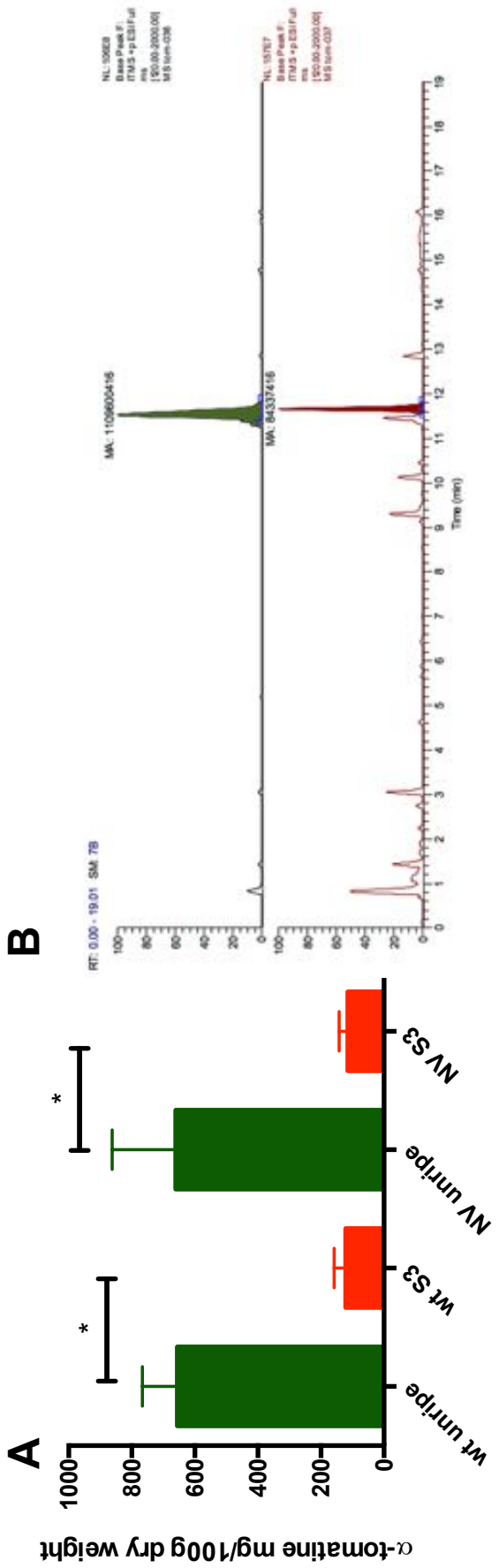
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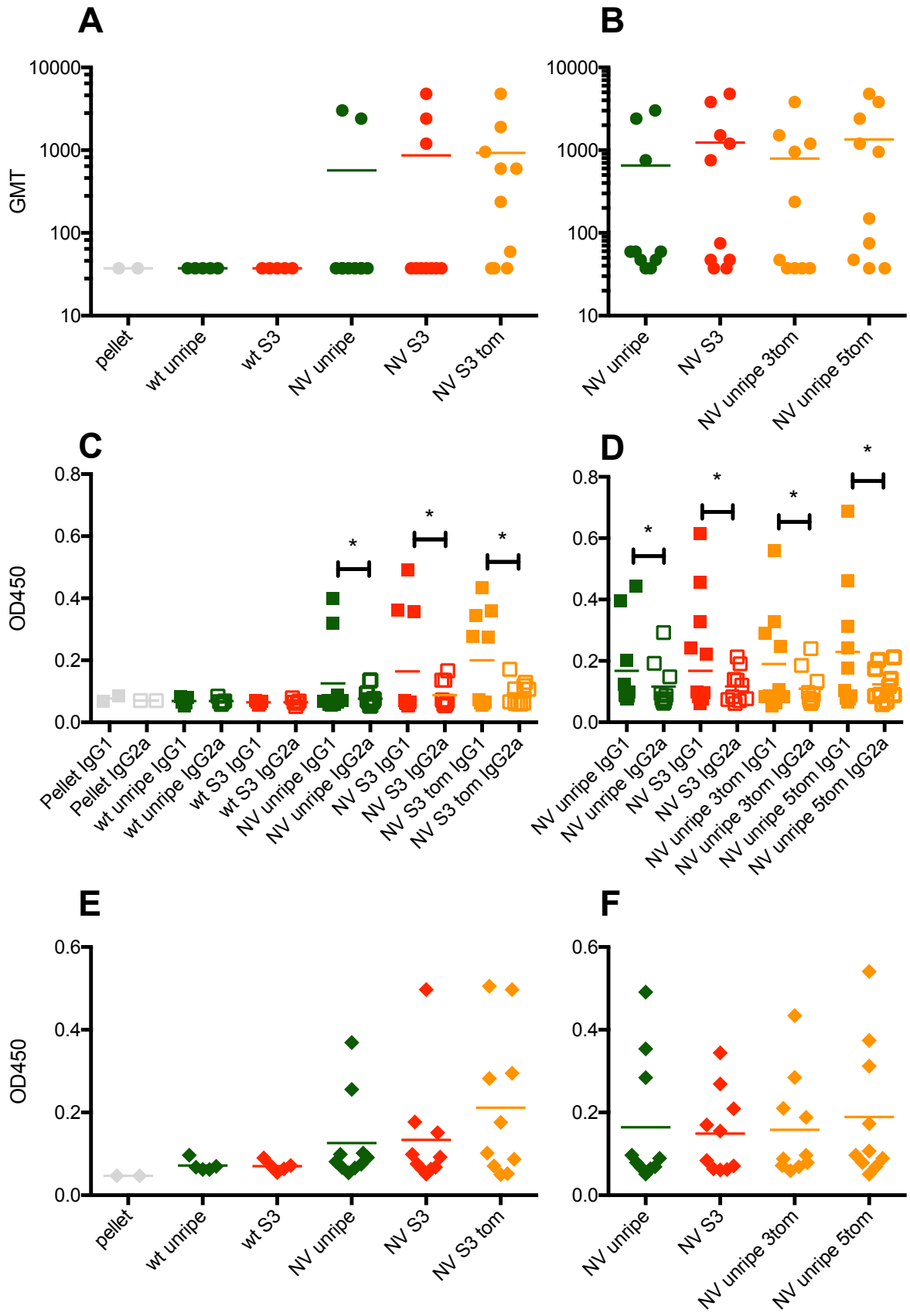
Figure 4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5. GENERAL DISCUSSION

5.1 Summary and outline of general discussion

Despite the latent demand for safe, effective and affordable vaccines for enteric diseases in low and middle-income countries, there have been few vaccines licenced for the treatment of diarrhoeal diseases.[1] Pre-clinical studies have repeatedly shown that oral delivery of plant cells expressing protein antigens from enteric pathogens can be both immunogenic and efficacious in a range of disease models.[2] Yet there have been relatively few candidate formulations that have progressed into early stage clinical trials and none that have progressed into later stages of clinical development.[3,4] Many of the factors limiting the progression of oral vaccines are related to the difficulty of inducing a protective immune response in the gastrointestinal tract (GIT). Administering vaccines orally requires the protection of antigens from degradation while imbuing them with sufficient immunological context that they are not ignored by the mucosal immune system.[5]

Thus, the goal of the work done for this thesis was to enhance the mucosal immunogenicity of vaccine antigens expressed and delivered orally in plant cells. I provide evidence that there are several plant-specific factors that can be optimised to improve immunogenicity, namely the choice of expression system and/or plant species (Chapter 2) and the subcellular localisation of antigen accumulation within the plant cell (Chapter 3). However, in the model system used (Chapter 4), it appears that the concentration of the tomato fruit metabolite α-tomatine does not influence oral immunogenicity.

This chapter provides an integrated summary of the data presented in each manuscript, further lines of investigation, a brief synthesis of where the data presented in this thesis sits in the greater body of research, and a short assessment of where plant-based vaccines can be improved as clinical candidates.

5.2 Impact of expression system on release and immune response

Table 5-1 Integrated summary of data presented in Chapter 2 on the expression and immunogenicity of LTB in different plant expression systems

	S. lycopersicum fruit	*P. parodii* hairy root cell culture	*N. benthamiana* leaf
Accumulation of LTB (direct bind ELISA)	3,700 μg/g	500 μg/g	1,200 μg/g
Bands observed on denatured western blot	~12 kDa	~12 kDa	~12 kDa
Bands observed on native western blot	~35 kDa	~35 kDa	~35 kDa
ARR stomach AH/PB	0.7 0.1	0.2 0.3	1.4 0.6
ARR duodenum AH/PB	1.2 0.25	0.5 0.25	0.7 0.2
ARR ileum AH/PB	0.2 0.35	0.05 0.2	0.15 0.3
ARR large intestine AH/PB	0.15 0.2	0.1 0.15	0.15 0.25
Predominant site of LTB release AH/PB	Duodenum Ileum	Duodenum Ileum	Stomach Stomach
Serum IgG titre (fold above background) AH/PB	5.6 3.4	4.6 4.9	67.5 17.0
Seroconversion (mice per group) AH/PB	4/5 1/5	3/5 3/5	4/5 5/5
Serum IgG1/IgG2a ratio AH/PB	$1.1^{1\#}$ $1.1^{1\#}$	4.1^{2*} 2.9^{2}	20.8^{3*} 8.6^{3}
Mucosal sIgA response rate (mice per group) AH/PB	1/5 1/5	3/5 4/5	5/5 3/5
Mucosal sIgA titre (fold above background) AH/PB	5 7	24 35	127 16

Accumulation of LTB as μg/g dry weight of leaf material, n.d.- none detected, DPI- days post infiltration, AH- aqueous apple and honey formulation (top row), PB- lipid peanut butter formulation (bottom row), ARR- antigen release ratio. $^{n}*$ indicates significantly different IgG1/IgG2a ratio, and $^{n\#}$ indicates non-significant IgG1:IgG2a ratio (see Chapter 2 for details of specific statistical tests).

The significance of the data presented in Chapter 2:

- This study is the first side-by-side comparison of the *in vivo* release and immunogenicity of a vaccine antigen expressed and delivered using different plant expression systems. The highest immunogenicity occurred when LTB was expressed and delivered in *N. benthamiana* leaves in an aqueous solution.
- This study leveraged the stability of the LTB protein as a marker of where plant cells release proteins during transit in the mouse GIT, and showed that the location of antigen release was different between expression systems and carrier formulations.

5.2.1 LTB yield in different expression systems

In this study, the same LTB coding sequence [6] was expressed using three different plant-based recombinant protein expression platforms; fruit from stable nuclear transformation of *S. lycopersicum* with *Agrobacterium tumefaciens*, hairy root cell cultures from stable nuclear transformation of *P. parodii* with *A. rhizogenes*, and leaves from transient non-replicating viral expression in *N. benthamiana* following transfection with *A. tumefaciens*. There was a greater than seven-fold difference in the yield of LTB between the three systems (see Table 5-1), with the highest yield measured in lyophilised tomato fruit.

The regulatory elements and DNA constructs used to drive expression of the LTB coding region were different between the expression systems, so no direct comparison of the yields were made. However, the yield of LTB in *S. lycopersicum* fruit was almost 100-fold higher than previously reported in tomato,[7] and approximately 20-fold higher than recovered from fresh fruit.[8] The factors associated with this high yield are unknown, but it is important to note that previous studies quantified pentameric, GM1-binding LTB, whereas our study quantified all LTB multimers. As not all LTB subunits are likely to be correctly folded into pentamers,[6] the quantification of LTB in our report was likely to be higher than previously reported. Furthermore, the use of a highly optimised tomato-specific promoter may have increased the transcription and ultimately the yield of LTB when compared to previous reports using constitutive viral promoters.[9,10] The yield of LTB in *N. benthamiana* leaves and *P. parodii root* cultures was similar to other reports of endoplasmic reticulum (ER)-localised LTB expressed in these systems.[11-13]

While none of the yields observed in this first study set any records for LTB expression in plant cells (most likely held by the 12% TSP yield of vacuolar-targeted LTB in maize endosperm),[14] sufficient antigen was available to perform the main goal of determining the release and immunogenicity of LTB in a mouse feeding model.

5.2.2 Release of LTB at different GIT locations

One of the most interesting, but difficult to interpret experiments presented in this thesis was the determination of the *in vivo* release of LTB in the mouse GIT. We used 'antigen release ratio' (ARR) as a comparison of the relative quantity of free LTB in the ingesta supernatant, divided by the quantity of LTB in the ingesta samples after mechanical rupture of intact plant cells. As expected, the ratio for all samples was below one (see Table 5-1), indicating that more LTB was released following further lysis of intact cells. The release of LTB from the plant cell during digestion is likely influenced by the properties of the plant cell wall, with the cell walls of the hairy root cell culture releasing protein at a slower rate than the more labile leaf or fruit cells.

Assuming that the LTB molecules produced in each expression system were equally resistant to proteolysis during formulation of the vaccine dose and when released into the gut lumen, it appears that generally, the aqueous formulations (made with apple juice and honey) increased the rate of release earlier in the GIT when compared to the lipid formulation (made with peanut oil and peanut butter). This was most evident in the leaf samples, where the ARR was higher in the early sections of the GIT compared to samples observed later in digestion. The specific factors that slowed the release of antigen from plant cells in lipid formulations was not determined, but may have involved encapsulation of plant cells in a lipid layer that protected the cell from proteolytic attack in the GIT. It is also important to note that the ARR does not account for the different proteolytic environments along the GIT that may degrade unencapsulated LTB at different rates. For example, the stomach has a lower pH and increased mechanical stress during digestion, and the duodenum and ilium have a higher proteolytic environment due to secretions from the pancreas.[15]

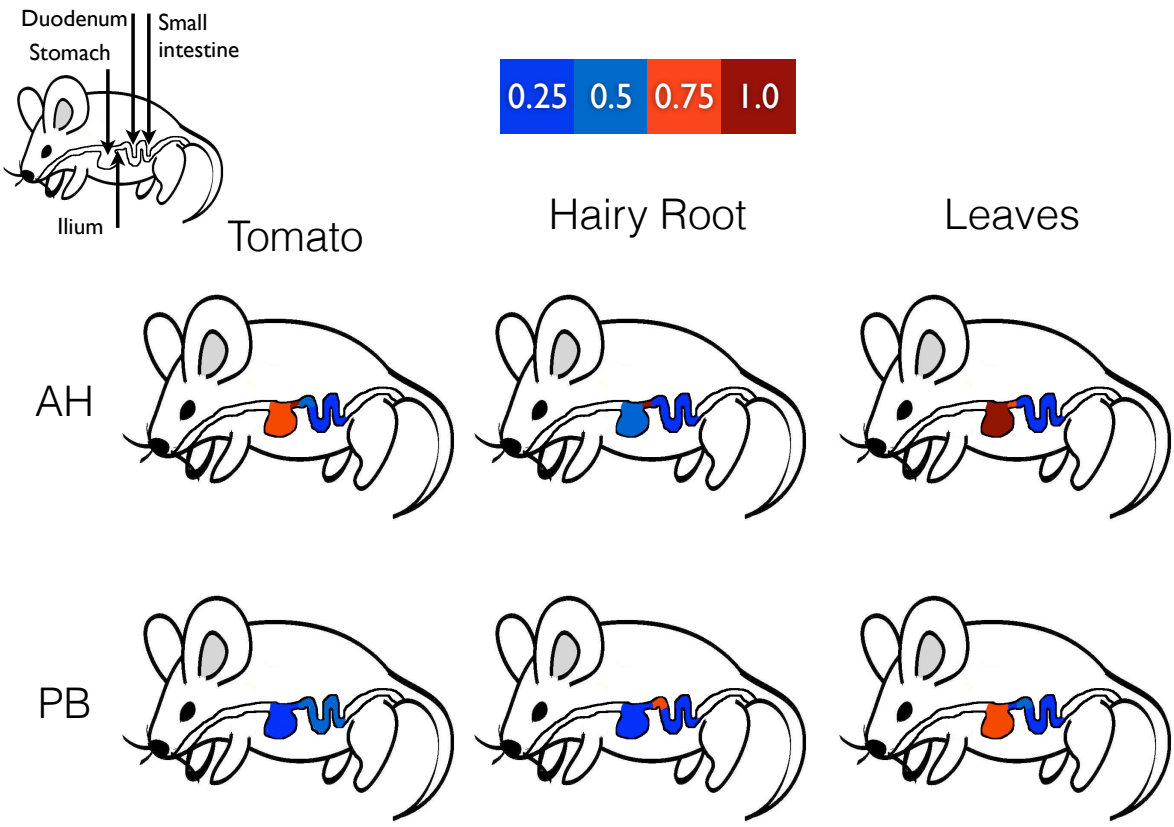

Figure 5-1 **Cartoon representation of LTB antigen release ratio at different locations along the mouse GIT.** Heat map of ARR at top of image in 0.25 increments. Higher ARR represents increased release and/or stability of LTB into gut lumen. Lower ARR represents lower LTB release or stability in gut lumen. AH- aqueous apple juice and honey formulation, PB- lipid peanut oil and peanut butter formulation.

Since the publication of our study, a report from Shaaltiel et al., using carrot cells to deliver β-glucocerebrosidase (prGCD) has provided further data on the kinetics of recombinant protein release and uptake from plant cells in the mouse and pig gut.[16] In this study, the low-pH stable prGCD protein expressed in lyophilised carrot cells was administered by gavage. The free prGCD in the stomach, small intestine and colon were measured over a period of 24 hours. Similarly to the *N. benthamiana* leaves in our study, most of the free prGCD released from carrot cells was observed in the stomach and small intestine.[16] Very little prGCD was observed in the colon and none in the faeces, consistent with our own observation that no LTB was detected in the mouse faeces. We assume that LTB was most likely bound to the epithelium or degraded by the time the plant material reached the colon. While these data show similar release characteristics to our study, interaction between the mucosal immune system and prGCD was not reported, and it is unclear if animals raised humoral or mucosal antibodies to the administration of the soluble protein.

The closely related cholera toxin B subunit (CTB) fused with green fluorescent protein (GFP) has also been shown to enter the blood stream following oral administration of transplastomic

N. tabacum leaves.[17] However, the region of the GIT where CTB is translocated from the lumen into the circulation is not known.[18] Interestingly, while the majority of plant cell wall degradation is a result of the communities of anaerobic bacteria, fungi, and protozoa in the mammalian large intestine,[19] both Shaaltiel et al., and our own study show that the majority of plant-encapsulated recombinant protein is released early in digestion within the stomach and small intestine, and that very little if any antigen makes its way through to the complex microbiome of the large intestine.

Combined, these studies in addition to ours suggest that leaf or protoplast cells release antigen relatively early during transit in the GIT, and the choice of plant cell type used to express proteins may offer a way to tailor delivery of proteins to specific regions of the GIT. However, it is not yet clear what plant-specific factors are responsible for the different release kinetics observed between expression systems and whether release is governed by cell walls or other plant cell characteristics.

5.2.3 Influence of expression system on mucosal immunogenicity

The choice of plant expression system used to express and deliver LTB strongly affected the humoral and mucosal immunogenicity. Tomato fruit was the least immunogenic system, with low serum IgG and mucosal IgA titres, and hairy root cells raised only moderately higher immune responses (see Table 5-1). LTB expressed and delivered in an aqueous formulation of *N. benthamiana* leaves was significantly more immunogenic than any of the other plant-derived or purified formulations.

The release of LTB from the leaf cells occurred earlier than the other systems, and may have increased the dose of LTB available to bind to the epithelium in the proximal regions of the GIT. However, as noted in Chapter 2, many different variables may have altered the immune response in this vaccination model:

- molecular composition of the LTB molecule including post translational modifications and tertiary pentamer formation,
- subcellular localisation of LTB within the plant cell,
- the different concentrations of LTB to plant biomass in the different expression systems,
- interaction of plant gelling agents including cell wall polysaccharides with aqueous and lipid formulations,
- the affect of plant macronutrients on mouse digestion,
- different mastication of the aqueous or lipid formulations.

For these reasons, it is difficult to identify the plant-specific factors that affected immunogenicity with any certainty.

To investigate whether the early release of antigen from *N. benthamiana* leaf cells in the mouse stomach was correlated with higher immunogenicity in a larger animal model, our group conducted a subsequent study where four doses of 5 mg LTB expressed in lyophilised *N. benthamiana* leaves or *P. parodii* hairy roots was administered to outbred male sheep (*Ovis aries*, Merion/Merino, see Appendix 1).[11] Due to the poor immunogenicity observed in the previous study, we did not use tomato-based formulations. The leaf and root material was delivered as a formulation of 19 g of plant material and 200 ml of an oil-based emulsion.[11] An emulsion was chosen in preference to an aqueous formulation to limit proteolytic degradation by rehydrated plant-cell proteases during dose formulation and administration. Unlike mice with a relatively short gastrointestinal transit time and simple structure,[20] the ovine digestive tract includes passage through the rumen, reticulum and omasum before reaching the abomasum which is most analogous to the mammalian stomach. Rather than directly sampling the antigen content of plant cells during transit in the sheep GIT, we chose to use antigen-specific antibody secreting cells (ACSs) of the abomasum and mesenteric lymph nodes (MLNs) to determine areas of the GIT that received the highest exposure to the plant delivered LTB. Concordant with the mouse data presented in Chapter 2, *N. benthamiana* leaf cells also appeared to release antigen early during transit, as 60% (3/5) sheep showed detectable levels of anti-LTB sIgA in the abomasum mucus, compared to the group fed the hairy root formulation where no abomasum response was observed. Moreover, gut wash samples and cultured ASCs of sheep administered the leaf formulation had anti-LTB IgG detectable at MLNs closer to the abomasum. At the trial end there was a generally low serum antibody titre to both formulations, with only 40% (2/5) sheep seroconverting to detectable anti-LTB IgG concentration with the leaf formulation and 20% (1/5) with the root formulation.

Interestingly, although MLNs contained LTB-specific IgG-secreting ASCs in sheep fed the leaf formulation, no IgA-secreting ASCs were detected in the MLN of these sheep. In contrast, there was almost no IgG-specific ASCs in sheep fed the root formulation, although one sheep had detectable IgA-expressing ASCs. Given the correlation between IgG and IgA response to orally-delivered LTB in previous trials in mice and pigs,[6,21-32] it is not clear what gave rise to this antibody class switching between plant formulations. However, with such small numbers of animals responding to these formulations, the ability to extrapolate this result is limited.

The increased immunogenicity of the leaf formulation compared to the root formulation in both mouse and sheep studies provides evidence that at least for low-pH and proteolytic resistant immunogens such as LTB, early release during transit provides increased immunogenicity.

5.2.4 Further experiments

- To determine if the plant expression system alters the quality of the LTB protein, further biochemical analysis should be conducted with LTB purified from each plant formulation. This should include quantification of the GM1-binding fraction, thermal or ligand-binding assays to determine stability, and HPLC/MS to identify any post-translational modifications to the LTB protein.
- The oral immunogenicity of LTB purified from each expression system formulated with water should be determined in order to understand if the LTB protein from each expression system was equally immunogenic.
- The adjuvant characteristics of the host plant species and tissue type should be determined by formulating purified recombinant LTB with wild type material from different plant expression systems and delivering orally.
- The characteristics of LTB encapsulation within more proteolytically resistant cell types should be determined by directly comparing the release and immunogenicity of LTB in easily-degraded *N. benthamiana* leaf cells with more robust tissues such as maize or rice endosperm.

5.2.5 Summary

The data presented in Chapter 2 and Appendix 1 provide some of the first evidence that the choice of plant expression system alters the oral immunogenicity of an enteric antigen. Our studies suggest that the leaf cells of *N. benthamiana* release the protein antigen LTB more rapidly during digestion than root tissue from *P. parodii* hairy root cell culture and tomato fruit, and that at least for oral delivery of LTB in mouse and sheep, this early release appears to improve immunogenicity. However, it is not clear if other factors such as the composition of the plant cell wall or the relative quality of the antigen may also have affected the immunogenicity of LTB delivered in different expression systems.

5.3 The influence of subcellular localisation on immunogenicity

Table 5-2 Integrated summary of data presented in Chapter 3 on the relative properties and immunogenicity of LTB trafficked to different subcellular locations of *N. benthamiana* leaf cells.

	LTB	LTB-KDEL	LTB-VTS	Zera®-LTB
Accumulation of LTB (GM1-ELISA/%TSP)	773 μg/g[1#] 0.66%	523 μg/g[1]* 0.33%	3,346 μg/g[1]* 2.29%	703 μg/g[1#] 0.43%
Necrosis of host plant at peak accumulation	High	Moderate	Moderate	ND
Peak accumulation (DPI)	5	5	6	>14
Nicotine accumulation	11.47 μg/g[2#]	11.27 μg/g[2#]	10.80 μg/g[2#]	10.23 μ/g[2#]
Bands on semi-denatured western blot	~11, 20, 28, 42, 50 kDa	~11, 20 kDa	~11, 20 kDa	~11, 20, 28, 42, 50 kDa
LTB in supernatant 30 mins SGF incubation (OD450)	0.59	0.26	0.43	0.25
Seroconversion	9/10	7/10	10/10	6/10
Mean serum IgG geometric titre	286[3#]	176[3#]	533[3]*	308[3#]
Mean serum IgG1/IgG2a ratio	2.28[4#]	1.69[4#]	2.50[4#]	1.26[4#]
Mucosal IgA response rate	5/10	6/10	9/10	6/10
Mean mucosal IgA (OD450)	0.37[b#]	0.27[b#]	0.69[b*]	0.38[b#]

LTB- heat-labile enterotoxin b subunit, KDEL- SEKDEL hexapeptide endoplasmic reticulum retention signal, VTS- vacuole transit peptide signal sequence from tobacco chintinase A, Zera®- Zera® proprietary protein body induction tag, accumulation presented as μg/g of GM1-binding LTB per μg dry weight of leaf material, %TSP- accumulation of LTB as a percentage of total soluble protein, DPI- days post infiltration, OD450- optical density of LTB-specific enzyme-linked immunosorbant assay, ND- none detected. [n]* indicates significantly different values, and [n#] indicates non-significant difference in values (see Chapter 3 for details on specific statistical tests).

The significance of the data presented in Chapter 3:

- This study is the first direct comparison of the oral immunogenicity of LTB localised to different regions of the plant cell.
- This study is the first report that trafficking a vaccine antigen to the plant protein storage vacuoles (PSVs) of *N. benthamiana* leaf cells improves yield and oral immunogenicity compared to other cellular locations.

5.3.1 Subcellular targeting of LTB

The data presented in Chapter 3 shows that the LTB protein can be successfully localised to the apoplastic space, ER, PSVs, and PBs of *N. benthamiana* leaf cells using genetically fused

plant signal sequences. The highest yield of GM1-binding LTB occurred when LTB was localised to PSVs (see Table 5-2).

Due to Australian import restrictions, the MagnICON® system used for all experiments did not use vectors encoding the movement protein (MP) of wild-type tobacco mosaic virus (TMV) required for cell-to-cell transfer of viral replicons.[33-35] Thus only palisade, mesophyll and vascular cell types are transfected by *A. tumefaciens*,[36] and concordantly it was only the palisade and mesophyll cells that showed detectable immuno-labelling of LTB when viewed by transmission electron microscopy (TEM, see Figure 5-2). It is not known if LTB traffics to different subcellular locations in other cell types.

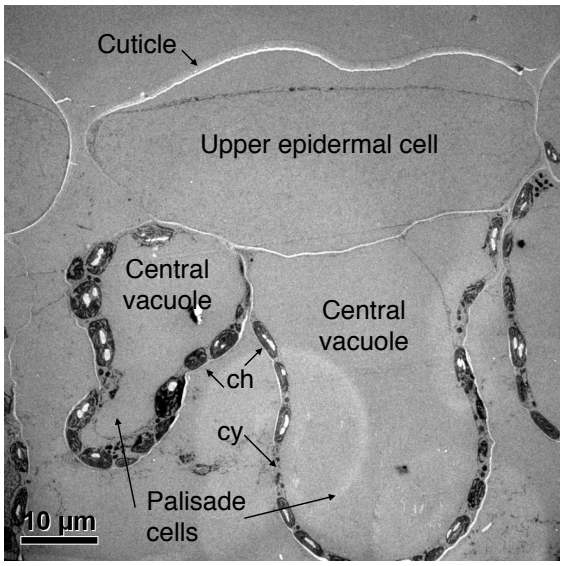

Figure 5-2 TEM transverse section of the upper portion of the *N. benthamiana* leaf highlighting the large central vacuole of the palisade cells. Due to the use of plasmid vectors without a MP sequence, the upper epidermal or lower epidermal cells were unlikely to have been transformed by transient infiltration and no gold labelling was observed in these cells. ch- chloroplasts, cy- cytoplasm.

A short thermal pulse of the total soluble protein extracted from leaf cells was used to determine if there was any variation in the thermal stability of LTB recovered from the different subcellular locations. LTB recovered from the apoplast and Zera®-PBs was separated into the correct size for monomers, dimers, trimers, tetramers, and pentamers (also seen in other studies of the native LTB protein extracted from plant cells).[12] This suggest that LTB molecules localised to the apoplast is more thermally resistant to degradation that than those targeted to other subcellular compartments.

These intermediate multimers were not observed in TSP from leaves where LTB was localised to the ER or the PSVs. It is unknown if these multimers are actually present within the plant cell or the result of the extraction and partial denaturing process. However, as only pentameric LTB is capable of binding gangliosides,[37] other multimers would not have been quantified by the GM1-binding ELISA used to determine vaccine dose.

It is interesting to propose a link between the process of LTB maturation as it is trafficked through the plant endomembrane system and the thermal stability of the recovered protein. The lowest thermal stability was observed when LTB-KDEL was arrested early during trafficking in the ER, and the highest thermal stability observed when the protein was allowed to traffic through the entire endomembrane system to the apoplast (see Figure 5-3). It is not clear how far the ER-derived PSV or PBs trafficked through the endomembrane system before entering the terminal storage organelles, but neither vacuolar or Zera® localised LTB showed the thermal stability of LTB localised to the apoplast. High yields of GM1-binding pentameric LTB, LTB-ST fusion proteins, and CTB have been recovered from transplastomic *N. benthamiana* leaves,[38-40] indicating that while transit through the plant endomembrane system improved the thermal stability of LTB in TSP, maturation in the ER or Golgi is not explicitly required to form the disulphide bonds required for pentamer formation. Thus, LTB may mature in the ER or other plant endomembrane systems due to other factors. The molecular composition of LTB in each subcellular compartment was not determined in this study, so it is unclear what post-translational modifications or other cellular processes may have led to the variation in thermal stability.

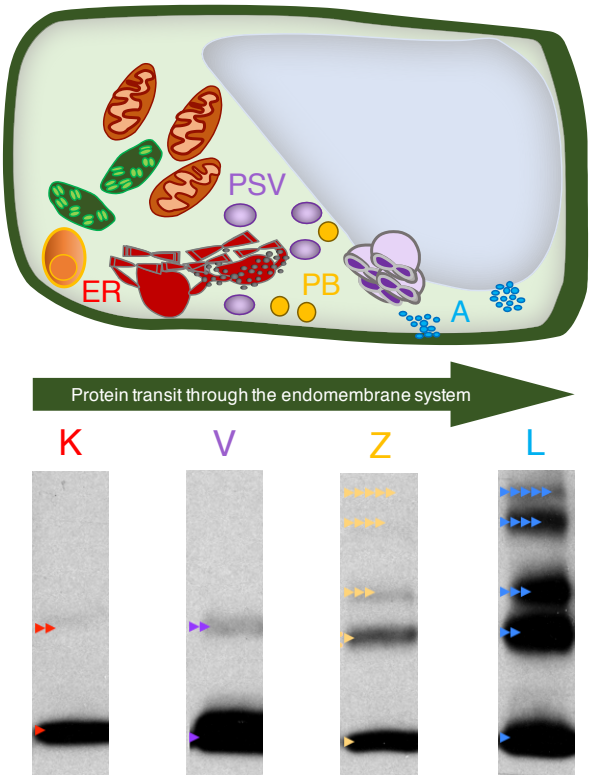

Figure 5-3 Transit of LTB through the plant endomembrane system may influence resistance to thermal degradation. The direction of protein trafficking is represented with an arrow, following translation in the cytoplasm and entry into the ER (left), through the endomembrane system to the apoplastic space (A, right). Thermally denatured LTB visualised by western blot forms monomers and dimers when localised to the ER (K) and PSVs (V), but multimers when further trafficked through the endomembrane system to PBs (Z) or the apoplast (A). Arrows indicate putative LTB multimers.

5.3.2 Subcellular release kinetics

The subcellular location of LTB accumulation within the *N. benthamiana* cell influences the kinetics of antigen release in the *in vitro* SGF assay (see Table 5-2). Over twice the functional GM1-binding LTB was present in the assay supernatant after 30 minutes of incubation with apoplast or PSV-localised LTB compared to ER or PB-localised LTB.

The detection of GM1-binding LTB in this assay is a function of both the rate of LTB released from the plant biomatrix into the supernatant, and the resistance of LTB to the pepsin-based proteolysis once it is released from the cell. The biochemical properties of LTB localised to different regions of the cell may also play a role in the resistance to degradation in the SGF assay. For example, LTB targeted to the apoplast showed the highest thermal stability and resistance to degradation in SGF. Accordingly, LTB localised to the ER was the least thermally stable and the least resistant to degradation in the SGF assay. However, both release and stability in SGF are likely to affect the kinetics of this system making it difficult to draw a direct conclusion regarding subcellular location and the rate of release of antigen into the SGF supernatant.

However, purified LTB mixed with leaf material expressing the unrelated GFP protein showed similar release kinetics and area under the curve as the formulations where LTB was encapsulated within the plant cell. This suggests that encapsulation within *N. benthamiana* cells doesn't provide significant protection from release during incubation in SGF. As a comparison, Chikwamba et al., observed that LTB localised to the starch granules of maize endosperm was resistant to degradation for up to 4 hours of incubation in an aqueous solution at 65°C.[41] Furthermore, approximately 75% of the CTB localised to PB-I in rice endosperm was protected from degradation following pepsin digest for 1-hour.[42] Purified 3Crp peptide and (water soluble) PB-II localised 3Crp were rapidly degraded in pepsin and pancreatin-based *in vitro* digestion, whereas PB-I localised peptide was stable for up to 18-hours,[43] and Japanese cedar pollen allergens localised to rice endosperm PB-Is were protected from degradation for up to 60 minutes in 0.1% pepsin.[44]

The short duration of LTB protection observed the SGF assay in Chapter 3 suggests that regardless of where LTB is localised within the cell, *N. benthamiana* leaves are unlikely to afford the same protection as the more proteolytically-resistant tissues of rice or maize endosperm.

5.3.3 Impact of subcellular accumulation of LTB on immunogenicity

The most significant finding in this study was that accumulation of the LTB antigen in different locations within the *N. benthamiana* leaf cell altered the humoral and mucosal immune response to the same dose of GM1-binding LTB delivered orally. The highest LTB-specific immunogenicity was observed in mice fed leaf cells with LTB targeted to PSVs (see Table 5-2).

While the complexity of the plant cell formulation makes it difficult to identify the specific mechanisms that altered the resulting immunogenicity of LTB, two likely factors for the difference are the intrinsic immunogenic properties of the LTB molecule, and the release and uptake of LTB during transit in the GIT.

With regard to the intrinsic immunogenic potential of LTB molecules, the first report of LTB expression *in planta* by Haq et al., observed lower humoral and mucosal immunogenicity of plant-derived LTB-KDEL compared to gavage with purified bacterial-derived LTB, and attributed the reduced immune response to inhibition by some part of the plant formulation.[21] In a subsequent study by the same research group, LTB with the native bacterial SP (presumably localised to the apoplast) expressed and delivered in potato tuber was significantly more immunogenic than purified, bacterial derived LTB.[6] The authors noted: "*The greater responses of mice that were fed transgenic tubers than those that were gavaged with 5 μg purified LT-B may be due to the higher dose of LT-B (20 and 50 μg) delivered in these tuber feeds*."[6] A later study using a prime-boost immunisation schedule showed that mice fed LTB-KDEL in potato tubers had a reduced immunogenicity compared to the same dose of purified bacterial LTB,[24] and subsequent studies have shown that LTB-KDEL in carrot cells [27] and lettuce leaf [29] are less immunogenic than purified recombinant LTB.

This raises the question of whether the fusion of different signalling sequences such as KDEL, the vacuolar transit peptide (VTS) or Zera® to the LTB protein structure alters the intrinsic immunogenicity of the native protein? It is known that the adjuvant and immunogenic properties of CT require direct interaction with the population of CD103+ dendritic cells (DCs) resident in the subepithelium dome (SED),[45] and it is feasible that any decrease in the rate of LTB attachment to epithelial cells and trafficking to these DCs may also decrease immunogenicity. Indeed, there are 8-known amino acid residues required for LTB binding to the GM1 gangliosides of the epithelium (see Figure 5-4), and it is not yet known how the addition of the KDEL, VTS or Zera® tags, or protein modifications following expression within the the plant endomembrane system affect the LTB tertiary structure or the ligand binding efficiency of the LTB quaternary, pentameric structure.

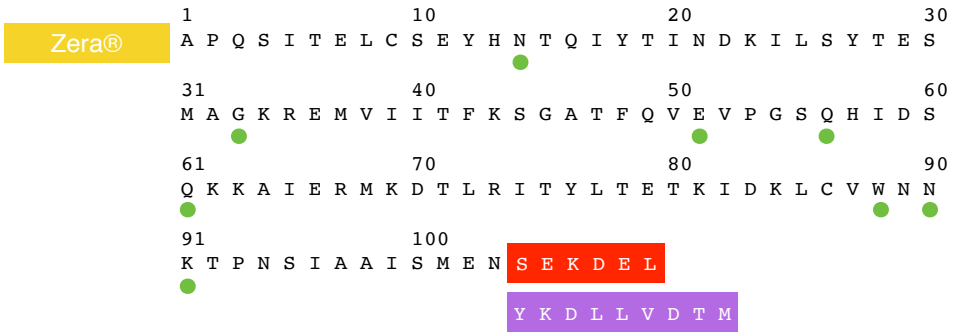

Figure 5-4 Amino acid residues of LTB involved in binding GM1 gangliosides (green circles, adapted from [46]). Zera ® tag (yellow) is fused N-terminal, and KDEL (red) and VTS (purple) sequences are fused C-terminal to the LTB protein sequence. The sequence above does not include the 21 amino acid N-terminal signal peptide that is cleaved upon entry into the ER.

The release of LTB from *N. benthamiana* leaf cells *in vitro* (Chapter 3) and *in vivo* (Chapter 2) is relatively rapid compared to other plant tissues including endosperm or hair root cells (see 5.3.1), and the rapid release of LTB in the stomach of mice or the abomasum of sheep was correlated with increased immunogenicity (see Chapter 2 and Appendix 1). However, it is not yet known if the kinetics of LTB release from the different subcellular compartments of the leaf cell observed in the SGF assay are correlated with similar kinetics during transit in the GIT, as the mechanical degradation of mastication and stomach peristalsis is not present in the simpler SGF assay.

It is also not yet known whether the *in vitro* release characteristics of LTB localised to different subcellular locations of the leaf cell caused the variation in mucosal immunogenicity observed in Chapter 3. Mice fed vacuolar-targeted LTB had the highest humoral and mucosal immunogenicity, but it is unclear what factors led to this result. It is likely that encapsulation within the PSVs reduced some of the proteolysis during digestion compared to other formulations where LTB was not as well protected (e.g. LTBK). Indeed, there was a correlation between subcellular locations that were more protective of the antigen in SGF (LTB and LTBV) and those that were more immunogenic *in vivo* (see Table 5-2).

Despite this correlation, thermal stability and release kinetics alone do not explain the higher immunogenicity of LTB in PSVs as LTB targeted to the apoplast was more resistant to thermal degradation and digestion in the SGF assay but less immunogenic than LTB localised to the PSVs. The release and potential uptake of particulate PSVs similar to PBs could be a mechanism for the improved immunogenicity of this formulation. However, LTB localised to Zera®-derived PBs was significantly less immunogenic than PSV-localised LTB, and may indicate that not all particulate formulations of LTB improve immunogenicity. Although PSVs of vegetative tissues are water soluble and not as resistant to proteolysis as starch

granules of maize [41] or PB-Is from rice endosperm,[44,47] they are similar in size to endosperm-derived PBs.[48-50] While there is evidence of antigen-carrying rice PBs being transcytosed across M-cells,[42,51] there is no such evidence for PSVs or Zera®-induced PBs. Thus, despite the significantly higher immunogenicity of LTB localised to PSVs of *N. benthamiana* cells compared to LTB localised to other organelles, the mechanism that led to this improved response remains uncertain.

5.3.4 Further experiments

- To determine if the trafficking of LTB within the plant endomembrane system was responsible for the differences in thermal and proteolytic protection observed *in vitro*, purified LTB from different subcellular locations should be characterised for post-translational modifications and GM1 binding efficiency.
- To remove the influence of the plant biomatrix on the intrinsic immunogenicity of the LTB molecules trafficked to different subcellular locations, LTB should be purified from each subcellular compartment and administered orally in an aqueous solution.
- Fluorescent or immunohistology markers should be used to track LTB uptake by M-cells to determine if there is any variation between LTB localised to the apoplast, ER, or particulate organelles such as PSVs or Zera®-induced PBs.
- The direct comparison of *in vitro* release and oral immunogenicity of LTB localised to different subcellular locations such as starch granules in maize or PB-Is in rice endosperm should be undertaken to determine if the encapsulation of LTB in proteolytic resistant formulations alters the immune response.

5.3.5 Summary

The trafficking and subcellular localisation within *N. benthamiana* leaf cells clearly affects the oral immunogenicity of LTB. However, there are several possible mechanisms that may have influenced the immunogenicity of LTB including different biochemical properties of the LTB proteins trafficked to different regions of the plant cell, the kinetics of LTB release during transit in the GIT, or differential antigen uptake and processing by the host mucosal immune system. In the model system presented here, the targeting of LTB to PSVs produced the highest accumulation of antigen and the most immunogenic formulation, and therefore warrants further investigation as a method for optimising the oral immunogenicity of other vaccine antigens.

5.4 The influence of α-tomatine on mucosal immunogenicity'

Table 5-3 Integrated summary of data presented in Chapter 4 on the relative properties and oral immunogenicity of NVCP expressed in fruit at different stages of ripening

	NV unripe	NV S3	NV S3 αtom	NV unripe 3αtom	NV unripe 5αtom
Mean fruit mass	15.13 g[1]*	80.41 g[1]*	n.a	n.a	n.a
Mean fruit diameter	33.84 mm[2]*	58.54 mm[2]*	n.a	n.a	n.a
Mean NVCP accumulation	189.4 μg/g[3#]	219.3 μg/g[3#]	n.a	n.a	n.a
Mean α-tomatine concentration	661.2 mg/100g [4]*	115.7 mg/100g [4]*	n.a	n.a	n.a
Bands on western blot	~28, 65 kDa	~28, 65 kDa	n.a	n.a	n.a
Dose of NVCP	50 μg	50 μg	50 μg	50 μg	50 μg
Endogenous α-tomatine per dose	1.98 mg	0.35 mg	0.35 mg	1.98 mg	1.98 mg
Exogenous α-tomatine per dose	-	-	1.63 mg	3.96 mg	7.92 mg
Total α-tomatine per dose	1.98 mg	0.35 mg	1.98 g	5.94 mg	9.90 mg
Seroconversion (trial 1/trial 2)	2/10 3/10	3/10 5/10	6/10	4/10	4/10
Serum geometric mean titre (trial 1/trial 2)	572[5#] 652[6#]	866[5#] 1232[6#]	926[5#]	790[6#]	1351[6#]
Mean serum IgG1/IgG2a ratio (trial 1/trial 2)	1.7 1.4	1.9 1.9	2.1	1.7	1.8
Mucosal IgA response rate (trial 1/trial 2)	2/10 3/10	3/10 5/10	5/10	4/10	4/10
Mean IgA response (trial 1/trial 2) OD450	0.13[7#] 0.16[8#]	0.13[7#] 0.15[8#]	0.21[7#]	0.16[8#]	0.19[8#]

Accumulation presented as mass/mass of dry weight fruit material, NVCP- Norwalk virus capsid protein, trial 1/trial 2- replicate experiments of the same formulation, IgG1- immunoglobulin G1, IgG2a- immunoglobulin G2a, NVLP- Norwalk virus-like particle, OD450- ELISA substrate optical density at 450 nM, n.a- not applicable. [n]* indicates significantly different values, and [n#] indicates non-significant difference in values (see Chapter 4 for details on specific statistical tests).

The strengths of the data presented in Chapter 4 are:

- This is the first report to quantify the mucosal adjuvant properties of the tomato metabolite α-tomatine in a vaccine formulation, showing it to be an ineffective endogenous or exogenous mucosal adjuvant for NVLPs delivered in tomato fruit.
- This study accurately quantified the α-tomatine concentration and Norwalk virus capsid protein (NVCP) expression on a per plant and fruit basis. There was no correlation between these variables suggesting that the presence of the NVCP transgene does not perturb the normal α-tomatine biosynthetic pathway.

5.4.1 Expression of NVCP in tomato

In the study presented in Chapter 4, NVCP was successfully expressed and isolated as VLPs from *S. lycopersicum* unripe and stage 3 (S3) fruit (see Table 5-3).

Expression of the NVCP in *N. benthamiana* leaves yielded a single, 58 kDa band identical in size to the VP1 protein of Norwalk virus.[52-56] However, in this study, two additional bands of ~25 and 65 kDa were observed in addition to a 58 kDa band at both stages of ripening. The composition of these additional bands is unclear. It is unlikely to be the addition of plant glycans as the NVCP sequence lacks an N-terminal SP or any known plant-specific signalling sequence.[57] Moreover, glycosylation of NVCP is not commonly observed in mammalian or insect cells.[58] If there was aberrant sorting of the NVCP protein into the plant secretory system, branched chain N-glycans may have been added at the three putative Asn-X-Ser or Asn-X-Thr sites present in the NVCP sequence. After treatment with PNGase, no change in the position or band density was observed indicating that the increase in molecular weight of the 65 kDa bands was not likely due to N-glycosylation. Modification with glycans does not explain the presence of the smaller 25 kDa band also observed by western blot. This band may be a cleavage product of the NVCP protein by plant-endogenous proteases or cleaved during the extraction of TSP. As noted in Chapter 4, the two other reports of NVCP expression in *S. lycopersicum* did not publish a western blot of the fruit lysate, therefore these additional bands may be a common but unreported trait of NVCP expression in *S. lycopersicum* fruit.

5.4.2 Accumulation of α-tomatine in tomato fruit

Many studies have utilised tomato fruit as a delivery vehicle for oral vaccine candidates,[59] yet this study is the first known characterisation of α-tomatine in a vaccine formulation. The accumulation of α-tomatine was higher in unripe fruit compared to S3 fruit from both wild type and psNV110-2 transgenic plants.

While performing initial exploratory work with the transgenic plant line used in Chapter 4, several experiments were conducted to determine if the concentration of NVCP correlated with the concentration of α-tomatine in individual plants or fruit. No correlation was observed between these two variables (see Figure 4-1 and 4-2), suggesting that the presence of the NVCP protein or selectable kanamycin marker did not perturb the endogenous glycoalkaloid biosynthesis pathway. However, our research group has also characterised the concentration of α-tomatine in a different transgenic line of tomato carrying a bacterial-derived multi-gene construct, and found higher concentrations of α-tomatine in fruit expressing higher

concentrations of the transgene construct (Claire Penney, personal communication). This line of fruit accumulates almost 2-fold higher α-tomatine in unripe fruit, and 1.5-fold higher α-tomatine in S3 fruit compared to growth matched fruit from a near isogenic line. Given the potential for acute toxicity of α-tomatine at moderate concentrations,[60] the accurate quantification of α-tomatine is important in understanding the toxicology of vaccine candidates using tomato fruit as a delivery vehicle.

5.4.3 Oral immunogenicity of tomato fruit and α-tomatine

No significant difference in mucosal immunogenicity was observed when lyophilised unripe or S3 lyophilised fruit expressing the NVCP were orally administered to mice. Nor was any difference in immunogenicity observed when purified α-tomatine was added to the fruit formulations. In fact, the humoral and mucosal immune response was relatively consistent across all treatment groups with no significant difference between any of the formulations tested (see Table 3).

Previous studies have shown that that the primary determinant of seroconversion to NVLPs is the available dose of antigen; higher doses generally induce more complete seroconversion.[10,61,62] The experimental conditions (three doses of 75 μg NVCP) used in Chapter 4 most closely resembled the conditions used in Zhang et al., where a group of mice were administered three doses of fruit containing 128 μg of NVCP.[10] Zhang et al., observed that 40% (2/5) mice seroconverted when fed lyophilised or air-dried fruit.[10] This incomplete seroconversion was similar to what was observed in Chapter 4 where 20-60% (2-6/10) of mice responded to NVCP delivered in different combinations of unripe or S3 fruit with or without exogenous α-tomatine (see Figure 5-3). Thus, the consistently low seroconversion across formulations with varying concentrations of endogenous or exogenous α-tomatine suggests that α-tomatine does not appear to increase the effective dose of NVLPs in this model system.

The effect of adjuvants including saponins on the magnitude of the humoral or mucosal immune response to NVLPs is less clear. Oral immunogenicity of NVLPs purified or delivered within plant cells can be boosted using adjuvants such as saponins from *Q. saponaria*,[9] genetically modified LT (R192G),[63] genetically modified cholera toxin CT-E29H,[64] CT,[52] or the TLR7 agonist gardiquimod.[65] However, there was no increase in immunogenicity to NVCP in lyophilised tomato powder when formulated with food-grade saponins.[9] In Chapter 4, no variation was observed in the IgG1/IgG2a ratio or magnitude of NVCP-specific serum IgG or mucosal IgA response between formulations of tomato fruit

with different concentrations of endogenous or exogenous α-tomatine. This suggests that at least for NVCP expressed and delivered in tomato fruit, α-tomatine is not effective at increasing the quality or magnitude of the immune response.

Given there was no significant difference in the seroconversion, magnitude or quality of the immune response with varying concentrations of α-tomatine in the tomato formulations, α-tomatine does not appear to improve the immunological presentation of the co-delivered NVLPs in this experimental setting. Despite its known systemic adjuvant properties,[66] it is unclear whether the inherent activity or bioavailability of the α-tomatine molecule prevents its mucosal effect. Similar saponins function by direct interaction with DCs and macrophages,[66-68] but most dietary α-tomatine forms an insoluble complex with dietary cholesterol and is excreted in the faeces,[69] and therefore may not be sufficiently bioavailable to the APCs of GALT to exert an affect. However, other saponins such as QS-21 are effective mucosal adjuvants even though they also share the same cholesterol-binding structure and dietary excretion.[70,71]

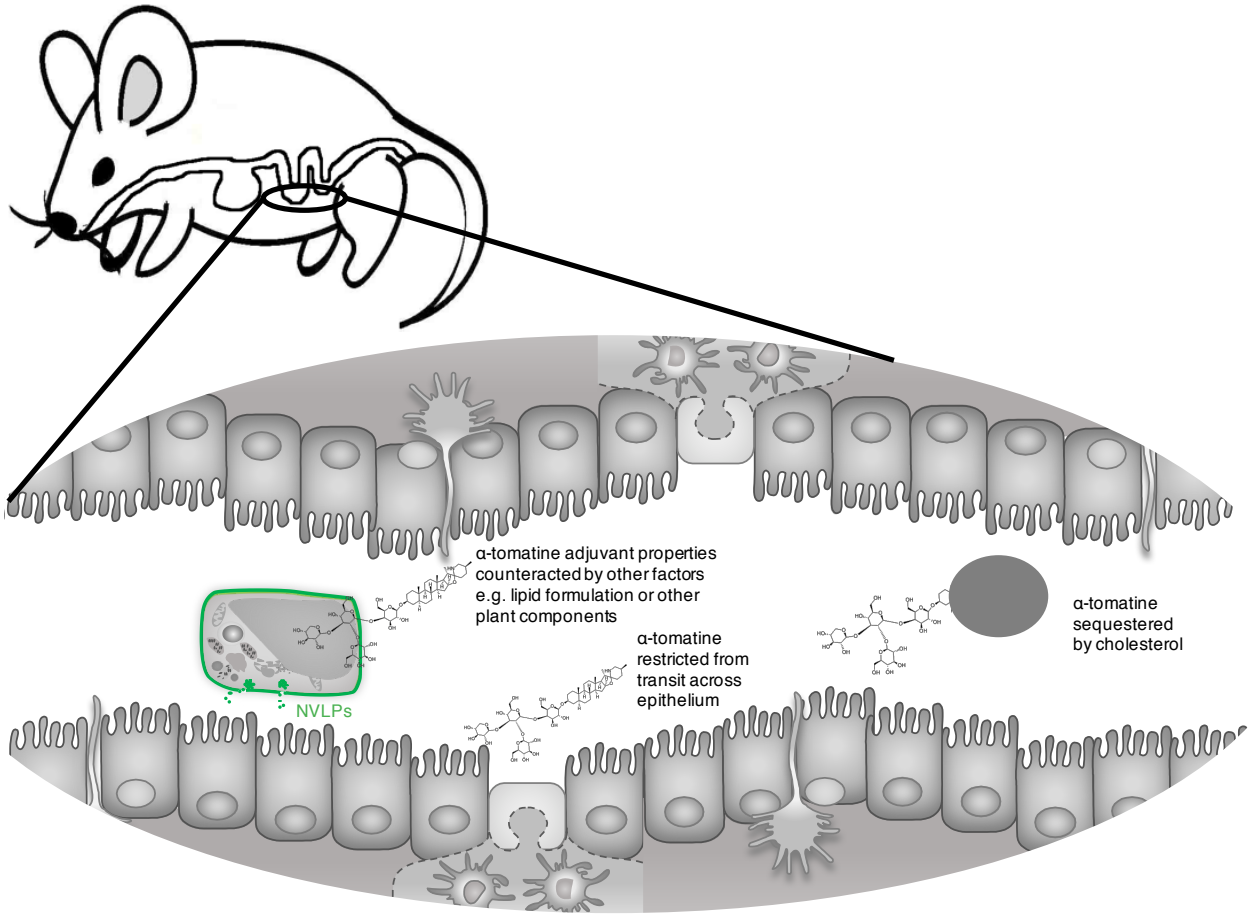

Figure 5-5 α-tomatine does not influence the oral immunogenicity of NVLPs delivered in lyophilised tomato fruit formulations. This may be due to interaction of endogenous or exogenous α-tomatine with other factors of the plant biomatrix causing limited bioavailability or counteracting the adjuvant properties, or α-tomatine forming indigestible complexes with cholesterol in the GIT.

It is also unclear whether the lipid based formulation used in this system or the other metabolites of tomato fruit may be counteracting any adjuvant properties of α-tomatine. Lycopene present in ripe tomato fruit is a relatively well characterised anti-inflammatory compound known to be bioactive when delivered orally,[72-75] and there are a wide variety of other bioactive metabolites that accumulate during tomato fruit ripening including carotenoids, xanthophylls, chlorophylls, tocopherols, ascorbic acid, flavonoids, phenolic acids, glycoalkaloids, saponins, and other glycosylated derivatives.[76] A detailed, pairwise comparison of these compounds with α-tomatine would be required to determine if the effects of α-tomatine are counteracted by other fruit metabolites.

5.4.4 Further experiments

- To determine if any aspect of the plant formulation masked any mucosal adjuvant affect of α-tomatine, the oral immunogenicity of purified α-tomatine with purified NVLPs or other mucosal antigens should be assessed.
- To determine if there is a minimum NVLP dose required for an adjuvant effect of α-tomatine to be observed, a series of experiments comparing the immunogenicity of varied doses of NVLPs with purified α-tomatine should be conducted.

5.4.5 Summary

Despite the inherent immunogenicity of VLPs,[77] all NoV vaccines candidates currently being investigated in clinical studies use at least one adjuvant in their formulation to improve the seroconversion and magnitude of the immune response.[78] Bioactive plant components such as saponins have been formulated with several plant-made and delivered vaccine candidates to improve mucosal immunogenicity,[79-81] but there has not been an investigation into whether the endogenous compounds of tomato fruit have the capacity to act as co-delivered adjuvants. In Zhang et al., the authors suggest "*α-tomatine, an alkaloid glycoside of tomato, may serve as a natural adjuvant potentiating immune responses in mice.*"[10] The results presented in Chapter 4 indicate that, at least in the model system used for this study, the presence of α-tomatine does not function as an effective natural adjuvant.

5.5 Optimising oral response to plant-made vaccines

The experiments presented in this thesis have focused on addressing two key issues of oral immunogenicity; delivery of antigens to the APCs of the GALT, and the use of mucosal adjuvants to improve the immunological context of orally-delivered antigens. The experiments undertaken provide several examples of the integral nature of the plant expression system effecting the mucosal immune response to antigens delivered in plant cells.

5.5.1 Improving delivery of orally-active vaccine antigens

Understanding the release and delivery of antigens from plant cells will allow for the rational optimisation of the plant expression system to maximise the immunogenicity of vaccine antigens. The variations in immunogenicity observed when the same antigen was delivered orally in different expression systems, formulations and subcellular locations as shown in Chapters 2 and 3, provides strong evidence that the choice of expression system is an important factor in the delivery of antigens to the APCs of the GALT. In contrast to the general consensus in the literature that increasing protection and encapsulation of antigens within plant cells is important for immunogenicity,[10,27,82-93] early release of LTB from plant cells appears to be correlated with strong immunogenicity in mouse and sheep models. It is unclear if early release is a specific requirement for LTB or antigens with inherent epithelial targeting capacity such as AB_5 enterotoxins, or may be common to a variety of mucosal antigens that are stable in the GIT.

It is important to note that the ideal location of antigen release during transit in the GIT is likely to vary based on antigen stability and the desired immune response. For example, the controlled release of orally delivered antigens in the colon induced a far higher humoral and cellular immune response in the genitorectal mucosa than similar formulations where the same antigen was released in the stomach or small intestine.[94] Importantly, changing the characteristics of the plant cell may allow for a tailored site of antigen release during digestion (see Figure 5-6). For example, increasing the concentration of plant-derived mucoadhesive polymers such as pectins may increase adhesion of the plant formulation to the epithelium,[62,95-97] and altering the biosynthetic pathways of the cell wall to modify its size, thickness or composition may increase the protection offered to recombinant antigens during transit in the GIT. [98-101] Also, expression of antigens in endosperm with thicker, enzymatically resistant cell walls and/or protein storage organelles may also lead to far longer transit in the GIT prior to antigen release.[43]

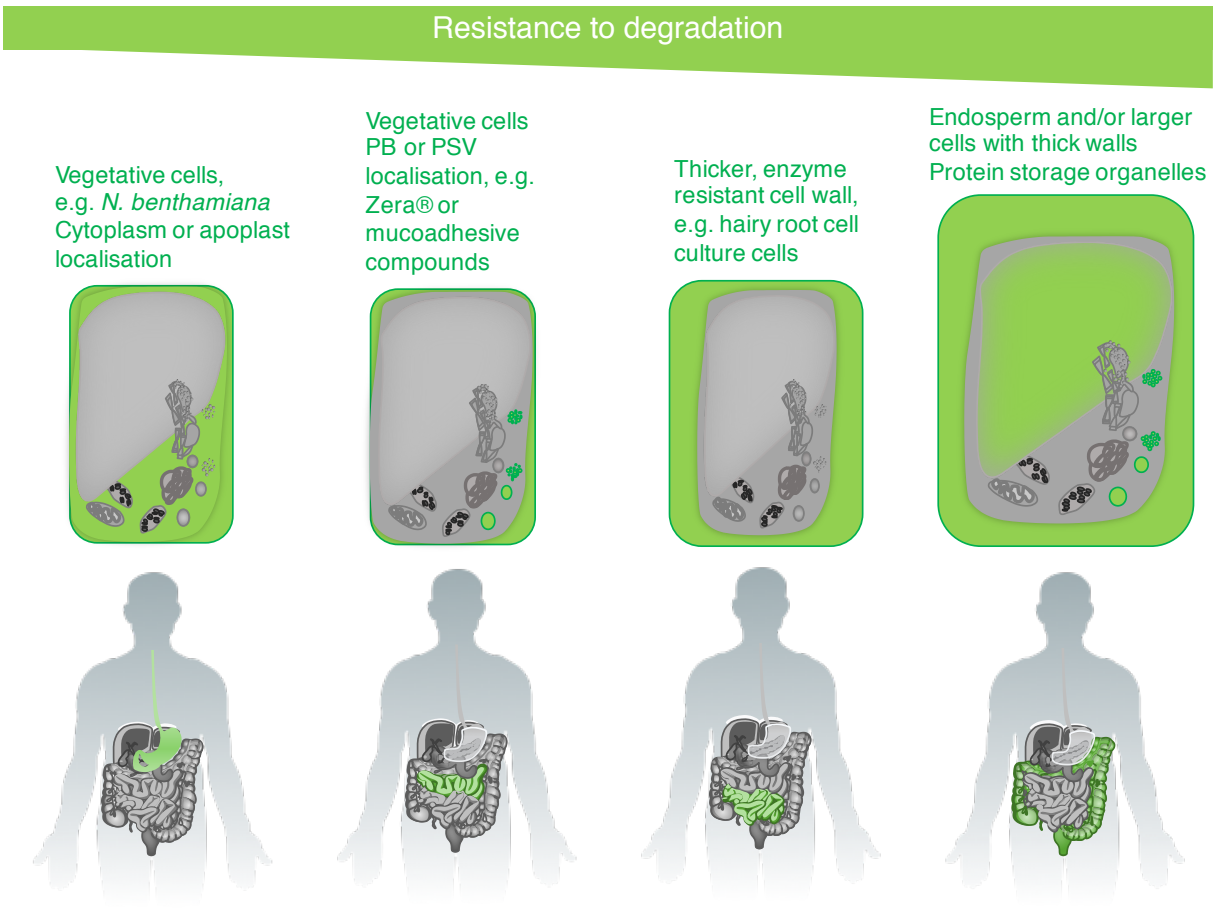

Figure 5-6 Plant cells characteristics may be tailored to the desired release characteristics for vaccine antigens. Leaf and other vegetative cells are likely to release cellular contents early during digestion and predominantly in the stomach. Plant cells containing mucoadhesive compounds or antigens encapsulated in PBs may slow the release of antigens from the plant biomass until they have transited into the small intestine. Also, plant cells with thicker and more enzyme-resistant cell walls may be able to transit further through the small intestine before releasing antigens. Finally, highly encapsulated and/or large cells may provide sufficient protection of antigens for extended transit in the GIT, potentially as far as the large intestine.

At present, the process of plant cell lysis and the factors affecting protein release during digestion are not particularly well characterised in any model system. Therefore, further exploration of this field will require a concerted effort to understand the mechanistic factors that influence the release characteristics of plant cells. This work should aim to understand the biomechanical, enzymatic, and commensal-related variables associated with antigen release in a variety of animal models. With a focus on ensuring that this understanding can be translated into larger non-human primates and eventually clinical studies. These studies would need to include an investigation of the batch to batch bioavailability and release kinetics, as well as the development of methods to effectively track and report on the site of antigen release *in vivo*, independent of the antigen stability in the GIT.[102] Indeed, the recent report of Shaaltiel et al., has provided one of the first clear examples of this detailed characterisation, where carrot and *N. tabacum* BY-2 cells were tested for the range of pH conditions and protease activity that the cell wall is able to withstand before releasing the cellular contents.[16]

It is also important that the formulations used for these studies are sufficiently characterised and applicable to real-world pharmaceutical formulations, including the use of GMP-grade excipients and stabilisers. For example, it is important that these formulations are based on dose forms that are currently licensed for human use including capsules, tablets, or powders, rather than the 'edible vaccine' formulations used in this thesis. Kashima et al., have recently published the GMP specifications for the master seed bank, drug substance and drug formulation of CTB-expressing rice powder, providing an excellent and timely example of the level of product qualification and standardisation required to reliably investigate the downstream effects of these complex formulations.[103]

5.5.2 Increasing the immunological context of antigen presentation

The data presented throughout this thesis indicates that while the choice of plant expression system influences the immune response to vaccination, the role of the plant-specific biomatrix on the presentation of co-delivered antigens to the GALT is difficult to determine due to the complexity of plant cell formulations. In an effort to isolate the impact of a single plant-derived metabolite of tomato fruit, the influence of α-tomatine on the oral immunogenicity of NVLPs expressed and delivered in tomato fruit was investigated using a mouse model. Despite the hypothesis that the presence of this known systemic adjuvant improves the immunogenicity of the co-delivered antigen, no variation in immunogenicity was observed across a range of endogenous and exogenous formulations containing varying levels of α-tomatine. Although α-tomatine was not an effective oral adjuvant in the model system used in this thesis, expression of antigens in other plant species with known bioactive metabolites such as saponins in *P. ginseng*,[104] or inulins [105] may still be a viable method for utilising the 'natural' adjuvant capacity of plant cells to increase oral immunogenicity (see Figure 5-7). In addition to the endogenous adjuvants of the plant cell, co-expression of orally active cytokines such as IFN-γ [106,107] or TLR agonists such as flagellin [108,109] may also improve the immunogenicity of co-delivered vaccine antigens in plant formulations without intrinsic immunostimulatory properties.

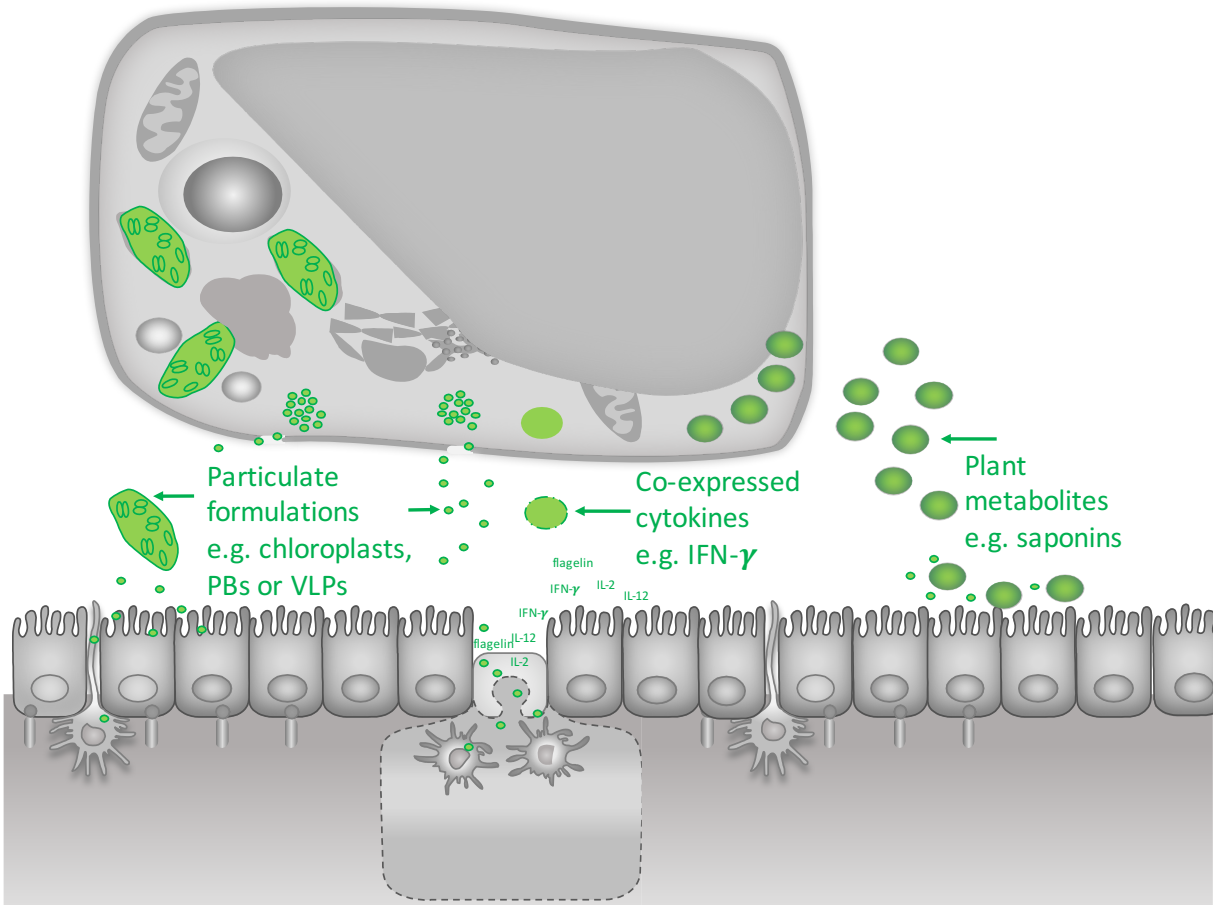

Figure 5-7 Improving the immunological context of antigens expressed in plant cells. Mechanisms for enhancing the immunological recognition and response to plant-delivered antigens includes formulation as particulates including protein bodies (PBs) or transplastomic chloroplasts, co-expression of immunomodulatory cytokines such as interferon γ (IFN-γ), or plant-intrinsic metabolites such as saponins.

Another strategy for improving the immunological context of orally delivered antigens in plant cells is the formulation of antigens into particulates. The capacity of 1-10 μm particles to be efficiently transcytosed by both M-cells [110] and DCs [111] appear to make particles an ideal solution for efficiently delivering otherwise low or non-immunogenic antigens across the epithelium. Particulate formulations may include transplastomic encapsulation of antigens,[112] PBs induced by fusion tags such as Zera® or by localisation of antigens in PBs endogenous to storage organs, or expression of native [77,113-119] or chimeric VLPs.[118,120] However, the moderate immunogenicity of LTB localised in Zera®-induced PBs observed in Chapter 3 questions whether Zera®-based PBs increase oral immunogenicity. Recent studies have used Zera® to express a range of antigens in PBs, but the oral immunogenicity of these formulations has yet to be determined.[116,121-123] Furthermore, the high immunogenicity of LTB localised to PSVs suggests that the use of this subcellular location may enhance immunogenicity improved uptake by M-cells or other, unknown mechanisms.

While many of the plant-specific mechanisms for improving the immunological context of antigen presentation noted in this thesis have been suggested previously in the literature,[124,125]

the integration of these ideas into the designs of new vaccine formulations has been relatively slow. Given that endogenous adjuvant capacity of plant-based expression is a relatively untapped resource for improving oral immunogenicity of plant made vaccines, advancing the understanding of how to best utilise this intrinsic capacity is likely to be one of the key tools to improve the immune response to antigens delivered in plant cells.

5.6 Developing plant-made vaccines into clinical candidates

Although the results presented in this thesis provide evidence that plant-specific factors are important in the use of plant cells as a delivery system, the model antigens, expression systems and delivery method used in these proof-of-concept studies are not candidates for the prevention of human disease regardless of their immunogenicity. Neither LTB or NVCP alone raise an immune response correlate with protection from their host pathogen in field trials where ETEC or *Norovirus* are endemic.[126,127] Specifically, protective immunity requires response to LT or LTB and additional components of the bacterial pathogen including colonisation factor antigens (CFAs) and heat-stable toxins.[126] Additionally, the rapid antigenic drift of the *Norovirus* RNA genome makes it unlikely that any VLP-based vaccine will provide more than 1-2 seasons of protection,[128,129] which is not compatible with expression in *S. lycopersicum* that takes at least a year to transform, grow and characterise the fruit. Other plant systems may be able to overcome some of the inherent limitations of these systems by expressing multiple ETEC antigens including CFAs,[31,32,130] or by rapidly manufacturing NVLPs using transient expression systems.[54] While LTB expressed in *N. benthamiana* or NVCP expressed in tomato are not likely to be clinical candidates, as model systems they have provided a deeper understanding of the plant-specific factors that influence immunogenicity.

The most important factor in determining the capacity for plants systems to be used in clinical applications is their compatibility with the regulatory frameworks for licensure as drugs. While the assessment of the complex regulatory frameworks that govern the use and licensure of human therapeutic products are beyond the scope of this thesis, all major regulatory frameworks are generally compatible with the use of plants for the manufacture and delivery of vaccines so long as they characterised in a similar manner as therapeutic products produced in other systems.[131,132] Any plant-made oral vaccine candidate developed within the existing frameworks for vaccines will require a far more detailed characterisation of the antigen and formulation than was used for the studies in this thesis.[90] The recent advancements in the Good Manufacturing Processing (GMP)-grade plant-based manufacture of several vaccine candidates provides a clear example of how plant-based production can be made compatible with the existing regulatory frameworks.[54,93,103,133] The use of food-grade expression systems in these GMP production systems, particularly those of stable transgenic crops, also leverage the capacity of plant cells to be administered orally after only minimal processing.[134]

5.7 Conclusion

The use of plants as vaccine delivery vehicles for antigens from enteric pathogens has been discussed in the literature for over 30 years,[135] but to date there are still no licenced products using plants cells as both expression and delivery systems. In part, this is due to the hugely complex task of determining the plant-specific factors responsible for inducing a consistent and protective immune response to vaccination. Recent advancements in the basic immunology of the gut has provided a clearer understanding of the mechanisms for inducing a protective immune response, and now provides some of the mechanistic understanding required to rationally optimise the plant based formulations for improved immunogenicity.

In this thesis, I have shown that the choice of plant expression system not only influences the immune response to an enteric antigen, but can be further optimised to enhance immunogenicity. In Chapter 2, the vaccine formulation and choice of expression system (tomato fruit, hairy root cell culture, or *N. benthamiana* leaf) dramatically altered the release characteristics and immunogenicity of the LTB protein in a mouse oral feeding model. Furthermore, in Chapter 3 the subcellular location of LTB within *N. benthamiana* leaf cells was shown to impact the biochemical characteristics and release kinetics *in vitro* and the immune response *in vivo,* with the optimal location for antigen accumulation being the protein storage vacuoles. However, in Chapter 4, the intrinsic or exogenous concentration of the saponin-like plant metabolite α-tomatine in formulations of lyophilised tomato fruit had no influence on the rate or magnitude of the immune response to Norwalk VLPs despite the known systemic adjuvant qualities of this compound.

Together, these results indicate that the choice and optimisation of the plant expression system are key factors in the effective use of plant cells as delivery vehicles. This understanding will hopefully allow a more considered selection of the plant host system based on the influence on the resulting immune response. Improved health outcomes are now being realised using purified recombinant therapeutic proteins expressed in plant cells, yet the understanding of how to optimise the complex interactions of the plant cell with the mucosal immune system is only now beginning to be understood. The time it takes us to characterise and optimise the immunogenicity of antigens delivered in plant cells will ultimately determine how rapidly this technology will live up to its potential to help in the global fight against diarrhoeal diseases.

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7. APPENDICES

7.1 Appendix 1 Sheep immunogenicity paper

Pelosi, A., Piedrafita, D., De Guzman, G., **Shepherd, RP**., Hamill, JH., Meeusen, E., Walmsley, AM. 2012. *The Effect of Plant Tissue and Vaccine Formulation on the Oral Immunogenicity of a Model Plant-Made Antigen in Sheep.* PLOS One, *7*(12), e52907.

This paper reports the immunogenicity of LTB in *N. benthamiana* leaves or *P. parodii* hairy root cells when orally administered to sheep.

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The Effect of Plant Tissue and Vaccine Formulation on the Oral Immunogenicity of a Model Plant-Made Antigen in Sheep

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Abstract

Antigen-specific antibody responses against a model antigen (the B subunit of the heat labile toxin of enterotoxigenic *Escherichia coli*, LTB) were studied in sheep following oral immunisation with plant-made and delivered vaccines. Delivery from a root-based vehicle resulted in antigen-specific immune responses in mucosal secretions of the abomasum and small intestine and mesenteric lymph nodes. Immune responses from the corresponding leaf-based vaccine were more robust and included stimulation of antigen-specific antibodies in mucosal secretions of the abomasum. These findings suggest that oral delivery of a plant bioencapsulated antigen can survive passage through the rumen to elicit mucosal and systemic immune responses in sheep. Moreover, the plant tissue used as the vaccine delivery vehicle affects the magnitude of these responses.

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Introduction

Vaccines administered via mucosal routes are sought-after because they can induce both mucosal and systemic immune responses to protect against infections caused by pathogens entering and colonising mucosal surfaces such as the gastrointestinal tract (GIT). Mucosal, humoral responses are characterised by secretory antibodies of which the IgA isotype is the most prominent and IgG less abundant [1,2]. An effective mucosal vaccine must deliver antigen to mucosal inductive sites including the mucosal lymphoid tissue (MALT) or sub-epithelial dendritic cells (DCs) when MALT is absent [1,2]. Activated DCs then transport the antigen via the lymphatics to draining mesenteric lymph nodes (MLN) where antigen is presented and a specific immune response mounted. Unfortunately, mucosal immune responses are often variable, particularly when vaccines are delivered orally, exposing the antigen to likely enzymatic degradation in the acidic gastric environment [3]. Vaccine delivery from plant tissues may overcome or at the very least mitigate the hostile gastric environment. Evidence points to antigens bioencapsulated within a plant cell being better protected from the enzymatic degradation of the GIT, prolonging release and presentation of the intact antigen to immune responsive sites of the gut associated lymphoid tissues (GALT) [3]. In addition, plant-made vaccines have a reduced risk of contamination with animal pathogens [4,5] and are stable at room temperature when stored as seed or freeze-dried material thus reducing the reliance for a cold chain [6,7].

The heat labile toxin (LT) of enterotoxigenic *Escherichia coli* is a well characterised, mucosal antigen often used as an adjuvant [8,9] or carrier protein [10]. LT comprises a single, active ADP-ribosylation subunit (LTA) and a non-toxic, pentameric subunit (LTB) [11,12] that selectively binds GM1 ganglioside receptors in the mucosal epithelium of the GIT [13,14]. LTB is stable in the hostile environment of the GIT [15], can be produced in transgenic plants and elicits potent antigen-specific immune responses when delivered orally from various plant tissues [3,10,16,17,18,19,20]. As such, LTB was chosen as a model antigen to study immunogenicity of orally delivered plant-made vaccines in ruminant species.

In an earlier study we examined different plant tissues as potential vehicles for oral delivery of recombinant LTB (rLTB) in the mouse GIT [3]. Our findings indicated that the plant tissue type used as the vaccine delivery vehicle affected the timing of antigen release, occurring earlier when delivered from leaf whilst being delayed from root [3]. In this same study, the orally delivered plant-made vaccines produced more robust immune responses when formulated in a lipid (oil) based, rather than an aqueous based medium [3]. On the basis of these preliminary studies in mice, the aim of the present study was to determine whether orally delivered plant-made vaccines survive passage through the more complex ruminant digestive system and induce

Table 1. Oral immunisation treatments and number of sheep assigned to each group.

Treatment	Sheep
Control hairy root (CtHR)	3 (Sheep #50, 28, 54)
Control leaf (CtLeaf)	2 (Sheep #37, 73)
Transgenic hairy root containing 5mg rLTB (LTB-HR)	5 (Sheep #29, 30, 31, 42, 75)
Transgenic leaf containing 5mg rLTB (LTB-Leaf)	5 (Sheep #36, 47, 57, 64, 69)

doi:10.1371/journal.pone.0052907.t001

immune responses in sheep. Leaf- and root-based LTB vaccines, each formulated in a lipid matrix, were compared and antigen-specific antibody responses localised to specific sites in the sheep GIT and mucosal immune system.

Materials and Methods

Plant materials

Hairy root cultures of transgenic *Petunia parodii* (petunia) plants producing rLTB were generated and maintained as described previously [3,21]. Control petunia hairy root cultures were stably transformed with the pBinPlus empty vector [21,22]. For vaccine batch processing, hairy root cultures were harvested 22 days after subculture, snap frozen in liquid N_2 then freeze-dried using a Dynavac freeze drier (Model FD12) for 48 h with a maximum shelf temperature of 20°C. *Nicotiana benthamiana* leaves transiently expressing apoplast-targeted LTB or GFP were produced as described previously [3]. Leaves were harvested at 7–10 days post-infiltration, snap-frozen in liquid N_2 then freeze-dried using a Dynavac freeze drier (Model FD12) for 48 h with a maximum shelf temperature of 20°C. Freeze-dried plant materials were powdered using a commercial coffee grinder and sieved to standardise particle size to 0.5–1 mm^2. Accumulation of rLTB pentamer, the functional form required for binding to GM1-gangliosides on the mucosal surface of the gut epithelium, was confirmed in *N. bethamiana* leaves and petunia hairy roots as per [3]. In each case, the hairy root and leaf vaccine batches accumulated 300 μg/g dwt rLTB.

Capture enzyme-linked immunosorbent assay (ELISA) to determine rLTB in vaccine batches

Crude protein was extracted from freeze-dried plant material by homogenising in 1:60 (w/v) PBST [PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) supplemented with 0.05% Tween 20] with two 3 mm tungsten carbide beads for 1 min at a frequency of 28/s in a Qiagen Mixer Mill. The homogenate was cleared by centrifugation at 13,000 rpm at 4°C for 5 min.

LTB-specific capture ELISA was performed using Costar 9018 96-well microtitre plates (Corning Life Sciences) coated with 50 μl/well of chicken anti-cholera enterotoxin subunit B (CTB) antibody (Sigma-Aldrich) diluted 1:5,000 in PBS. Plates were sealed and incubated at 4°C overnight. Unless stated otherwise, all subsequent incubations were performed at 37°C for 1 h and antibodies diluted in 1% dry skim milk powder (DM) in PBST. Following all incubations, plates were washed three times with PBST.

Plates were blocked with 5% DM in PBST before a 2 h room temperature (22–25°C) incubation with serially diluted crude plant extract starting with 1:100 in PBS. Plates were then incubated with 1:2,000 rabbit anti-LTB (Benchmark Biolabs), then 1:15,000 goat anti-rabbit IgG HRP conjugate (Sigma-Aldrich). Bound LTB-specific antibodies were visualised using TMB-peroxidase substrate (Bio-Rad Laboratories) according to manufacturer's instructions. The amount of rLTB in the freeze-dried plant materials was calculated against a *Pichia pastoris*-made rLTB (Sigma-Aldrich)

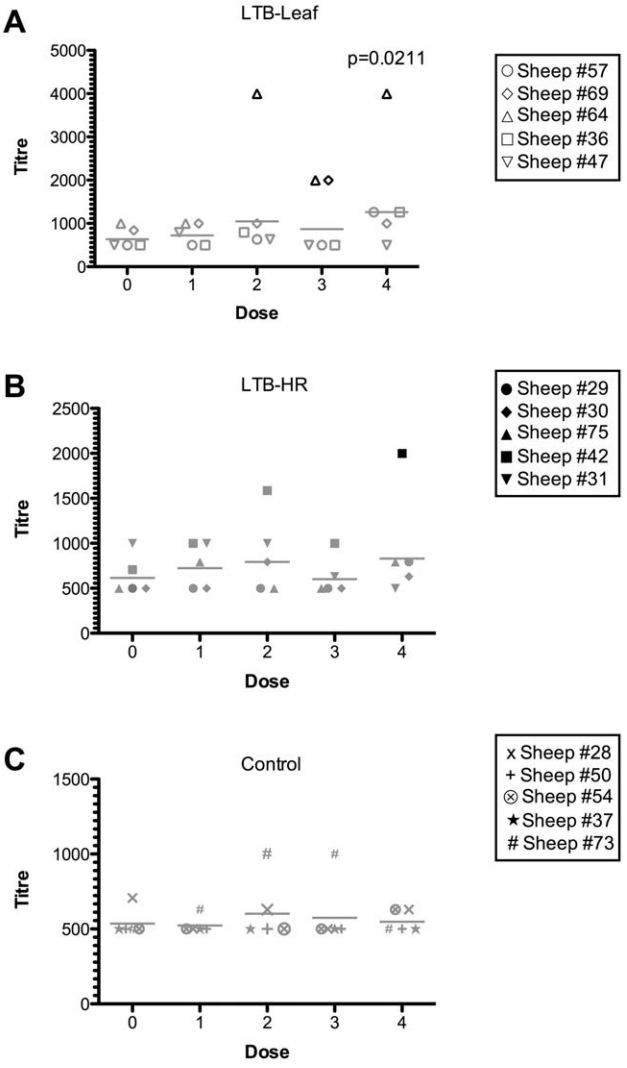

Figure 1. LTB-specific IgG antibody titres in serum collected from sheep before immunisation with LTB-Leaf (A), LTB-HR (B) or control vaccines (C). Black symbols denote positive responders defined as sheep with antibody titres at least three standard deviations above the control mean, non-responders are indicated by grey symbols. The horizontal lines represent geometric means, statistical analysis (Student's t-test determined a significant difference between the means of the control and LTB-Leaf groups after four doses, $p<0.05$).
doi:10.1371/journal.pone.0052907.g001

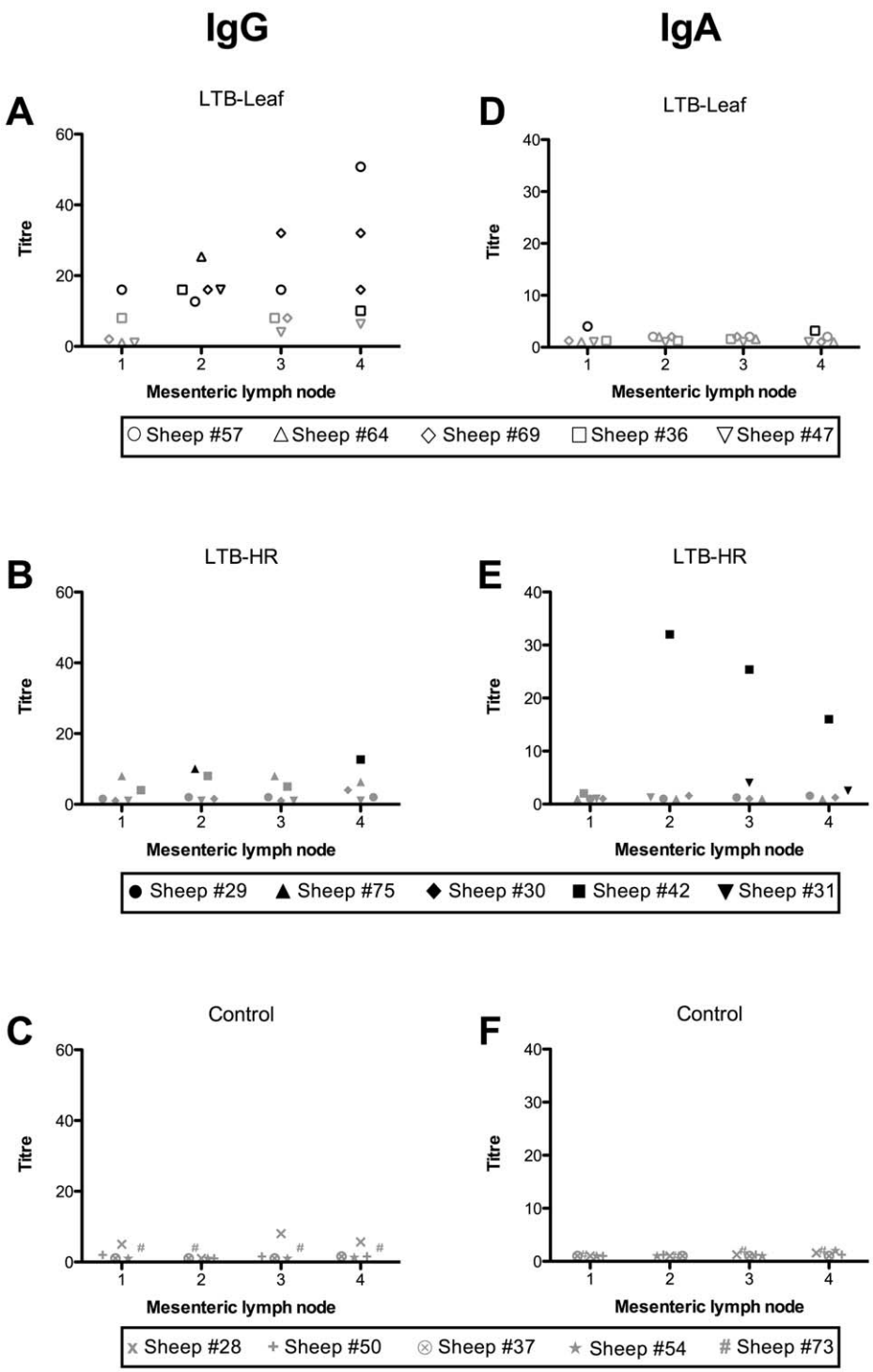

Figure 2. LTB-specific antibody titres in MLNs collected from successive sites along the small intestine of the sheep GIT following oral immunisation with four doses of LTB-Leaf (A and D, IgG and IgA respectively), LTB-HR (B and E, IgG and IgA respectively) or control (C and F, IgG and IgA respectively) plant materials. MLN 1 was sampled from the abomasum/duodenum junction, MLN 2–4 were the next three lymph nodes sampled from the first 0.5 m of the small intestine. Black symbols denote positive responders defined as sheep with antibody titres at least three standard deviations above the control mean, non-responders are indicated by grey symbols.
doi:10.1371/journal.pone.0052907.g002

standard. Accumulation of the functional pentameric form of rLTB was confirmed by western blot [3].

Mucosal vaccination of sheep

Outbred, male sheep (*Ovis aries*, Merion/Merino) aged between 4.5 to 12 months were obtained from the Commercial Registered Pfizer Animal Health Woodend Farm and housed at the Monash

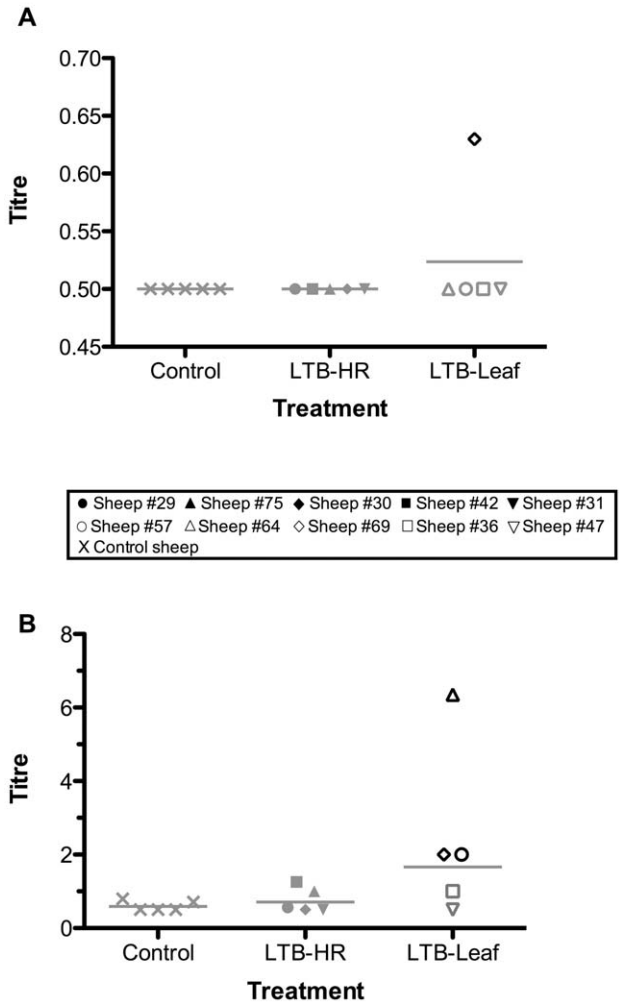

Figure 3. LTB-specific IgG (A) and IgA (B) antibody titres in abomasum mucus following oral immunisation with four doses of control or LTB-transgenic plant materials. The horizontal lines represent geometric means. Black symbols denote positive responders defined as sheep with antibody titres at least three standard deviations above the control mean, non-responders are indicated by grey symbols.
doi:10.1371/journal.pone.0052907.g003

University Werribee Animal Facility under conditions approved by the Monash University Animal Ethics Committee (AEC SOBSA/P/2009/98). Sheep were provided with water and standard feed ad lib and fasted 16 h before oral immunisation. Sheep were randomly assigned into four groups of 2–5 animals each (Table 1). A single sheep from the transgenic rLTB expressing leaf vaccine group (LTB-Leaf) developed balanopsthitis (pizzle rot) 14 days after beginning the trial and was treated with a testosterone implant. This sheep was not excluded from analyses.

Sheep were immunised on days 0, 14 and 28 followed by a boost dose on day 38, four days before sacrifice. Vaccine materials were formulated immediately before delivery by mixing 19 g freeze-dried plant material with 200 ml of an oil based emulsion (125 ml peanut oil:75 ml dH_2O). When receiving the transgenic rLTB plant-based vaccines (LTB-HR or LTB-Leaf), each dose was sufficient to deliver 5 mg rLTB. Sheep receiving the CtHR or CtLeaf vaccines were immunised with the equivalent volume of formulated control plant materials. The formulated vaccines were administered orally to sheep by gavage directly into the rumen to simulate drenching, a common delivery system used routinely to worm domestic sheep flocks. At trial termination (day 42), sheep were humanely killed by intravenous injection with a lethal dose of lethobabarb (100 mg/kg bodyweight).

Collection and processing of biological specimens

Serum collection. Blood samples were taken from the jugular vein using an 18 G needle immediately before the first immunisation (pre-immune), 14 days after each of the first three doses and four days (at trial termination) after the boost. The blood was clotted at room temperature (20–22°C) overnight and serum separated by centrifugation at 400 *g* for 10 min and stored at −20°C until required for LTB-specific antibody detection by ELISA.

Sampling and in vitro culture of mesenteric lymph nodes. At post-mortem, four lymph nodes were taken from the mesentery, the first at the abomasum/duodenum junction (MLN 1) and the next three along the first 0.5 m of the small intestine (MLN 2–4). MLNs were subjected to an antigen-specific antibody secreting cell (ASC) assay for detection of LTB-specific antibody responses using a protocol modified from those previously described [23,24]. MLNs were dissected into small pieces and cultured in 24-well flat-bottomed tissue culture plates. One MLN and 1 ml complete DMEM medium (Gibco) containing 10% (v/v) heat-inactivated foetal calf serum (Thermo), 100 U/ml penicillin (Gibco), 0.1 mg/ml streptomycin (Gibco) and 2 mM glutamine (Gibco) per well were incubated at 37°C in a humidified incubator with 5% CO_2 for 24 h. Culture supernatants (ASC supernatants) were collected and stored at −20°C and the presence of LTB-specific antibodies determined by ELISA.

Sampling the mucosa of the abomasum. The mucosal lining of the abomasum was sampled by scraping the inside surface with a glass slide. Mucus scrapings were prepared for ELISA as described by [25]. Abomasal scrapings were washed off the slide into a 50 ml tube with 3 ml PBST supplemented with 2x Roche Complete Protease Inhibitor Cocktail tablets (PBST2I). The supernatant was collected following centrifugation at 9000 *g* for 15 min at 4°C and stored at −20°C until required.

Small intestine washes to sample intestinal secretions. Four sections of the small intestine were excised, each section measured 0.5 m in length and was taken 3 m apart, beginning at the abomasum/duodenum junction (section 1, 0–0.5 m). Sections 2–4 were sampled at 3.5–4 m, 7–7.5 m and 10.5–11 m respectively. Each segment was flushed with 20 ml saline then incubated for 30 min with 10 ml saline and gentle rocking. Each end of the intestinal segments was clamped during washes to prevent leakage. Washes containing intestinal secretions were collected and stored at −20°C until required.

Faecal sampling. Faecal samples were collected before vaccination on day 0 and again at day 16 and 36 h after immunisation with the second oral dose allowing administered vaccine material to complete transit through the sheep GIT [26]. Faecal matter was homogenised in 1 ml/g PBST2I with two 3 mm tungsten carbide beads for 1 min at a frequency of 28/s in a Qiagen Mixer Mill. The homogenate was cleared by centrifugation at 13,000 rpm at 4°C for 10 min and capture ELISA performed (as described above) to detect and quantify LTB in the supernatant.

ELISA to determine LTB-specific IgG and IgA antibody titre

LTB-specific ELISA was used to assess IgG and IgA antibody responses in immunised sheep. Costar 9018 96-well microtitre plates (Corning Life Sciences) were coated with 50 µl/well chicken CTB antibody (Sigma-Aldrich) diluted 1:5,000 in PBS. Plates were

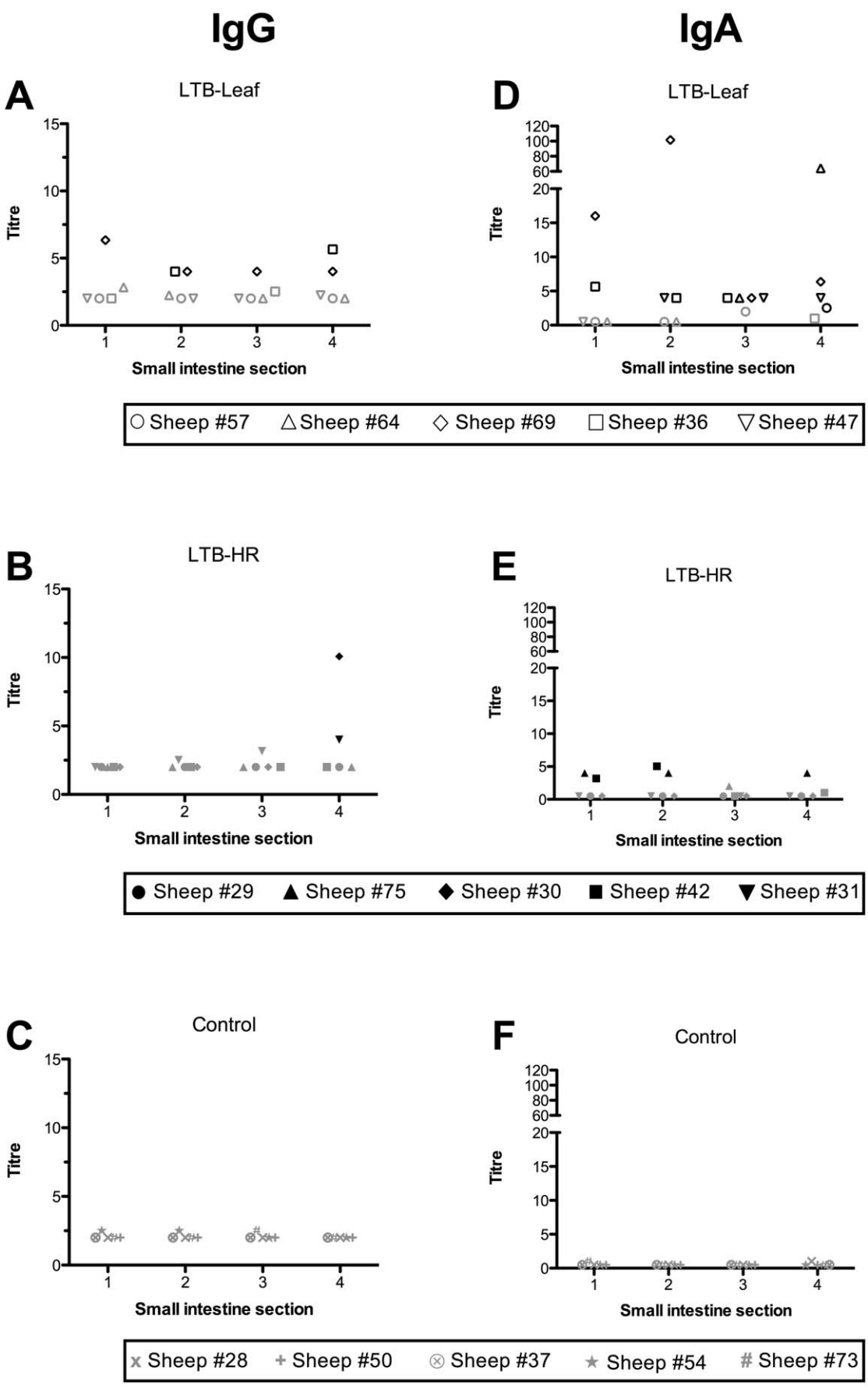
IgG
IgA
A
LTB-Leaf
D
LTB-Leaf
Titre
Small intestine section
○ Sheep #57
△ Sheep #64
◇ Sheep #69
□ Sheep #36
▽ Sheep #47
B
LTB-HR
E
LTB-HR
● Sheep #29
▲ Sheep #75
◆ Sheep #30
■ Sheep #42
▼ Sheep #31
C
Control
F
Control
x Sheep #28
+ Sheep #50
⊗ Sheep #37
★ Sheep #54
Sheep #73

Figure 4. LTB-specific antibody titres detected in intestinal washes performed at four sites along the first 11 m of the sheep small intestine following oral immunisation with four doses of LTB-Leaf (A and D, IgG and IgA respectively), LTB-HR (B and E, IgG and IgA respectively) or control (C and F, IgG and IgA respectively). Sections 1, 2, 3 and 4 are defined as the first 0–0.5 m, 3.5–4 m, 7–7.5 m and 10.5–11 m respectively from the abomasum/duodenum junction. Black symbols denote positive responders defined as sheep with antibody titres at least three standard deviations above the control mean, non-responders are indicated by grey symbols.
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sealed and incubated at 4°C overnight. Three washes with PBST were performed following each incubation. Plates were blocked with 5% DM in PBST at 37°C for 1 h before a further incubation for 1 h at 37°C with 0.25 μg/ml *Pichia*-made rLTB (Sigma-Aldrich). Serial dilutions of the various biological samples were made on the plate with starting dilutions in PBST as follows – 1:1000 or undiluted serum for IgG or IgA determination respectively, 1:2 ASC supernatant, 1:4 small intestine wash and undiluted abomasum mucus. Plates were incubated overnight at 4°C before incubation with 1:2,000 mouse anti-sheep/goat IgG HRP conjugate (Enzo Life Sciences), or rabbit anti-sheep IgA HRP conjugate (Novus Biologicals) at 37°C for 2 h. Bound LTB-specific antibodies were visualised using TMB-peroxidase substrate (Bio-Rad Laboratories) according to manufacturer's instructions. Endpoint antibody tire was reported as the highest dilution with an absorbance of four standard deviation units above background (mean absorbance of at least three wells lacking biological sample). All measurements were performed in triplicate, the geometric mean titre determined and data subjected to statistical analysis using the non-parametic one way analysis of variance (ANOVA) and student's t-test. Data from sheep receiving control vaccine treatments (CtHR and CtLeaf) were combined for analysis. An antigen-specific antibody response exceeding the geometric mean titre of the control group (background) by at least three standard deviations was considered a positive response.

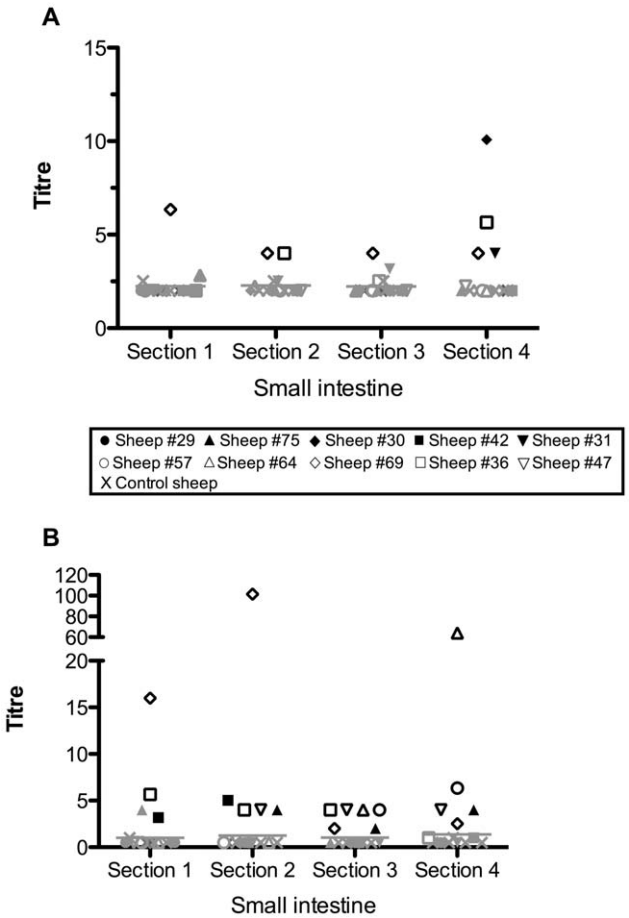

Figure 5. Relative abundance of LTB-specific IgG (A) and IgA (B) at different sections of the sheep small intestine following oral immunisation with four doses of control or LTB-transgenic plant materials. The horizontal lines represent geometric means. Black symbols denote positive responders defined as sheep with antibody titres at least three standard deviations above the control mean, non-responders are indicated by grey symbols.
doi:10.1371/journal.pone.0052907.g005

Results

Plant Materials

Two different plant species *N. benthamiana* and *Petunia parodii* (petunia) were chosen due to their lack of use in the animal or human food chain to reduce the chance of contamination of the food chain and due to their ease of transformation. Although this resulted in more than one variable in the study our previous study demonstrated their optimal nature for oral delivery to mice [3] and we hence sought to delineate their ability to orally deliver to ruminants.

LTB-specific antibody responses in serum

Immunisation of sheep with the LTB-Leaf vaccine resulted in a higher number of sheep with positive antigen-specific IgG-serum responses than those receiving the LTB-HR vaccine (Fig. 1). The mean titre observed for sheep immunised with the LTB-Leaf vaccine was significantly different from controls after four vaccine doses. In one of the five LTB-Leaf immunised sheep (Sheep #64), the maximum IgG-serum response was observed after two immunisations (Fig. 1A) and was not increased by an additional two doses. After three doses, the number of reactive LTB-Leaf immunised sheep was doubled, but this response waned in one animal (Sheep #69) by trial's end. In contrast, four doses of the LTB-HR vaccine were required to produce a single animal (Sheep #42) with reactive serum (Fig. 1B). LTB-specific IgA antibodies were not detected in sera, irrespective of the vaccine or number of doses administered. The baseline antibody titres observed in pre-immune serum could be attributed to a low level of *E. coli* colonisation in animals, which were not housed in germ-free conditions.

LTB-specific antibody responses in antibody secreting cells of mesenteric lymph nodes

Detection of antibody production in serum following oral immunisation may not be indicative of immune responses at mucosal sites [24]. The ASC assay was adopted as a potentially more sensitive method for detection of antigen-specific antibody production from MLNs draining the intestinal tissue. Unlike the serum analysis, both IgG and IgA antibody isotypes were detected in MLN-derived ASC supernatants taken from LTB-HR or LTB-Leaf immunised sheep (Fig. 2).

All five sheep immunised with the LTB-Leaf vaccine assayed positive for an LTB-specific ASC-IgG response at one or more of the MLN sites sampled (Fig. 2A). One sheep from the LTB-Leaf group (Sheep #57) exhibited a positive ASC-IgG response at all

four MLNs. This same sheep, along with Sheep #36 were also positive for an ASC-IgA response at MLNs 1 and 2 respectively (Fig. 2D). Of the LTB-HR immunised sheep, Sheep #42 and 31 displayed at least one positive ASC response for both IgG and IgA isotypes with maximum IgA titres recorded for Sheep #42 at three MLN sites (Fig. 2E).

LTB-specific antibody responses in the abomasal mucosa and secretions of the small intestine

Induction of LTB-specific antibody responses in the mucosa of the abomasum was identified only after immunisation with the LTB-Leaf vaccine (Fig. 3). At this site three sheep were identified as positive responders with IgA titres above those observed for the control group (Fig. 3B). One of these sheep (Sheep #69) also exhibited an elevated IgG titre (Fig. 3A).

LTB-specific IgG antibody was detected in intestinal washes of two of the five sheep immunised with the LTB-Leaf vaccine (Fig. 4A). In one of these sheep (Sheep #69) the response was detected at all four sections sampled from the small intestine (Fig. 4A). The number of antigen-specific IgG positive LTB-Leaf immunised sheep increased from one to two when washes were taken at sections 2 and 4 (3.5–4 m and 10.5–11 m respectively) of the small intestine (Fig. 4A). It was at the most distant site sampled that two IgG positive LTB-HR immunised sheep were also identified (Fig. 4B). All sheep immunised with the LTB-Leaf vaccine also exhibited a positive IgA response at one or more sites sampled along the small intestine (Fig. 4D). LTB-specific IgA responses in the small intestine were stimulated above controls in two LTB-HR immunised sheep at all sections except section 3 (7–7.5 m; Fig. 4E); one of these sheep (Sheep #75,) was also positive at section 4 (10.5–11 m; Fig. 4E). Of the sites sampled along the small intestine, the most immunologically responsive with respect to immunoglobulin production was section 4 (10.5–11 m) for IgG (Fig. 5A), whilst IgA was more widespread, observed at sections 2 to 4 (3.5–11 m; Fig. 5B).

Detection of LTB in faeces

Faecal samples were assayed for LTB to determine whether the vaccine plant materials had resisted breakdown during passage through the sheep GIT. LTB was not detected in faecal samples taken from pre- and post-immune sheep from control, LTB-HR or LTB-Leaf groups (data not shown).

Discussion

The pharmaceutical industry is constantly assessing methods for improved delivery for vaccines, pharmaceuticals and nutraceuticals. The oral route increases ease of delivery, is less expensive, and encourages increased compliance by eliminating the need for needles. Moreover, oral delivery is particularly desired for immunising free-ranging domestic animals that are typically ruminants. Numerous studies have reported immunogenicity of orally delivered plant-made vaccines in humans and small animal models, but few have demonstrated their efficacy in ruminants [27,28,29,30]. We have previously determined that the way plant-made vaccine material is delivered influences immunological outcomes in mice [3]. We therefore now investigate how plant-made vaccine material delivery influences immunological outcomes in sheep, an important end user ruminant and also a model for other ruminants such as goat and cattle.

LTB was chosen as our model antigen because it can be produced in a wide variety of plant systems [3,16,19,20], is stable under acidic conditions [31] and in the GIT [15] and has immunogenic properties when delivered orally. Its affinity for binding the GM1 receptor to mediate transepithelial flux from the lumen into the abluminal environment also makes LTB a potentially important component as an immune modulator in the design of subunit vaccines. Similarly, the plant system used to orally deliver a vaccine candidate merits careful consideration. Destruction of pH-sensitive antigens in the acidic environment of the sheep abomasum could be avoided if delivered from a root-based vaccine to manipulate release into the small intestine. In the present study, mucosal (abomasal, intestinal and ASC-derived IgA and IgG) and systemic (serum IgG) immune responses were achieved in sheep orally immunised with plant-made LTB vaccines delivered from root and leaf material. Local antibody detection at mucosal sites was more sensitive than serum. Of the LTB-HR and LTB-Leaf vaccines delivered, the latter stimulated more robust antigen-specific antibody responses at mucosal sites of the GIT, including the stomach and small intestine, in serum and MLNs.

Vaccine materials were formulated in oil and administered directly into the rumen of the sheep via a tube inserted down the oesophagus. The delivered plant materials were sieved to achieve a uniform particle size of 0.5–1 mm^2 to better protect the antigen from degradation by minimising the time spent in the rumen. In sheep, particles with diameters larger than 1.18 mm transit through the rumen slower than smaller particles [32]; this has also been found in cattle with increased forage particle size improving fibre digestibility by increasing retention time in the rumen [33].

From the rumen, the vaccine transits through the reticulum and omasum before reaching the abomasum (true stomach) where enzymatic digestion of protein, carbohydrates and lipids is initiated. It is anticipated that breakdown of the plant cells encapsulating the rLTB antigen begins in the rumen and continues in the reticulum, the principal sites for cellulose digestion in ruminant species. It was in the abomasum mucus that antibody responses were first observed following administration of the LTB-Leaf vaccine. This suggests that as the leaf material begins to degrade the antigen remains sufficiently protected during rumination, presumably by the lipid coating provided by the oil formulation matrix. In contrast, the lack of antibody response in abomasum mucus from the LTB-HR vaccine suggests that root tissue may be comparatively more resistant to rumination and enzymatic digestion resulting in delayed antigen release.

Although GALT is absent in the abomasum, immune responses can be induced when the mucosal epithelium is penetrated [2]. LTB is particularly efficient in crossing the epithelium from the lumen primarily via binding to ganglioside GM1 along with other mammalian galactoglycoprotein receptors [13,14]. Moreover, direct sampling of antigen from the mucosal lumen may also occur via intra- and sub-epithelial DCs [2,34]. Once the antigen has traversed the mucosal epithelium it is transported by DCs via the lymphatics to draining MLNs where antigen-specific B cells are generated and then returned to mucosal sites via the blood stream [2,35].

From the abomasum, the vaccine materials enter the small intestine. By this stage breakdown of the plant cells and formulation matrix should be completed, releasing the remainder of its antigenic cargo. It was in the small intestine that the most robust mucosal immune responses were detected from both the LTB-Leaf and LTB-HR vaccines, the leaf material producing elevated IgA titres compared to other treatments in all five sheep receiving this vaccine. It was of interest that section 4, the section further through the GIT, was the site where the most robust antigen-specific IgG responses were found while IgA responses expanded to earlier sites (sections 2 to 4). The consistency in the

immune response observed at the small intestine, particularly for the LTB-Leaf group, is noteworthy given the potential for variable responses when using an outbred sample of sheep.

LTB-specific IgA antibodies were absent in all sera, irrespective of vaccine treatment or number of doses administered. This is not unexpected as detection of antibody production in serum following mucosal immunisation can be typically difficult particularly when responses are low [24]. An alternative approach, previously validated in several studies, was utilised to detect antibodies secreted by MLNs using the ASC assay [23,36]. Elevated IgA titres were detected in the MLNs of two LTB-Leaf- and LTB-HR-vaccinated sheep as compared to other treatments. In addition, MLN 2 was identified as the most active site for generating an IgG response with all LTB-Leaf- (two more than that identified from serum) and one LTB-HR-vaccinated sheep exhibiting stimulated titres. It is interesting to note that the different plant vehicles induced different isotype responses at the MLNs with root-delivered LTB elevating IgA titres in contrast to the stimulated IgG titres observed for the leaf-delivered counterpart.

Whilst most of the immune inductive sites of the GIT are located in the GALT of the small intestine, the potency of the LTB-Leaf vaccine benefitted from an early release in the abomasum perhaps due to the stability of LTB and the resulting prolonged antigen exposure at mucosal surfaces and priming distal sites in the small intestine. Antibody responses at the tonsils or other lymphoid tissues of the oral and nasopharyngeal cavities were not sampled in this study but should not be discounted as additional sites within the mucosal epithelium that could be exploited for induction of immune responses from plant-made vaccines. Plant material in its nature is fibrous and as such is often regurgitated from the rumen during fermentation for further mechanical breakdown by chewing and can result in repeated and sustained exposure of the plant-delivered antigen to the tonsils priming more distal sites of the GIT or respiratory system [28].

It is apparent that both the leaf- and root-based vaccine preparations protected the antigenic load sufficiently during rumination and enzymatic digestion to enable its delivery to relevant immune responsive sites. Furthermore, the type of plant tissue used can manipulate timing of antigen release. In our experience, antigen release from both leaf- and root-based vaccines has been consistent across sheep (present study) and mouse [3] animal models. In each case the leaf-based vaccine facilitated early antigen release in the true stomach of orally immunised sheep and mice, whilst the root-based vaccine delayed release to the small intestine. Improved antigen release and antibody responses from root-based vaccine delivery vehicles may be served by different plant species, altered culture conditions or harvest times.

The plant material used to deliver LTB orally to sheep affected immunogenicity. This finding suggests that a delicate balance between protecting the vaccine antigen against digestive degradation and enabling release for presentation of the antigen at immune responsive sites needs to be struck to maximise vaccine efficacy. Although *N. benthamiana* leaf material provided the optimal oral delivery vehicle for induction of mucosal immune responses to LTB in both monogastric (mouse) and ruminant (sheep) models, it is anticipated that plant choice will need to be assessed on a case by case basis, taking into account antigen stability.

Optimising oral delivery of plant-made, valuable proteins will have broad ramifications to animal as well as human health. Oral delivery will facilitate treatment of free-ranging domesticated and native animal populations that may otherwise go untreated, broaden opportunities for existing pharmaceuticals and create opportunities for new compounds and target populations.

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Author Contributions

Conceived and designed the experiments: AP DP RS EM AW. Performed the experiments: AP GDG RS. Analyzed the data: AP DP RS EM AW. Contributed reagents/materials/analysis tools: AP GDG RS EM AW. Wrote the paper: AP.

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7.2 Appendix 2 Delivery review

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This brief review discusses some of the factors associated with delivery of plant made vaccines.

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Research review paper

Delivery of plant-made vaccines and therapeutics

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ABSTRACT

As commercial approval of the first, purified, plant-based biopharmaceuticals for parenteral delivery to humans approaches, improved strategies for delivery of plant-made vaccines and therapeutics are required to ensure their further development and to fulfil the prospect of supplying a global solution for affordable medicines. To ensure that this occurs, research should investigate and characterise the host immune system in addition to the effects of adjuvants and carrier vehicles on consistency and efficacy of vaccination. In this review we explore the basic understandings of pharmaceutical delivery and its effect on immunogenicity in an effort to advance the plant-made pharmaceutical platform.

Contents

1. Introduction

The strong global demand for low-cost pharmaceuticals is the impetus driving molecular farming, particularly in developing nations where the demand is highest yet access to therapeutics for political, economic and logistical reasons is limited (Drake and Thangaraj, 2010; Penney et al., 2011). Plant-based systems potentially provide a low cost alternative for the production of recombinant proteins. The regulatory

Abbreviations: APCs, Antigen presenting cells; cGMP, Current good manufacturing practices; DCs, Dentritic cells; Ig, Immunoglobulin; ISCOM, Immunostimulatory complex; M-cells, Microfold cells; MHC, Major histocompatibility complex; Th, T-helper; TSP, Total soluble protein; VLP, Virus-like particle.

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guidelines for the manufacture of plant-made pharmaceutical proteins are now defined and quality standards achievable since plant-based recombinant proteins can be purified or processed to meet criteria for current good manufacturing practices (cGMP) for either parenteral or mucosal delivery. Whilst the commercial manufacture of the first highly-purified, parenteral-delivered plant-made therapeutics is close to reality, the promise of an affordable, stable and easily administered product given by oral delivery still merits pursuing. In this review we discuss the existing strategies, merits and pitfalls for parenteral and mucosal delivery of plant-made vaccines and therapeutics. We explore some of the rapidly advancing basic understandings of delivery of therapeutics in an effort to merge the worlds of drug delivery, immunology and plant sciences.

2. Plants as bioreactors for recombinant pharmaceutical proteins

Therapeutic proteins have been produced in plants since 1986 when human growth hormone was expressed in engineered tobacco and sunflower callus cultures (Barta et al., 1986). The first description of a plant-made vaccine antigen appeared in a patent application filed in 1990 by inventors Curtiss and Cardineau who expressed *Streptococcus mutans* surface protein A in transgenic tobacco plants (Curtiss and Cardineau, 1990). Two years later the expression of hepatitis B surface antigen, also in tobacco, was published in what was to be the first report of a plant-made antigen in a peer-reviewed journal (Mason et al., 1992). Fourteen years later, the first plant-made vaccine received regulatory approval. Chickmate Newcastle disease virus vaccine for chickens (Vermij, 2006) was approved by the US Department of Agriculture Centre for Veterinary Biologics in 2006. The vaccine was produced in a contained, tobacco, plant cell production system, the cells lysed and delivered by subcutaneous injection to chickens. Although this landmark example demonstrated the feasibility of licencing plant-made biopharmaceuticals within the current regulatory framework, it remains the only licenced plant-made vaccine.

One of the greatest hurdles of *in planta* protein expression has been achieving sufficient yields of the protein of interest to warrant further development. Whilst the challenge to improve yields has been addressed for many proteins with recombinant protein yields of up to 80% of total soluble protein (TSP) reported using transient viral based systems (Marillonnet et al., 2004), 51% of TSP reported for stable chloroplast transformation (Lentz et al., 2010) and up to 37% of TSP in seed derived from stable nuclear transformation (De Jaeger et al., 2002), protein-specific solutions may be required in some cases. Recombinant protein yields need to be assessed on a case-by-case basis, a strategy that may work for the production on one protein may not be applicable to another. Efforts devoted to improving recombinant protein yields *in planta* have led to the development of novel and varied strategies, including stable or transient, nuclear or plastid production systems, subcellular organelle targeting, codon optimisation, or choice of promoter, plant tissue or species (Ling et al., 2010; Streatfield and Howard, 2003). If sufficient yields can be obtained the next challenge facing development of plant-made vaccines and therapeutics is an understanding of the optimal routes of delivery, which can deliver therapeutics in bioactive forms that, are safe, consistent and efficacious.

3. Parenteral delivery

To date injection remains the most effective route of vaccine administration. There are some perceived disadvantages of parenteral delivery including the need to purify vaccines to standards compliant with cGMP, which often results in a soluble product that requires cold chain logistics to maintain product integrity during storage and transport. Not only does this increase the financial burden of therapy but can be logistically problematic given that regions of the world that are in greatest need, experience a warm–hot climate and/or may be inaccessible by vehicle. Injected therapeutics require administration by health care professionals and impose risks of needle-stick injury and contamination by infectious agents if appropriate practices are not adhered to. There is also reduced patient compliance due to the physical discomfort and even fear involved with needle delivery.

Parenteral delivery refers to a route of administration that usually involves one or more injections to penetrate the skin or mucosal membrane, bypassing the gastrointestinal tract. This includes subcutaneous, intramuscular, intradermal, intraperitoneal, intralymphatic and intravenous injection. In human drug delivery, intramuscular and intravenous are the predominant delivery routes due to their convenience of administration and in the case of intravenous, speed of circulation. The epidermal and dermal layers of the skin comprise an important network of frontline immunity including antigen presenting cells (APCs) such as dendritic cells (DCs) (Langerhans cells), keratinocytes and lymphocytes. Also between these two layers is a dense network of lymphatic vessels draining into lymph nodes (Bos and Kapsenberg, 1993; Kupper and Fuhlbrigge, 2004). Immunogens injected into the skin are delivered from the peripheral tissue at the site of injection to the lymph nodes in a size dependent manner by either direct drainage or cellular transport (Manolova et al., 2008). DCs residing at both the periphery and lymph nodes are key players in trafficking the antigen and directing protective immune responses. Traffic of larger molecules or particles (500–2000 nm) is mediated by Langerhans cells. Once activated the Langerhans cells mature, take up, process and transport the immunogen to lymph nodes. In contrast, smaller molecules or particles (20–200 nm), including free antigens and virus-like particles (VLPs) drain feely to the lymph nodes where they are taken up by the residing DCs.

The route of parenteral administration may dictate the type and the quality of immune response elicited based on the type of APCs encountered. Intraperitoneal immunisation for example, is more often associated with uptake by macrophages, whilst intradermal immunisation is associated with uptake by DCs (Newman et al., 2002). Comparison of subcutaneous, intradermal, intramuscular and intralymphatic administration of ovalbium (two doses of 20 μg), suggests that whilst all routes efficiently deliver antigen to lymphoid organs to evoke antigen-specific T-helper (Th) 2-type immune responses, Th1 responses were more sensitive to the route of administration with only weak responses observed for subcutaneous, intradermal and intramuscular routes (Mohanan et al., 2010). The inclusion of adjuvants improved the strength of the elicited immune responses across all administration routes. Earlier studies indicate that the Th response is dose sensitive with low doses preferentially stimulating Th2 responses whilst Th1 responses require higher doses (Hosken et al., 1995). These observations suggest that whilst adjuvants may generally improve immune responses, improved Th1 responses may benefit from increased doses when administering antigens *via* subcutaneous, intradermal or intramuscular routes (Mohanan et al., 2010).

The first parenterally (subcutaneous) delivered plant-made vaccine therapeutic to undergo a human Phase I clinical trial was an idiotype vaccine for treatment of non-Hodgkin's lymphoma (McCormick et al., 2008). Patient-specific tumour-derived single-chain variable fragments were transiently expressed in *Nicotiana benthamiana* leaves using a plant-virus based vector (McCormick et al., 2003). Production and purification to cGMP standards occurred within 12–16 weeks of receiving patient biopsy samples, comparing favourably with animal cell approaches. A rapid production system means that vaccines could be administered to newly diagnosed patients before exposure to immunosuppressive treatments such as chemotherapy. This unique feature suggests a niche application for plant-made, patient-specific, recombinant, idiotype vaccines for the treatment of tumours. Subcutaneous administration of the plant-made, patient-specific, non-Hodgkin's lymphoma vaccines were safe, well tolerated and in most cases vaccine-

specific humoral responses and/or cell responses were reported (McCormick et al., 2008).

3.1. Processing and purification

All plant biomass for use as a therapeutic or vaccine, particularly if delivered parenterally, will require some form of processing in order to obtain a homogeneous batch with a uniform distribution of the antigen or therapeutic to ensure accurate dosage. Plant biomass will also require a level of processing compliant with cGMP of relevant regulatory agencies such as the US Food and Drug Administration, European Medicines Agency or the World Health Organization, to yield a safe and well defined product. The feasibility for purification of commercial quantities of recombinant plant-made proteins of pharmaceutical value has been demonstrated previously for avidin (Hood et al., 1997), aprotinin (Zhong et al., 1999), monoclonal antibodies (Baez et al., 2000), bovine trypsin (Woodard et al., 2003), human insulin (Boothe et al., 2010; Nykiforuk et al., 2006) and apolipoprotein AI (Nykiforuk et al., 2011).

The minimally processed extract granted regulatory approval by the US Department of Agriculture Centre for Veterinary Biologics for the Newcastle disease virus vaccine has set the standard for plant-made biopharmaceuticals for veterinary purposes. Standards for human dosing however are more stringent. For parenteral delivery to humans, plant biomass will need to be processed to a well defined and highly purified product. Both the US Food and Drug Administration and European Medicines Agency have established strict guidelines to ensure safety and efficacy of the final product (EMEA, 2008; FDA, 2002). Standard fractionation procedures currently used in the food industry can be adapted and applied to some plant tissues. Seeds and grains for instance can be dehulled using standard grain mills in the early stages of processing. In most cases the engineered biopharmaceutical will be extracted from the plant biomass into an aqueous solution using standard protocols before chromatography and filtration are applied to purify the final therapeutic agent and ensure its sterility for parenteral delivery. It is essential that the desired recombinant protein is stable throughout the processing and purification phases of production. This may be more challenging if the plant tissue is subjected to heat, acidic pH or solvents during processing. Once purified all therapeutics will then need to be subjected to analytical characterisation to evaluate product purity, concentration and homogeneity.

Although plant production systems are free from human and animal pathogens (unlike conventional mammalian cell culture systems) there is an inherent bioburden acquired during development, growth and maintenance. Consequently, processing is required to take place under stringent conditions and containment procedures adhered to wherever possible (EMEA, 2008; FDA, 2002). The final product must be evaluated for impurities and contaminants, including those originating from (i) the host tissue such as extraneous plant proteins (other than the recombinant protein of interest), proteases, plant DNA, secondary metabolites, and post-translationally modified derivatives of the desired product; and (ii) those introduced during production and processing such as soil, fertilisers, pesticides, solvents, endotoxin, toxic metals and microorganisms (mycoplasma, bacteria and fungi). Potential allergens, teratogens, carcinogens, toxins should be all but removed to ensure that any remaining impurities in the final product are within a safe range.

The final processed product will be held to the same standards of quality and efficacy as those imposed for established bacterial, yeast and mammalian cell-based recombinant protein production platforms. Ideally, the product will be stable for a prolonged period of time at ambient temperature, but more likely will require cold storage. The final formulation, whether lipid or aqueous based, encapsulated by or conjugated to carrier particles, or with the inclusion of stabilisers and/or protease inhibitors will influence both efficacy and thermostability. Finally, appropriate analytical assays and/or trials should be performed to demonstrate efficacy and stability of the recombinant plant-made biopharmaceutical.

3.2. Case studies

Some of the cost–benefits of using plants for the commercial production of biopharmaceuticals may be offset by the need to process plant biomass during manufacture. However, case examples taken from commercial biotechnology enterprises SemBioSys Genetics Inc. and Medicago Inc. have demonstrated the practical potential and applicability of two very different plant systems for rapid production of affordable parenteral-delivered vaccines and therapeutics.

SemBioSys Genetics Inc. has produced a pharmaceutical-grade plant-made human insulin (SB-1000) to cGMP specifications using a stable seed-based production platform whereby the engineered protein of interest is targeted to the oil bodies of safflower seeds *via* a fusion to a single chain antibody directed against the 18 kDa oleosin of *Arabidopsis thaliana* (Boothe et al., 2009, 2010; Nykiforuk et al., 2006). A simplified, cost-effective extraction procedure is then used to purify the desired protein from the oil bodies before separation from the oleosin fusion partner by acid cleavage (Boothe et al., 2010; Nykiforuk et al., 2006, 2011). A comprehensive analysis of purified SB-1000 showed it to be free from host-specific impurities and its toxicokinetic characteristics, pathology, clinical signs and injection site changes were indistinguishable from current widely used commercial forms of human insulin when injected subcutaneously into rats and monkeys (Boothe et al., 2009, 2010). A Phase I/II human clinical trial completed in 2009, demonstrated the safety and bioequivalence of SB-1000 to the commercially available forms and has since been submitted to the FDA for drug approval, suggesting the commercial availability of plant-made insulin may be imminent. The applicability of the SemBioSys Genetics Inc. oil body-based production platform to other therapeutic proteins has also been demonstrated with intravenous delivery of a safflower seed-derived recombinant apolipoprotein AI Milano complexed with a phospholipid carrier, resulting in the activation of cholesterol mobilisation and the reduction of atherosclerosis in a preliminary mouse trial (Chyu et al., 2010).

Medicago Inc. has developed a plant-made VLP vaccine against avian H5N1 influenza using a manufacturing platform based on the transient expression of H5 hemagglutinin protein in agroinfiltrated *N. benthamiana* leaves (D'Aoust et al., 2010; Landry et al., 2010). VLPs are recovered from the plant biomass by routine homogenisation, centrifugation, filtration and chromatography. The purified VLPs are then formulated and filter sterilised ready for delivery. The entire process from the identification of pandemic strains to formulated doses can be completed within three weeks. A human Phase I clinical trial showed that two intramuscular doses of alum-adjuvanted plant-made H5 VLP vaccine was safe and well tolerated. Positive responses for microneutralisation assays suggested the presence of antigen-specific antibodies in 95.8% of subjects receiving two doses of 10 or 20 μg (Landry et al., 2010). Immunogenicity and safety of two 20 μg doses of this vaccine has been demonstrated in a Phase II clinical trial (Medicago, 2011). Of the 120 healthy individuals receiving the Medicago vaccine, up to 77% developed immune responses to the H5N1 virus. No serious adverse reactions have been reported, with local site reactions and systemic side effects comparable to the placebo control. The clinical data is encouraging given that a two dose regime of 10 μg is recommended for the commercial inactivated, adjuvanted Panflu® H5N1 vaccine produced in chicken embryos and marketed for human use in China by Sinovac Biotech Co., Ltd. (Lin et al., 2006; Wu et al., 2010).

3.3. Formulation for improved parenteral delivery of plant-made pharmaceuticals

The way forward in the development of plant-made vaccines and therapeutics is to improve bioavailability of the delivered material. This can be achieved through rational and innovative design strategies for better protection and targeting of relevant cellular pathways and immune responsive sites. Also, less invasive alternatives to traditional

injections in the form of micro needles should be explored as a means of parenteral delivery with improved patient compliance.

3.3.1. Adjuvants and biodegradable carrier vehicles

Adjuvants are defined as molecules that modulate the immune response when co-administered with an antigen, but are themselves not immunogenic. They are often used to improve upon weak immunogenic responses associated with subunit vaccines and can direct the immune system to favour Th1 or Th2 responses (Moingeon et al., 2001). Although their mode of action is often unclear, adjuvants can improve immune responses by acting as a depot to guide antigens to relevant sites, protect them from degradation, control release and activate APCs (Granell et al., 2010). They can be naturally occurring in plants, such as lectins and saponins, or can be produced *in planta* as recombinant proteins. A benefit of using plants as a production platform is that they are capable of expressing, processing and assembling complex, recombinant immunogenic complexes. These self-adjuvanting complexes may comprise ligands for pattern recognition receptors (Farran et al., 2010), antigen and self-specific antibodies (Chargelegue et al., 2005), or cytokines (Gora-Sochacka et al., 2010; Merdano et al., 2010).

Adjuvants can be co-delivered with an antigen at the time of administration or incorporated with the antigen into particulate delivery systems. Biodegradable particle carries or delivery vehicles are composed of various materials such as lipids, proteins, starch, polysaccharides or polyesters, and like adjuvants are immunogenically inert (Bachmann and Jennings, 2010). Incorporation of antigens and adjuvant into the delivery vehicle ensures that both reach the same population of APCs (Xiang et al., 2006). The therapeutic cargo can be either encapsulated by the particle by entrapment, or linked to the surface by chemical conjugation or physical adsorption (Oyewumi et al., 2010). The method chosen to associate antigen and particle should be that which affords minimal risk of damage to the antigen. Encapsulated antigens are afforded protection from extracellular proteases during trafficking to the target immune responsive sites. Ideally the carrier particle will erode at a rate sufficient to sustain prolonged exposure to APCs including DCs, macrophages and monocytes. Consequently, the choice of material used to form the particle is critical to maximise bioavailability of its cargo. A fine balancing act is required to avoid overprotection of the encapsulated antigen, inhibiting release of the therapeutic, or premature release if protection is inadequate. In either case the reduced bioavailability of the therapeutic would result in weakened host immune responses. If controlled and sustained release can be achieved it may permit single dose immunisation schedules.

Particulate carriers may offer better targeting for parenteral delivery. In this respect, size is important. Nanoparticles ($\leq$100 nm) are able to move through biological barriers such as membranes and along blood and lymph vessels after injection more efficiently than larger particles (Bachmann and Jennings, 2010; Oyewumi et al., 2010). Alternatively, incorporation of ligands for pattern recognition receptors, such as toll-like receptors, into the design of the particle carrier can facilitate delivery to specific immune responsive sites or cells (Bachmann and Jennings, 2010). Structures such as liposomes, VLPs, virosomes and immunostimulatory complexes (ISCOMs) combine the advantages of both particulate carrier and adjuvant. Moreover, they are particularly well recognised by APCs because they have characteristics, including size, shape and surface properties that are similar to viral and bacterial pathogens that the immune system has evolved to attack (Bachmann and Jennings, 2010; Peek et al., 2008). VLPs for example comprise viral envelope proteins that enable them to self-assemble to closely resemble viruses, but lack the genetic material rendering them non-invasive but readily identifiable by the host immune system. VLP-based vaccines have the added benefit of having been successfully produced in plants (Landry et al., 2010).

ISCOMs are comprised of antigen, cholesterol, phospholipid and an adjuvant of Quil A saponin from the bark of *Quillaja saponaria.* They are within the size range of viruses (~40 nm) and are readily taken up by DCs, macrophages and monocytes. Furthermore, they are stable for up to three months at 37 °C, two years at 2–8 °C, can sustain multiple freeze–thaw cycles and are manufactured at pH 6.2 in phosphate buffered saline (Xiang et al., 2006). Clinical studies have shown that ISCOM adjuvanted viral vaccines are well tolerated both orally and parenterally in humans and stimulate both cellular and humoral immune responses against antigens for human immunodeficiency virus, herpes simplex virus, human papilloma virus, hepatitis C virus, influenza, as well as cancer (Peek et al., 2008). These attributes may make ISCOMs an ideal formulation partner for purified plant-made vaccines, providing a means not only to enhance immunogenicity but may also confer the stability required to avoid the cold chain when distributing to warmer climates.

3.3.2. Non-biodegradable particles

An alternative to biodegradable, particulate carriers is solid nanoparticle carriers. It has been hypothesised that if gold, silver, latex, silica or polystyrene particles remain in tissues for extended periods of time, antigen presentation will also be prolonged resulting in strengthened immune responses (Peek et al., 2008). The potential for solid nanoparticles as carriers for parenteral delivery of therapeutics (Paciotti et al., 2004) and vaccines (Chen et al., 2010; Greenwood et al., 2008) has been demonstrated in mice.

Metal nanoparticles can be engineered *in planta* in diverse species including alfalfa, cucumber, red clover, ryegrass, sunflower and oregano or from plant extracts (Ahmad et al., 2010; Chandran et al., 2006; Starnes et al., 2010). The system is flexible, allowing size and geometry of metal particles to be manipulated by simply altering plant growth conditions (Starnes et al., 2010). Antigens or peptides can be coupled to the metal particle but unfortunately few conjugation sites are available, often resulting in inadequate epitope presentation. Additionally, the coupling processes are inconsistent and can result in aggregates and precipitates (Chen et al., 2010).

3.3.3. Microneedles

A promising alternative to injections are dissolving microneedle patches developed by Sullivan et al. in 2010 (Sullivan et al., 2010). The microneedles are 650 μm in length and made from a biocompatible water soluble polymer (polyvinylpyrrolidone). The microneedles deliver the encapsulated vaccine through the epidermis to a depth that optimises exposure to APCs in the skin. Upon application, the microneedles become inserted into the skin and dissolve to within 89% of its mass in 5 min. As the needles dissolve the vaccine load is released and deposited within the epidermis. Studies in mice showed that delivery of a lyopholised inactivated influenza virus vaccine using the microneedle patch produced antibody and cellular immune responses equivalent to a single intramuscular injection (Sullivan et al., 2010). In challenge studies, the microneedle patch out performed intramuscular injection (Sullivan et al., 2010). Although both routes of administration were protective 30 days after vaccination, at 90 days mice immunised by microneedle patch showed a 1000-fold better clearance of the virus, had better cellular immune responses and more antibody-secreting cells in the spleen and lungs than those immunised intramuscularly.

Microneedle technology overcomes many of the problems associated with injections. The patches are stable at ambient temperature, by dissolving upon application they eliminate the risk of needle-stick injury and biohazard waste disposal, and they can be self-administered overcoming needle phobia and improving patient compliance. The technology should be applicable to any plant-made antigens or therapeutic that will withstand lyophilisation and encapsulation during polymerisation. However, no matter the method, the antigen to be entrapped or carried, requires purification. This purification and

association with the delivery vehicle adds extra time and cost to the processing of the vaccine and therefore would most likely prohibit use in the developing world and some veterinary industries such as poultry.

4. Mucosal delivery of therapeutics and vaccines

The mucosal surfaces are a popular site for delivering therapeutic small molecules due to the ease of administration and speed of uptake across the large surface areas. However, development of orally delivered peptide and protein therapeutics has been hampered by the inherent proteolytic and hydrolytic environment of the mucosal surfaces. The physical encapsulation within plant cells has been suggested as a means by which protein therapeutics may be protected during transit in the gastrointestinal tract (Mason et al., 2002), and several studies have indicated that plants are also successful at delivering recombinant proteins and therapeutics orally (Limaye et al., 2006; Pelosi et al., in press). However, in addition to transit across the epithelium, a strong tolerising response to therapeutic proteins including proinsulin and clotting factor IX has been observed (Ruhlman et al., 2007; Verma et al., 2010). In a conundrum, the principal mucosal immunoglobulin (Ig) IgA, involved in protective mucosal immune responses (in its secretary form) also appears to be extensively implicated in tolerising responses to mucosally delivered antigens (Smits et al., 2009) when not secreted. The mechanism that determines whether a tolerising or immunogenic response is made is unknown and poses the conundrum, how can therapeutics be delivered orally without inducing an undesirable immune response?

One of the oldest aims of plant-produced therapeutics is to activate the mucosal immune response to enable immunisation against infectious disease. Whilst parenteral delivery of vaccine antigens is capable of generating protective systemic immune responses, the administration of vaccines at mucosal surfaces is often immunologically superior at protecting mucosal sites than needle-based delivery (MacPherson et al., 2008; Neutra and Kozlowski, 2006). However, due to the inherent problems determining the dose, release kinetics, and host variability, there have been limited oral vaccines reaching the market. Recent advances in understanding the complex environment of the mucosal immune response system are driving improvement of the efficacy, consistency and versatility of mucosa vaccination.

4.1. Immunology of mucosal vaccine delivery

4.1.1. Geography of the mucosal immune response

Due to the perceived advantages of plant-made vaccines for oral delivery, the focus of this discussion will be limited to the gastrointestinal mucosal surfaces (Czerkinsky and Holmgren, 2010). In addition to a multitude of innate defences including a thick mucin-rich glycocalyx and strongly hydrolytic environment, the gastrointestinal tract employs an adaptive immune response finely balanced to differentiate between dietary or commensal antigen and pathogenic invasions. Sub-unit vaccination *via* the mucosal surface must specifically activate an immune response akin to pathogen invasion, without the processes normally associated with pathogenesis such as inflammation or toxicity. The continuous gut surface is comprised of a single layer of columnar epithelial cells bound by tight junctions. The mucosal sentinel organs, the lymphoid follicles called Payer's patches, are distributed along the length of the gastrointestinal tract (Dukes and Bussey, 1926), and include specialised follicular-associated epithelial cells, APCs, and effector B and T cells (Neutra et al., 2001). Follicular-associated epithelial cells have a reduced glycocalyx and a smooth luminal-facing membrane, and are referred to as microfold cells, or M-cells. These cells are adapted for transcytosing luminal antigens and whole microbes, and releasing them at their basal surface (Corr et al., 2008). These cells have invaginated basal surfaces where gut-specific lineages of infiltrating DCs and macrophages reside (Kelsall, 2008; Owen and Jones, 1974). Once activated, these APCs move to the basolateral membrane where they can interact with germinal centres of B and T cells to generate effector, memory or tolerogenic responses (Owen and Jones, 1974). In addition to antigen sampling by M-cells, other enterocytes are capable of sampling the lumen including M-cells located outside the Payer's patches (Jang et al., 2004), DCs which send dendrites through the epithelium to sample bacteria directly (Rescigno et al., 2001) and the columnar enterocytes transcytose lumen contents to the sub-epithelium layer (Ménard et al., 2010).

4.1.2. Friend versus foe: the spectre of mucosal tolerance

Another important role of the mucosal immune system is that it must be able to survey and distinguish the resident microflora from invading pathogens. As the mechanisms by which the mucosal immune system balances the homeostasis between defence and digestive activity are slowly unravelled, relevant immune responsive cells and signals identified, and the complexities of antigen presentation to epithelial and immune cells elucidated, our ability to engineer plants to deliver desired immunological outcomes will also improve.

Cells and organs of the mucosal immune system must differentiate between allowing coexistence of mutualistic microbiota of the mucosa for *ex-vivo* metabolic functions, and defence of the luminal space from invading or toxic pathogens. The ability of the mucosal immune system to actively down-regulate inflammation following repeated oral administration of soluble monomeric antigens (Faria and Weiner, 2005; Richman et al., 1978) is not surprising given the relative innocuous nature of nutritional antigens. The ability of orally delivered plant-made vaccines to trigger this down-regulation is sometimes observed when attempting to deliver therapeutic proteins *via* the oral route. The discovery of a distinct subset of cells capable of suppressing inflammation elucidated a possible mechanism by which this oral tolerance may occur (Sakaguchi et al., 1985). Mucosal tolerance is therefore important to two opposing therapeutic desires. Tolerance hampers the efforts to develop oral vaccines wishing to mount immune responses but extensive effort has also been directed at harnessing this tolerance pathway for treatment of inflammatory diseases such as inflammatory bowel disease and allergic encephalomyelitis. The translation between animal models and human immunotherapies has been stymied by varied responses observed between antigen, routes of administration, dose concentration and frequency, and animal model (Faria and Weiner, 2005).

4.2. Plants as delivery vehicles for mucosal vaccines

There have been many pre-clinical trials with minimally processed plant cells (Streatfield, 2006), but only a few trials in which unpurified plant materials have been orally administered to humans. These include two trials using the B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* delivered in potato tubers (Tacket et al., 1998) and maize seed (Tacket et al., 2004), the viral capsid protein of Norwalk virus delivered in potato tubers (Tacket et al., 2000), hepatitis B surface antigen from transgenic lettuce leaves (Kapusta et al., 1999) and potato tubers (Thanavala et al., 2005), and lettuce leaves expressing a genetic fusion of peptides from the rabies virus glycoprotein and nucleoprotein with the viral coat protein of alfalfa mosaic virus (Yusibov et al., 2002). In all trials, serum and mucosal immune responses were variable however no toxic safety concerns were observed.

Protection during digestion of antigens may be facilitated by plant cells themselves (Chikwamba et al., 2002), and modern plant expression techniques can extend this encapsulation either *via* the aggregation of proteins into chloroplasts (Limaye et al., 2006), protein bodies (Alvarez et al., 2010), non-lytic vacuoles (Di Sansebastiano et al., 1998), or within the endomembrane system of the plant cell (Vitale and Pedrazzini, 2005). Even the addition of a non-reactive bystander protein in formulation with common mucosal adjuvants was sufficient to

ameliorate non-specific proteolysis of the adjuvant and retain higher quantities of the protein of interest after a 3 hour exposure to rat stomach fluid (Reuter et al., 2009).

4.3. Improving delivery and efficacy of plant-made protein therapies at the mucosal surface

With many contradictory outcomes observed across studies with different antigens, adjuvants, schedules, disease models, and species, it may be important to focus development on some of the consistent pathways that have been shown to be effective, and are deliverable from a plant platform. Whilst there are still no oral adjuvants licenced for human use, many well-tolerated and orally-active adjuvants are either derived from, or amenable to expression in plant systems. We will discuss the most salient adjuvants here, but many more are reviewed by Granell et al. (2010).

4.3.1. Receptor-targeting ligands

There has been significant investigation into identifying receptor-mediated pathways for targeting epithelial cells, rather than simply flooding the luminal space with formulated antigen and relying on standard antigen uptake processes. Whilst not highly immunogenic itself (Lavelle et al., 2000), the plant lectin UAE-1 from *Ulex Europaeu-I* has been extensively characterised for binding the apical surfaces of epithelial and endothelial cells and is one of the classical markers of epithelial cells (Ching et al., 1988), leading to the suggestion for a role as a localising molecule for vaccine development (Chen et al., 1996). When used as an epithelial localising agent in the formulation of poly DL-lactide-co-glycolide microparticles containing human immunodeficiency virus peptides, UAE-1-coated particles generated a more rapid response than uncoated particles when administered *via* the oral route, and an increased rate and intensity of response to human immunodeficiency virus peptides were observed when delivered *via* the intranasal route (Manocha et al., 2005). UAE-1 and other plant lectins have also been shown to be effective targeting molecules for many types of delivery vehicle (Granell et al., 2010). An advantage of plant biotechnology is that recombinant lectin can be expressed in plants as a genetic fusion with an antigen of interest. The potential for such a strategy was successfully demonstrated by Medina-Bolivar et al. (2003) who expressed ricinB in tobacco as a fusion with GFP. In this study, ricinB retained its specificity to bind galactose/galactosamine cell surface receptors and deliver its fusion partner to the mucosal surface to elicit systemic and mucosal immune responses. Other receptor-targeting ligands include invasin from *Yersina* sp. and the RGD motif of fibronectin. Both have been shown to increase the binding of latex nanoparticles to human immune follicle-associated epithelium (Gullberg et al., 2006) and induce an improved immune response to poly DL-lactide-co-glycolide particles containing ovalbium (Garinot et al., 2007).

4.3.2. The AB5 toxins as adjuvants

Cholera toxin was first suggested as an oral adjuvant after it was found to prevent oral tolerance to another otherwise tolerogenic protein, keyhole limpet hemocyanin, when co-administered orally to mice (Elson and Ealding, 1984). Mutants that fully or partially abrogate the catalytic function of the A or B ganglioside-binding subunits have also been extensively investigated as both adjuvants and carrier proteins. Moreover, the B subunit of heat-labile enterotoxin from enterotoxigenic *E. coli* was one of the first vaccine antigens or adjuvants expressed in plant cells (Mason et al., 1998). Paradoxically, when chemically conjugated to other antigens the cholera toxin subunit B can in some cases induce oral tolerance (Sun et al., 1994). The relationship between tolerance or immune induction and dose, schedule, conjugation method, antigen and species have not yet been elucidated, and there is growing evidence that suggests that the B subunits may directly stimulate the innate immune system in the absence of the A subunit, steering a Th2 and Th17 response instead of the canonical whole toxin Th1 response (Hajishengallis et al., 2005; Liang and Hajishengallis, 2010). The interaction between these two subunits has been explored using chimeric proteins with only the holotoxin-interacting domain of the A subunit fused to strotococal adhesion antigen (Hajishengallis et al., 1995), and in similar fusions (Gockel and Russell, 2005; Martin et al., 2006) including tuberculosis antigens early secretory antigenic target 6 and antigen 85b *in planta* (Claire Penney, unpublished data), no tolerogenic effects have been observed following oral immunisation in mice.

4.3.3. Saponins

Saponins are a diverse group of metabolic glycosides present in many higher plants (Sparg et al., 2004). Of importance to therapeutic and vaccine delivery is the ability of many saponins to potentiate systemic and oral delivery of antigens (Grossinklaus et al., 2004; Sjölander and Cox, 1998; Sun et al., 2009). Crudely processed saponin extracts may be too toxic for parenteral delivery in humans, yet crudely prepared saponins from the *Q. saponaria* Molina have been used extensively in injected veterinary vaccines since the 1950s (Doel, 2003), and have been investigated for their adjuvant function during oral delivery (Grossinklaus et al., 2004). Early animal studies indicated that many viral proteins formulated with Quil A containing ISCOMs became highly potent at inducing IgA, IgG2a and cytotoxic T-lymphocyte responses upon oral delivery (Mowat et al., 1993). Investigation of saponins as adjuvants during oral delivery suggests that saponin and ISCOMs are more rapidly transcytosed by APCs including DCs and macrophages compared to antigen alone, and are more rapidly transported to the draining lymph nodes than soluble protein (Cox et al., 1998). Despite being already produced in plants and well tolerated orally, saponins and ISCOMs have been infrequently investigated in plant-vaccine trials, with only four trials using saponins delivered orally. When orally co-administered with a crudely purified plant-made measles virus H protein vaccine, food-grade *Quillaja* bark extract was a more efficient adjuvant than the non-toxic enterotoxigenic *E. coli* heat labile toxin mutant, LTR192G, cholera toxin/cholera toxin B subunit (Pickering et al., 2006). This response may be due to a possible adjuvant role for saponins in increasing the permeability of the intestinal epithelium to facilitate transit of high molecular compounds across the mucosa (Gee et al., 1996).

For low-cost oral vaccine purposes, it would be ideal to express an antigen complex in a host plant capable of direct or indirect modulation. The principal saponin in unripe *Lycoperscium* fruit and leaves is the glycoside alpha-tomatine (Fontaine and Irving, 1948; Yamanaka et al., 2008), which exhibits diverse bioactivity similar to *Quillaja*-derived saponins (Friedman, 2002), making it a potential candidate for oral vaccine formulation. Alpha-tomatine has been shown to act as a potent adjuvant to steer oral and subcutaneous delivery of the Norwalk virus capsid protein expressed in tomato and delivered as a crude, dried preparation towards a Th1, IgG2a dominant response in rats (Robert Shepherd, unpublished data).

The large hollow tubular structures of 100–160 nm width and up to 3000 nm length (Yang et al., 2002) formed by alpha-tomatine have been suggested to act not only as an antigen 'depot' slowing release of antigen into the major histocompatibility complex (MHC) class II pathway following phagocytosis by APCs, but to also directly stimulate loading of antigen onto MHC class I molecules (Morrow et al., 2004), and the potent cholesterol-binding ability of alpha-tomatine may be trafficked *via* lipid rafts on epithelial cells (Friedman et al., 2000).

4.3.4. Genetic-encoded particles: virus like particles

In addition to receptor-mediated and encapsulated strategies for transporting antigens across epithelial membranes, non-replicating VLPs may also be a key tool in generating immunity instead of tolerance at the mucosal surface. By their inherent adaptation to environmental stresses, many non-enveloped mammalian mucosal viruses are also

stable at low pH and are resistant to proteolytic degradation. When expressed in plant cells, many recombinant VLPs have been shown to correctly assemble into tertiary structures indistinguishable from their native conformation. Of those mammalian VLPs produced in plant systems, many have been shown to be orally immunogenic in rodents, including the hepatitis B surface antigen VLPs (Mason et al., 1992), Norwalk VLPs (Mason et al., 1996), and human papilloma VLPs (Warzecha et al., 2003). Of these, Norwalk and hepatitis B surface antigen are also orally immunogenic in humans (Tacket et al., 2000; Thanavala et al., 2005).

5. Concluding remarks

Having overcome initial limitations of low yields and a level of pessimism in the field, it is clear that safe and effective purified plant-made biopharmaceuticals can be produced at large scale for administration by parenteral routes. SemBioSys Genetics Inc., Medicago Inc., Protalix BioTherapeutics and ORF Genetics amongst others, have shown that it is possible to rapidly produce purified plant-made pharmaceuticals that can compete in today's market to meet increasing global demands (Penney et al., 2011). Unfortunately, purified biopharmaceuticals carry the burden of requiring a cold chain for long-term storage and transport. In addition to a general phobia that surrounds injections particularly in children, needle-based administration require healthcare professionals, adding to the overall cost of delivery. Furthermore there are potential biohazard risks from incorrect disposal of used equipment, reuse and needle-stick injuries.

One of the greatest advantages plants have over other systems is that they cannot only produce pharmaceuticals but their cells can potentially be exploited as vehicles for oral delivery. Plant cells themselves may facilitate one of the greatest hurdles of oral delivery and protect antigens from proteolysis (Limaye et al., 2006; Mason et al., 2002), but little effort has been put into directly comparing the release characteristics of different expression platforms. Indeed, significant deliberation regarding which plant models are preferable for the production of biopharmaceuticals has been driven from a production rather than delivery standpoint, yet there are vast immunological differences that require consideration including sensitisation (Gizzarelli et al., 2006; Oakes et al., 2009) and toxicity (Friedman et al., 1996) that remain unaddressed. Moreover, antigen expression in plants needs to rephrase the immunological context in which they are presented to the mucosal immune system to avoid tolerance, anergy or ignorance. Focussing on where, when, and with what co-signalling molecules antigens reach the immune cells of the gut with, will be key to elucidating what has been the 'black box' of oral delivery.

Advancement in mucosal delivery strategies will benefit from a clearer understanding of the key cells, tissues, pathways and microenvironments of the host mucosal immune system. By combining an efficient epithelium targeting strategy, with a delivery vehicle appropriate to the desired immunological outcome, inconsistency to mucosal delivery may be reduced, and administered dose reduced. For vaccine applications, mimicking highly immunogenic pathogens using adjuvant-integrated VLPs or particles (Helgeby et al., 2006) expressed *in planta* may be a short-cut to developing strong, consistent and most importantly protective immunity (Bachmann and Jennings, 2010; Querec et al., 2009).

By providing clear guidelines and standards, relevant agencies have cleared the regulatory landscape for purified plant biopharmaceuticals, pushing commercialisation ever closer to reality (Rybicki, 2009). Unfortunately, the provision of orally produced and delivered low-cost therapeutics and vaccines is where plant-made therapeutics reach their zenith as a technology, yet remains the least advanced field of development. Only by understanding the mechanisms of the mucosal immune system and its manipulation, may plants finally fulfil the prospect of supplying a global solution for affordable medicines.

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7.3 Appendix 3 Immunoassay book chapter

De Guzman, G., **Shepherd, RP**. Walmsley, AM. 2010. In G Shaw, *Immunoassays in Agricultural Biotechnology*, John Wiley and Sons, Hoboken, USA.

This chapter includes details on the general optimisation process undertaken for immunoassays used to quantify antigen concentration in plants and immunogenicity in animals.

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CHAPTER 14

IMMUNOASSAYS IN VETERINARY PLANT-MADE VACCINES

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14.1 INTRODUCTION

Plant-made vaccines are a type of subunit vaccine where the reactor is the whole plant, or cultured plant cells, or organs. Plant-made vaccines can be produced and delivered in a number of ways; however, no matter the means, the potential of expressing and delivering a protective vaccine antigen in plants has sparked interest, wonderment,

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and incredulity, resulting in increasing research dedicated to advancing the fledgling biotechnology. A basic introduction to plant-made vaccines is given by Walmsley and Arntzen (2000), while a thorough review of plant-made vaccines for veterinary applications is given by Floss et al. (2007).

Producing a plant-made vaccine begins by selecting a suitable antigen. The corresponding gene is cloned into an expression cassette that contains plant regulatory sequences that drive gene expression. This cassette is then used in plant transformation. *Agrobacterium*-mediated transformation, stable or with assistance from plant viral elements for magnified transient transformation, is usually the preferred method for transformation of the plant cell nucleus. *Agrobacterium* is a plant pathogen that in the process of infection, transfers a segment of its DNA (T-DNA) into the nucleus of the host. Molecular biologists have taken advantage of this process to transfer genes of interest, in plant expression cassettes, into plant genomes. Transfer of the T-DNA from the bacterium into the host's genome occurs upon incubation of the transgenic *Agrobacterium* with plant cells. In transient expression systems, the infected plant tissues are harvested 2–9 days later and yield significant quantities of the protein of interest. For stable transformation, the transformed cells are positively selected during tissue culture using a marker or resistance gene and regenerated into transgenic plants or multiplied into plant cell lines. The time taken to regenerate a transgenic line is species dependent and ranges from 6 weeks to 18 months. Stably or transiently transformed, plant tissues are selected for further development based on authenticity of the plant-made subunit protein to the native form, and its concentration in plant tissues. The materials are amplified either in the greenhouse or fermentation reactors (plant cell and organ cultures), fully characterized and then tested for immunogenicity in animal trials.

In the years spanning the present day and the first mention of plant-made vaccines in peer-reviewed literature (Mason et al., 1992), the dogma of plant-made vaccine technology has evolved from being food items ("edible vaccines" in 1992) to prescribed fruit (1998) to plant-derived pharmaceuticals (2001). This change in dogma was brought about by the gradual realization that both agricultural and pharmaceutical regulations needed to be adhered to in order for this technology to produce a commodity. That is, not only is the growth and economical production of genetically modified organisms needing to be addressed, but also the adherence to good laboratory and manufacturing practices (GLP and GMP) and demonstration of consistency of vaccine batch production and performance.

On January 31, 2006, Dow AgroSciences LLC announced that in collaboration with Arizona State University and Benchmark Biolabs, Inc. (Lincoln, NE) they had received the world's first regulatory approval for a plant-made vaccine (USDA APHIS, 2006). The developed plant-made vaccine combating Newcastle Disease Virus (NDV) was made using a contained, plant-cell production system. The plant-made NDV vaccine is produced by a stably transformed plant cell line that is grown as a suspension culture in a conventional bioreactor system. The resulting plant cell cultures are harvested and partially purified before the antigen is formulated into the final vaccine. Chickens vaccinated subcutaneously with the plant-made, Newcastle Disease Virus vaccine, proved to be protected against lethal challenge by a highly virulent NDV strain. The dose response capable of greater than 90% protection ranged between 3 and 33 μg/dose with overall protection of 95% (Mihaliak et al., 2005). A

formulation was advanced through the United States of America Department of Agriculture (USDA) Center for Veterinary Biologics' (CVB) regulatory approval process in a feat that demonstrated plant-made vaccines could be developed within an existing regulatory framework.

Many antigens have been successfully expressed in plant systems so the question is not whether plants can act as vaccine productions systems but whether they are consistent and economically feasible vaccine expression systems. Many factors will play important roles in the quest for plant-made vaccine feasibility. However, development of dependable immunoassays will play no small role in this achievement by reliably characterizing and quantifying protective antigens in plant cells and also determining the type, strength, location, and duration of the antigen-specific immune response induced. Other chapters in this book have discussed various aspects regarding immunoassay development (Chapters 4 and 5), validation (Chapter 6), and applications in GE product development (Chapter 10) of plant-derived proteins. This chapter provides a series of conditions, protocols, and trouble-shooting exercises to enable development of dependable immunoassays for detecting plant-made antigens and resultant induced immune responses.

14.2 PLANT-MADE ANTIGEN EXTRACTION

Functional proteins (as opposed to structural) ultimately act due to their tertiary configuration enabling specific binding to different compounds, molecules, or proteins. To enable detection and quantification of functional plant-made antigens, they first need to be extracted from plant tissues in a manner that allows their tertiary structure to be maintained. This extraction process, though seemingly simplistic, is one of the most critical aspects of proteomic analysis and is often problematic in plants due to an abundance of proteases and other interfering structures and compounds such as the plant cell wall, storage polysaccharides, various secondary metabolites, such as phenolic compounds, often low vaccine antigen expression (Nanda et al., 1975). Therefore, there are several factors needing consideration for optimal extraction of authentic and functional plant-made antigen.

14.2.1 Factors Influencing Effective Antigen Extraction

The nature of the antigen itself plays a major role in extraction from plant tissues. Protein extraction should therefore not be a standard procedure but investigated on a case by case basis. Knowing the range of pH and temperatures at which the protein is stable and when and where it is expressed in the plant cell is invaluable. Does it have transmembrane domains and therefore require detergent dispersal? Is it secreted into the apoplast and perhaps present in the media? Variables that should be taken into consideration include: optimal harvest point, extraction buffer, and physical disruption method (Figure 14.1).

14.2.1.1 Optimal Harvest Point Expression of heterologous proteins in plant cells is effected by a myriad of factors both environmental and genetic including temperature, water availability, age of plant material, location of transgene insertion

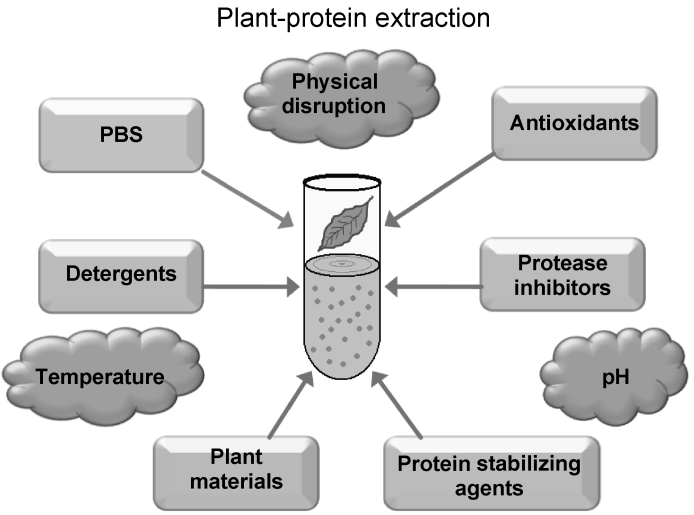

Figure 14.1 Plant protein extraction.

into the genome, and the promoter elements used. No matter the transformation method, investigations should be first made into what point of culture or plant development antigen expression is optimal. For example, our investigations in tomato have found that tomato fruit developmental stage accounts for 60.54% of total variance in antigen expression, and that harvest at the optimal fruit developmental stage resulted in up to 10-fold increase in detectable antigen.

14.2.1.2 Extraction Buffers Below are the buffer constituents and their roles:

Phosphate buffered saline (PBS) (137 mM NaCl, 8 mM Na_2HPO_4, 2 mM KH_2PO4, 2.7 mM KCl, and a pH of 7.4) is more effective than H_2O in maintaining the integrity of protein structures since it buffers changes in pH.

Nonfat dried skim milk powder (NFSM) can be added to act as an adsorbent of plant secondary metabolites such as phenolics that may react with and hamper the extraction of the protein of interest. NFSM also competes with the antigen for degradation by proteases and therefore increases the amount of antigen detected.

Protease inhibitors result in higher antigen detected (Dogan et al., 2000) since more intact antigen is present. Addition of leupeptin, aprotinin, E-64, pepstatin, and pefabloc to extraction buffers has resulted in increased detectable antigen. Commercial protease inhibitor cocktails are available, such as Complete protease inhibitor cocktail from Roche Pty. Ltd, and several plant-specific products from Sigma Aldrich. Phenylmethylsulfonyl fluoride (PMSF) has also been extensively used as a protease inhibitor in the past; however, the commercial cocktails are as effective without having the toxicity of PMSF.

Sodium ascorbate 1–20% (w/v) improves levels of monoclonal-reactive antigen 4–12-fold. Being an antioxidant, it prevents the extraction buffer and extracted proteins from reacting with atmospheric oxygen. Using sodium ascorbate rather than ascorbic acid is recommended because it does not change the pH of the buffer.

Glycerol at 10–20% (v/v) is often used to stabilize active proteins in solution (Smith et al., 2002). It also aids protein stability after samples are freeze-thawed.

14.2.1.3 pH In addition to the constituents of the extraction buffers, the pH of the buffer can greatly affect not only the amount of proteins extracted, but also protein integrity. The pH affects the charge and therefore solubility of the protein of interest. For example, the pH of the extraction buffer should not be at or near the protein of interest's isoelectric point as proteins are least soluble in any given solvent at this point. Generally, it has been seen that an increase in pH of sample extractions leads to an increase in solubilization of proteins; however, it is recommended that the pH should not exceed 8 to avoid an adverse effect on the biological activity of the proteins (Nanda et al., 1975). A pH of 7–8 is generally desired for most extraction protocols.

14.2.1.4 Temperature Extreme temperatures denature proteins. In general, a low temperature is desired to inhibit endogenous protease activity and slow the degradation of desired protein products. It is recommended to keep extraction procedures close to 4°C. However, certain antigens are more stable at higher temperatures, for example, 50°C gave optimal protein extraction yields of hepatitis B surface antigen (HbsAg) (Dogan et al., 2000).

14.2.2 Antigen Release

Since detergent helps to disrupt membranes, addition of detergent can often increase antigen extracted if the antigen is localized within a subcellular organelle. It is noteworthy that high detergent concentration may interfere with ELISA and that the amount of antigenic protein extracted is not affected by the concentration of detergent but the detergent to cell ratio (Smith et al., 2002).

Samples of callus, leaf or root are often physically disrupted to determine antigen production. However, care must be taken not to introduce extra variability by having vastly differing cell types in different samples. Different cell types not only produce different amounts of protein but may also differ in the ease of cell disruption and protein release (e.g., vein material versus lamina tissue). Therefore, taking samples from areas of the leaves/roots that contain similar cell types will greatly increase consistency in the amount of protein extracted.

There are two main tissue-grinding techniques for extraction of proteins of interest from plant tissues. These are manual and mechanical grinding. Manual grinding is generally performed with a pestle in either a mortar (large tissue mass) or in centrifuge tubes (small tissue mass). The main issue with manual grinding is consistency between sample extractions. In general, manual grinding is less consistent between extractions than mechanical grinding because of operator variability. Mechanical grinding involves addition of glass/ceramic/metallic beads alongside the sample and extraction buffer to the extraction vessel and using a vortex/mixer bead mill apparatus to consistently agitate the vessel (with respect to time and force applied). Other tissue grinding mills can use blades to cut up the material. The use of machines for grinding plant tissues not only eliminates operator variation but also

greatly reduces time spent and labor involved performing extraction. The type of grinding technique performed is largely dependent on the mass and the number of samples that are to be extracted. For large masses of tissues or small sample sizes, it is more efficient to grind the tissue manually, whereas when working with large sample size or small tissue masses, mechanical grinding is recommended.

14.2.2.1 Examples of Plant-Made Antigen Extraction Protocol

General Extraction Buffer: Phosphate-buffered saline (PBS) (0.15% Na_2HPO_4, 0.04% KH_2PO_4, 0.61% $NaCl_2$ w/v, pH 7.2), 10 mM EDTA, and 0.1% Triton X-100.

Possible buffer additions to increase antigen authenticity:

Protease inhibitor

1–20% (w/v) Ascorbic acid

5% (w/v) Nonfat skim milk powder

10–20% (v/v) Glycerol

Example: Tomato Fruit Extraction Buffer consists of 50 mM sodium phosphate, pH 6.6, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 µg/mL leupeptin, and 1 M phenylmethylsulfonyl fluroide.

14.2.2.2 Total Soluble Protein Extraction

A general protocol of total soluble protein extraction is listed in Table 14.1. Quantification of total soluble protein is often performed to enable comparison of the amount of antigen produced to units of total soluble protein and to gain insight into success of protein extraction. Protocols are usually described in Bradford reagent dye manuals such as Bio Rad's Protein Assay Dye Reagent Concentrate (Catalogue number 500-0006). After performing the required dilutions and reading absorbance. Plot and calculate the equation of the standard curve from the BSA readings (Figure 14.2). To ensure accurate protein content determination, the standard curve equation should contain at least five BSA dilution points, including a zero point, be in the linear region of the curve and have an r^2 value of 0.95 or higher. The linear region of standard curves gives the most accurate portrayal of concentration as absorbance is directly proportional to concentration and the assay is not restricted by biological or instrumental limitations. Using the equation of the BSA standard curve and the OD readings of the extracted samples enables calculation of the total soluble protein content.

TABLE 14.1 TSP Extraction Procedure

Step	Procedure
1	Snap freeze plant material and add ice cold extraction buffer
2	Homogenize sample
3	Centrifuge at 20,000 × *g* for 10–20 min
4	Store extract supernatant after adding 10–20% glycerol at -20°C

See Figure 14.3 for an example of a BSA standard curve and TSP concentration.

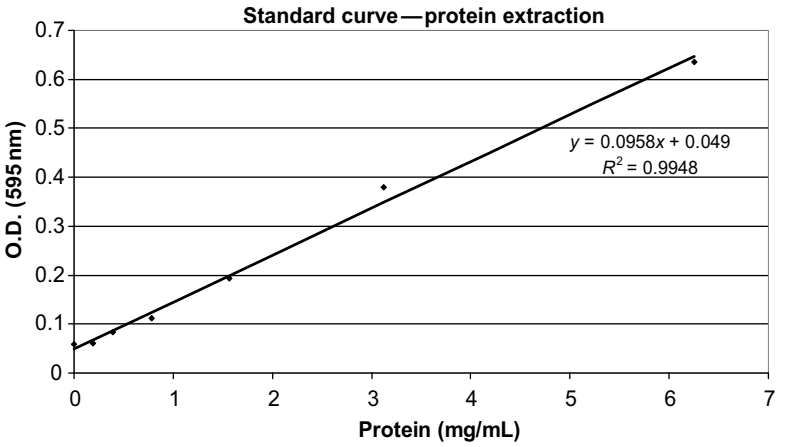

Figure 14.2 Example BSA standard curve.

14.3 ANTIGEN DETECTION AND QUANTIFICATION IN PLANT TISSUES

There are a number of methods that enable detection and quantification of a particular antigen in plant extracts. The technique selection relies on the information required such as qualitative (How big is the protein? Is it glycosylated, cleaved, or multimeric?) or quantitative; the number and type of antigen-specific antibodies available, whether a standard protein is available to act as a reference for quantification, and the required sensitivity of your assay to enable detection of your antigen of interest.

14.3.1 Dot Blots

Dot blotting in its simplest form is a qualitative assay where a 0.5–5 μL of extract is applied to a membrane and then immunohistochemically detected using appropriately derived antibodies. Dot blots are generally used for determining optimal conditions for Western blot analysis including blocking conditions, antibody dilutions and wash buffers. By comparing intensity of sample signals with a serial dilution of a standard antigen, dot blots can be semiquantitative. Since dot blots do not separate the samples according to protein size as accomplished through PolyAcrylamide Gel Electrophoresis (PAGE), less time is required. However, dot blots do not differentiate between specific and nonspecific binding. Therefore, negative controls should always be loaded.

14.3.2 Organ/Tissue Blots

Squash blots were initially used to detect virus infection in plant leaves. Recently, they have been used as a rapid method for detecting recombinant proteins in plant tissues with the advantage of indicating site of protein accumulation. However, organ/tissue

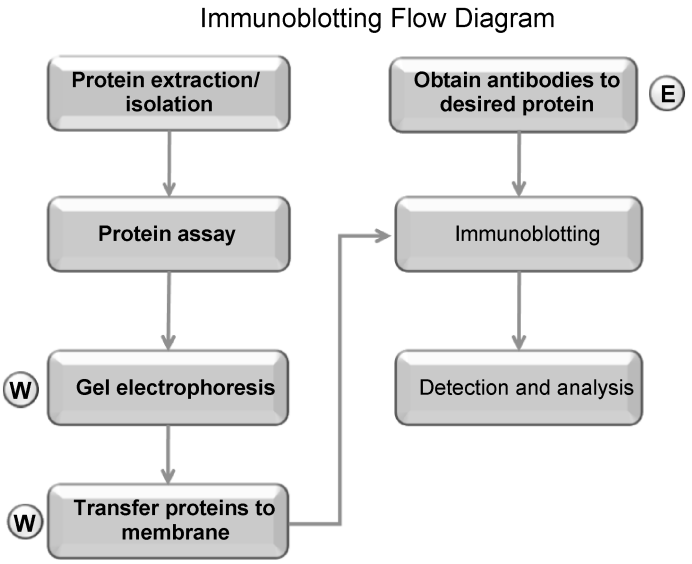

Figure 14.3 Immunoblotting flow diagram.

blots are not particularly sensitive. Therefore, the protein of interest must be produced abundantly.

14.3.3 Western Blot Analysis

Western blot or immunoblot analysis first involves separating extracted proteins according to size and charge using PAGE. The separated proteins are then transferred and immobilized onto a membrane and detected using antigen-specific antibodies (Figure 14.3; Table 14.2). Western analysis can detect native or denature proteins and determine if the protein of interest is the correct size, being degraded, is glycosylated or forms oligomers. Western analysis is usually qualitative, although if known amounts of standard protein are run along side the samples, an estimate of the amount of the protein of interest present can be made through comparing intensity of the signal. If protein extracts from a negative, wild-type sample also runs along side the samples, differentiation can be made between specific and nonspecific binding.

14.3.4 ELISA

Direct-bind ELISA can detect nanogram to picogram quantities of a protein of interest. In this assay, the protein of interest is directly adsorbed onto the surface of a polystyrene plate. The plate is then blocked with a blocking buffer to prevent unwanted binding proteins to the plastic surface. An antibody (primary or detector antibody) specific to the protein of interest is applied. This primary antibody may either be directly conjugated with a marker enzyme such as an alkaline phosphotase, or horseradish peroxidase, or another secondary antibody-conjugate may be used to

TABLE 14.2 General Protocol for Western Analysis

Step	Procedure
1	Determine total soluble protein content and dilute samples to contain same amount of TSP
2	Add protein-loading buffer and denature by boiling for 10 min, then snap cool on ice
3	Load into an acrylamide gel, along with molecular mass ladder. Run at 30 milliamps per gel until the gel front is approximately 5–10 mm from the end of the gel
4	Equilibrate the gel and transfer to membrane
5	Block membrane in PBST + NFSM for 1 h at 37°C or overnight at 4°C
6	Rinse the membrane with PBST then incubate the membrane with the optimal dilution of primary antibody in PBST + NFSM for 1 h at room temperature
7	Rinse membrane with PBST then incubate with the optimal dilution of your secondary antibody (with conjugate) in 10 mL PBST + NFDM at room temperature for 1 h
8	Rinse the membrane then visualize using film and ECL + kit as per manufacturer's directions

For detailed procedure see Walmsley et al. (2003).

detect the primary antibody. A quantitative analysis of the total number of antibody–enzyme conjugate molecules present on the plate surface is performed using a chromogenic or fluorescent substrate.

While direct-bind ELISA is sufficient to detect high abundance of proteins of interest in samples, low abundance molecules may not bind to the plate in sufficient numbers or may be sterically hindered in complex sample mixtures such that binding to the plastic surface is not enabled. Capture ELISA (or indirect, sandwich ELISA) can often detect picogram to microgram quantities of molecules in more complex mixtures (Figure 14.4).

In this assay, a monoclonal- or polyclonal-antibody specific to the antigen of interest is adsorbed onto the surface of the polystyrene plate. The plate is then blocked with a blocking buffer to prevent unwanted binding of molecules to the plastic surface. Samples are then added to the plate in a series of dilutions with buffer as with the direct-bind ELISA. The antigen in the samples specifically binds to the coating antibody. Other nonspecific molecules in the samples are washed away. All subsequent steps are performed in a similar manner to direct-bind ELISA.

A variety of factors may influence the choice of direct-bind or capture ELISA. These include whether or not whole serum or purified antibodies from a heterologous species are available to use as the capture antibody, the level of background noise associated with the specific interaction of different samples with the plastic adsorption surface, the sensitivity of the assay required, and the time limitations of the assay procedure (as the addition or the capture antibody requires an additional binding step).

14.3.4.1 ELISA Protocol Examples of general protocols for direct and indirect ELISAs are listed in Tables 14.3 and 14.4.

Coating Buffer Coating buffers are used in ELISA analysis to stabilize protein tertiary structure and allow strong adsorption to the polystyrene plate matrix.

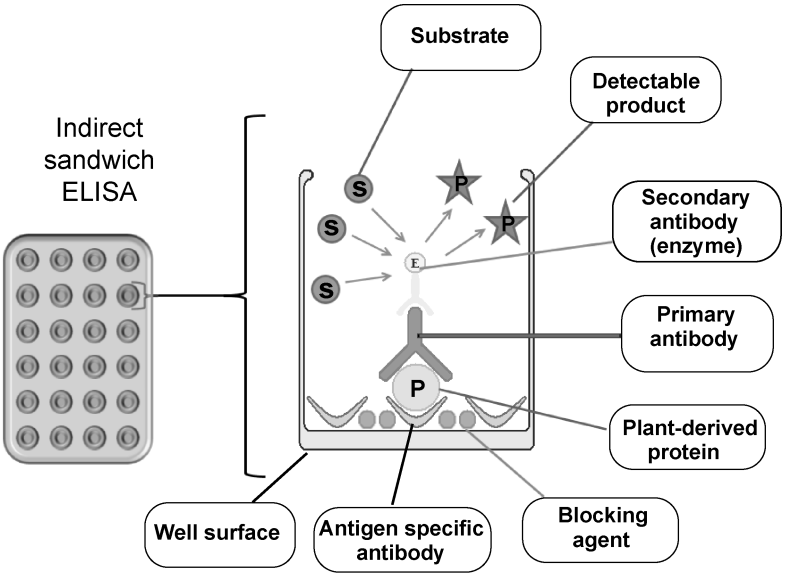

Figure 14.4 Indirect sandwich ELISA diagram.

TABLE 14.3 Direct ELISA Protocol

Step	Procedure
1	Coat a 96-well plate with 1–10 µL per well of plant extract diluted in PBS/coating buffer
2	Perform twofold serial dilutions of each sample down the plate. PBS + 0.05% Tween (PBST) + 1% NFSM
3	Make the standard curve by diluting the antigen stock to 50 ng/mL, then perform twofold dilutions down the plate in PBS + 0.05% Tween (PBST) + 1% NFSM
4	Incubate for 1 h at 37°C of overnight at 4°C
5	Wash three times with PBST
6	Block wells with 200 µL/well of 5% NFSM in PBST at 37°C for 1 h
7	Wash three times with PBST
8	Add 50 µL/well of primary antibody diluted in 1% PBST + NFSM. Incubate at 37°C for 1 h
9	Wash three times with PBST
10	Add 50 µL/well of secondary antibody diluted in 1% PBST + NFSM. Incubate at 37°C for 1 h
11	Wash four times with PBST
12	Add 50 µL/well of TMB substrate. Incubate at room temperature for 5 min
13	Add 50 µL/well of 1 N H_2SO_4
14	Read absorbance at 450 nm
15	Construct standard curve and calculate sample antigen concentrations

TABLE 14.4 Indirect ELISA Protocol

Step	Procedure
1	Coat plates with 50 µL/well of diluted capture antibody (10–100 µg/mL) in coating buffer. Incubate overnight at room temperature
2	Wash three times with PBST
3	For the standard curve, dilute stock to 50 ng/mL
4	Perform twofold dilutions down the plate in PBST/1% NFSM
5	Coat plates with 1–10 µL/well of plant extract and make up to 100 µL with PBS
6	Perform twofold serial dilutions down the plate
7	Incubate shaking for 2 h at room temperature or overnight at 4°C
8	Wash plate three times with PBST (0.05% Tween)
9	Block wells with 200 µL/well of 5% NFSM in PBST (0.05% Tween-20), 37°C for 1 h
10	Wash three times with PBST
11	Add 50 µL/well of primary antibody diluted in 1% NFSM in PBST. Incubate at 37°C for 1 h
12	Wash three times with PBST
13	Add 50 µL/well of secondary antibody diluted in 1% NFSM in PBST. Incubate at 37°C for 1 h
14	Wash four times with PBST
15	Add 50 µL/well of TMB substrate. Incubate at room temperature 5 min
16	Add 50 µL/well of 1 N H_2SO_4
17	Read absorbance at 450 nm
18	Construct standard curve and calculate sample antigen concentrations

A general schematic for an indirect ELISA can be found in Figure 14.8.

A good starting point for determining the optimal binding conditions for antibody or antigen adsorption onto the solid-phase of the 96-well capture is to simply use PBS as the coating buffer. Several other buffers are reported in the literature. These include carbonate buffers (0.1 M carbonate or bicarbonate in H_2O at pH 9.6) or buffers containing protamine sulfate. The coating buffer used can significantly affect the amount of specific protein ultimately detected (Figure 14.4). It is therefore important to ensure that the buffer is optimized.

Blocking Agents Many reagents can be used to block surfaces of plates or membranes to which proteins of interest are bound. This blocking step is required to minimize the nonspecific binding of antigens and antibodies in subsequent steps, and to reduce the background of the assay. Nonfat dried skim milk powder made up in PBS works very well and is relatively inexpensive. However, caution should be used in situations where blocking agents are derived from the same species as will be subsequently tested, that is, do not use NFSM if you will be testing the IgM response to vaccination in cattle. Other common blocking agents include bovine serum albumin at 1–3% (w/v) in PBS (He et al., 2008).

Pipette Tips and Multichannel Pipette Although ELISA analysis can be performed using a single channel pipette, a multichannel pipette (when properly used)

reduces pipetting error, time-spent pipetting, and operator fatigue. Good pipetting technique is essential for the success of ELISA analysis. Due to the lack of internal reference, small errors introduced by inaccurate or imprecise technique may introduce significant noise in the final analysis. Always inspect the pipette and tips for correct seal, and ensure that consistent dispensing technique is used. Electronic multichannel pipettes are ideal for this purpose as they generally have a higher precision of dispensed volume than can be achieved by hand. Fully read the manufacturer's instructions to determine the range of volume to be dispensed.

Shaking Platform and/or Incubator While not essential, increased temperature during reactions and/or shaking will help to reach reaction equilibrium conditions faster. For example, a typical incubation step may be to place plate with reagents at room temperature for 2 h. Typically, with shaking at 90–120 rpm or incubation at 37°C, this can be decreased to 30–60 min. When designing a protocol for immune-response detection, these variables need to be standardized early during protocol development and followed strictly. Large variation between assay runs is possible when these conditions are not adhered to.

Adsorbent Polystyrene 96-Well Flat-Bottom Plates These plates can be procured from any manufacturer, but it is important that they are specifically formulated for protein adsorption and are generally sold as being specific to ELISA. It is best to standardize protocols for a specific plate model as differences in binding do occur. This will allow confidence in later comparison of assay results between samples, and will provide a consistent background signal between experiments. Also remember that vinyl plate covers will prevent evaporation of samples and may avoid spills.

Monoclonal or Polyclonal Antibody In ELISA, it is important to ensure that the animal species from which the antibodies are raised for capture, primary or secondary binding are all different. This will minimize the chance of cross-reactivity between already adsorbed but still exposed antibody motifs (e.g., to investigate the immune response of chickens to an influenza vaccine, antibodies specific to the influenza antigen should be produced in guinea pig, for example and guinea pig capture or conjugate antibody is not subsequently used in the detection for the antibody isotypes). Commercial, recombinant, primary antibodies should be made up into a range of 1 mg/mL of buffer (PBS), and a starting solution of 0.1–0.5 μg/mL (1:2000–1:10,000) is recommended. A starting dilution is often suggested by the manufacturer. It is also important for antibody dilutions used in Western blot and ELISA to be optimized. However, for whole serum, good starting dilutions to try for a primary antibody are 1:1000 or 1:5000 and for a secondary antibody 1:10,000–1:15,000.

Some commercially available primary antibodies are tailored for use in either ELISA or Western blot. Ensure that the primary antibody you are using was raised in a manner appropriate to your method. For example, in ELISA, the antibody should be raised using the native antigen and not raised from denatured linear protein as is commonly used in nonnative Western blot and immunoblot procedures. This infor-

mation should be available in the antibody product sheet or upon request from the manufacturer.

Purified or Recombinant Reference Antigen Antigens may be purified from their host source, or manufactured in a heterologous recombinant system. Antigens are recommended to be stored at concentrations of 1 mg/mL and should be diluted to a range of 1–0.1 μg/mL (1:1000–1:10,000 dilution). Best-practice ELISA strategies use a reference antigen from the pathogen or viral host rather than purified from the tested system. For example, after vaccinating chickens with recombinant plant-produced proteins, the reference antigen should ideally be produced in a host system (e.g., avian cell line) and not in plant cells. This gives a clear indication that the antibodies raised during vaccination are able to bind and potentially neutralize the native or near-native antigens.

Commercial Colorimetric Substrate This is generally purchased as a premixed solution from a commercial supplier. Due to variation in propriety solutions, it is best practice to standardize this reagent during assay development to minimize variation in subsequent assays. Commonly used substrates include: 0.004–0.02% H_2O_2 plus *ortho*-phenylene diamine (OPD), tetra-methylbenzidine (TMB), 2,2′-azino di-ethylbenzonthiazoline-sulfonic acid (ABTS), 5-aminosialic acid (5AS), or di-aminobenzidine (DAB) for use with horseradish peroxidase enzyme labels, or 2.5 mM *para*-nitrophenyl phosphate (pnpp) for alkaline phosophotase enzyme labels, or 3 mM *o*-nitrophenyl beta-D-galactopyranoside (ONPG) or urea and bromocresol dyes for urease enzymatic markers.

Spectrophotometer There are many models of spectophotometers capable of reading fluorometric or colorimetric results from Bradford and ELISA. The complexity of the model you use is reliant on what other research is performed within your laboratory. If the spectrophotometer is to be used only for reading Bradford's and ELISA plates then it should be compatible with 96-well plates and fitted with filters specific to your preferred substrate. There are also many software packages available to read and analyze results and these usually come with the spectrophotometer.

14.3.5 Optimizing Antibody Dilutions for Western Analysis

Reduced wastage of reagents, reduced background, and increased sensitivity are three desirable advantages of optimizing antibody dilutions used in Western analysis. Dot blots using a range of antigen concentrations and different primary and secondary antibody dilutions and permutations should be used to determine the antibody dilution combination that is able to detect the least amount of antigen using the highest dilution of antibodies. Manufacturers of reagents kits often used to detect Western analysis (e.g., Stratagene's ECL + Kit), often include a general protocol in their instruction booklet. Good starting points for antibody dilutions for Western optimization are three primary antibody dilutions (1 in 1000, 1 in 2500, 1 in 5000) and three secondary antibody dilutions (1 in 10,000; 1 in 25,000; 1 in 100,000). Remember to treat the

antigen of interest during dot blot optimization the same way as you would during Western analysis, that is, in its native or denatured state. A general protocol for Western analysis is given in Table 14.2.

14.3.6 Optimizing Antibody Dilutions for ELISA Analysis

As per Western analysis, antibody dilutions used in ELISA analysis should be optimized to decrease the amount of reagents used, decrease background, and increase sensitivity. Depending on how optimization is performed, this important step can also flag possible undesirable interactions of the antibodies used (interactions not based on antigen presence). This possibility is accounted for in the optimization protocol described by Bruyns et al. (1998). Good starting points for antibody dilutions for ELISA optimization are four primary antibody dilutions (1 in 500, 1 in 1000, 1 in 2500, 1 in 5000) and four secondary antibody dilutions (1 in 5000; 1 in 10,000; 1 in 25,000; 1 in 100,000). Make sure the amount of secondary antibody is in excess to antigen to ensure the assay is quantitative and that each blank only gives background signal. A general schematic of indirect ELISA can be found in Figure 14.4 and a protocol in Table 14.4. An example of coating buffer: in every 300 mL of buffer, it contains 0.48 g $NaHCO_3$, 0.88 g $NaHCO_3$, and 0.06 g NaN_3 (pH 9.6). It is usually stored at 2–8°C.

14.4 IMMUNOASSAYS FOR DETERMINING RESPONSE TO VETERINARIAN PLANT-MADE VACCINES

The overall aim of vaccination is the development of an immunological memory toward a particular antigen/pathogen to enable a rapid immunological response against the antigen/pathogen upon future exposure. Determining if a vaccination trial is efficacious ultimately relies upon the survival of animals when challenged with the live pathogen. Due to the cost, complexity, and ethical implications of challenge trials (as well as the difficulty in obtaining approval for human trials), proxy markers of a protective immune response are often developed and utilized. It is important to note that these markers are unable to ultimately indicate if vaccination is effective, but instead indicate whether an avenue of vaccine development is worth pursuing, or if there is an advantage to existing vaccines. These markers are often the type of cytokines or antibodies induced following effective vaccination. Cell-mediated responses to pathogens are usually indicated by increased cytokine and chemokine production, while humoral responses to intercellular pathogens are usually indicated by specific antibodies. For example, an inflammatory (Th1) response is usually required for an intracellular pathogen, such as a virus, and this response can be indicated by increase production of interferon gamma (INF-γ). A Th2 response is usually required by an intercellular pathogen such as bacteria that produce toxins. A Th2 response is often indicated by chemokines interleukin-10 (IL-10) and IL-4 and antibody specifically produced toward the pathogen.

This section will focus on characterization of Th2 responses and antigen-specific antibody analysis post-vaccination with protein antigens. These assays

require only minimal experimental apparatus, and do not require the expensive and technically demanding cell culture techniques used for characterization of cellular response. All that is required for determination of the humoral response is samples of blood, fecal and/or mucosal surface washes collected over the vaccination schedule.

Antibodies secreted from lymphocytes are retained in the serum, or are secreted to the mucosal surfaces of the gastrointestinal, respiratory or genital tract where they act as the first line of immune defense in neutralizing toxins and pathogens. The total antibody titer (how many individual antibodies molecules are present), and the specific antibody structural isotypes present in the serum or mucosal surface may provide a marker of the intensity and specificity of the immune response to vaccination with the plant-made protein. To quantify the concentration of antibodies in the blood or fecal samples raised by vaccination, ELISA is performed to measure the total number of dilutions required to reduce the signal to the background for the assay. This measurement does not rely on an external standard, and as such indicates the ultimate intensity of immune response. This antigen-specific antibody response is determined using either a direct noncovalent adsorption of the antigen to the plastic substrate of a 96-well plate, or as a capture ELISA as where an antigen-specific antibody is bound to the plate and subsequently used to capture and present the antigen to the sample serum.

14.4.1 Endpoint Determination

The goal of the end point ELISA is to have the total antibody titer to be exhausted within the range of the plate, while at the same time maximizing the resolution of response detection. This is a balancing act that requires careful optimization of the starting dilution of serum or fecal matter, and will be dependent on the dynamic range of immune responses expected. It is suggested to either have the dilution series running down or across an entire column or row of a 96-well plate. This will give the highest chance of being able to assess the end point across the high dynamic range of results that are to be expected for "real-world" biological data sets.

14.4.2 Blood Sample

Whole blood should be taken from the animal in accordance with prescribed animal ethics. After collection, the blood should be transferred to a suitable centrifuge tube and kept at room temperature for an hour to allow coagulation. As soon as feasible (within hours), centrifuge collected blood at $6000 \times g$ for 5 min until the erythrocytes and lymphocytes have separated from the serum. Longer spinning of cells may be required for larger quantities of blood. Make sure not to spin at too high a force as shearing of erythrocytes will release the hemoglobin into the supernatant. A maximal force of around $8000 \times g$ is recommended. Release of the cellular contents into the supernatant dramatically increases extraneous binding of primary and/or secondary antibodies, and increases the ELISA signal regardless of antigen-specific antibody concentration. Aspirate the upper supernatant and store at -20 or -80°C for long-term storage. Serum can be stored at 4°C for short periods of time. Typically, only 100–200 μL of blood is required for a series of end point analysis assays. However, if replicates or repeated measurements are required for the assay, greater than 1 mL of

serum may be required (particularly, if low antibody titers are observed). This is not commonly a problem in large animals, but if small animal such as mice are assayed, it may be an important consideration when planning experimental protocols for control and middle time point bleeds.

14.4.3 Fecal Sample

Antibodies, particularly secreted gut IgAs can be detected in fecal matter by resuspending the dry or wet matter in PBS, and extracting the total soluble protein fraction. For dry fecal matter, start by making a 1:10 (w/v) suspension of fecal matter in PBST containing 0.1% Tween-20 and protease inhibitors. For hydrated fecal matter, begin extraction with a 1:5 (w/v) suspension. Homogenize samples using a mechanical grinding device or by adding a ceramic or tungsten bead to a flat bottom tube and using a lab vortex or shaker plate for 30–60 s. Centrifuge suspensions for 10 min at 12,000 × *g* at 4°C, collect the supernatant and centrifuge the suspensions again for 10 min at 12,000 × *g* at 4°C. Quantify the total soluble protein component, and store samples at − 20°C until assay. Load approximately 500 μg of total soluble protein onto plate when performing ELISA.

14.4.4 Detection with Direct and Indirect ELISAs

Direct coating plates may require more antigen to achieve similar signals to that received from indirect sandwich ELISAs and should always be applied to the plate in coating buffer without a blocking agent to maximize the number of bound motifs (Kulkarni et al., 2008).

A Primary antibody specific to the subject animal and isotype to be detected is required. For example, to determine the response of antigen-specific IgG1 antibodies in a sheep trial, an antisheep IgG1 produced in goat, mouse, or rabbit may be used. Again, this antibody needs to be produced in a different host to the subject species. Some monoclonal secondary antibodies are manufactured with covalently attached conjugate enzymes and these can be detected directly by addition of a suitable substrate. More commonly, a monoclonal recombinant antibody specific to the serum antibody (i.e., antisheep IgG1 produced in goat) is used to detect the sheep IgG1 molecules, then an antisheep Ig antibody-conjugate produced in another species (i.e., mouse, anti-goat Ig) is used to detect all bound goat antibodies. Care should be taken when ordering these antibodies pairs to ensure that they are compatible and in ready supply. The need to change suppliers of a secondary or conjugate antibody may change the background of the ELISA signal, and alter the specific end point titers observed. Many commercial primary or secondary conjugated antibodies are provided at around 1.0 mg/mL and can be diluted to 0.1–0.5 μg/mL (1:200–01:10,000) or as manufacturer recommends. A general protocol for direct-bind ELISA can be found in Table 14.5 and indirect-bind ELISA in Table 14.6.

Due to the lack of internal references in end point-titer analysis, strict data interpretation and statistical analysis should be used to ensure robust and reproducible results. The key to end point ELISA data analysis is the robust determination of the background for the assay. Background is present in all immune assays and is the signal

TABLE 14.5 Immune Response Direct ELISA Protocol

Step	Protocol
1	Coat an ELISA plate with 50 µL/well of antigen diluted in PBS, incubate for 2 h at 37°C or for overnight at 4°C
2	Wash plate three times with PBST (0.05% Tween-20)
3	Block plate with 300 µL/well of blocking buffer, incubate at room temperature for 1 h
4	Wash three times with PBST
5	Add 50 µL of sample to top row of plate and serially dilute down the plate
6	Incubate at room temperature for 1 h
7	Wash three times with PBST
8	Add 50 µL/well of the primary antibody in blocking buffer, incubate at room temperature for 1 h
9	Wash three times with PBST
10	Add 50 µL/well of the secondary antibody–enzyme (HRP) conjugate in blocking buffer, incubate at room temperature for 1 h
11	Wash five times with PBST
12	Add 50 µL/well of substrate according to manufacturers instructions, develop for 30 min at room temperature
13	Add 50 µL/well of substrate stop solution
14	Read plate at 450 nm

TABLE 14.6 Immune Response Indirect ELISA Protocol

Step	Protocol
1	Coat a plate with 50 µL/well of capture antibody diluted in PBS, incubate at 37°C for 2 h or for overnight at 4°C
2	Wash three times with PBST
3	Block plate with 300 µL/well of blocking buffer, incubate at room temperature for 1 h
4	Wash plate three times with PBST
5	Add 50 µL per well of antigen diluted in PBS, incubate at room temperature for 1 h
6	Wash three times with PBST
7	Add 50 µL of serum samples to top row of plate, serially dilute down the plate, incubate at room temperature for 1 h
8	Wash three times with PBST
9	Add 50 µL/well of the primary antibody in blocking buffer, incubate at room temperature for 1 h
10	Wash three times with PBST
11	Add 50 µL/well of the secondary antibody enzyme (HRP) conjugate in blocking buffer, incubate at room temperature for 1 h
12	Wash plate five times with PBST
13	Add 50 µL/well of substrate to plate according to manufacturers instructions, develop for 30 min at room temperature
14	Add 50 µL/well of substrate stop solution
15	Read plate OD values specific to substrate at 450 nm

produced through non-specific binding of antibodies to the plate surface or non-target ligands. The literature on immunoassays differs on what is considered "above background" signal, and the two most common methods are an absolute absorbance above which will be considered as valid, or an adaptive method where the mean signal of multiple nonserum or seronegative control groups are used to determine the threshold of validity.

Recently, the ability of several data analysis software packages to plot individual data points in single columns while also providing a mean figure have become popular. These plots are ideal as they allow the viewer to interpret the raw absorbance data to visually gauge how high and variable the background is. It is important to note that if titer data is to be statistically analyzed for significance above background or control group levels, then raw dilution figures cannot be used to perform standard parametric statistical analysis. The data in its raw form (e.g., titer of 1/100) is not normally distributed, rather the twofold relationship between dilution series follows a geometric distribution. Most software packages used for data analysis have methods of manipulating geometric datasets, and it is important to ensure that valid statistical tests are performed only when this limitation has been considered.

Problems are usually encountered with ELISA or Western blot analysis if antibody concentrations have not been optimized or the antibodies are inappropriate. A dot blot protocol and ELISA plate design have been given to optimize antibody concentration for Western blot and ELISA. The goal of performing these optimization protocols is to maximize the sensitivity of antibody detection, while promoting a robust and reproducible assay. Also, always know the source of your antibodies and what they were raised against. This can save considerable angst, for example, if the detection/primary antibody was raised against the native protein, it may not recognize the linear conformation. For Western analysis, another thing to consider is the isoelectric point of target protein. Should the isoelectric point of target protein be reached close to pH 8.3 (the pH of the transfer buffer), the protein of interest will have no charge to enable transfer to the membrane. Should this be the case, do not equilibrate the gel but use the charge on the protein of interest given by SDS in the running gel.

14.5 OTHER TECHNOLOGIES AND TECHNIQUES

The aim of this chapter is to discuss immunoassay methodologies for the production, characterization, and *in vivo* testing of plant-made vaccines for veterinary purposes. We have limited the protocols and discussion to techniques that are achievable with general laboratory equipment present in an agricultural molecular biology laboratory, and have excluded techniques that require significant investment in specialized equipment and training. However, the end goal of any vaccination program is to characterize the intensity and type of immune response raised, and while we have discussed the antibody humoral response to vaccination, cell-culture and biochemical analysis tools are available for assessing induced cytokines. Cytokine production may be determined in a cell-free or cell-culture method. Most simply, ELISA can be used to determine the concentration of cytokines present in the periphery. However, due to

the low concentrations and relative instability of cytokine proteins, these signals are often difficult to detect within the sensitivity of this assay. There are several cytokine detection kits on the market that have been validated to resolve these low cytokine concentrations, but the majority of these kits are not useful for veterinarian purposes, only for human or rodent targets.

Due to the low concentrations of some circulating cytokines, tissue culture techniques can be employed to enrich a culture for lymphocytes expressing these molecules. These cells can be isolated from the lymphatic tissues of the animal under sterile conditions, separated from other cells types, and grown in culture. The enrichment of lymphocytes may be of a general nature, for example, removal of animal spleens and the selective hypotonic lysis of the erythrocytes, or may be performed by cell sorting. Cell sorting is generally performed in automated flow cytometry systems where specific cell-surface receptors are tagged with fluorescent marker and sorted by their presence or lack of the fluorescent marker. These systems have the added benefit of being able to process a higher throughput of samples and can be used to rapidly sort cells based on single or multiple surface markers. Smaller scale sorting is also possible with paramagnetic bead systems which can be used to pull-down cells expressing specific markers, and there are several commercially available kits that can be used to prepare small-scale enrichments of cells using these systems. There are also several small-scale cell enrichment columns available, but unfortunately, at present these have only been verified with cells from model organisms.

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