finalreport

Project code: **AHW.025**

Prepared by: **Dr Timothy J Mahony**

Department of Primary Industries and Fisheries

Date published: **June 2006**

ISBN: 9781741914405

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Development of non-injectable vaccine technologies for red-meat industries

In submitting this report, you agree that Meat & Livestock Australia Limited may publish the report in whole or in part as it considers appropriate.

This publication is published by Meat & Livestock Australia Limited ABN 39 081 678 364 (MLA). Care is taken to ensure the accuracy of information in the publication. Reproduction in whole or in part of this publication is prohibited without the prior written consent of MLA.

Abstract

Infectious diseases negatively impact on the profitability of red-meat enterprises. While these impacts can be reduced through the use of effective vaccines, current vaccine delivery technologies are not optimal. The majority of currently registered vaccines are delivered through injection and while effective, there are negative impacts. For example, the formation of lesions at the site of vaccine injection may require trimming at slaughter thus reducing carcass value. Further, injectable vaccines typically require multiple doses that require re-mustering that increases labour overheads, particularly in extensive enterprises. The oral delivery of vaccines presents a valuable opportunity to address these negative factors of injection based vaccination strategies. As a first step in the application of oral vaccination to ruminants this study has demonstrated the production of vaccine components in transgenic plants. The development of a vaccinated strategy using a plant-based production of vaccine antigens was chosen due to the potential benefits of eliminating the likelihood of vaccine contamination by adventitious infectious agents, elimination of cold storage chains and use of a cost efficient system for vaccine production. Preliminary trials in which prototype a vaccine (freeze-dried transgenic plant material) were fed to sheep demonstrated immunological activity to the expressed antigen. However, more extensive animal testing failed to induce measurable responses in the vaccinated animals which has been attributed to poor antigen expression in most the lines of transgenic plants. Despite the inability to consistently demonstrate successful oral vaccination of sheep with transgenic plant material the results of this study do warrant further investigation that once fully optimised could provide an alternative vaccination strategy for the red-meat sector.

Executive Summary

Vaccines are one of the principle mechanisms utilised to reduce the impact of infectious diseases in livestock industries. Several factors determine the effectiveness of a vaccine. One is the vaccine formulation which is determined during the development phase of the vaccine, and apart from the end cost of the vaccine does not impact on production costs. Another is the delivery mechanism which is probably the most significant factor when considering the end user of the vaccine, namely the producer. The majority of current vaccines are delivered by injection. While injection is a very effective delivery mechanism it has significant drawbacks. Animals must be mustered and restrained to ensure safety of both the animals and workers involved. This adds to the labour costs. Furthermore, injectable vaccines generally require multiple doses, thus further increasing the associated labour costs. Perhaps the most significant disadvantage of injectable vaccines is the potential for lesion formation at the injection site. At the time of slaughter these lesions must be trimmed from the carcass and discarded. The economic losses due to this trimming were estimated to be $8.95 (CAN) per head of cattle for the Canadian beef industry in 1997.

A better option is to take vaccines to the animal, rather than taking the animal to the vaccine. This can be achieved by oral delivery of vaccines in block or lick formulations, or the addition of vaccine material to foodstuffs. The vaccine to be delivered may consist of either transgenic plant-derived material or viral vector vaccines.

The concept of plant-derived vaccination is now well accepted and the scientific literature contains many examples where plant-derived vaccines have conferred viral resistance in animals. However, these have been in either laboratory animals (mice and rats) or other non-ruminant animals. The delivery of plant-derived vaccines to ruminants presents difficulties due to the structure of the digestive system. For monogastrics, most research groups have targeted the intestinal lymphoid tissue. For ruminants, a potential target for oral vaccines is the oro-pharyngeal tissue. Typically, orally delivered vaccines do not provoke large immune responses and multiple does are required. This is not a problem for vaccines delivered in a feed formulation.

Plants also offer considerable advantages as a vaccine production system. Plants are a cost efficient production system requiring only soil, water and light to grow. While this is an oversimplification, compared to the high costs of constructing a manufacturing facility required for production systems based on mammalian cell culture, plants are highly attractive. Further as plants are not susceptible to mammalian pathogens the risk of vaccine contamination and associated components by adventitious infectious agents, such as non-cytopathic pestivirus or prions, is eliminated.

The application of oral vaccination with plant material also provides a cost effective delivery mechanism in difficult to access areas. Further, studies looking at the stability of human vaccine components in freeze-dried plant material have demonstrated prolonged stability at environmental temperatures thus eliminating the requirement of cold-chain storage.

To develop an effective vaccine that can be administered orally to ruminants the antigen needs to survive passage through the rumen to stimulate the immunological tissues of the small intestine. In order to facilitate this in this study, viral structures which are naturally resistant to the rumen environment were tested. Segments of the bovine parvovirus were expressed and these proteins auto-assembled into virus-like-particles (VLP). The VLP do not carry any part of the viral genome

and as a result are neither infectious nor pathogenic. Typically the proteins which form VLP are immunostimulatory and act as natural adjuvants to enhance the responses of the immune system.

This project has been demonstrated that transgenic plants can be utilised to produce antigens from economically important pathogens of cattle. Further, preliminary sheep feeding studies demonstrated that parvovirus VLP was able to elicit specific immunological responses in some animals. These promising results were not supported in more extensive animal testing. The primary reason for this is thought to be due to inconsistent expression of the antigen between transgenic plant lines resulting in suboptimal levels of antigen being fed to the animals. In spite of this the studies presented have advanced the development of an effective oral vaccination strategy for ruminants by demonstrated the feasibility of the process. Future studies are required where the expression of the parvovirus VLP is optimised to maximise expression in transgenic plants, and also to engineer the VLP to carry antigens from economically important pathogens. The activity of these hybrid VLPs would then need to be assessed in functional disease models, including challenge models, to demonstrate protection from infection.

The successful application of oral vaccination in red meat industries is a long term objective (>10 years). However, in three years this project has been able to demonstrate that oral vaccination of ruminants is achievable with a model antigen (parvovirus VLP) under controlled experimental conditions. To be a routinely available technology, further studies are required to demonstrate effective vaccination in an economically important disease model. This would then need further validation in pen and field trials. Consideration must also be taken into account that approval would be needed for the production of the transgenic plants on a much larger scale. In this study sufficient material could be produced in a controlled environment (glasshouse) however if a vaccine was being produced for use in the beef industry as a whole or even a sector, such as feedlots, then a much larger scale transgenic plant production system would be required. Currently the production of transgenic plants on this type of scale is subject to strict regulation.

All red-meat producers stand to benefit from the findings of this study. While demonstrating protection from infection was beyond the scope of this study, the study provided evidence that this is achievable. Fully validated oral vaccination will be of particular use in extensive meat production systems by providing easier compliance with vaccination regimes leading to better disease control. Intensive producers (such as feedlots) will also benefit as this sector currently utilises injectable vaccines and as a result is at risk of losses associated with lesion formation. In addition, excessive handling of animals has been associated with production loses.

Contents

Page

1 Background 7
2 Project Objectives 8
3 Methodology 8
3.1 Antigen Development 8
3.1.1 Antigen Cloning 8
3.1.2 Bacterial expression 9
3.1.3 Baculovirus Expression 9
3.1.4 Mammalian Expression 9
3.1.5 Plant Expression 9
3.1.6 Analysis of Antigen Expression in Plants 10
3.2 Animal Experiments 10
3.2.1 Experiment 1 – Assessment of oropharyngeal tissue as oral vaccine target. 10
3.2.2 Experiment 2 – Assessment of oral vaccination using plant material. 11
3.2.3 Experiment 3 – Assessment of prime/boost strategies. 12
3.2.4 Experiment 4 – Validation of oral vaccine with plant-derived antigen. 13
4 Results and Discussion 14
4.1 Antigen Expression 14
4.1.1 Analysis of Antigen Expression 14
4.1.2 Self-assembly of parvovirus VP2 in virus-like-particles 14
4.2 Assessment of transgenic plants for antigen production 16
4.2.1 Expression of GFP in transgenic plants 16
4.2.2 Expression of E2 in transgenic tobacco plants 20
4.2.3 Production of BPV in transgenic plants 21
4.3 Animal Experiments 23
4.3.1 Experiment 1 – Assessment of oropharyngeal tissue as a delivery route 23
4.3.2 Experiment 2 – Oral vaccination with transgenic tobacco 26
4.3.3 Experiment 3 – Assessment of prime/boost strategies 28
4.3.4 Experiment 4 – Validation of oral vaccine 30
5 Success in Achieving Objectives 31

5.1 Demonstrate production of vaccine components in plants....31
5.2 Demonstrate immunological response to oral delivered antigen(s) in a model ruminant system....31
5.3 Develop non-injectable vaccination strategies for ruminants....32

6 Impact on Meat and Livestock Industry – now & in five years time....32

7 Conclusions and Recommendations....32

7.1 General Conclusions....32
7.2 Future Studies....34

8 Bibliography....35

1 Background

Red meat producers currently utilise a number of vaccine delivery technologies, such as injectable and intranasal vaccines. Generally there are two major factors that determine the effectiveness of a vaccine. The first is the vaccine formulation which is determined during the development phase of the vaccine and apart from the end cost of the vaccine does not impact on production costs. The second is the delivery mechanism which is probably the most significant factor when considering the end user of the vaccine, namely the producer. The majority of current vaccines are delivered by injection. While injection is a very effective delivery mechanism it has significant drawbacks. Animals must be mustered and restrained to ensure safety of both the animals and workers involved. This adds to the labour costs. Furthermore, injectable vaccines generally require multiple doses thus further increasing the associated labour costs. The reluctance of producers to re-muster to deliver subsequent doses results in reduced compliance with vaccination programs, and animals with suboptimal immunisation levels are left susceptible to the development of diseases.

One of the most significant disadvantages of injectable vaccines is the potential for lesion formation at the injection site. At the time of slaughter these lesions must be trimmed from the carcass and discarded. The economic losses due to this trimming were estimated to be $8.95 (CAN) per head of cattle in the Canadian beef industry during 1997 (van Donkersgoed *et al.*, 1997). There is also the potential for needle breakages and subsequent contamination of the carcass.

Intra-nasal delivery of the vaccine via a live viral vector vaccine is an option which we are developing with MLA funding for the cattle feedlot market (FLOT.203). This method provides good protection with a single does of vaccine and also targets the mucosal immune system, which is of primary importance in preventing infection by pathogens of the upper respiratory tract. However, we consider that intra-nasal vaccine administration on-property is likely to be difficult and will not gain a high level of producer acceptance.

A potentially better option is to take vaccines to the animal, rather than taking the animal to the vaccine. This can be achieved by oral delivery of vaccines in block or lick formulations, or the addition of vaccine material to foodstuffs. The vaccine to be delivered may consist of either transgenic plant-derived material or a viral vector vaccine formulation.

The concept of plant-derived vaccination is now well accepted and the scientific literature contains many examples where plant derived vaccines have conferred pathogen resistance in animals (Arntzen *et al.*, 2005; Dus Santos and Wigdorovitz, 2005). However, these have been in humans, laboratory animals (mice) and pigs (Thanavala *et al.*, 2005; Legocki *et al.*, 2005; Howard, 2004; Lamphear et al., 2004; Richter *et al.*, 2000). The delivery of plant-derived vaccines to ruminants presents difficulties due to the structure of the digestive system. For monogastrics, most research groups have targeted the intestinal lymphoid tissue (Walmsley and Arntzen, 2000: 2003; Rigano *et al.*, 2003). For ruminants, a potential target for oral vaccines is the oropharyngeal tissue. Typically, orally delivered vaccines do not provoke large immune responses and multiple doses are often required. This is not a problem for vaccines delivered in a feed formulation.

To successfully vaccinate cattle, sheep and other ruminants with plant-derived vaccines, technologies are required to circumvent the ruminant digestive system. One strategy to be utilised is viral structures which are naturally resistant to the rumen environment and are also able to stimulate the immune system. When the segment of the virus genome that encodes for viral proteins that form the outer structure of some viruses are expressed the proteins auto-assemble into virus-like-particles (VLP). The VLP do not carry any part of the viral genome and as a result are neither

infectious nor pathogenic. Typically the proteins which form the VLP are immunostimulatory and act as natural adjuvants to enhance the immune system. By adding the genetic material that encodes antigens from target pathogen to the genes encoding the VLP, a hybrid VLP could be produced that should elicit a specific immune response to not only the VLP but also the added antigens.

An important feature of oral delivery of vaccines is the type of immune response that is induced. Many of the important pathogens which infect domestic ruminants enter via the mucosal surfaces. To ensure that a vaccine effectively protects the host it is important that a mucosal immune response is induced. It has generally been found that the best method for inducing an immune response is to vaccinate at the mucosal surfaces. With oral delivery of plant-derived vaccines a mucosal response will result, thus giving protection against primary infection by important pathogens infecting by the oral, respiratory or reproductive routes.

Oral vaccination of ruminants will be investigated using bovine viral diarrhoea virus (BVDV) infection of a sheep as an experimental model. Pestivirus is a pathogen of both cattle and sheep, causing mucosal disease and Border disease respectively in these species. While pestivirus is not a major sheep disease it is an excellent model system for testing the ability of vaccines to induce mucosal and systemic humoral immune responses. The cost of an experimental model using sheep is significantly cheaper compared to an analogous model in cattle.

2 Project Objectives

1. Demonstrate production of vaccine components in plants

2. Demonstrate immunological response to oral delivered antigen(s) in a model ruminant system

3. Develop non-injectable vaccination strategies for ruminants

3 Methodology

3.1 Antigen Development

3.1.1 Antigen Cloning

The following antigens from livestock selected pathogens were chosen for expression analyses in various expression systems. The antigen genes listed below were amplified from the designated starting material using standard polymerase chain reaction (PCR) protocols.

Bovine viral diarrhoea virus (BVDV)
A synthetic gene for BVDV Glycoprotein E2 was constructed as part of project FLOT.203. The sequence of this gene was modified from the native version of the gene for optimal expression in mammalian cells. The open reading frame corresponding to E2 was amplified from existing constructs using PCR.

Bovine parvovirus (BPV)
BPV was kindly provided by Dr Jan Smith, James Cook University of North Queensland. The gene encoding for the viral protein 2 (VP2) was amplified by PCR from DNA isolated from the virus supernatant for direct cloning.

Bovine enterovirus (BEV)

The genetic sequences for Bovine enterovirus (BEV) structural proteins and 3C3D were amplified from clones previously report by McCarthy *et al*. (1999).

Mannheimia haemolytica
The gene encoding for leukotoxin (*ltx*) was amplified directly from cultured *M. haemolytica* kindly provided Dr Carol Stephens (Department of Primary Industries & Fisheries).

Control Antigen
Green fluorescent protein (GFP, control antigen) amplified from expression of the plasmid pEGFP-N1 (Clontech).

To simplify the cloning and expression of the six genes used as part of this study the cloning system was utilised. A detailed explanation of this system is beyond the scope of this report, however the full details can been found on the Invitrogen homepage (www.invitrogen.com). Briefly, this a multifaceted cloning system that enables the rapid shuttling of genes in to vectors required for gene expression in different expression systems. For example, as part of the antigen development process for this project it was proposed to express genes in bacteria, baculovirus, mammalian cells and two plant species. Using traditional approaches to cloning and expression we would have needed to clone the gene into a plasmid (vector) that contained the necessary genetic elements for gene expression. Using the Gateway® system once a gene is cloned it can then be transferred directly into an expression vector for a specific system. In order to facilitate the generation of primary clones for gene transfer, PCR-generated amplicons were cloned using pENTR-TOPO (Invitrogen).

3.1.2 Bacterial expression

The expression of the genes in bacterial cells was facilitated by transfer of the antigens into the following vectors pDEST-14 or pBAD-DEST49. Following confirmation that the antigen gene had been successfully transferred to the plasmids these were used to transform *Escherichia coli* expression strains using standard techniques.

3.1.3 Baculovirus Expression

The expression of antigen genes in insect cells was done using baculovirus vectors following transfer to the vector pDEST-8. Recombinant baculoviruses where then generated using the Baculo-direct system (Invitrogen) according to the manufacturer's instructions.

3.1.4 Mammalian Expression

Expression of antigen genes in mammalian cells was facilitated via transfer into either pcDNA-DEST-49 or pcDNA-DEST-57. These vectors promote the expression of the antigen as a fusion protein with green fluorescent protein (GFP). The vector pcDNA-DEST-49 permits expression of the antigen fused to the N-terminus of GFP, while the vector pcDNA-DEST-57 permits expression of the antigen fused to the C-terminus of GFP. Expression was assessed following transfection of the plasmid containing the gene of interest into rabbit kidney cells (RK13). Antigen expression was assessed using fluorescent microscopy and Western blot analysis.

3.1.5 Plant Expression

Two plant species were utilised in these studies. The first system used in this study was tobacco (*Nicotiana benthamiana*) which is a laboratory model system that enables construction of transgenic

plants within a six month time frame to ensure transgenes are operating correctly in plants. The second plant system used in this study was lucerne, which requires a 12 month period for the generation of transgenic plants.

At the commencement of this project there were no Gateway® enabled plant transformation vectors available. Previously, to permit plant expression there were multiple cloning steps required to transfer a gene of interest from an *E. coli* vector to an agrobacterial transfer vector for use in plant transformation. As such, a Gateway® vector was constructed that permits direct transfer of genes into the Agrobacterial transfer vector. Following successful transformation of Agrobacterium with the respective antigens, transgenic plants were constructed as described below.

Plant tissues (either callus and/or embryo) were transferred to media to initiate shoot development while still selecting for genetic transformed plants. Shoots were cut off and transferred to individual tissue culture (TC) vessels for rooting. TC plants were screened for the presence of the genes of interest by PCR on crude plant extracts prior to transfer into glasshouse.

3.1.6 Analysis of Antigen Expression in Plants

To determine if the transgenes were being expressed in the generated plant lines three analyses were conducted:

1. The presence of the transgene in the plant genome was determined using PCR,
2. Expression of the transgene was analysed using 3'-RACE PCR of the mRNA of each transgene,
3. The presence of the antigen was analysed using Western blotting of whole protein extracts.

3.2 Animal Experiments

All animal trials were conducted at the Animal Research Institute (Yeerongpilly) using sheep sourced from local sale yards. The trial protocols were approved by the Department of Primary Industries and Fisheries Staff Access Animal Ethics Committee, and the relevant approval numbers are listed.

3.2.1 Experiment 1 – Assessment of oropharyngeal tissue as oral vaccine target.

The following immunisation trial was conducted to determine if the oropharyngeal tissue of sheep could be utilised as a target tissue for the oral vaccination of sheep, as described below.

Animal ethics approval number ARI-010-03-2005.

Ten sheep were divided randomly into treatment groups (2 per group). On the day of vaccination (Day 1) immediately prior to vaccination the following samples were collected from all animals:

i. Rectal temperature
ii. 20 to 40 ml blood
iii. Bilateral nasal swabs.

The pairs of sheep were then vaccinated as described below:
Group 1: E2 (expressed in bacteria) injected subcutaneously (with adjuvant).
Group 2: BPV (expressed in baculovirus) injected subcutaneously (no adjuvant).
Group 3: BPV (expressed in baculovirus) injected into oropharyngeal tissue.
Group 4: BPV (expressed in baculovirus) rubbed onto oropharyngeal tissue.
Group 5: Control group no vaccinations administered.
(Groups 3 and 4 were sedated prior to vaccination.)

The vaccinations were repeated on days 21 and 42 following the primary immunisation. The following samples were collected from all animals prior to administering the immunisations:

i. Rectal temperature
ii. 20 to 40 ml blood
iii. Bilateral nasal swabs

On day 63 the trial concluded following the collection of 200 ml of sera and nasal swabs from each animal.

3.2.2 Experiment 2 – Assessment of oral vaccination using plant material.

To assess if the BPV VLP being produced in the transgenic tobacco plants was immunologically active the following immunisation trial was conducted. Animals for experimental activity were divided into two treatment groups for testing with transgenic plant material processed at different levels, and one sentinel group of control animals. Experiment 2 was conducted concurrently with Experiment 1, hence only one control group was included, Group 5, as described in Section 3.2.1.

Animal ethics approval number ARI22-05-2005.

Four sheep were divided randomly into two groups. On the day of vaccination (Day 1) immediately prior to vaccination the following samples were collected from all animals:

i. Rectal temperature
ii. 50 ml of blood
iii. Bilateral nasal swabs

The animals were then orally vaccinated with the following preparations of transgenic plant material:

Group 1: Liquid homogenate of freeze-dried plant material - BPV VLP. The homogenate was prepared by resuspending freeze-dried plant material in a minimal volume of phosphate buffered saline (PBS) and mixing to remove large clumps of material.

Group 2: Transgenic plant freeze dried and ground into fine particles - BPV VLP. The homogenate was prepared by grinding the freeze-dried plant material mixed with liquid nitrogen with a mortar and pestle and resuspending in a minimal volume of PBS.

The vaccinations were performed by introducing the plant homogenates to the back of the throat. Each animal received 60 ml of homogenate, which equates to approximately 30g of original plant material. The homogenate was aspirated into the rear of the oral cavity and massaged onto the tissue using a gloved finger.

The vaccinations were repeated on days 21 and 42 following the primary immunisation. The following data & samples were collected from all animals prior to these immunisations animals:

i. Rectal temperature
ii. 20 to 40 ml blood
iii. Bilateral nasal swabs

The experiment concluded on day 63 following the collection of 200 ml of sera and nasal swabs from each animal.

3.2.3 Experiment 3 – Assessment of prime/boost strategies.

The following experiment was conducted to determine if an antigen produced in plants could be utilised as part of a prime/boost vaccination strategy. Due to the large amount of material required to immunise twelve sheep multiple times, parvovirus antigen expressed by *E. coli* was utilised.

Animal ethics approval number SA2005-10-60.

Twelve sheep were divided into 6 treatment groups on day 1.
Immediately prior to vaccination the following data & samples were collected:

i. Rectal temperatures.
ii. Blood (20 ml).
iii. Bi-lateral nasal swabs.

The animals were then vaccinated as described in Table 1.

Table 1: Prime/Boost Vaccination Schedules for Treatment Groups

Group (# Animals)	Primary vaccination (day 1)	Boost at 3 weeks (21 days)	Boost at 6 weeks[1] (42 days)
1 (2)	**Nil**	**Nil**	**Nil**
2 (2)	**A[1]**	**A**	**A**
3 (2)	**B[2]**	**A**	**A**
4 (2)	**C[3]**	**A**	**A**
5 (2)	**C**	**C**	**C**
6 (2)	**B**	**B**	**B**
Total Animals: 12			

[1]Treatment A: Oral delivery of up to 50μg of antigen
[2]Treatment B: Injection of up to 50μg of antigen. Antigen delivered subcutaneously with adjuvant MPL + TDM CWS Adjuvant System (MTCAS).
[3]Treatment C: Intranasal installation of 50μg of antigen. Antigen applied in solution to the nasal passage in liquid formulation, no adjuvant.

Booster vaccinations were administered on days 21 and 42. The following data & samples were collected prior to vaccination:

i. 40ml of blood
ii. Bi-lateral nasal swabs
iii. Rectal Temperatures

The experiment ended on day 63 when 200 ml of blood was collected for serological studies.

3.2.4 Experiment 4 – Validation of oral vaccine with plant-derived antigen.

Analyses of the sera samples from Experiment 2 (Section 3.2.2) indicated that one of the two sheep that were fed transgenic plant material (freeze-dried and ground into a paste in liquid nitrogen) developed parvovirus-specific antibodies. The purpose of Experiment 4 was to validate this finding with a larger number of animals and add statistical weight to this finding.

Animal ethics approval number SA 20060412.

Twelve sheep were randomly assigned to 2 treatment groups immediately prior to vaccination and the following data and samples collected:

i. Rectal temperatures.
ii. Collect of 20ml blood.
iii. Bi-lateral nasal swabs.

Animals were then fed 100ml of plant slurry (equivalent to 60g freeze-dried tobacco, ground into fine particles):
Group A: Control plant material (Transgenic tobacco expressing BVDV E2)
Group B: Treatment group: (Transgenic tobacco expressing BPV VLP.

Vaccinations were repeated on days 14, 28, and 42. At this time the following samples & data were also collected:

i. 40 ml of blood.
ii. Bi-lateral nasal swabs.
iii. Rectal Temperatures.

At the conclusion of the experiment (Day 56) 200 ml of blood was collected for serological studies from each animal. The sera were then tested for antibodies specific to parvovirus VP2.

4 Results and Discussion

4.1 Antigen Expression

4.1.1 Analysis of Antigen Expression

Table 2 summarises the analyses of the expression of the antigens used in this study in the various expression systems.

Table 2: Summary of antigen expression in different expression systems

	Expression system				
	E. coli	Baculovirus	Tobacco	Lucerne	Mammalian
E2 (BVDV)	N[1]	Y	Y[2]	Y[2]	ND
BEV Struct	N	Y	Y[2]	ND	ND
BEV 3C3D	N	Y	Y[2]	ND	ND
VP2 (parvo)	Y	Y	Y[2]	Y[2]	ND
Leukotoxin	N	ND	ND	ND	Y

N – No expression detected;
Y – Expression confirmed;
ND – Not Done
[1]The E2 gene did not express when tested as a single open reading frame in the *E. coli* expression system. However, fusion of the E2 gene to the C-terminus of thioredoxin in the expression vector pBAD-49 facilitated expression in *E. coli*.
[2]Gene expression in the transgenic plants has been confirmed by reverse transcriptase PCR. The level of protein expression appears to be below the level of the detection limits of western blotting. There is also considerable background on these blots that prevent confirmation of protein expression.

4.1.2 Self-assembly of parvovirus VP2 in virus-like-particles

As illustrated in Table 2 the VP2 gene of parvovirus was readily expressed in all of the systems tested. Analysis of the VP2 protein in *E. coli* indicated that it was expressed as an insoluble protein that was secreted into intracellular inclusion bodies. This was not unexpected, as this is a common problem when over-expressing proteins in *E. coli*. To determine if VP2 was able to self-assemble into virus-like-particles (VLP), the gene was also expressed in insect cells using baculovirus expression vectors. Excellent expression of VP2 was detected in insect cells.

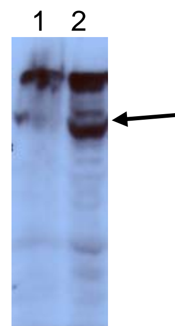

Figure 1: Western blotting analysis of parvovirus VP2 protein expressed in insect cells. Lane 1: insoluble protein extract from infected insect cells; Lane 2: Soluble protein extracts from infected insect cells. Expressed VP2 (indicated by arrow) was detected using an anti-6His monoclonal antibody.

Following confirmation of VP2 expression in insect cells the soluble protein extract was fractionated on sucrose gradients to determine if self-assembly into VLP was occurring. Figure 2A and 2B, illustrates the expressed VP2 was indeed forming VLP. The VLP was characterised by a dark centre, which is the empty viral particle. The outer structure of the VLP was lighter in colour and is formed by the expressed VP2 protein. The VLP structures observed were considered typical of native parvovirus particles, as the VLP ranged in size from 20 to 25 nm in diameter.

While expression and self-assembly was evident in insect cells, we were concerned that the overall yield of VLP from insect cells was low, particularly if large amounts were required for animal immunisation experiments. To improve the yield of BVP VLP we solubilised the bacterial-expressed VP2 under strong denaturing conditions. The VP2 protein was then renatured and examined using electron microscopy to determine if it would self-assemble into VLP under laboratory conditions. Figure 2C illustrates that the bacterial-expressed VP2 self-assembled into structures consistent with the insect VLP, with dark centres and lighter outer structures formed by the expressed VP2 protein. However the bacterial-expressed VLP were much more variable in size, ranging from 20 to 75 nm. Another notable difference was the bacterial VLP were less regular in shape compared to the insect VLP (Figure 2).

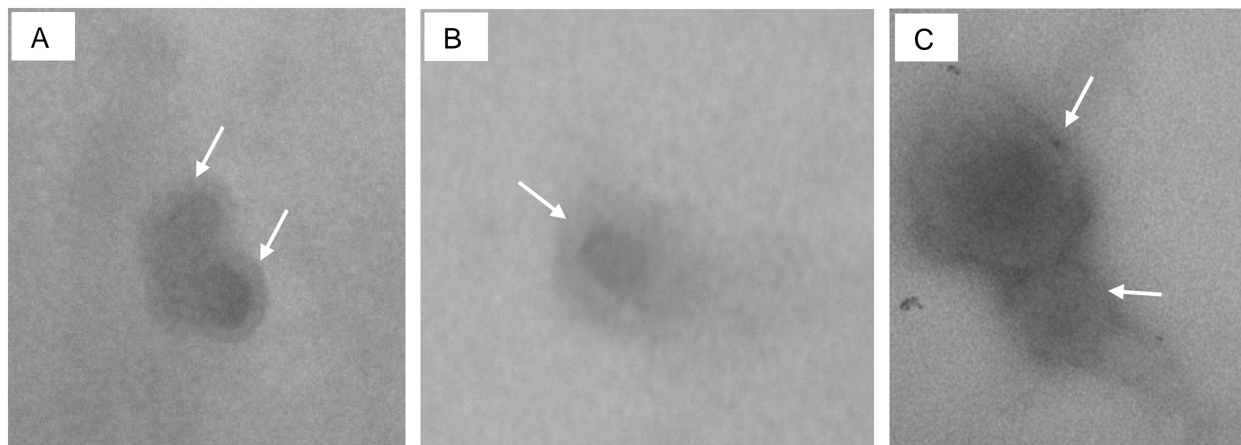

Figure 2: Electron micrograph of parvovirus virus-like-particles (VLP) produced through the over expression of VP2 in insect and bacterial cells. The VLP is characterised by a dark centre, which is the empty viral particle. The outer structure of the VLP is lighter in colour and is formed by the expressed VP2 protein. Panel A depicts two closely associated VLP produced in insect cells. Panel B depicts a single VLP that is slightly deformed compared to those shown in panel A, also expressed in insect cells. The estimated diameter of the insect VLP was 20 to 25 nm which is within the published range for parvoviruses at 18 to 26 nm. Panel C depicts two VLP expressed in bacterial cells. While these bacterial VLP were visually very similar to insect VLP they were highly variable in size, ranging from 30 to 75 nm in diameter. White arrows indicate the individual VLP structures.

4.2 Assessment of transgenic plants for antigen production

Following the selection of transgenic plant lines three strategies were used to assess the potential antigen expression levels. These were:

1. PCR on genomic DNA to determine the presence of the transgene,
2. cDNA analyses to estimate the level of transcript (mRNA) present, and
3. Western blotting of total protein extracts for the detection of specific antigens.

Data is only presented here for the GFP (control), E2 and BPV antigens. However similar analyses were conducted for the 3C3D and BEV structural antigen transformed plants.

4.2.1 Expression of GFP in transgenic plants

Previous studies in our laboratory have demonstrated that GFP can be readily expressed in the transgenic plant systems that were utilised in this study. Further, excellent reagents are available for the detection of the GFP protein in extracts.

Genomic DNA was extracted from putative transgenic tobacco and lucerne plant lines for PCR analyses. Figure 3 illustrates the typical efficiency for the transformation of tobacco plants with a GFP transgene. Though the PCR method utilised is not strictly quantitative, it is clear that there are differences between the plant lines. Tobacco Line GFP5 (Fig 3, Lane 8) appears to have a higher number of gene copies, compared to Tobacco Line GFP6 (Fig 3, Lane 9). Similar results where obtained for the lines of transgenic lucerne transformed with the GFP transgene (data not shown).

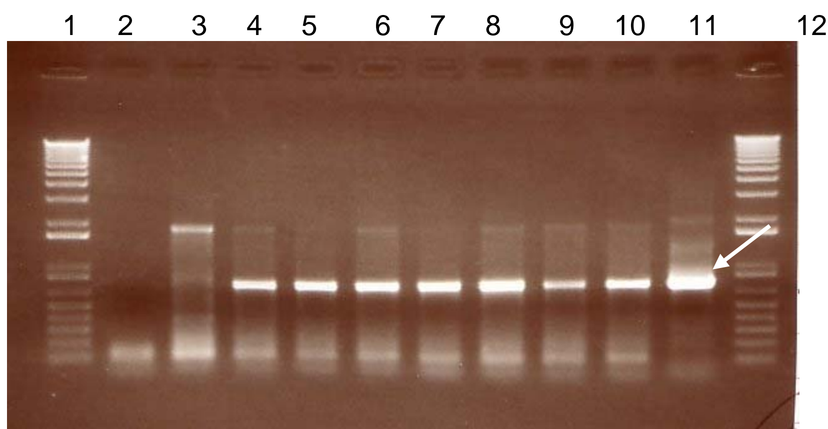

Figure 3: Amplification of the gene for green fluorescent protein (GFP) from genomic DNA of transgenic tobacco plants. Arrow indicates amplicon of the correct size in the control reaction.
Lane 1. Molecular weight marker; Lane 2: No template control; Lane 3: Agrobacterium control; Lane 4: Tobacco Line GFP1; Lane 5: Tobacco Line GFP2; Lane 6: Tobacco Line GFP3; Lane 7: Tobacco Line GFP4; Lane 8: Tobacco Line GFP5; Lane 9: Tobacco Line GFP6; Lane 10: Tobacco Line GFP7; Lane 11: GFP positive control; Lane 12: Molecular weight marker.

Following confirmation of the insertion of the GFP transgene into the plant's genome, expression of the transgenes was assessed at both the mRNA and protein levels. The level of gene expression was first determined using 3'-RACE PCR. This process utilises the polyA tail of mRNA to attach a unique primer to the end of cDNA. The unique primer can then be utilised in conjunction with a gene specific-primer to specifically amplify transcripts of the gene of interest (in this case, GFP). Figure 4 illustrates the 3'-RACE amplification of the GFP transcripts from four lines of tobacco and four lines of transgenic lucerne. Clearly, there is significantly more variation in the level of GFP mRNA expression compared to the level of genome transformation (Fig. 3). The transgenic lines of tobacco analysed using 3'-RACE correspond to those analysed using genomic PCR described above (Fig. 3 & Fig. 4).

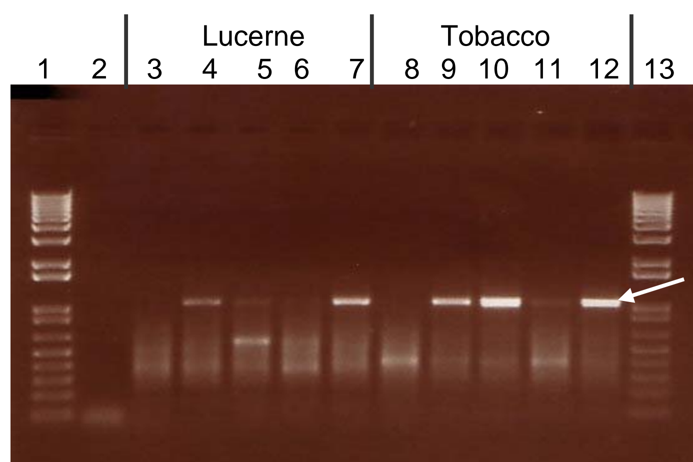

Figure 4: Assessment of the level of the green fluorescent protein (GFP) mRNA expression in lines of transgenic lucerne and tobacco. Amplification was performed using 3'-RACE methodology. Arrow denotes correct amplicon of the correct size.
Lane 1: Molecular weight marker; Lane 2: No template control; Lane 3: Lucerne Control; Lane 4: Lucerne Line GFP2; Lane 5: Lucerne Line GFP3; Lane 6: Lucerne Line GFP4; Lane 7: Lucerne Line GFP5; Lane 8. Tobacco Control; Lane 9: Tobacco Line GFP1; Lane 10: Tobacco Line GFP2; Lane 11: Tobacco Line GFP3; Lane 12: Tobacco Line GFP4; Lane 13: Molecular weight marker.

The reasons for the high variability observed between the different lines are unknown at this time. One possible explanation is that the level and direction of the transgene insertion into the plant genomes occurs at random. Consequently, if transgenes are expressed that are complementary, this can induce RNA interference (RNAi) that results in specific degradation of homologous mRNA molecules. This phenomenon was first recognised in transgenic plants and has recently been described in other biological systems. RNAi mechanisms are part of the natural host defence mechanism against RNA viruses. For example, amplification of the GFP gene from Tobacco Line GFP3 at the genomic level was successful (Fig 3, Lane 6) however at the transcript level using 3'-RACE there was poor amplification (Fig. 4, Lane 11). In comparison, both amplicons for Tobacco Line GFP2 were high (Fig. 3, Lane 5 and Fig. 5, Lane 10). The investigation of the exact reasons for these discrepancies was beyond the scope of this study but these types of variations are not uncommon in transgenic plant studies.

Following confirmation of the presence of GFP specific mRNA, experiments were conducted to confirm the production of the GFP protein. This was achieved by using GFP-specific antibodies to detect the presence of the GFP protein in total protein extracts from the various transgenic plant

lines. These analyses were conducted for both transgenic lucerne and tobacco lines. Western blot analyses were conducted using an anti-GFP specific monoclonal antibody and also with an anti-6-His tag monoclonal antibody that was added to the open reading frame of the GFP protein. The 6-His tag was added to all of the genes utilised in this study, these types of tags can be used to detect recombinant proteins with a single antibody, and also to purify the recombinant protein if required.

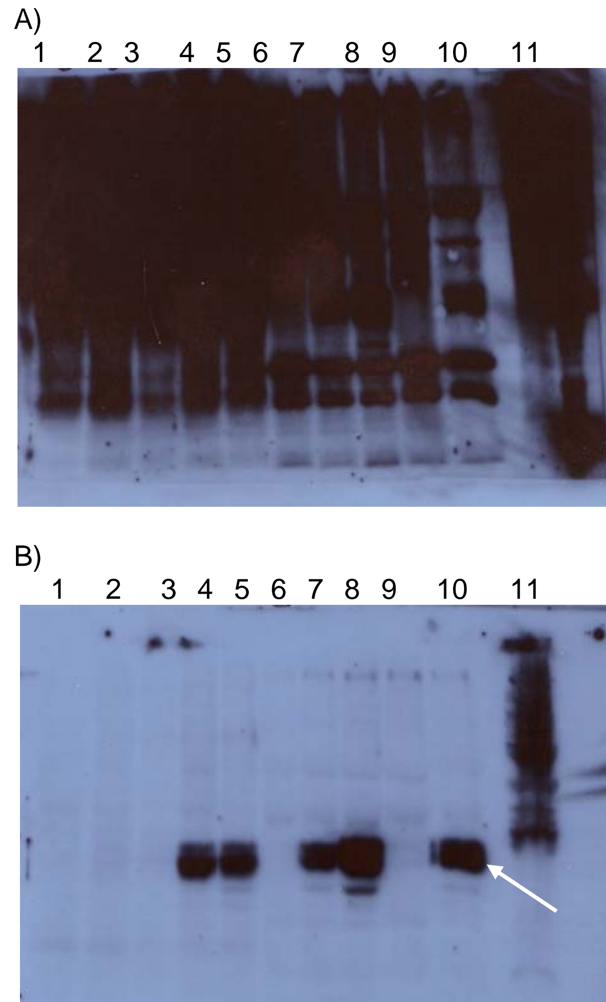

Figure 5: Detection of green fluorescent protein (GFP) protein in whole plant protein extracts using western blotting. Arrow indicates expected band.

A) Detection of GFP with a monoclonal antibody against 6-His tag.
B) Detection of GFP with a monoclonal antibody against GFP.

Lane 1: Lucerne Control; Lane 2: Lucerne Line GFP2; Lane 3: Lucerne Line GFP3; Lane 4: Lucerne Line GFP4; Lane 5: Lucerne Line GFP5; Lane 6: Tobacco Control; Lane 7: Tobacco Line GFP1; Lane 8: Tobacco Line GFP2; Lane 9: Tobacco Line GFP3; Lane 10: Tobacco Line GFP4; 11. GFP positive control.

The specific detection of GFP protein was evident in a number of the lines of transgenic plants tested with the anti-GFP antibody only (Figure 5B). Figure 5A illustrates the extremely high background observed when probing the western blots with the anti-6-His monoclonal antibody (for both transgenic tobacco and lucerne). The 6-His tag consists of six consecutive histidine residues fused to the carboxyl terminus of the GFP protein. The high background prevented the identification of the recombinant protein using this antibody. This outcome was not expected, as we have successfully utilised the 6-His tag and the monoclonal detection system in other expression systems (bacterial, insect and mammalian). In an effort to resolved the high background observed with the 6-His monoclonal detection system attempts were made to optimise the various components of the system. This included testing of differing concentrations of primary antibody (6-His monoclonal) and secondary antibody (anti-mouse IgG antibody), and different blocking conditions for the Western blot membrane. None of these variations were able to resolve the issue of high background.

There was a strong correlation between the level of protein GFP detected for the tobacco lines and the results of the 3'-RACE analyses of the mRNA transcripts (Fig. 4 & Fig. 5). The level of 3'-RACE amplification of the tobacco lines GFP1, GFP2, GFP3, and GFP4 (Fig 4, Lanes 9 to 12) clearly reflected the level of GFP protein detected via western blot analysis of these lines (Fig 5A, Lanes 7 to 10). Tobacco Lines GFP2 and GFP4 had the strongest signals in both analyses, while in comparison Line GFP1 had slightly lower levels of products in both analyses. Tobacco Line GFP3 had a barely discernable 3'-RACE amplicon (Fig. 4 Lane 11) and no detectable GFP protein (Fig. 5B).

Similar correlations between transcript level and protein expression level were observed in the analyses of the transgenic lucerne lines. Although the lucerne 3'-RACE amplifications were not as strong as the corresponding amplifications from the transgenic tobacco (Fig. 4), there were no discernable differences between the different plants at the protein level (Fig. 5B). Lucerne Line GFP5 indicated the highest level of 3'-RACE amplicon, which corresponded to high levels of protein in western blot analysis. Contrary to this were the results for Lucerne Line GFP4, where no amplicon was observed in the 3'-RACE amplification (Fig. 4, Lane 6) but high levels of GFP protein were detected via western blotting (Fig. 5B, Lane 4). The reasons for this discrepancy are not clear. One possibility is that the mRNA purification may have been less efficient from the lucerne plants compared to the tobacco plants, resulting in the generally low levels of 3'-RACE amplicons observed. This may also reflect that lucerne is a field crop while the species of tobacco used in this study has been adapted for experimental uses.

The correlation between the levels of GFP protein and GFP transcripts for transgenic tobacco provided high confidence that 3'-RACE amplifications were strong predictors of the level of protein in transgenic plant lines. This was an important finding as at this stage of the project we did not have access to specific antibodies for the antigen genes being assessed. Initially, we had planned to analyse the level of antigen in the transgenic plants expressing different antigens using the anti-6-His monoclonal antibody. However, based on the analysis of the GFP lines we were unsure of how useful this antibody would be (Fig. 5A). Any correlation between GFP protein and GFP transcripts in lucerne plants was less obvious, indicating that any extrapolations made between transcript and protein in lucerne would be unsound. The reasons for this in lucerne, particularly in light of the tobacco results, are unclear. One possibility is the extraction procedures utilised for both mRNA and protein were more efficient for tobacco compared to lucerne. It may be necessary further to optimise both these extraction procedures for application to lucerne in order to improve the correlation between mRNA and protein analyses.

4.2.2 Expression of E2 in transgenic tobacco plants

The generation of transgenic tobacco lines carrying the BVDV E2 transgene was confirmed using PCR amplification from purified genomic DNA (Fig. 6). The amplification from each plant line was variable. This may be the result of more transgenes being incorporated into the genomes of some lines compared to others. Excellent transformation efficiencies were obtained as the majority of lines tested were PCR positive for the presence of the E2 transgene.

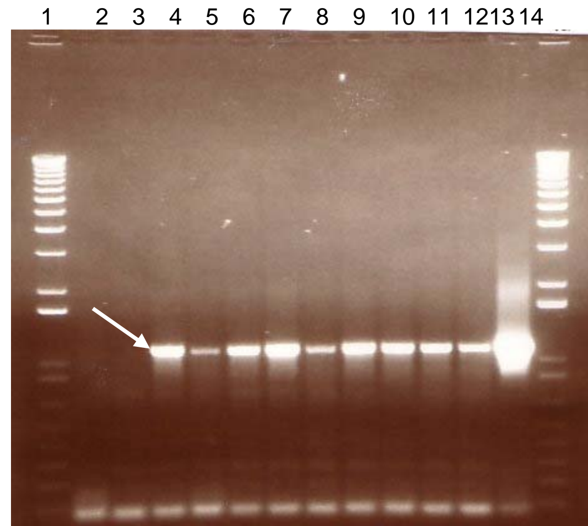

Figure 6: Amplification of the bovine viral diarrhoea virus E2 gene from genomic DNA extracted from transgenic tobacco plants. The arrow indicates the expected amplicon (1200 bp.)
Lane 1: Molecular weight marker; Lane 2: No DNA control; Lane 3: Tobacco DNA control; Lane 4: Tobacco Line E1; Lane 5: Tobacco Line E2; Lane 6: Tobacco Line E3; Lane 7: Tobacco Line E4; Lane 8: Tobacco Line E5; Lane 9: Tobacco Line E6; Lane 10: Tobacco Line E7; Lane 11: Tobacco Line E8; Lane 12: Tobacco Line E9; Lane 13: E2 positive control (plasmid DNA); Lane 14: Molecular weight marker. The arrow indicates the amplicon at the expected size.

Confirming the presence of the E2 transgene in the genomic DNA of the plant lines was the first step in determining the level of gene expression, and hence, the potential amount of antigen within the plant material.

Following confirmation of the presence of the E2 transgene in the genome of the plants, 3'-RACE analysis was conducted on extracted RNA. As illustrated by Fig. 4, the amount of E2 transcript present in each line was highly variable. This is indicates that the amount of E2 protein could also vary considerably between the different lines. However, the detection of the transcript for the E2 transgene in these transgenic plant lines provided high confidence that the plants contained the antigen.

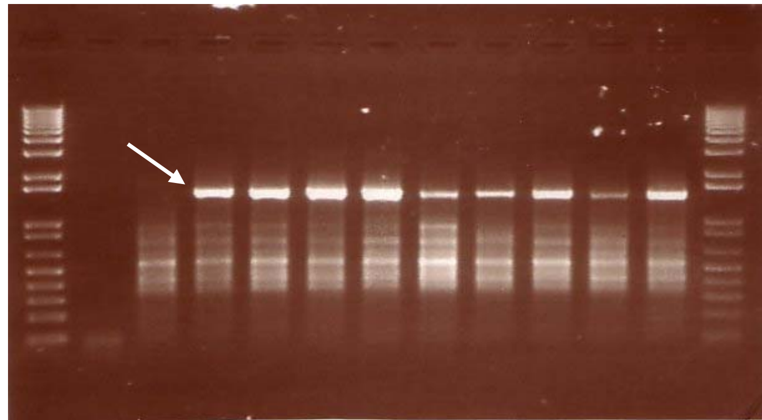

Figure 7: Amplification of the bovine viral diarrhoea virus E2 cDNA using 3'-RACE from transgenic tobacco.
Lane 1: Molecular weight marker; Lane 2: No cDNA control; Lane 3: Tobacco cDNA control; Lane 4: Tobacco Line E1; Lane 5: Tobacco Line E2; Lane 6: Tobacco Line E3; Lane 7: Tobacco Line E4; Lane 8: Tobacco Line E6; Lane 9: Tobacco Line E7; Lane 10: Tobacco Line E8; Lane 11: Tobacco Line E9; Lane 12: Tobacco Line E11; Lane 13: Molecular weight marker. The arrow indicates the amplicon at the expected size.

No specific antibodies were available for the detection of the E2 protein in these transgenic plants. A 6-His tag has been added to the transgene to facilitate this analysis however, as a result of the high background observed following the analysis of the GFP expressing plants (see Fig. 5A) with the anti-6-His monoclonal it was not possible to utilise this antibody to detect the E2 protein (data not shown).

4.2.3 Production of BPV in transgenic plants

In general, the frequency of plant lines testing positive for the presence of the BPV VP2 transgene was reduced compared to the E2 transgene (Fig. 6 and Fig. 8). The reasons for this difference are not known at this time.

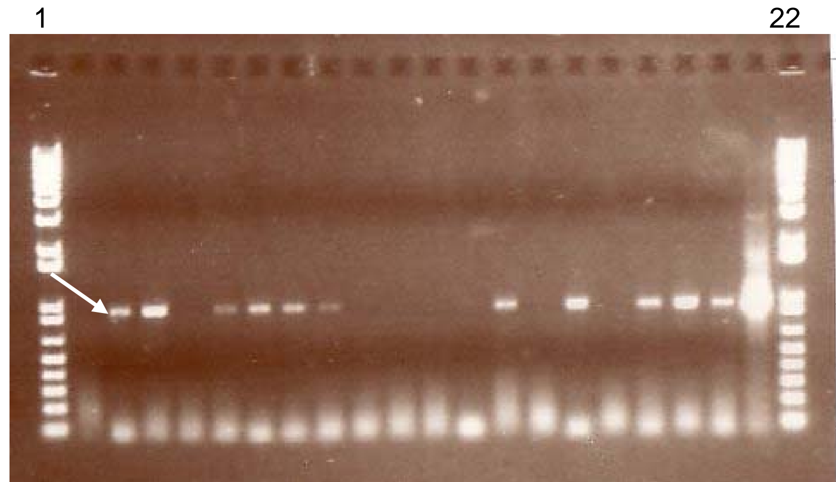

Figure 8: Amplification of the bovine parvovirus VP2 gene from genomic DNA extracted from transgenic tobacco plants . The arrow indicates the expected amplicon.
Lane 1: Molecular weight marker; Lane 2: No cDNA control; Lane 3: Tobacco Line BPV6; Lane 4: Tobacco Line BPV7; Lane 5: Tobacco Line BPV8; Lane 6: Tobacco Line BPV9; Lane 7: Tobacco Line BPV10; Lane 8: Tobacco Line BPV11; Lane 9: Tobacco Line BPV12; Lane 10: Tobacco Line BPV13; Lane 11: Tobacco Line BPV14; Lane 12: Tobacco Line BPV15; Lane 13: Tobacco Line BPV16; Lane 14: Tobacco Line BPV17; Lane 15: Tobacco Line BPV18; Lane 16: Tobacco Line BPV19; Lane 17: Tobacco Line BPV20; Lane 18: Tobacco Line BPV21; Lane 19: Tobacco Line BPV22; Lane 20: Tobacco Line BPV23; Lane 21: BPV VP2 control DNA; Lane 22: Molecular weight marker.

Following confirmation of the presence of the BPV transgene, the level mRNA was assessed using 3'-RACE amplification of positive plant-lines. As illustrated by Figure 9 all of the lines tested were positive for the BPV transcript, though similar to the results of the genomic amplifications, the level of transcript was variable between these lines. Significant non-specific amplification was also evident in these assays which may have reduced the overall amplification efficiency of the VP2 transcript (Fig. 9).

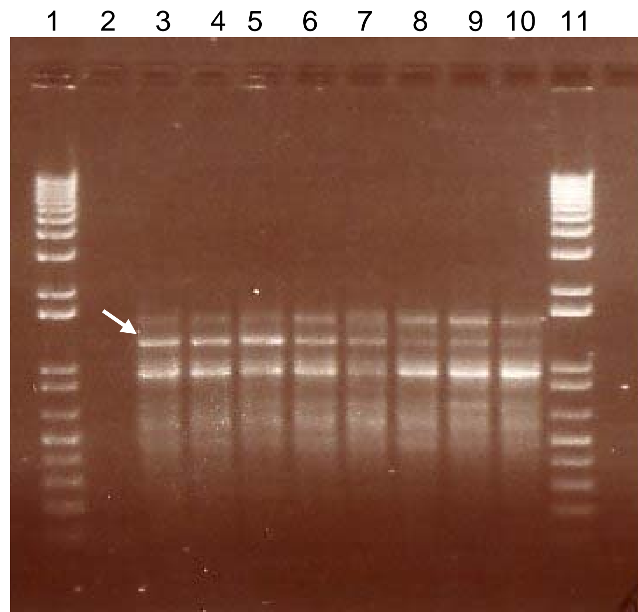

Figure 9: Amplification of the gene for bovine parvovirus VP2 cDNA using 3'-RACE from transgenic tobacco. The arrow indicates the amplicon at the expected size.
Lane 1: Molecular weight marker; Lane 2: No cDNA control; Lane 3: Tobacco Line BPV3; Lane 4: Tobacco Line BPV4; Lane 6: Tobacco Line BPV6; Lane 6: Tobacco Line BPV10; Lane 7: Tobacco Line BPV14; Lane 8: Tobacco Line BPV16; Lane 9: Tobacco Line BPV18; Lane 10: Tobacco Line BPV23; Lane 11: Molecular weight marker.

The detection of the BPV VP2 transcript in these transgenic plants provided high confidence that the plants would also contained the VP2 antigen. Attempts to detect the presence of the protein using a anti-6-His monoclonal antibody did not identify a protein band that could be conclusively identified as BPV VP2 (data not shown).

4.3 Animal Experiments

4.3.1 Experiment 1 – Assessment of oropharyngeal tissue as a delivery route

The first animal experiment was conducted to determine if exposure of a model antigen to the oropharyngeal tissue of sheep was sufficient to elicit a specific immunological response. The antigen utilised in this experiment was the BPV VLP expressed in the baculovirus expression system.

Two control groups were used. Group 1, the positive control, were vaccinated intramuscularly with BVDV E2 (with adjuvant) on three occasions to demonstrate the immunological competence of the sheep in response to baculovirus-expressed antigens. The negative control group, Group 2, received no vaccination and enabled evaluation of seroconversion of the trial sheep due to infection with an antigenically related virus as opposed to seroconversion as a result of the vaccinations. No evidence was found to indicate that a virus, antigenically related to BPV, infected Group 2 during the course of this experiment. Therefore all detected sero-conversions were attributed to the delivered antigens.

To demonstrate that the baculovirus expression system was able to produce immunogenic antigens for sheep, two sheep were injected intramuscularly with the control antigen BVDV E2. Using western blot analysis, it was shown that both of the immunised sheep had developed antibodies for the E2 antigen (Fig. 10). There were clearly apparent specific antibodies for E2 in the serum collected from Sheep-804 (Fig. 10). The second animal, Sheep-799, also developed specific antibodies to E2 though the reaction was not as strong in comparison to Sheep-804. There was no evidence of E2 specific IgA antibodies in the nasal swabs collected from either animal.

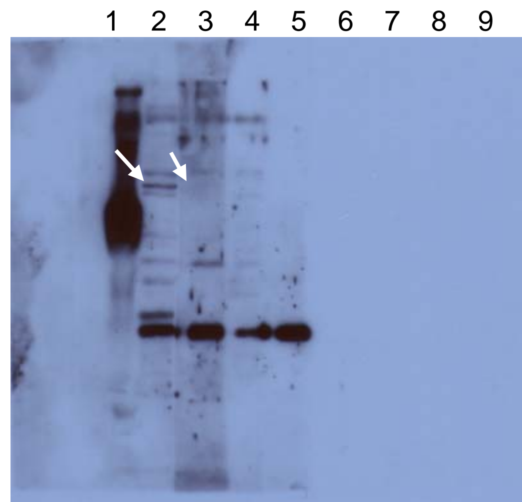

Figure 10: Western blot analysis of sera and nasal swabs samples were collected from sheep vaccinated intramuscularly with BVDV E2 (with adjuvant) for IgG and IgA respectively. Lane 1: Molecular Weight Markers; Lane 2: Sheep 804 Immune serum; Lane 3: Sheep 799 Immune serum; Lane 4: Sheep 804 pre-immune serum; Lane 5: Sheep 799 pre-immune serum; Lane 6: Sheep 804 Nasal Swab pre-vaccine; Lane 7: Sheep 804 Nasal Swab post-vaccine; Lane 8: Sheep 799 Nasal Swab pre-vaccine; Lane 9: Sheep 799 Nasal Swab post-vaccine. Arrows indicate bands of the expected size for BVDV E2.

The results of the immunisations with the BPV VLP were more equivocal than those for the BVDV E2 due to high background binding of the sheep sera (Fig. 11). Of the three groups vaccinated using alternative routes, one of the two animals developed parvovirus-specific antibodies when compared to pre-immune serum of the same animal (Fig. 11).

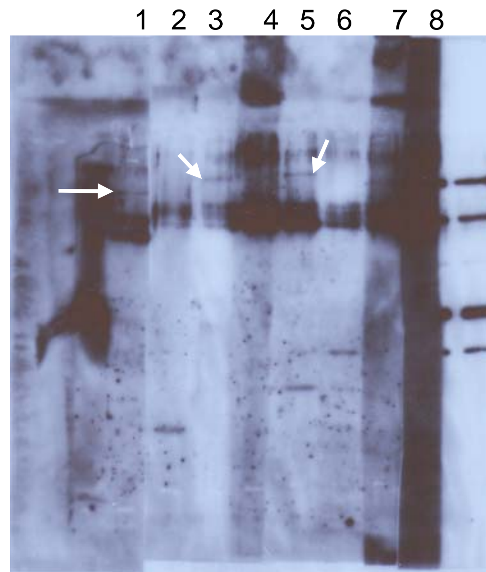

Figure 11: Western blot analyses of sheep sera following vaccination with parvovirus VLP via three different routes. Arrows denote VP2 protein specifically-recognised sheep serum corresponding to the expected size of BPV VP2 in lanes 1, 3 and 5. Routes of immunisation were: Injection subcutaneously (Lane 1 & 2); Injection into oropharyngeal tissues (Lane 3 & 4); Rubbed onto tissues (Lanes 5 & 6); No vaccination (Lane 7 & 8).

Table 3 summarises the results illustrated in Fig. 10 and Fig. 11. The development of specific anti-E2 antibodies in both animals immunised intramuscularly with adjuvant was not surprising as this is a well-proven delivery route. Interestingly, the parvovirus VLP were immunogenic in the absence of adjuvant, independent of the route of immunisation. This was an important finding in the context of this study as one of the primary objectives was to demonstrate that VLP are self-adjuvanting i.e. they were able to stimulate the immune system in the absence of adjuvant. This is an essential element of any oral vaccination strategy, where the incorporation of an adjuvant may not always be economically feasible for livestock industries. Another important finding was that the oropharyngeal tissue appeared to be immunologically active regardless of the route of immunisation (either injection or contact). These results indicate that targeting of the oropharyngeal tissue may increase the likelihood of successfully applying oral vaccination against a broad range of pathogens. This is because the antigens do not have to survive passage through the digestive system until they reach the small intestine, which is a requirement of most oral vaccination strategies.

It is not clear why only one of the two animals developed specific immunological responses to the parvovirus VLP regardless of the route of administration (Table 3). One possible explanation is the amount of antigen delivered was low, thus the individual responses to the VLP may have been dependent on the genetics of the individual animals. Immunisation with higher amounts of VLP could have increased the proportion of animals developing specific antibodies. This was not feasible in this experiment due to the time consuming process required for purification of the VLP. Increasing the number of immunisations might also increase the likelihood of animals developing specific antibodies. It is also possible that those animals that were immunised all developed specific antibody responses that were below the detection limits of western blotting. Other more sensitive

test were investigated but not used. Enzyme-linked immunoassay (ELISA) is perhaps the most sensitive test available for measuring specific antibodies. It was not used in this study due to high background with the sheep sera which reduced the specificity of the test to unacceptable levels (data not shown). The use of the serum samples to neutralise bovine parvovirus growth in cells was also investigate. However parvoviruses are very difficult to grow *in vitro* and as a result it was not possible to employ this test either.

Despite only one animal in each treatment group developing VLP-specific antibodies, it is highly improbably that the antibodies would develop by chance as none of the unimmunised animals developed antibodies, indicating there was no natural infection of the animals in this experiment by an immunologically related pathogen. Further, none of the serum samples collected from all animals prior to immunisation contained parvovirus-specific antibodies. These factors led us to conclude that the immunological responses detected in this experiment were a result of the immunisations.

Table 3: Summary of the immunological responses of the animals vaccinated in Experiment 1

Antigen	Route	Adjuvant	Antibody positive
E2	**Intramuscular**	**Yes**	**2/2[2]**
Parvovirus VLP	**Subcutaneously**	**No**	**1/2[2]**
Parvovirus VLP	**Injected into oropharyngeal tissue**	**No**	**1/2[2]**
Parvovirus VLP	**Rubbed onto oropharyngeal tissue**	**No**	**1/2[2]**
Nil[1]	**Not applicable**	**No**	**0/2[2]**

[1]Control animals used to determine if any infectious agent that was serologically related to bovine parvovirus infected animals during the course of the experiment.
[2]Number of animals showing a detectable antigen-specific response compared to the total number of animals within the treatment group.

4.3.2 Experiment 2 – Oral vaccination with transgenic tobacco

Following confirmation that the BPV VLP was immunogenic in the absence of adjuvant, a feeding trial was conducted using transgenic tobacco. As mentioned previously, one of the most important factors in the successful application of oral vaccination is likely to be striking a balance between antigen availability and antigen destruction by the ruminant digestive system. With this in mind the production of antigen components in plants may aid antigen survival and availability to the immune system due to the difficulties in digestion of plant material.

Two mechanisms are available to increase antigen survival and availability:

1. Limiting the amount of processing of the antigen-carrying plant material, thus as the plant material is digested the antigen becomes available for immune recognition over a longer period.
2. The use of antigenic structures that are naturally resistant to digestion which in this study are parvovirus VLP.

With these factors in mind, a feeding trial was conducted with BPV VLP transgenic tobacco material that was subjected to two levels of processing. The first treatment was freeze-drying followed by grinding of the resulting plant material into fine particles. The second treatment was similar to the first, except the plant material was frozen in liquid nitrogen and ground into a fine paste with a mortar

and pestle in the presence of liquid nitrogen. Laboratory experiments indicated that much higher yields of protein were obtained when plant material was ground in liquid nitrogen indicating that the plant cells were ruptured more efficiently (data not shown).

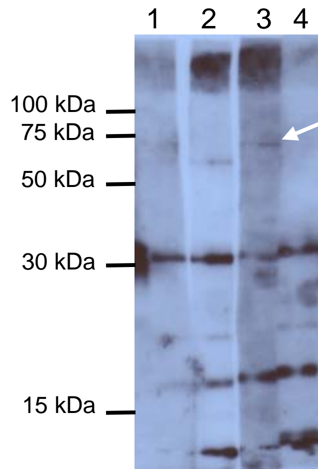

Figure 12: Assessment of the immunological response of sheep feed transgenic plant material expressing bovine parvovirus virus-like-particles. Two sheep were fed freeze-dried tobacco that had been ground in to fine particles (Lanes 1 & 2); Two sheep were fed freeze-dried tobacco that had been ground in to fine particles in liquid nitrogen (Lane 3 & 4). The arrow (white) indicates a protein band recognised by serum from Sheep 858 that corresponds to the anticipated molecular weight of bovine parvovirus VP2 (74 kDa).

Sheep were fed three doses of plant slurry at three week intervals and sera were collected three weeks after the final dose. Western blot analysis indicated that serum from 1 of the 2 animals that were fed material ground in the presence of liquid nitrogen contained antibodies that specifically recognised a band corresponding to BPV VP2 (Fig. 12). No VP2-specific antibodies could be detected in animals fed material that was ground up in the absence of liquid nitrogen (Fig. 12). Importantly, no VP2-specific antibodies could be detected in the serum from any of the sera samples collected prior to immunisation. Similarly, no VP2-specific antibodies could be detected in the 2 negative control animals (same control animals as Experiment 1). A summary of the immunological responses of this experiment are illustrated in Table 4.

Table 4: Summary of the immunological responses of the animals vaccinated in Experiment 2

Antigen	Route	Adjuvant	Antibody positive
Parvovirus VLP	**Freeze-dried plant material/ground into particles**	**No**	**0/2[2]**
Parvovirus VLP	**Freeze-dried plant material/ground into particles in liquid nitrogen**	**No**	**1/2[2]**

Nil[1]	Not applicable	Not applicable	0/2[2]

[1]Control animals used to determine if any infectious agent that was serologically related to bovine parvovirus infected animals during the course of the experiment.
[2]Number of animals showing a detectable antigen-specific response compared to the total number of animals within the treatment group.

The results of this experiment were encouraging as a specific immunological response was detected in one of the four animals fed transgenic plant material. Interestingly, it appears that processing of transgenic plant material containing the antigen of interest may play a critical role in eliciting antigen-specific immunological responses. In this experiment a specific response was only detected when the plant material was ground up in liquid nitrogen which presumably aided the release of the BPV VLP from the plant cells thus increasing the availability of the antigen for immunological recognition.

The low amount of antigen present in the transgenic plants may also have contributed to the low rate of immune recognition. For example, if there was a higher concentration of antigen in the material fed to sheep, more antigen may have been released even if the plant material was inefficiently digested.

Despite the promising, but preliminary, results presented care must be taken not to over-interpret these results due to the low level of sero-conversion detected. The results however, did warrant further investigation. The rate of sero-conversion may be improved through the following strategies:

1. Increased antigen loading – either with plants expressing higher levels of antigen, or increasing the dose.
2. Increased number of doses may also promote the rate of immunological recognition

4.3.3 Experiment 3 – Assessment of prime/boost strategies

Two prime/boost strategies were evaluated to assess if oral delivery of antigen could be utilised as supplementary strategy for the vaccination of ruminants. Utilising this type of strategy would allow animals to be vaccinated using a standard technology (prime), such as injection or intranasal, when they arrive at a property. The correct priming is important, as the level of antigen delivered can be effectively controlled to ensure immune recognition. Subsequent immunisations (boost), where the antigen level is not as crucial for immune responsiveness, could then be delivered using transgenic plant material where the dose cannot be so effectively controlled. The premise of successful prime/boost strategies is that the initial immunisation “alerts” the immune system to the antigen. The subsequent immunisations reinforce the immune response to that antigen, but require less antigen to do so.

Two prime/boost strategies were investigated in this experiment:
1. Prime/boost strategy A: Vaccinated animals received, one intranasal dose on day 1, one subcutaneous injection on day 21 followed by an oral vaccination on day 42.
2. Prime-Boost strategy B: Animals were vaccinated with two subcutaneous injections on days 1 and 21 followed by an oral vaccination on day 42.

Two non-injectable vaccination strategies were also investigated in this experiment
1. Non-injectable strategy A: Three vaccinations administered oral
2. Non-injectable strategy B: Three vaccinations administered intra nasally

Two control groups were also included in this experiment:
1. Negative Control Group A: No vaccinations.
2. Positive Control Group B: Animals were vaccinated with BPV VLP injected subcutaneous with adjuvant.

Two weeks after the final vaccinations serum samples were collected from all animals and analysed for the presence of VLP-specific antibodies using western blotting. Figure 13 illustrated the immunological responses of the animals in this experiment to the BVP VLP. The only animals that generated measurable anti-VLP antibodies were those from Control Group B, i.e. three subcutaneous injections.

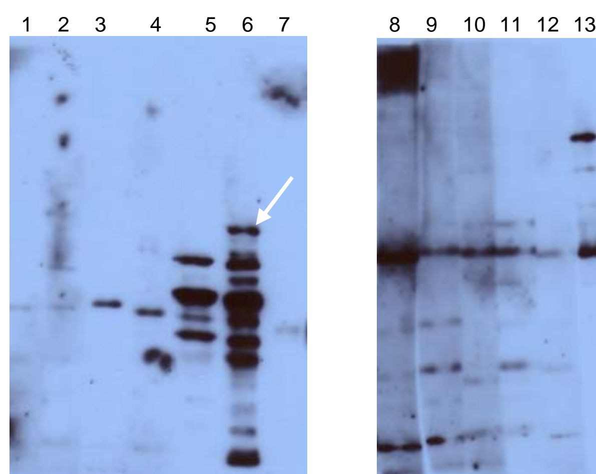

Figure 13: Western blot analysis from sheep sera vaccinated using different strategies. A single dose regardless of route of administration was 50μg of VLP expressed in *E. coli*. **Prime-Boost strategy A**: Vaccinated animals received, one intranasal dose on day 1, one subcutaneous injection on day 21 followed by an oral vaccination on day 42. Sera were collected two weeks after the final vaccination from all animals. **Prime-Boost strategy B**: Vaccinated animals received two subcutaneous injections on days 1 and 21, followed by an oral vaccination on day 42. Sera were collected two weeks after the final vaccination from all animals. **Control Group A**: Animals were not vaccinated; **Control Group B**: Animals were vaccinated subcutaneously on days 1, 21, and 41. Sera were collected two weeks after the final vaccination from all animals.
Lane 1: Control Animal 857; Lane 2: Control Animal 853; Lane 3: Prime/boost strategy A Animal 862; Lane 4: Prime/boost strategy A Animal 871; Lane 5: VLP injected subcutaneous with adjuvant Animal 872; Lane 6: VLP injected subcutaneous with adjuvant Animal 863; Lane 7: VLP delivered intranasal (3 doses) Animal 864; Lane 8: VLP delivered orally (3 doses) Animal 873; Lane 9: VLP delivered orally (3 doses) Animal 855; Lane 10: Prime/boost strategy B Animal 854; Lane 11: Prime/boost strategy B Animal 860; Lane 12: VLP delivered intranasal (3 doses) no number; Lane 13: Control antigen.

Table 5 summarises the immunological responses of the animals vaccinated in this experiment. A possible explanation to why only the two animals injected with VLP responded to immunisation antigen is that the antigen was produced in a bacterial expression system. As discussed previously we were able to demonstrate that expressed BPV VP2 protein self-assembles in to VLP (Section 4.1.2, Fig. 2). The size of the VLP produced in insects cells were of a similar size to native BPV,

however electron microscopy of VLP derived from VP2 expressed in bacterial cells were larger and less regular in shape by comparison. The variation observed for the bacterial VLP indicates that these structures may not have assembled correctly. As a result the bacterial VLP was likely to be less stable compared to the insect cell derived VLP. This increased instability could have reduced the immunogenic potential of these structures when administered via the different routes during this experiment.

The eliciting of specific anti-VLP responses in Control Group B animals (Table 5), immunised with VLP with adjuvant subcutaneously, mimics the generation of anti-E2 antibodies in Experiment 1. In this case the VLP functions as a standard antigen where the co-delivery of the adjuvant plays a critical role in stimulating the required immune responses. However, when the VLP is administered in the absence of adjuvant it is required to be self-adjuvanting, a property that is strongly dependent on the overall structure of the VLP. Thus it would appear that the bacterially expressed VLP was less stable compared to the insect derived VLP and would have broken down quickly *in vivo* and be less likely to stimulate specific immunological responses.

Table 5: Summary of the immunological responses of the animals vaccinated in Experiment 3

Group	Route	Adjuvant	Antibody positive
Prime/Boost Strategy A	**Intranasal/subcutaneous/oral**	**No**	**0/2[2]**
Prime/Boost Strategy B	**Subcutaneous/subcutaneous/oral**	**No**	**0/2[2]**
Non-injectable A	**Oral (X3)**	**No**	**0/2[2]**
Non-injectable B	**Intranasal (X3)**	**No**	**0/2[2]**
Control Group A[1]	**N/A**	**N/A**	**0/2[2]**
Control Group B	**Subcutaneous (X3)**	**Yes**	**0/2[2]**

[1]Control animals used to determine if any infectious agent that was serologically related to bovine parvovirus infected animals during the course of the experiment.
[2]Number of animals showing a detectable antigen-specific response compared to the total number of animals within the treatment group.

Poor stability of the bacterial derived VLP would also explain the lack of specific responses in the two non-injectable strategies tested in this experiment. As discussed in previous sections the correct structure of the VLP is essential for the particle to survive the digestive system. Incorrectly formed VLP may expose the VP2 protein to protease digestion reducing the likelihood of stimulating specific immune responses at these mucosal surfaces.

4.3.4 Experiment 4 – Validation of oral vaccine

The aim of Experiment 4 was to confirm that transgenic plant material expressing a BPV VLP was able to elicit specific immunological responses when delivered orally.

In Experiment 2 (Section 4.3.2) we were able to demonstrate that one of two animals vaccinated with transgenic plant material containing the bovine parvovirus VLP developed a specific immunological response. The purpose of Experiment 4 was to confirm this positive result in a larger group of animals. Unfortunately, we were not able to confirm this result as we did not detect antigen-specific immunological responses in any of the six sheep that were fed the transgenic plant material.

In comparison to the previous experiment, we actually increased the number of doses from three to four in order to increase the likelihood of generating an immunological response. However this did not affect detectable antibody levels. We believe that the primary reason for there being no measurable responses was due to the low level of antigen (VLP) present in the plant material. As previously discussed the low level of antigen may be reduce the likelihood of the immune system mounting an antigen-specific response, due to the differences in genetics within a population. If we were able to increase the level of antigen expression in the plants, the animals might have generated detectable responses. Also the low amount of antigen may have resulted in the induction of a “tolerance response” which is a mechanism used by the immune system to prevent the generation of immunological responses to potential antigens in food stuffs. Preventing the induction of tolerance is thought be a critical step in successful oral vaccination strategies.

While this final experiment was not successful, overall we consider the project to have significantly advanced the development of oral vaccination strategies for ruminants.

5 Success in Achieving Objectives

5.1 Demonstrate production of vaccine components in plants

We were able to demonstrate the production of antigens (potential vaccine components) in two plant species. We confirmed expression of the bovine pestivirus, bovine enterovirus and bovine parvovirus genes in tobacco, and bovine pestivirus and bovine parvovirus in lucerne plants. Interestingly, the bovine parvovirus was readily expressed in all of the systems tested. We also investigated the expression of the leukotoxin gene of the bacterial pathogen *Mannheimia haemolytica*. Expression of this gene was not tested in transgenic plants as it was only expressed in mammalian cells when fused to green fluorescent protein.

The achievement of this objective together with further development could provide vaccines to red meat industries that are:

- Cost-effective to produce,
- Cost-effective to deliver in difficult areas,
- Eliminate cold-chain storage,
- Eliminate inclusion of adventitious agents (such as prions and noncytopathic viruses),
- Eliminate lesions at injection site.

5.2 Demonstrate immunological response to oral delivered antigen(s) in a model ruminant system

We were able to demonstrate an immunological response to an oral delivered antigen (bovine parvovirus virus-like-particles) in sheep. In an initial experiment one of the two sheep that were fed transgenic plant material (ground in liquid nitrogen) developed specific antibodies to the antigen. While it might be expected that both of these animals should have responded, it is possible that low antigen

levels in the plant material may have contributed to this variation between animals. In addition, at low levels of antigen exposure variability in immunological responses may become a more determining factor in the development of a measurable immunological response.

The detection of a positive immunological response in one sheep that was fed transgenic plant material expressing the parvovirus VLP is, to our knowledge, a world first. To confirm the results of this preliminary study a second trial was conducted that used a larger number of animals in the vaccinated group (six) and also the amount of antigen that was delivered. No immunological responses were detected in this larger group. The reasons for this are not readily apparent but may be due to one or more of the following reasons:

a) The level of the parvovirus antigen being produced was low and could not be accurately assessed at the protein level. As a result of this the amount of antigen delivered was suboptimal.
b) Variation in individual responsiveness to vaccination due to genetics. It is possible that with low antigen loads that the genetics of a population may be important, i.e. whether the immune system responds to immunisation or not. Increasing the amount of antigen should help to alleviate this.

5.3 Develop non-injectable vaccination strategies for ruminants

We have established the legitimacy of non-injectable vaccination strategy for ruminants. The demonstration of the immunogenic property of parvovirus VLP when delivered orally to sheep provides a basis for oral vaccination in ruminants. Further work is required to move from these proof-of-principle studies conducted as part of this project to the application of this technology in a disease model.

6 Impact on Meat and Livestock Industry – now & in five years time

The outcomes of this project will not impact on Meat and Livestock Industries within the next five years. The primary objectives of this project were to explore the development of new vaccine production and delivery technologies for red meat producers. The project has been successful in demonstrating that plants can be used as a production system for vaccine formulations in ruminants.

Additional work is required before direct impacts could be achieved for these industries. This includes the application of the investigated technologies in an economically important disease model.

7 Conclusions and Recommendations

7.1 General Conclusions

In the relatively short time frame of this project we have been able to make significant progress towards the development of a non-injectable vaccine technology for application to red-meat industries. Future studies are required to advance the technologies developed within this project for use in red-meat industries.

Due to time constraints, we were unable to fully optimise the genes used in this study to maximise their expression in plants. Other studies have shown that when expressing genes in heterologous

systems alteration of the codon bias and removal of undesirable genetic signals can significantly improve gene expression (e.g. Schmitt *et al*., 1999; Ashraf *et al*., 2005; Kheyar *et al*., 2005). Unfortunately, at this time there are no specific guides to enable maximal gene expression in any given system, therefore the requirements of a particular antigen gene must be determined empirically on case-by-case basis, which was beyond the scope of this study. Increasing antigen expression, however, is likely to improve immunological recognition and subsequent responses generated in vaccinated animals.

One issue with oral antigen delivery is that there is a risk that the immune system will not receive significant stimulation leading to tolerance, which occurs for many foreign molecules in food. If the immune system is stimulated repeatedly by high doses of a protein it is more likely to mount an immune response. The use of VLP technology also decreases the likelihood of tolerance occurring, as the physical nature of the VLP increases immunogenicity. Despite this, it is still considered likely that a stronger immune response would have been detected in this study if antigen (VLP) concentrations were higher.

In addition, transient expression systems may have also yielded higher expression of the genes of interest, which may have provided improved immunological potency during *in vivo* studies. An example of this would be to utilise a plant viral vector to infect plants (McCormick *et al*., 2006). It is likely that this would result in high levels of antigen expression in a transient manner. There would be considerable regulatory issues regarding the testing of this type of plant material *in vivo*. It may also prove more difficult to commercialise these types of vaccines in the current regulatory environment.

At the commencement of this project the debate of the use of genetically modified (GM) crops, including for use as vaccine factories, was being hotly debated. At this time the use of standard crops was considered desirable as no new technology would be required for planting, harvesting and processing. For example, lucerne was selected as it is a known food source for cattle and sheep and is widely grown in Australia. Since that time however there has been a shift in this debate. One of the key factors that has emerged in the GM debate is “segregation”, in other words the use of GM crops has become more acceptable if it is possible to distinguish between GM and non-GM. A major driving factor for this has been providing consumers with a choice between GM and non-GM food, or in the case of this study it might be meat from cattle vaccinated with a GM vaccine or vaccinated with a traditional vaccine. In this context, tobacco or another non-feed crop may actually be the crop of choice for the production of livestock vaccines as it would prevent non-GM lucerne crops becoming contaminated with GM varieties.

In addition, tobacco is not a particularly palatable crop hence it is unlikely to be consumed by accident. While use of tobacco may require additional processing which would add to the cost of the end vaccine, it is still likely any vaccine would be competitively priced compared to traditional vaccines.

Perhaps the most significant development in application of plants for the production of vaccines has been the registration of the first animal vaccine produced in plants in the USA in early 2006 by Dow AgroSciences (http://www.dowagro.com/newsroom/corporatenews/2006/20060131b.htm). The vaccine was made for the prevention of Newcastle disease in poultry and is made in plant-cell cultures, purified and delivered through injection.

7.2 Future Studies

One of the most exciting discoveries of this project was the demonstration that bovine parvovirus VLP were able to stimulate an immunological response when administered orally to sheep. The logical progression of this study is to generate hybrid VLP where the neutralising epitopes of an economically important pathogen are fused to the parvovirus VP2 protein. When used for the purposes of vaccination the hybrid VLP should stimulate an immunological response to both parvovirus and the other epitopes of the pathogen. The generation of this hybrid VLP, however, is not likely to be a trivial exercise as it is unlikely that all of the VP2 molecules that form the VLP would be able to carry the epitope without causing structural defects in the VLP. These defects could render the VLP either highly unstable and/or poorly immunogenic. Thus, the most likely approach would be to have a VLP made up from VP2 molecules both with and without the additional epitope. The exact ratio of these molecules could require empirical determination on a case-by-case basis.

8 Bibliography

Arntzen, C., Plotkin, S., Dodet, B. (2005) Plant-derived Vaccines and Antibodies: Potential and Limitations. Vaccine, **23**:1753-1756.

Ashraf, S., Singh, P.K., Yadav, D.K., Shahnawaz, M., Mishra, S., Sawant, S.V. and Tuli, R. (2005) High level expression of surface glycoprotein of rabies virus in tobacco leaves and its immunoprotective activity in mice. J. Biotechnol. **119**:1-14.

Dus Santos, M.J. and Wigdorovitz, A. (2005) Transgenic plants for the production of veterinary vaccines. Immunol. Cell Biol. **83**:229-238.

Kheyar, A., Jabrane, A., Zhu, C., Cleroux, P., Massie, B., Dea, S. and Gagnon, C.A. (2005) Alternative codon usage of PRRS virus ORF5 gene increases eucaryotic expression of GP(5) glycoprotein and improves immune response in challenged pigs. Vaccine. **23**:4016-22.

Howard, J.A. (2004) Commercialization of plant-based vaccines from research and development to manufacturing. Anim. Health Res. Rev. **5**:243-245.

Lamphear, B.J., Jilka, J.M., Kesl, L., Welter, M., Howard, J.A. and Streatfield, S.J. (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. Vaccine. **22**:2420-2424.

Legocki, A.B., Miedzinska, K., Czaplinska, M., Plucieniczak, A. and Wedrychowicz, H. (2005) Immunoprotective properties of transgenic plants expressing E2 glycoprotein from CSFV and cysteine protease from *Fasciola hepatica*. Vaccine. **23**:1844-1846.

McCarthy, F.M., Smith, G.A. and Mattick, J.S. (1999) Molecular characterisation of Australian bovine enteroviruses. Vet. Microbiol. **68**:71-81.

McCormick, A.A., Corbo, T.A., Wykoff-Clary, S., Nguyen, L.V., Smith, M.L., Palmer, K.E. and Pogue, G.P. (2006) TMV-peptide fusion vaccines induce cell-mediated immune responses and tumor protection in two murine models. Vaccine **24**:6414-6423.

Richter, L.J., Thanavala, Y., Arntzen, C.J. and Mason, H.S. (2000) Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nature Biotech. **18**:1167-1171.

Rigano, M.M., Sala, F., Arntzen, C.J. and Walmsley, A.M. (2003) Targeting of plant-derived vaccine antigens to immunoresponsive mucosal sites. Vaccine. **21**:809-811.

Schmitt, J., Becher, P., Thiel, H.J. and Keil, G.M. (1999) Expression of bovine viral diarrhoea virus glycoprotein E2 by bovine herpesvirus-1 from a synthetic ORF and incorporation of E2 into recombinant virions. J Gen Virol. **80**:2839-2848.

Thanavala, Y., Mahoney, M., Pal, S., Scott, A., Richter, L., Natarajan, N., Goodwin, P., Arntzen, C.J. and Mason, H.S. (2005). Immunogenicity in Humans of An Edible Vaccine for Hepatitis B. PNAS **102**:3378-3382.

Van Donkersgoed, J., Dixon, S., Brand, G. and VanderKop, M. (1997) A survey of injection site lesions in fed cattle in Canada. Can Vet J. **38**:767-72.

Walmsley, A.M. and Arntzen, C.J. (2003) Plant cell factories and mucosal vaccines. Curr. Opin. Biotechnol. **14**:145-150.

Walmsley, A.M. and Arntzen, C.J. (2000) Plants for delivery of edible vaccines. Curr. Opin. Biotechnol. **11**:126-129.