Chapter 15

EDIBLE VACCINES IN PLANTS FOR LIVESTOCK PATHOGENS

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Abstract Edible vaccines derived from plants offer clear advantages to the livestock industry with respect to cost and ease of delivery. From a consumer perspective, the widespread adoption by the industry of efficient, low-cost, edible plant-based vaccines offers the prospect of reduced exposure to animal pathogens in food products, as well as in the water supply. A major obstacle encountered in the initial development of plant-based vaccines has been the very low levels of antigens expressed in transgenic plants. Some of the strategies adopted to meet this obstacle include synthetic forms of the antigen genes, fusion proteins and inducible expression systems. However, even with augmented levels of antigen in plant tissue, a successful edible vaccine in plants will probably require in addition strategies for reducing degradation in the gut and enhancing immunogenicity. It may even be possible to develop DNA-based vaccines in plants.

1. INTRODUCTION

Animal health is a major concern in any livestock operation regardless of species or the size and nature of the operation. The recent outbreak of foot and mouth disease in Europe demonstrates how vulnerable the livestock industries are to pathogens and how costly it can be when existing practices fail. Public

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concern is even more heightened when farm animal health problems become human health problems. The negative impact of "mad cow" disease on public perceptions of the industry, its products and practices is worldwide, deep and probably long-term. There is also increased awareness of the involvement of livestock operations in human illness related to toxigenic *E. coli* and cryptosporidium. The intensification of animal agriculture, characterized by very large operations and crowded, stressful conditions, is seen by many to increase the probability of disease in these animals, as well as in adjacent human populations. Finally, the use of antibiotics by the industry to enhance animal health and productivity is facing mounting criticism for its potential role in developing resistance to antibiotics of significance to human health.

It is apparent to many that if the livestock industries are to survive, increased emphasis on animal health will be necessary. This will involve, among other things, the development of new approaches to enhancing the immunity of animals to pathogens. As many of these pathogens infect the host via mucosal tissues, such as those in the lungs and intestines, strategies for generating a protective mucosal immune response are very attractive. Conventional approaches to vaccines, which utilize attenuated or killed strains of a viral pathogen, for example, will likely continue to be a mainstay of the industry. Problems with such vaccines, such as reversion of attenuated strains to virulence, has prompted development of alternative systems, such as DNA vaccines or those based on adenovirus (Babiuk and Tikoo, 2000). Another option is the development of edible vaccines in plants, to be consumed either directly or in some processed form.

2. ADVANTAGES OF EDIBLE PLANT-BASED VACCINES TO INDUSTRY AND THE CONSUMER

The commercial advantages of plant-based production systems for therapeutic proteins, such as high volume/low cost, ease of scale-up and storage, and absence of animal pathogens, have been well-publicized. However, there are particular advantages to using edible plant material as vaccines for livestock. There is a growing desire to eliminate, where possible, the use of needles for vaccines. Injections are time-consuming, labour-intensive and unpleasant to administer, especially with large animals. Producers also refer to problems in the administration of such injections by their labour force, which is generally

unskilled in veterinary techniques. Such problems are highlighted by reports of broken needles in meat purchased by consumers. Although infrequent, these incidents are widely broadcast in the media, much to the detriment of the industry. Producers and processors also refer to the losses incurred due to lower carcass quality because of needle marks in the meat.

The oral consumption of antigens as a component of plant tissue may have considerable advantages from an efficacy standpoint, as well. The kind of strong mucosal immune response needed for resistance to many of the pathogens which infect animals through such tissues is most effectively generated by presentation of antigens to those same tissues through what is generally referred to as a common mucosal response (McGhee et al. 1999). The generation of such immunity by the presentation to intestinal tissues of large amounts of antigen in edible plant material is a very attractive strategy for achieving this. Crop plants and existing cropping/processing technologies associated with the various crop species offer very cost-efficient systems for production and delivery of large mounts of antigen. The potential advantage of orally consuming an antigen as part of intact plant tissue, rather than in a purified form, was demonstrated by Modelska et al. (1998); in this study, a rabies antigen expressed as part of a plant virus particle induced a much stronger immune response when fed to mice in spinach leaves than when fed as virus particles purified from leaf tissue.

From a consumer perspective, the widespread adoption by the industry of efficient, low-cost, edible plant-based vaccines offers the prospect of reduced exposure to animal pathogens in food products, as well as in the water supply. In addition, there is the benefit of the perception by the public of enhanced animal health and welfare by means of a less intrusive, more "natural" approach of "medicinal plants". Vaccines produced in crop plants and incorporated as a feed component represent a combination of plants as a traditional source of nutrients and modern pharmaceuticals for prevention of disease.

3. OBSTACLES TO THE DEVELOPMENT OF EDIBLE PLANT-BASED VACCINES

3.1 Expression Levels of Antigens in Plants

The first obstacle faced by those attempting to develop this technology is achieving an adequate level of antigen protein in plants. There is a growing list of antigens which have been expressed in plants, and a striking feature of all of the data is the low levels of protein reported. There is still only a handful of antigens expressed in plants for the purpose of developing vaccines for livestock, and levels are either not reported (na) or very low also (Table 18).

Table 18. Expression of antigen genes from livestock pathogens in plants

Pathogen	Gene	Plant Species	Expr. Lev[a] (max)	Biological Activity[b]	Reference
foot and mouth virus	epitope of VP1	cowpea	na	na	Usha et al. 1993
	VP1	arabidopsis	na	I,P(inj mice)	Carrillo et al. 1998
		alfalfa	na	I,P(inj/or mice)	Wigdorovitz et al. 1999
transm. gastroent. virus(swine)	S(spike)	arabidopsis	0.06	I(inj mice)	Gomez et al. 1998
	S(trunc.)	tobacco	0.1	I(inj swine)	Tuboly et al. 2000
	S(trunc. synthetic)	alfalfa, tobacco	0.1	I(inj mice)	Yu et al. 1999
	D epitope of S gene fused to plant prot	alfalfa, tobacco	0.2	I(inj mice)	Bailey, 2000
	S(synthetic)	maize	na	P(or swine)	Jilka(ch. 10, this vol)
porcine resp/reprod virus	ORF5	alfalfa, tobacco	0.2	na	Zhang, 1999
shipping fever (Mann. hemolytica)	Lkt50 (leucotoxin) (trunc/fused to GFP))	white clover	1.0	I(inj rabbits)	Lee et al., 2000

[a] Percent soluble protein. [b]I = immunogenic, P = protective, inj = injected, or = orally delivered.

Although many feel that for commercial purposes the antigen should be present in plant tissue in the 1.0% range of total soluble protein, such a figure is fairly meaningless as to the final efficacy of an oral vaccine produced from such tissue. First, it is very difficult to translate concentrations of an antigen in plant tissue to effective concentrations at the mucosal surface; for example, an antigen at a level of 1% of soluble protein in starting plant material will be present at a much lower level in the total biomass passing through the intestine, even if the diet consists entirely of the plant material containing the vaccine, which may not practical. Ultimately, however, the concentration required in the starting plant tissue is determined by the amount needed at the mucosal surface to induce an optimum immune response, and the amount lost prior to delivery to those surfaces. Antigens vary a great deal in their immunogenicity. The B subunit of the heat labile toxin (LTB) of E coli, for example, is known to be a potent oral immunogen (Haq et al., 1995), and therefore protective immunization may not require the levels of such an antigen that are needed for another antigen. Furthermore, the structure of the antigen protein in plant tissue may affect levels reached, as well as immunogenicity. The formation of viral particles, e.g. Norwalk virus (Mason et al., 1996), or other polymeric structures, such as the pentameric ring of LTB (Haq et al., 1995), may reduce susceptibility to degradation and interfere less with cell structure and functioning than other antigen proteins which may associated freely with other cellular components. Undoubtedly, the formation of stable, inert Bt protein crystals in chloroplasts was a major factor enabling the very high accumulation of this protein in leaf tissue (DeCosa et al., 2001). In addition, the ability to target expression to a specific plant tissue which would allow subsequent processing or concentration, has implications for the levels needed for commercial production. Finally, the amount of antigen needed in the plant tissue is very much dependent on the degree of degradation experienced by that antigen in the digestive tract prior to delivery to the target tissues in the animal.

3.2 Degradation of Antigens in the Gut

The emphasis to date on enhancing expression of antigens in plants for the purpose of edible vaccines reflects the difficulties associated with what is only the first step in developing this technology. It is surprising how little attention has been paid to what may be an even greater obstacle to successful oral immunization - break-down of the antigen proteins in the stomach and intestine. The pH of the stomach of carnivores and omnivores is quite low, and this

acidity, in conjunction with the presence of proteolytic and hydrolytic enzymes, results in the reduction of many food constituents to smaller components for absorption by tissues lining the gut (Sanderson and Walker, 1999).The complexity of the digestive environment, as well as the variability between animals and even within animals over time, make this obstacle difficult to predict reliably and overcome. For example, there are many significant differences between the structure and function of the digestive tract of a young piglet vs that of a lactating sow regarding pH, the presence of proteinases, other digestive enzymes, mucins, antimicrobial peptides and antibodies. Other factors such as diet, microbial populations, housing conditions, use of antibiotics can also have significant impact on gut function, and especially on the break-down and utilization of feed components. Given the variations that can exist between animals in a livestock operation regarding the physiological condition and function of the gastrointestinal tract, devising a feed component and feeding strategy which will effectively vaccinate all animals in that operation has to be considered a major hurdle for the development of this technology.

Perhaps because of the complexity of the variables and interactions involved in gastrointestinal degradation, as well as the difficulties inherent in addressing this obstacle, those working in this field have chosen to attempt feeding trials despite these unknowns and the low levels of antigen present in their plant material. Although the results of some of these experiments are surprisingly positive (Wigdorovitz et al. 1999; Richter et al., 2000;), it must be conceded that we are still some distance from the desired level of protection from pathogens necessary for commercialization. Even if the levels of antigen in plants can be significantly increased over current levels, it is unlikely that simply producing an antigen in plants and feeding the plant material will ultimately succeed without developing strategies for reducing degradation in the gut and for enhancing immunogenicity.

3.3 Immunogenicity of Antigens Produced in Plants and Delivered Orally

The levels of antigen in the starting plant tissue, losses due to length and conditions of storage and processing of plant tissue, and degradation in the gut will determine how much antigen reaches the mucosal surfaces in the animal. However, even if those obstacles are adequately addressed, there is no guarantee of the kind or extent of the immune response in the animal ingesting the antigen. The immunogenicity of an antigen protein in plant tissue will depend on many

factors. First, a protein which is immunogenic as a component of a live, replicating virus, for example, may not be as immunogenic when expressed as a separate protein in plant tissue. The response of the intestinal mucosa to orally ingested antigen may range from absence of a specific immune response, such as when a pathogen is cleared by non-specific innate mechanisms, to more specific B and T cell responses in the mucosal and systemic branches (Strobel and Mowat, 1998). The specific response depends on the nature of the antigen, how it is administered and what cells are involved in uptake.

Generally, antigen entering the body through the intestinal mucosa does so via epithelial cells or M cells (Hershberg and Meyer, 2000; Neutra, 1998). Following entry, the antigen will encounter a range of immune cells, and, depending on how it is processed during internalization, will be taken up by professional antigen presenting cells (Neutra, 1998) or presented directly to T cells (Mayer and Blumberg, 1999). The nature of the immune response will be determined by the specific cytokines produced by the CD4+ helper cells to which the antigen is presented (Kelsall and Strober, 1999). The response may be largely localized to the common mucosal immune system, characterized mainly by secretion of sIgA at immune effector sites (Strobel and Mowat, 1998). Alternately, the primary mucosally-induced response will consist of IgG antibodies in the systemic compartment (Strober et al. 1998). In other cases, the major immune response may be the generation of IgE antibodies, characteristic of allergic reactions (Lorentz et al., 2000). There is currently insufficient understanding of the immune response to orally-administered antigens to enable us to manipulate the process, but clearly, strategies to meet this final challenge are needed to develop edible vaccines in plants. Such strategies will have to address the difference in the presentation to the immune system between an antigen as a component of plant tissue versus an antigen as part of a replicating pathogen during an infection. Another hurdle is delivering the appropriate dosage of an antigen to absorption sites in the digestive tract using current feeding practices in the industry. The importance of this consideration cannot be overlooked in light of the evidence for generating oral tolerance by feeding an antigen expressed in plants (Ma et al., 1997). Finally, plants are known to contain many compounds with immunomodulator properties, which could inhibit a mucosal response to associated compounds in the diet or dominate the immune response by virtue of their strongly immunogenic properties (Mason et al., 1998).

4. STRATEGIES FOR PRODUCTION AND DELIVERY OF EDIBLE VACCINES IN PLANTS

4.1 Expression of Antigens Using Native Gene Constructs

Most antigens expressed to date in plants have been encoded by the native gene, i.e. a cDNA or bacterial clone, modified appropriately for expression in plants by the addition of regulatory and targeting sequences. Almost all of the antigens produced to this time in plants for oral immunization of farm animals are viral in origin (Table 1). Expression levels in plants containing the native cDNA clones of the foot and mouth viral gene VP1 were very low (Carrillo et al. 1998; Wigdorovitz et al. 1999), as was that reported by Gomez et al. (1998) for swine transmissible gastroenteritis virus (TGEV).

The initial work with TGEV in our labs involved two constructs containing the full-length cDNA coding for the spike (S) protein on the surface of the virus particle. Specifically, the full length cDNA clone (4300 bp) was released from the recombinant plasmid pTS (Tuboly et al., 1994) by *Eco*RI digestion, rendered blunt-ended with Klenow fragment, and digested with *Bam*HI. The resulting fragment was cloned into the binary vector pBI121 (Jefferson, 1987) following digestion of the vector with *Sac*I (adjacent to the 5' end of the nos terminator), end-filling with Klenow and digestion with *Bam*HI, creating vector pS1 (Figure 47). In pS3, an enhanced 35S promoter and 35S terminator from pFF19G (Timmermans et al., 1990) were fused to the full-length S gene to which an endoplasmic reticulum targeting sequence had been added. This form of the gene was created by fusing the 3.8 kb *Bam/Xba* fragment from the 5' end of the cDNA to a 0.6 kb *Xba/Pst* fragment containing the remainder of the coding region of the S gene and a SEKDEL tail. This terminal portion of the coding region had been amplified by PCR from the cDNA using a primer starting at nt 3826 containing an *Xba*I site (5'-GCTTCAGATG GTGATCG-3') and a 49 nt primer (5 '-TGCACTGCAG TCATAGCTCA TCTTTCTCAG AATGGAGGTG CACTTTTTC-3'), containing sequentially the end of the coding region of the S gene, the SEKDEL coding region, a stop codon and a *Pst*I site. This construct was transferred to pBI121 which had been digested with *Hind* III/*Eco*RI.

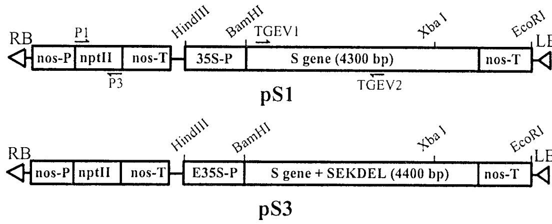

Figure 47. Constructs containing the native cDNA for the S gene. nos-P: nopaline synthase promoter; npt II: neomycinphosphotransferase II; nos-T: nos terminator; 35S-P: CaMV 35S promoter; E35S-P: enhanced CaMV 35S promoter; 35S-T: CaMV 35S terminator. Positions of primers for amplifying the NPT-II gene (P1, P3) and the S gene (TGEV1, TGEV2) are indicated by small arrows parallel to the vector sequence.

The native S gene contains a putative signal peptide-coding region, and such signals of genes from mammals and mammalian viruses have been shown to be correctly recognized in transgenic plants, resulting in targeting to the endoplasmic reticulum (e.g. Khoudi et al. 1999). The above two vectors represent two common and alternate strategies for expression of xenoproteins in plants, i.e. secretion from the cell (pS1) and retention in the endomembrane system (pS3).

Of the approximately 150 tobacco and alfalfa plants containing the full length native cDNA for the S gene controlled by the 35S promoter (pS1), only 3 contained mRNA detectable by Northern blots and this consisted mainly of a faint band of about 1200 nt in size (Figure 48B, lane 2). Addition of an enhanced 35S promoter to this cDNA resulted in higher levels of S mRNA than with the 35S promoter and in a greater proportion of transgenic plants (12 out of 12), but still of the 1200 nt size. Southern blot analysis of these transgenic plants indicated that all plants contained full-length copies of the gene (Figure 48A). A Western blot of protein from plants containing these two constructs did not detect any S protein (Figure 48C, lanes 2,3 and 4).

Analysis of transcripts in transgenic plants by RT-PCR produced bands of expected size for the NPT-II-gene with the use of an internal primer (P3) or oligo-dT primer. However, when RNA extracts from the same plants containing the native cDNA clone of the S gene (vector pS3) were used as the template for reverse transcriptase, a band was produced only with an internal primer (TGEV2) and not with an oligo-dT primer.

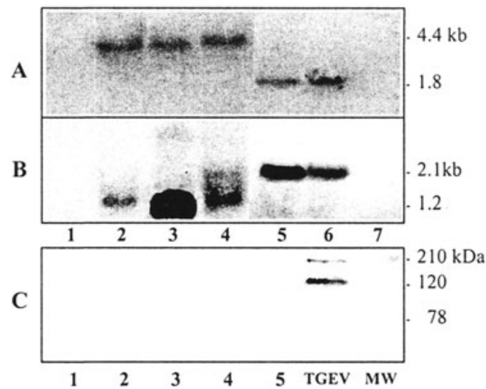

Figure 48. Analysis of transgenic plants containing the S gene by Southern (A), Northern (B) and Western (C) blots. Transgenic tobacco (lanes 2-5) and alfalfa (lane 6) contain the cDNA of the native S gene: pS1 (35S promoter, lane 2), pS2 (pollen-specific promoter, lane 3), pS3 (enhanced 35S promoter, lane 4) and the synthetic S gene, pSS1 (enhanced 35S promoter, lane 5, 6); DNA from plants containing vectors pS1, pS2 and pS3 was digested with BamHI and EcoRI and plant DNA containing the synthetic gene was digested with XbaI and SacI.. Lanes 1 and 7 are the control non-transgenic tobacco and alfalfa, respectively. TGEV: TGEV-infected swine testicle cell lysate; MW: protein molecular weight standards.

Reports of truncated transcripts present as distinct bands on a Northern blot are uncommon in plant literature. Analysis of two short mRNA fragments of 600 and 900 nt in transgenic plants containing a wild-type insecticidal gene from bacteria gene revealed that they were polyadenylated, and probably resulted from premature termination at an upstream cryptic poly(A+)-like signal present in the gene (Diehn et al., 1998). The native S gene contains 8 potential premature polyadenylation signals (Figure 49), five of which are concentrated in the region from nt 838 to 1615, and most of the form AATAAT, reported to be a very strong substitute of the more typical polyadenylation signal AATAAA (Rothnie, 1996). These AT-rich near-upstream-elements, NUEs, occur typically 10 to 30 bases upstream of the polyadenylation site in plants, and for efficient formation of 3' ends are accompanied by TG-rich far-upstream-elements, FUEs, located 40 to 150 bp upstream of the polyadenylation site (Hunt, 1994; Rothnie, 1996). Five FUEs were found in the S gene, but most occur downstream of the main cluster of NUEs (Figure3). However, several less typical NUEs were positioned downstream of some of the these FUEs: AATATA(2085, 2182, 2657,2917), AATAGA (2688), AATACA (2948). Despite the presence and possible effects of premature poly(A+) signals in the S gene, we were unable to

detect polyadenylated S gene mRNA of any size in our extracts using RT-PCR and oligo-dT primers, although full-length polyadenylated mRNA for the NPT-II gene was detected in parallel experiments.

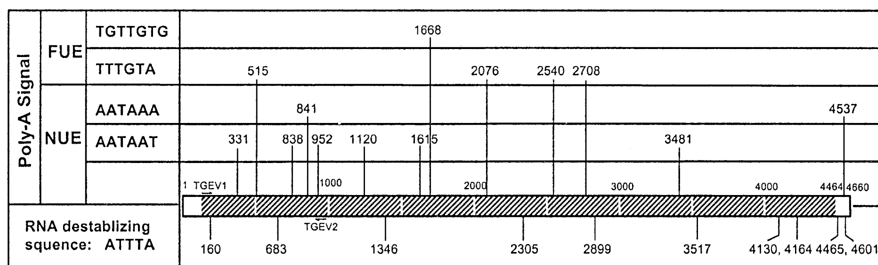

Figure 49. Potential mRNA processing signals in the native S gene. Poly-A signals are divided into near upstream elements (NUEs) and far upstream elements (FUEs). Arrows indicate positions of primers used for PCR analysis: TGEV1(138-155 nt) and TGEV2(977-994 nt). Hatched area represents protein coding region of the gene.

The above data suggest that the mRNA observed from the native S gene is not polyadenylated. There are very few reports with examples of discrete bands of non-polyadenylated truncated transcripts in the plant literature. Truncated mRNA from the soybean small subunit gene SRS4 of Rubisco have been detected by Northern blots by Tanzer and Meagher (1995). Analysis of these fragments demonstrated that all contained the intact 5' end of the transcript and none were polyadenylated. The authors concluded that the fragments resulted from endonuclease digestion of the full-length transcript and that the location of the cleavage site was determined more by secondary structure than specific sequences in the transcript. Such discrete mRNA degradation intermediates are not the normal products of mRNA degradation in plants, nor in the better-characterized systems of mammals and yeast (Gutierrez et al., 1999).

It is possible that the predominant 1200 nt band of S gene mRNA is also the result of endonuclease activity, but unlike the SRS4 fragments, which are derived from the degradation of full-length message, the S mRNA is probably not derived from full-length transcripts, as these could not be detected. We propose rather that the truncated and non-polyadenylated S gene mRNA results from the failure of the elongation stage of transcription. Such failure may involve endonuclease activity, such as that associated with Pol II during

transcriptional arrest (Shilatifard, 1998), as well as subsequent 3' to 5' exonuclease activity presumed to be part of the mRNA degradation pathway for plants (Gutierrez et al., 1999). However, extensive exonuclease activity would likely result in a smear of mRNA which was not evident in the Northern blots (Figure 48B).

There is very little information on the factors affecting transcription elongation in plants. Based on the information available from other eukaryotes such as yeast, there are many genes encoding unique factors which control this process (Shilatifard, 1998; Reines et al., 1999). As truncated transcripts were not evident in plants containing the synthetic S gene, we assume that elongation failure with the native S gene was due to DNA-dependent arrest. In yeast and mammalian systems, there are elongation factors such as SII which assist Pol II in overcoming arrest sites by means of a reiterative process of cleavage and re-extension (Shilatifard, 1998). It may be that the kind and/or degree of pausing at a particular site in the S gene caused destabilization of the elongation complex and subsequent cleavage with no re-extension. In a similar experiment involving the expression of a malarial gene in yeast, Milek et al. (2000) also reported truncated transcripts, but did not mention whether the transcripts were polyadenylated ; when the malarial gene was re-synthesized to optimize codon usage and reduce AT content, only full-length mRNA was detected.

The specific features of the native S gene which might cause transcriptional arrest are unknown, but must include those altered in the synthetic form of the gene (see below), which did not result in truncated transcripts. The first of these is the high AT content (63%) of the native gene, which increases the probability of cryptic mRNA processing sites that are AT rich, such as poly(A+) sites. Aside from the numerous poly(A+) sites in the first half of the gene, there are also in the same region some T-rich sequences which could block elongation by Pol II (Shilatifard, 1998). For example, T occurs in 31 of the 65 nt starting at position 906, in 26 of the 54 nt starting at position 1045, 7 of the 10 nt starting at position 1173, and 31 of the 60 nt starting at position 1385 (Figure 49). The S gene also contains 10 sequences of the form ATTTA, which has been shown to target mRNA for rapid decay when located in multiple copies in the 3' untranslated region of mammalian genes, and has produced similar effects when introduced into plant genes (Ohme-Takagi et al.,1993; Gutierrez et al., 1999); 2 of the 10 copies of this sequence occur in the 3' untranslated region of the S gene (Figure 49).

Given that we could not detect polyadenylated mRNA for the native S gene in any transgenic plants containing that gene, it is not surprising that S protein was not detectable on Western blots with protein extracts from any of these plants, which prompted us to adopt alternative strategies with different gene constructs. It is interesting that expression of the full-length S gene in insect cells employing baculovirus vectors has also been at very low levels (Tuboly et al.1994)

4.2 Synthetic Genes

The very low levels of expression frequently observed for foreign proteins in plants may be due to a variety of factors affecting transcription, translation, and stability of the protein. Some of these factors can be predicted from the DNA sequence, and steps taken to eliminate them. An example of this is the 100-fold increase in expression of a bacterial insecticidal protein gene in maize following modification of the gene to eliminate features such as premature poly A signals, high AT content and unusual codons (Perlak et al., 1991).

In our labs, we adopted a similar strategy for expressing the S gene of TGEV when it became clear that the native form of the gene would not provide the levels of antigen needed for oral immunization. As it was our intention to use alfalfa as our crop production vehicle, codon usage in the synthetic S gene fragment was optimized for dicot species (Ikemura, 1993; Murray et al., 1989), and GC base content was increased to 49% from the original 37%. Possible mRNA destabilizing segments, such as ATTTA, were removed, and unstable codons such as xCG and xTA (Grantham and Perrin, 1986; Murray et al., 1989) were avoided. The translational initiation site was optimized for expression in plants using the sequence ACC<u>ATG</u>G (Koziel et al., 1996). Although non-plant signal sequences appear to be generally recognized in plants, the putative 16-aa signal peptide of the native gene was replaced by a 25-aa tobacco pathogenesis-related protein signal peptide, MNFLKSFPFLOFGQYFVAVTHA, to ensure secretion of the S protein from the cell. This strategy and particular signal sequence has been utilized to enhance the synthesis of foreign proteins in plants (Sijmons et al., 1990; Firek et al., 1993; Verwoerd et al., 1995). The remainder of the coding region of the synthetic gene encoded 579 aa of the N-terminal part of the native mature S protein to which was added a six-histidine tag for purification from plant extracts (Figure 50).

The resulting 1856 bp synthetic S gene DNA was 83% identical to the same coding region in the native S gene cDNA. It should be noted that this synthetic form of the gene codes for much less than the 1447 aa of the entire native protein, but does include the major immunogenic epitopes of the full-length protein.

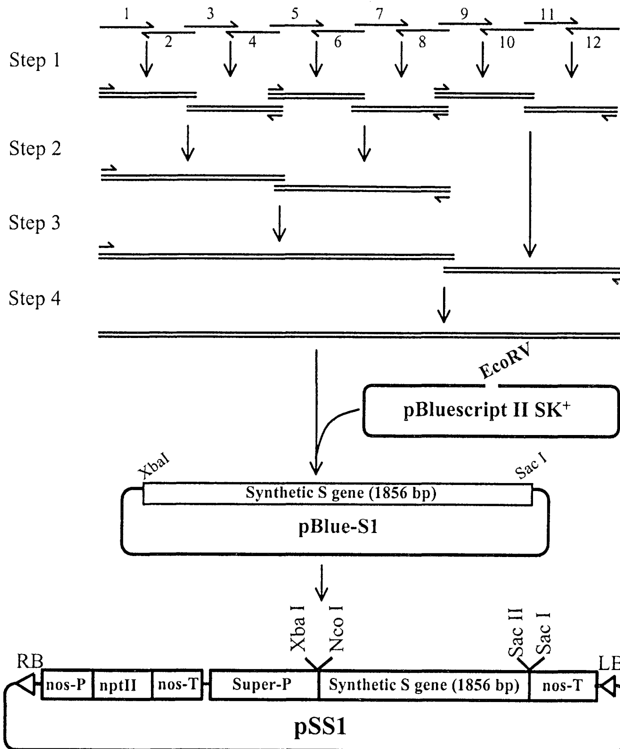

Figure 50. Synthesis of the redesigned 1856 bp S gene by overlap extension PCR and construction of the expression vector pSS1. The starting synthetic oligos for Step 1 are numbered 1-12.

The modified and truncated version of the *S* gene was synthesized by overlap PCR (Ho et al., 1989; Rouwendal et al., 1997), using twelve oligomers, 175-180 nt in length, synthesized by ACGT Corp., Toronto, Canada, and purified by polyacrylamide gel electrophoresis. The oligomers were designed such that six, numbered 1, 3, 5, 7, 9, 11 comprised the sense strand of the designed gene and six, numbered 2, 4, 6, 8, 10, 12, comprised the antisense strand, with the ends of adjacent oligomers overlapping by 23 to 25 nt (Figure

50). Four restriction enzyme sites, *Xba*I and *Nco*I at the 5'-end, *Sac*II and *Sac*I at the 3'-end, were added for cloning into different expression vectors.

Step 1: Six amplification reactions were carried out using pair-wise combinations of the overlapping oligomers: 1+2 (nt 1-323), 3+4 (nt 301-628), 5+6 (nt 605-934), 7+8 (nt 908-1236), 9+10 (nt 1213-1540), and 11+12 (nt 1515-1850). Each reaction (50 μL) contained 0.5 μM of both oligomers, 0.2 mM of dNTP mixture, and 2.5 U of *Pfu* DNA poymerase (Stratagene). The reaction was performed by denaturing the DNA at 94°C for 5 min, followed by 20 cycles of: 94°C for 45 sec, 50°C for 45 sec and 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products of ~ 330 bp each were purified from an agarose gel following electrophoresis.

Step 2: Three overlap PCR reactions were carried out, each containing two contiguous and overlapping products from step 1: bp 1-323 + bp 301-628, bp 605-934 + bp 908-1236, and bp1213-1540 + bp 1515-1850. An initial amplification step consisted of three cycles at 94°C for 3 min, 50°C for 1 min, and 72°C for 8 min. This was followed by the addition of two primers, 18 to 24 nt in length, to a final concentration of 0.5 μM in each reaction. The primers were designed to amplify the entire length of the combined parent fragments in that reaction, i.e. primers P1 and P628c for the first fragment reaction, P605 and P1236c for the middle fragment , and P1213 and P1850c for the end fragment, the number of each primer indicating the position of the nucleotide at the 5' end of the primer. A denaturation step at 94°C for 3 min was followed by 20 cycles of: 94°C for 45 sec, 50°C for 45 sec and 72°C for 1 min, and the reaction was terminated with a step at 72°C for 10 min. The amplification products were separated by gel electrophoresis and the bands of expected size were cut out and purified as templates for further amplifications.

Step 3: The PCR products, bp 1-628 plus bp 605-1236 were combined and amplified as in step 2, using primers P1 and P1236c.

Step 4: The purified 1245 bp PCR fragment from step 3 and 628 bp product from step 2 were coupled, using primers P1 and P1850c to amplify the entire S gene fragment under the same conditions as in steps 2 and 3.

The synthetic *S* gene fragment was purified from an agarose gel and inserted into pBluescript II SK$^+$ at an *Eco*RV site. Seventeen clones were sequenced, and, as all contained one or more sequence errors, the final correct version of the S gene fragment was created by assembly of fragments from these clones and site-directed mutagenesis (Gene Editor, Promega, USA). The binary vector pSS1 was constructed by excising the synthetic S gene from pBluescript using

*Xba*I and *Sac*I and inserting it into pBSN1 (Ni et al., 1995), cut with the same enzymes. The promoter for the synthetic gene in this vector, composed of elements of the mannopine and octopine synthase gene promoters from Agrobacterium, is reported to result in high expression levels in various plant tissues and is frequently referred to as the "super" promoter.

A single mRNA transcript of predicted size was evident in most transgenic tobacco and alfalfa plants carrying the synthetic S gene (Figure 48B). The elimination of the poly(A+) sites and the ATTTA sequences, as well as a reduction of AT (principally T) content from 63% to 51% in the synthetic gene were the major alterations made to the native S gene, and probably account for the presence of full-length transcripts from the synthesized gene. The alterations in codon usage would not likely reduce DNA-dependent arrest of transcript elongation, aside from the effects on AT content.

Most of the S protein detected by immunolocalization in tobacco leaf tissue containing the synthetic S gene was extracellular (data not shown). Interestingly, the labelled protein was located predominantly in the cell wall rather than in the extracellular space, where other foreign proteins have been reported to be primarily deposited when fused to the same PR signal peptide (Sijmons et al., 1990; Firek et al., 1993; Verwoerd et al., 1995). We can only speculate on the features of the S protein which might account for this, but the sequestering of an antigen in the cell wall matrix may provide certain advantages for reducing degradation and enhancing immunogenicity. The levels of S protein in these plants was higher than in those with the native gene to the extent that it was detectable on a Western blot (Figure 48C), reaching a maximum of about 0.01% of total soluble protein. Similar increases in protein levels of xenoproteins in plants following reconstruction of the coding region have been reported for another antigen (Mason et al., 1998) and for the insecticidal Bt protein (Koziel et al., 1993). However, such levels may be inadequate for some applications requiring purification or direct feeding, especially where large amounts of antigen are required and degradation of the foreign protein occurs in the plant tissue/extract (Khoudi et al., 1999) or in the gut.

It is clear from these results that there are as-yet-unexplained features of genes which may limit the synthesis and/or accumulation of transgenic proteins in plants. It is also becoming clear that synthetic genes and codon optimization may not adequately address these features. In contrast to our experience with the S gene of TGEV, we have expressed an unmodified cDNA clone coding for the ORF5 antigen of the porcine respiratory and reproductive syndrome (PRRS)

virus in tobacco and alfalfa at levels considerably in excess of those attained with the synthetic S gene (J. Zhang, 2001). Fortunately, there are many examples from microbial expression systems of alternate or additional strategies which can increase foreign gene expression, such as fusion proteins and inducible promoters.

4.3 Fusion Proteins

The strategy of translational fusions to increase levels of a foreign protein or peptide has not been adopted as widely in plant production systems as in those which are microbially-based. This is due at least in part to the long history of research in microbial systems, particularly for the purpose of producing heterologous proteins. The fusion of antigens to other proteins for expression in plants has taken one of three forms: fusion to a non-plant protein, fusion to a protein of plant origin, and fusion to a protein of a plant virus.

Lee et al. (2001) fused a truncated form of a bacterial leucotoxin to the amino or carboxyl terminus of an ER-targeted green fluorescent protein (GFP) in two separate constructs. Expression of the leucotoxin was detected in clover plants containing the construct in which the antigen was inserted between the signal peptide and the amino terminus of GFP and not in the other construct. The maximum level of the fusion protein reached with this construct containing the 35S promoter was about 1% of soluble protein. Whether such a fusion was necessary to reach such expression levels in plants cannot be determined as no attempt was made to express an unfused form of the protein.

In our lab, we have been experimenting with fusing portions of the S gene, the major antigen of transmissible gastroenteritis virus (TGEV) of swine, to plant proteins to enhance expression. Attempts to express the full-length cDNA clone of this gene using various promoters, including the 35S CaMV promoter did not result in levels of the S protein detectable on Western blots, although antibodies to the viral protein were induced by injecting plant tissue extracts into pigs and mice. As an alternative, portions of the S gene encoding a linear epitope (D) responsible for generating neutralizing antibodies to the virus, were fused to two plant genes truncated at the 3' end.

One of these proteins was a beta amylase to which an extended D epitope of 390 bp was attached (Tuboly et al., 2000) (Figure 51). The other plant gene used as a fusion partner codes for a pollen-specific protein from alfalfa (Qiu et al., 1997). PO2 is a highy glycosylated protein with properties typical of an arabinogalactan, and possibly plays a role in pollen hydration. It has no

homologies to any allergen, enzyme or structural protein and was thought to be a relatively inert protein which might accumulate to high levels when secreted out of the cell. The coding region for the carboxy terminal 21 amino acids of PO2 was replaced with one encoding a nine-amino acid epitope D of TGEV, and an additional nine-amino acid peptide thought to enhance antibody binding (Posthumus et al., 1990); codon usage was optimized in this fragment for expression in dicots.

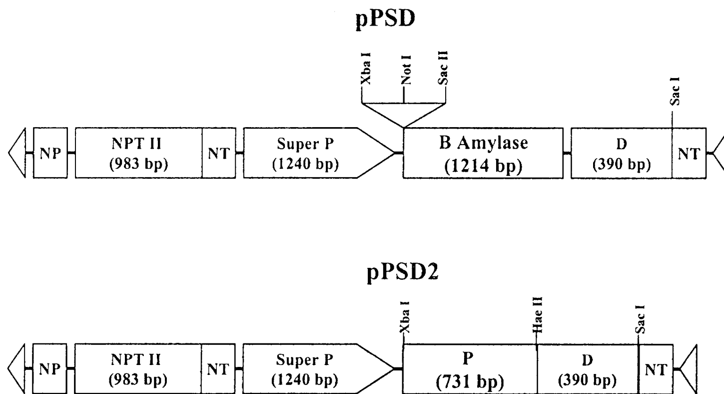

Figure 51. Binary vectors containing a swine viral epitope fused to plant protein genes. NP: nopaline synthase promoter; NPT II: neomycinphosphotransferase II; NT: nos terminator; Super P: mannopine/octopine synthase promoter; D: D epitope region of the S gene; P: truncated PO2 gene from alfalfa.

Both fusion proteins were driven by a chimeric mannopine/octopine synthase promoter (Ni et al., 1995), referred to by some as the "super-promoter". In the case of the fusion to PO2, the fusion product was detected with antibodies to PO2, and the presence of the TGEV epitope was confirmed by the injection of plant extracts into mice, resulting in the production of neutralizing antibodies to the virus (Bailey, 2000). The highest expression levels achieved with this construct was in the 0.2% range in alfalfa plants, but were much lower in tobacco plants. This is in marked contrast to plants containing the full-length cDNA clone for the S antigen of the virus, wherein the S protein could not be detected, and to plants containing a truncated, synthetic version of the S gene in which the S protein constituted only 0.01% of total soluble protein.

Following the initial example of fusing a foot and mouth viral epitope to a protein of a plant virus (Usha et al., 1993), there have been several reports of this approach to expressing viral antigens in plants (rev. by Walmsley and

Arntzen, 2000). Although the particulate structure of antigens produced by this method is very desirable for inducing an immune response, the ability of plant viral particles to survive the gastrointestinal tract is questionable and, as yet untested. However, intranasal administration of such particles has elicited a protective immune response in a mouse model of hepatitis (Koo et al, 1999).

4.4 Inducible Systems

The very low levels of expression frequently observed for foreign proteins in plants may be due to a variety of gene-specific factors which affect transcription, translation, and stability of the protein. Alternatively, the foreign protein may have inhibitory effects on the growth and development of the plant, resulting in the selection of low-expressing plants during regeneration. Mason et al. (1998) enhanced the levels of the B subunit of the E coli heat labile toxin (LTB) in potato shoots and tubers using a synthetic form of the gene driven by the 35S promoter. However, they also noted that as LTB levels increased, rate of shoot growth and tuber yield dropped in plants grown in greenhouse conditions. When LTB synthesis was controlled by a tuber-specific promoter, shoot growth was normal, but tuber development was impaired. Similarly, Richter et al. (2000) noted poor shoot growth and low tuber yield in potato as they increased levels of hepatitis surface antigen B by adding plant terminator sequences to their construct driven by an enhanced 35S promoter. .

The negative physiological impact of foreign genes in transgenic plants was not widely anticipated by those working in this field, as proteins from organisms as distant as mammalian pathogens have little homology with structural or enzymatic proteins of plants. However, with the accumulation of sequence data from many organisms, it is becoming clear that many protein motifs are widely shared across organisms. An example is the particular amino acid pattern of six cysteines in a protein, first noted in genes for epidermal growth factor (EGF) in mammals, and subsequently determined to be essential for the characteristic three-dimensional structure and biological activity of this molecule. There is an abundant and growing number of DNA sequences from many organisms which contain EGF motifs. EGF motifs have been detected in the extracellular domain of a wall-associated kinase gene from arabidopsis (He et al.1996.), as well as in a putative vacuolar sorting receptor (Miller et al. 1999). EGF-binding proteins have been detected in plant extracts (Komatsu et al.1996) and recently membrane-associated proteins have been discovered in plants with homology to EGF receptor proteins (Ahmed et al.1997).These

observations raise the possibility that plant proteins with EGF-like domains may have some physiological function in plants, and that expression of mammalian forms of EGF in plants may interfere with that function. This is perhaps the reason for the lack of success recorded by Higo et al. (1993) in expressing a human EGF gene in tobacco and for similar problems in our lab with such a protein in transgenic plants. In addition, there are many other types of non-specific and unpredictable interactions possible between a foreign protein and: other plant proteins, such as enzymes and receptors; plant membranes, such as those of the endoplasmic reticulum, Golgi apparatus, vacuole and plasmalemma; or the host of other molecules critical to the growth and development of the plant

The toxic or inhibitory effects of a foreign protein are particularly problematic when expression of the gene is controlled by constitutive promoters such as the 35S promoter from cauliflower mosaic virus (Mason et al.,1998; Richter et al., 2000). Although tissue-specific promoters may reduce such effects generally throughout the plant, the toxic effect of the protein on the development and productivity of the target tissue, such as tubers (e.g. Mason et al. 1998) or seeds, could probably not be avoided and would likely be more serious as such promoters usually provide higher expression levels in the targeted tissue than do constitutive promoters . For example, the levels of Bt protein in maize leaves were much higher on average across a sample of transgenic plants when the Bt gene was controlled by the PEP carboxylase promoter than the 35S promoter (Koziel et al., 1993). Another disadvantage of a constitutive promoter is the metabolic cost of synthesizing the transgenic protein in all tissues at all stages of growth. If the only tissue to be harvested is the leaves, for example, it is inefficient and wasteful for the plant to produce the foreign protein in other tissues. In this case, leaf-specific promoters or promoters induced in the leaves by some treatment would restrict synthesis to only the harvested tissue.

For these reasons there is growing interest in developing inducible expression systems suitable for practical application to crop plants cultivated in a conventional field setting (Zuo and Chua, 2000). Ideally, synthesis of the foreign protein could be induced by simple, low-cost application of a treatment to plants in the field, resulting in high levels of the protein within hours up to a day or so. Leaf tissue is the most obvious target for such rapid induction, as it would present the largest biomass available for induction and probably the most amenable to rapid metabolic change.

We have used subtraction libraries to clone genes that are induced in alfalfa grown in field conditions, following application of specific treatments. We are concentrating on three clones for which we cannot detect mRNA in above-ground plant tissue from plants growing in the field under normal conditions. All three clones are induced by a specific treatment applied to plants in the field; one of the clones is also induced by a heat treatment and another is induced by wounding. Induction of the third gene appears to be exclusive to a particular treatment.

There are a number of advantages to such an approach. The potential deleterious effects of expressing a foreign protein on the growth and development of a plant are avoided. The synthesis of the transgenic product can be timed to coincide with optimal conditions of growth and stage of development of the crop plant. Induction may be targeted to specific harvestable tissues, thereby avoiding synthesis in other tissues which entails additional metabolic costs and perhaps regulatory issues, as well. It may be, as well, that the possibility of gene silencing could be reduced if expression of the transgene is delayed to a late stage of growth in the plant rather than in all tissues at all times. Finally, inducible transgene systems offer a method of biological containment, i.e. the foreign protein is not present in the crop until the application of the inducing treatment, at which time the crop is harvested.

4.5 Reducing Degradation and Enhancing Immunogenicity

To address concerns regarding degradation of the protein, one might select proteins which can resist such degradation, either because they are adapted to that environment or because of their intrinsic properties. Examples of the former are the various surface antigens of intestinal pathogens, and an example of the latter is the beta-conglycinins of soybean (Astwood et al., 1996), perhaps a candidate for a carrier protein.

There are other strategies one can adopt, as well, to reduce degradation, such as localization in cell walls. Plant cell walls, depending on species, tissue and environment can produce a limitless array of physicochemical structures which can protect a protein in planta, as well as in the digestive tract of animals that ingest the plant tissue. For the purpose of oral vaccination, the gradual breakdown of the cell wall during digestion could result in a slow continual release of antigen as the tissue passes through the gut. The association of the antigen with resulting particulate structures could also enhance the immune response to the antigen.

Dietary context is another very significant factor affecting both degradation and antigenicity. It represents the entirety of biochemical components of the feed, many of which have varied effects on the immunophysiological functions of the intestinal mucosa, as well as on the digestion process itself. An edible vaccine strategy must include considerations not only of the effects of the plant tissue containing the antigen, but also the effects of other components of the diet before, during and after oral vaccination. Experiments by Modelska et al. (1998) demonstrate the effects of feeding an antigen as part of plant tissue. Mice were fed spinach leaves containing a alfalfa mosaic virus modified to express two rabies virus epitopes. The levels of secreted IgA in such mice were twice those in mice that had been orally intubated with purified virus particles at a dose ten-fold that received by those eating the spinach leaves directly. Either the virus particles were protected physically by the associated cell walls and membranes, or biochemically by various protein and non-protein compounds of plant origin, or by both mechanisms. Equally as important may be the immunological, adjuvant-type effects of these physical and biochemical components provided by the leaf tissue.

The amount and type of processing can also affect the amount and condition of peptides in a plant-derived product that reach the target tissues of the gastrointestinal tract. Some seed-derived feed ingredients, such as soya meal, are heat-treated during processing at temperatures ranging from 60° to 80° C for periods ranging from a few to several minutes, depending on the process. It has been shown in many nutritional studies that such heating is necessary to inactivate enzyme inhibitors and other antinutritional factors to enable utilization of these components in animal diets. The potential for denaturation of a therapeutic protein using such a production and delivery system is obvious, but it not may be as extensive as anticipated since incorporation of the peptide within plant tissues may reduce the extent of denaturation compared to subjecting such proteins to heat as isolated molecules.

In addition, there is extensive experimental literature documenting the beneficial effects of heating plant-derived feedstuffs to enhance utilization of the protein component especially (Conrad and Klopfenstin, 1988). This process of rendering proteins less digestible is particularly attractive for ruminant utilization of the high protein content of alfalfa, much of which is rapidly degraded microbially in the rumen and lost as methane and urea. Controlled heating converts much of the readily soluble and digestible plant protein to "bypass protein" which escapes rapid degradation in the rumen, and is broken

down more slowly in passage through the intestinal tract. This approach could be conceivably be utilized to reduce degradation of antigens expressed in plant tissue for oral delivery not only to reduce degradation, but also to enhance immunogenicity.

The delivery of oral vaccines in plant tissue or in some form derived from plants presents unique opportunities for mucosal immunization, as well as obstacles. As mentioned above, a problem faced by any subunit vaccine is the extent and kind of immune response induced by an antigen stripped of its association with a particulate, replicating structure typical of a pathogen. The possibility of inducing oral tolerance to an antigen delivered as part of a potato, for example, has been demonstrated by Ma et al. (1997). The ability to control this response has not yet been developed, although a number of strategies have been adopted to produce the desired "vaccinated" response. For example, the selection of antigens which in plants assemble into particulate structures, such as the pentameric ring of LTB (Haq et al., 1995) or the viral particle of Norwalk virus (Mason et al. 1996) should enhance the oral immunogenicity of those antigens (Florence and Jani, 1993). Incorporation of mucosal adjuvants along with an antigen produced in plant material could also increase the immune response. For example, Richter et al. (2000) added cholera toxin B (CTB) to transgenic potato tissue containing hepatitis B surface antigen, but a wide variety of other protein and non-protein compounds could serve a similar function, including polycations, lipid conjugates, streptomycin, bacterial adhesins and lectins of diverse origins (Mahon et al. 1998; De Aizpurura and Russell-Jones, 1998). Plants are especially rich in lectins, some of which are potent mucosal adjuvants while others are not. Intranasal co-administration of mistletoe lectin with ovalbumen (OVA) enhanced secretion of IgA to OVA from mucosal surfaces of rat, whereas phytohemagglutinin did not increase levels over those elicited by the antigen alone (Lavelle et al. 2001). An example of potent non-protein adjuvants from plants is the class of compounds referred to as saponins, which are found in many plant species, but are especially prominent in *Quillaia saponaria* (Sjolander et al., 1998). In this connection, the attachment of antigens to oil-bodies produced in oilseeds (Parmenter et al., 1996) may provide another mechanism for stimulating immunity, depending on the plant source of the oil-bodies.

It is also possible to draw on the extensive literature dealing with mammalian genes affecting the mucosal immune response to engineer plants for edible vaccines. For example, the role of cytokines in modulating T-cell

response towards tolerance to an orallly-administered antigen might be exploited by co-producing specific cytokines in plant tissue. Recently, a number of neuropeptides endogenous to the gut have been identified which can affect T-cell response and levels of antibody secretion when orally co-administered with an antigen (Pascual et al.1998).

5. EDIBLE DNA VACCINES IN PLANTS

Recombinant DNA vaccines have shown great promise in animal models for inducing long-lived cell- and humorally-mediated immunity to pathogens (Apostolopoulos and Plebanski, 2000). A particularly attractive feature of this approach is the possibility of creating multivalent vaccines by incorporating multiple genes for antigens in one or more plasmid molecules. An extension of this principle could involve , as well, inclusion of genes for immunomodulatory proteins, such as cytokines, in the DNA construct. The potential of this approach to immunizing livestock against diseases is under active investigation, including its application to enteric diseases (van Drunen Littel-van den Hurk et al.,2001). However, there has been little mention in the literature of plants as vehicles for orally-delivered DNA vaccines. There are some features of plants which may make such an approach more feasible than would be apparent at first glance. First, with the advent of chloroplast transformation, it is now possible to grow plant tissues which contain many thousands of copies of a transgene per cell (DeCosa et al. 2001). Second, as mentioned above, plant cell walls provide a form of encapsulation, the specific features of which can vary, depending on the species, tissue and environment. It is also possible to modify cell wall structures by various processes, such as heating. An example of organism-mediated delivery of DNA to intestinal target tissues in the intestine was provided by Darji et al.(2000) who utilized attenuated strains of *Salmonella*. The possibility of using plant tissue in a similar fashion is strengthened by the observations of Schubbert et al.(1998) who demonstrated that oral administration of naked DNA alone was sufficient to result in the uptake of such DNA in cells of the spleen and liver, as well as macrophages. Histological analyses and in situ hybridization assays provided evidence of incorporation of the foreign DNA into the chromosomal DNA of the mouse and replication of this DNA during cell division resulting in transformed tissue sectors. It may be that such tissues are amenable to oral vaccination with DNA in plant cells containing not only

many copies of mammalian genes and the appropriate regulatory sequences, but also other mammalian genes to enhance a mucosal immune response.

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