

## INVITED REVIEW:

### TARGETING AND EXPRESSION OF ANTIGENIC PROTEINS IN TRANSGENIC PLANTS FOR PRODUCTION OF EDIBLE ORAL VACCINES

SCHUYLER S. KORBAN\*

*Department of Natural Resources & Environmental Sciences, 310 ERML, University of Illinois, Urbana, IL 61801*

(Received 7 December 2001; accepted 9 January 2002; editor M. E. Horn)

#### SUMMARY

Exploiting plants as biological bioreactors for production and delivery of edible oral subunit vaccines is a promising application of biotechnology. Efforts to enhance expression levels of transgenes coding for antigenic proteins by exploiting promoters, targeting sequences, and enhancer elements have produced rather low quantities of the antigen in plant tissues, but enough to induce immune responses in feeding studies. This review will cover components of various gene constructs used in developing plant-based vaccines against a myriad of viral and bacterial diseases. Specifically, it will focus on sequences that are involved in targeting the antigen to mucosal tissues of the intestinal tract, thus enhancing the immunogenicity of the plant-based vaccine as well as those components that result in higher accumulation of the protein within the plant.

*Key words:* biopharmaceuticals; molecular farming; recombinant proteins; cholera toxin B; *Escherichia coli*; heat-labile enterotoxin; respiratory syncytial virus; hepatitis B surface antigen.

#### INTRODUCTION

Exploiting plants to produce medicinal products has become a well-recognized and important field of biopharmaceutical science that shows a promising economic potential. In recent years, the tools of genetic engineering have allowed development of transgenic plants that can express various recombinant biopharmaceutical compounds, including viral and bacterial antigens, antibodies, and various therapeutic human and animal proteins (Buetow and Korban, 2000; Giddings et al., 2000; Daniell et al., 2001). Production of recombinant proteins in plants can be achieved either directly by stable transformation of plants with the recombinant protein gene(s) or indirectly via transient expression of plant viruses, transfected with the recombinant protein gene(s), replicating within a plant (Buetow and Korban, 2000; Giddings et al., 2000; Daniell et al., 2001). Thus far, several plant species, including potato, tobacco, tomato, *Arabidopsis*, soybean, alfalfa, lettuce, lupine, rice, wheat, apple, black-eyed bean, cowpea, and corn, have been used for production of recombinant therapeutic proteins using either approach and whereby the recombinant protein is expressed in either vegetative and/or reproductive tissues. This review, however, will focus primarily on therapeutic recombinant proteins produced via stable transformation, as summarized in Table 1.

At this time, transformed plants accumulate relatively small amounts of foreign proteins (0.001–0.3%; Yu et al., 2000), thus limiting the application of plants for production and delivery of

various therapeutic human and animal proteins. Recently, efforts have been made to increase the level of accumulation of these proteins in transgenic plants by developing constructs whereby antigenic protein-encoding genes are fused to transcriptional and/or translational enhancer elements and driven by various promoters, either constitutive or tissue-specific. In addition, efforts have been made to add peptide sequences to these constructs that either target the protein to cellular organelles or serve as carrier molecules and adjuvants for effective binding and targeting of these antigenic proteins to mucosal cells and tissues of the mammalian host. In this review, the role of various components of gene constructs used in developing transgenic plants expressing therapeutic proteins that serve as plant-based subunit vaccines will be presented. These plant-based vaccines are administered orally, and are targeted to induce an immune response in mucosal tissues, as these tissues serve as the first line of defense against invading pathogenic organisms entering the body through oral, respiratory, and reproductive tracts. Thus, targeting sequences used in constructs to enhance the antigenicity of the plant-derived vaccine against these mucosal tissues will be covered. Then, this will be followed by an overview of components of gene constructs used to increase the accumulation of the antigenic proteins in plant cells that serve as biological bioreactors.

#### TARGETING SEQUENCES FOR ENHANCED PRIMING OF MUCOSAL TISSUES

The nontoxic cholera toxin B (CTB) subunit pentamer has been shown to function as an effective carrier molecule for foreign

\*Author to whom correspondence should be addressed: Email s-korban@uiuc.edu

TABLE 1  
SUMMARY OF ANTIGENS, ALONG WITH THEIR CONSTRUCTS, PRODUCED IN TRANSGENIC PLANTS AS CANDIDATE VACCINES

Pathogen or disease	Construct					References
	Plant	Promoter <sup>a</sup>	Antigen	Targeting/enhancers/signal peptides/terminator sequences		
Canine parvovirus	<i>Arabidopsis</i>	35S	Capsid protein 2L21	-	Gil et al., 2001	
<i>Vibrio cholerae</i>	Potato	<i>mas</i> P2	Cholera toxin B subunit (CTB)	SEKDEL	Arakawa et al., 1998	
Cholera, enterotoxigenic <i>E. coli</i> , rotavirus	Potato	<i>mas</i> P1- <i>mas</i> P2	CTA2:CFA/I-CTB:NSP4	SEKDEL + CTA leader- SEKDEL + CTB leader	Yu and Langridge, 2001	
Diabetes (autoimmune)	Potato	<i>mas</i> P2	Insulin	C. terminus of CTB	Arakawa et al., 1998	
<i>E. coli</i> heat-labile enterotoxin B subunit (LT-B)	Corn	-	LTB	Codon-optimized version of barley $\alpha$ -amylase signal sequence	Streatfield et al., 2001	
	Potato, tobacco	35S	LTB	SEKDEL + 5' TEV leader + 3' vspB	Haq et al., 1995	
	Potato	35S	s-LTB (optimized)	SEKDEL + 5' TEV leader + 3' vspB	Mason et al., 1998	
Foot-and-mouth disease virus	<i>Arabidopsis</i>	35S	Structural protein VP1	5' TEV	Carrillo et al., 1998	
Hepatitis B	Tobacco	35S	HB surface antigen (HbsAg)	5' TEV leader	Mason et al., 1992	
	Lupine, lettuce	35S	HbsAg		Kapusta et al., 1999	
	Potato	35S	HbsAg	5' TEV leader + <i>vspB</i> ; <i>pinII</i> ; <i>vspA</i> ; <i>vsp<math>\alpha</math>L</i> ; SEKDEL; TPSS	Richter et al., 2000	
Herpes simplex virus 2 (HSV-2)	Soybean	35S	Glycoprotein B antibody	Tobacco extensin signal peptide	Zeitlin et al., 1998	
Human carcinoembryonic antigen (CEA) producing tumors	Rice, wheat	Ubi-1; double-35S (for rice only)	Single-chain Fv (ScFv) antibody	5'-UTR-CHS + codon-optimized leader peptide (LPH) from TMV + SEKDEL or His <sub>6</sub> tag	Stöger et al., 2000	
Human cytomegalovirus (HCMV)	Tobacco	Gt3	Glycoprotein B	Gt3 signal peptide sequence	Tackaberry et al., 1999	
Measles virus (MV)	Tobacco	35S	Hemagglutinin (H) protein	5' TEV leader + SEKDEL + signal peptide (SP) of tobacco <i>Pr1a</i> gene	Huang et al., 2001	
Norwalk virus (NV)	Tobacco	35S	Capsid protein	5' TEV	Mason et al., 1996	
Organophosphate poisoning	Potato	Patatin	Capsid protein	5' TEV	Mor et al., 2001	
Rabbit hemorrhagic disease virus (RHDV)	Tomato	35S	Human acetyl-cholinesterase (AChE)	5' TEV leader + 3' vspB	Castañón et al., 1999	
Rabies virus	Potato	35S	Structural protein VP60	Strong transcriptional enhancer	McGarvey et al., 1995	
Respiratory syncytial virus (RSV)	Tomato	35S	Glycoprotein G		Sandhu et al., 2000	
<i>Streptococcus mutans</i>	Tomato	E-8	F protein		Ma et al., 1995	
Swine transmissible gastroenteritis coronavirus (TGEV)	Tobacco	35S	Secretory immunoglobulin (SigA-G)		Gómez et al., 1998	
	<i>Arabidopsis</i>	35S	Spike (S) glycoprotein		Gómez et al., 2000	
	Potato	35S	S		Tuboly et al., 2000	
	Tobacco	Super P	S (optimized codon)	Signal peptide sequence of tobacco PR protein	Streatfield et al., 2001	
	Corn		S (optimized codon)	Maize codon optimized version of barley $\alpha$ -amylase signal sequence		

<sup>a</sup> 35S, CaMV 35S; *mas*, mannopine synthase; Gt3, glutelin protein seed-specific promoter from rice; E-8, fruit ripening tissue-specific promoter from tomato; Super P, Super promoter; Ubi-1, maize ubiquitin promoter plus intron 1.

proteins, especially for plant-based vaccines priming the mucosal mammalian immune system, as it has a selective affinity to the sugar-lipid  $G_{M1}$ -ganglioside receptor molecules embedded in the intestinal epithelial cell (enterocyte) and M-cell membranes (Dertzbaugh and Elson, 1993a). The CTB subunit has been used in synthesizing therapeutic fusion proteins in potato against microbial enteropathogens and their toxins, cholera holotoxin (CT) and rotavirus (Arakawa et al., 1998, 2001). This is accomplished by linking the gene encoding the CTB subunit to a DNA fragment encoding an epitope of the rotavirus enterotoxin protein (NSP4) (Arakawa et al., 1998, 2001), among others.

The CTB fusion proteins are assembled into pentamers that exhibit the native antigenicity and biological activity of the CTB with a small increase in molecular weight in comparison to the bacterial CTB or the plant-synthesized CTB. This is due to the amino acids contributed by the epitope protein (e.g., 22 amino acids coding for NSP4) as well as any additional amino acids. For example, an endoplasmic reticulum (ER) retention signal (SEKDEL) (six amino acids) is often used in these constructs (Arakawa et al., 1998, 2001). This increase in molecular weight may alternatively be due to failure of the plant to cleave the signal sequence or to undetected differences in the glycosylation pattern of the plant-synthesized fusion protein (Arakawa et al., 2001). It is important to note that the addition of a polypeptide (coding for a desired epitope) to the N-terminus of the CTB subunit reduces  $G_{M1}$ -ganglioside binding affinity as it results in a steric hindrance of conformational protein folding (Dertzbaugh and Elston, 1993b). This is likely to interfere with CTB binding to the ganglioside receptors on the enterocyte membrane (Dertzbaugh and Elston, 1993b; Arakawa et al., 2001). In contrast, adding polypeptides to the C-terminus of the CTB subunit does not disrupt the affinity of CTB to its ligand (Arakawa et al., 2001).

Utilizing plant viruses, such as cowpea mosaic virus (CPMV), tobacco mosaic virus (TMV) and alfalfa mosaic virus (AIMV), as vectors for expression of antigenic proteins have been successfully used for developing plant-based vaccines against rabies virus (Yusibov et al., 1997; Modelska et al., 1998), *Pseudomonas aeruginosa* (Brennan et al., 1999a), hepatitis C virus (Nemchinov et al., 2000), mink enteritis (Dalsgaard et al., 1997), and *Staphylococcus aureus* (Brennan et al., 1999b), among others. By virtue of their ability to invade plants and rapidly replicate, these engineered viruses can accumulate large amounts of viral proteins, including desired engineered antigenic proteins (Yusibov and Koprowski, 1998; Buetow and Korban, 2000). Moreover, fusing peptides to viral coat proteins will allow assembly of engineered antigenic protein genes into chimeric virus particles with enhanced immunogenicity of antigenic determinants (Yusibov and Koprowski, 1998). For example, CPMV virions contain 60 copies of each of the large and small coat protein subunits, and thus 60 copies of the antigenic protein can be expressed and displayed on the surface of each chimeric virus particle. This has been clearly demonstrated with expression in tandem of peptides 10 and 18 of the outer-membrane protein F of *Pseudomonas aeruginosa* in CPMV chimeric virus particles (Brennan et al., 1999a). Moreover, using adjuvants such as QS-21, QuilA, Freund's complete adjuvant (FCA) and incomplete Freund's adjuvant (IFA) as carrier molecules for chimeric virus particles has been reported to enhance the immunogenicity of such plant-based vaccines (Brennan et al., 1999a, 1999b).

#### DNA SEQUENCES AND PROMOTERS FOR ENHANCED ACCUMULATION AND TARGETING OF ANTIGENIC PROTEIN IN PLANT TISSUES

The microsomal retention signal SEKDEL, a six-amino acid peptide (Ser-Glu-Lys-Asp-Glu-Leu), has been fused to the C-terminus of the binding subunit of *Escherichia coli* heat-labile enterotoxin (LT-B), and the construct has been used to transform both tobacco and potato plants (Haq et al., 1995). The LT-B transgene, driven by CaMV 35S, was also flanked by the tobacco etch virus 5' untranslated region (TEV 5'-UTR) and the polyadenylation signal of a soybean vegetative storage protein (3' *vspB*) gene. The 5' TEV leader sequence served as a translational enhancer, and the 3' *vspB* also mediated the 3' end processing of the transcript. The recovered transgenic tobacco and potato plants expressing the transgene carrying the SEKDEL retention signal showed significantly higher levels of the LT-B antigenic protein: a 1.2–2.8-fold increase in tobacco and 2–4-fold increase in potato. This suggested that the retention signal was involved in cellular compartmentalization of the fusion proteins, thus facilitating protein subunit oligomerization. Mason et al. (1998) later optimized the LT-B gene for plant mRNA processing by changing the 'AAT' codon coding for Asn to 'GTG' coding for Val, in order to accommodate a *NcoI* site surrounding the translation start site. The optimized synthetic gene, designated *sLT-B*, was introduced into a similar construct as described in Haq et al. (1995). Then, it was used in transforming potato plants, and found to accumulate 5–40-fold higher amounts of LT-B in leaf tissues than those lines carrying the bacterial (unmodified) LT-B gene (Mason et al., 1998). Arakawa et al. (2001) have indicated that the plant ER provides an intracellular environment in which chimeric monomers are accumulated and assembled into pentameric fusion proteins.

Streatfield et al. (2001) have utilized a maize codon-optimized version of a barley  $\alpha$ -amylase signal sequence, a cell secretion signal, to optimize antigenic protein accumulation in transgenic corn plants. This signal sequence has been incorporated at the N-terminus of the *Escherichia coli* heat-labile enterotoxin subunit B (LT-B) which causes diarrhea in children under 5 yr of age in developing countries, resulting in about 800 000 deaths every year. Similarly, this sequence is also added to the S protein of the swine transmissible gastroenteritis virus (TGEV), a highly contagious enteric disease which results in high mortality in piglets of less than 2 wk of age. This barley  $\alpha$ -amylase signal sequence is apparently cleaved upon antigenic protein export, resulting in high levels of protein accumulation in the cell walls of plant cells.

Castañón et al. (1999) developed transgenic potato plants expressing the major structural protein, VP60, of rabbit hemorrhagic disease virus (RHDV). The gene coding for VP60 was driven by either the cauliflower mosaic virus (CaMV) 35S promoter or a modified 35S that included two copies of a 343 bp region containing a strong transcriptional enhancer, but no detailed information on this enhancer was provided. Both promoters allowed production of detectable levels of the recombinant VP60. A range of 0.06–0.43  $\mu\text{g mg}^{-1}$  soluble protein was detected when the VP60 coding sequence was driven by the 35S promoter, compared to a range of 0.82–2.97  $\mu\text{g mg}^{-1}$  soluble protein when VP60 was driven by a modified 35S promoter. Thus, the modified 35S promoter resulted in the production of significantly higher levels of the recombinant protein in transgenic potato plants.

Lauterslager et al. (2001) also used a synthetic gene coding for *E.*

*coli* LT-B made by ligating fragments obtained following enzymatic conversion of two synthetic complementary oligonucleotides with overlapping 3' ends. They were optimized for retention of the protein in the ER by adding the SEKDEL peptide to the 3' end of the antigenic protein. The synthetic LT-B gene was driven by a class I patatin promoter to target the antigenic protein to potato tubers. The amount of the recombinant LT-B protein in transgenic potato lines reached  $17 \mu\text{g g}^{-1}$  fresh weight of tuber, corresponding to  $1.3 \text{ mol kg}^{-1}$  of monomeric recLT-B and  $0.26 \text{ mol kg}^{-1}$  of pentameric recLT-B.

To develop a plant-based vaccine against the respiratory syncytial virus (RSV), a human virus that infects humans of all ages, but is especially serious in premature babies and infants, resulting in pneumonia and bronchiolitis, Sandhu et al. (2000) targeted the antigenic RSV-F protein to the fruit of tomato. This was achieved by using a fruit ripening-specific promoter, E-8, also from tomato, to drive the expression of the RSV-F gene in transgenic tomato plants. The amount of the recombinant RSV-F protein in transgenic tomato lines reached  $32.5 \mu\text{g g}^{-1}$  fruit fresh weight. Earlier, Sandhu et al. (1999) reported that inserting the 5' untranslated leader sequence (37 bp) from alfalfa mosaic virus (AMV) RNA4 between the CaMV 35S promoter and the RSV-F gene increased transient viral expression in apple leaf protoplasts by 5.5-fold compared to the construct without this sequence. Moreover, adding a 268 bp transcriptional enhancer from the pea plastocyanin gene (*PetE*) upstream of the CaMV 35S promoter containing the AMV leader further increased RSV-F gene expression up to 7.7-fold compared to the construct lacking both AMV leader and *PetE* transcriptional enhancer.

In a recent study by Richter et al. (2000), several factors involved in the production of the hepatitis B surface antigen (HBsAg) in transgenic potato tubers were investigated. It was reported that the use of alternative polyadenylation signals to the commonly used nopaline synthase (NOS) 3' element in constructs, such as the 3' end of the soybean vegetative storage protein (*vspB*) gene and the 3' element from the potato *pinII* gene, increased the amount of HBsAg mRNA (up to  $16 \mu\text{g g}^{-1}$  potato tuber). Moreover, evidence was presented demonstrating that extension of the ER retention signal SEKDEL by adding a cleavable plant signal peptide, for example adding VSP to the N terminus of HBsAg, promoted more efficient ER targeting, leading to higher accumulation of the antigenic protein than a noncleaved HBV signal peptide. Richter et al. (2000) took advantage of an N-terminal chloroplast transit peptide from the small subunit of ribulose biphosphate carboxylase-oxygenase (Rubisco) to target the antigenic protein into the outer membrane of the plastid. However, this approach did not result in higher levels of HBsAg mRNA in transgenic plants, likely attributed to poor utilization or inefficient cleaving of the transit peptide. Alternatively, integration into the outer membrane of the plastid might disrupt disulfide cross-linking of the subunits, resulting in their degradation.

Working with the hemagglutinin (H) protein gene of measles virus, Huang et al. (2001) introduced three different constructs into tobacco, and found that the highest level of protein accumulation was observed in transgenic plants containing the H protein gene, driven by CaMV 35S virus, with a 5' TEV leader sequence along with the ER retention sequence SEKDEL.

Stöger et al. (2000) successfully expressed, in both rice and wheat, a single-chain Fv antibody (ScFv) against carcinoembryonic

antigen (CEA), a well-characterized tumor-associated marker antigen able to diagnose tumor onset. The ScFv constructs were engineered for recombinant antibody targeting either to the plant cell apoplast or to the ER by incorporating either a hexameric histidine ( $\text{His}_6$ ) tag or SEKDEL, respectively, to the C-terminus of the transgene. For both constructs, the transgene was driven either by a maize ubiquitin-1 promoter (with intron 1) (used for both wheat and rice transformation) or a double-CaMV 35S promoter (used only for rice transformation). Also, a 5' untranslated region (UTR) chalcone synthase (CHS) along with a codon-optimized (for plant expression) heavy-chain leader peptide, LPH, from the tobacco mosaic virus (TMV) virion-specific mAb 24, were placed upstream of the ScFv coding region. Accumulation of ScFv antibody levels was significantly higher in transgenic wheat and rice plants carrying the transgene along with the ER targeting sequence SEKDEL. These results indicated that ScFv recombinant protein yield was likely to be more stable or would be more correctly folded in the ER than in the apoplast. Expression levels of the recombinant protein were detected in those transgenic rice lines where the transgene was driven by the ubiquitin promoter and tagged with a C-terminal SEKDEL motif ( $29 \mu\text{g g}^{-1}$  in leaves and  $32 \mu\text{g g}^{-1}$  in seeds).

A multicomponent vaccine against three enteric diseases, cholera, rotavirus, and enterotoxigenic *E. coli* (ETEC), was recently developed in transgenic potato plants by Yu and Langridge (2001). In a C-terminal fusion, a 22-amino acid immunodominant epitope of the murine rotavirus enterotoxin, NSP4, was fused to the CTB subunit under control of the mannopine synthase (*mas*) P2 promoter. While in an N-terminal fusion, they fused the ETEC fimbrial colonization factor CFA/I to CTA2, and this fusion gene was cloned downstream of *mas* P1 promoter. Each cholera toxin fusion gene contained its own leader sequence and an ER retention signal (SEKDEL). To increase the flexibility of the fusion protein, a four-amino acid GPGP hinge region was inserted between the CTB and NSP4 peptides. The amount of the recombinant fusion proteins used in their immunological studies was reported as  $3.3 \mu\text{g g}^{-1}$  potato tuber. Nevertheless, orally immunized mice generated detectable levels of both serum and intestinal antibodies against the bacterial and viral pathogen antigens (Yu and Langridge, 2001).

## CONCLUSIONS

The use of the CTB subunit in gene constructs as a carrier to increase the immunogenicity of antigenic proteins by priming the mucosal immune system at the intestinal surface, appears to be a promising approach for enhancing the targeting of plant-based vaccines. This, along with the use of other adjuvants, may provide a viable strategy for effective delivery of plant-based vaccines.

Several approaches have been used to increase accumulation of antigenic proteins in transgenic plants. These include exploiting tissue-specific promoters, transcriptional and translational enhancers, ER targeting sequences, cleavable signal peptides, as well as 3' terminal sequences, all used in various combinations. Although modest success has been achieved in elevating the levels of antigenic proteins in various transgenic crops, higher levels of these proteins are needed for efficient commercialization of these therapeutic plant-derived products. The use of novel strong promoters, targeting sequences, and other transcriptional and/or translational sequences must be evaluated.

One area that requires critical attention is the possible role of

post-transcriptional gene silencing (PTGS) in the observed accumulated levels of antigenic proteins reported thus far in various transgenic plants. It is likely that PTGS, a homology-dependent process that reduces cytoplasmic RNA levels, is a limiting factor in transgene expression, and should be investigated (Vaucheret et al., 2001).

Recent studies on expression of various protein-based polymers and other proteins have shown up to 100-fold accumulation of these proteins when these are targeted to plastid rather than nuclear genomes of transgenic plants (Kota et al., 1999; Guda et al., 2000). Staub et al. (2000) targeted the expression of a recombinant therapeutic protein, human somatotropin (hST), into tobacco chloroplasts. The hST is used in the treatment of hypopituitary dwarfism in children with likelihood for use in the treatment of Turner syndrome, chronic renal failure, HIV wasting syndrome, and possibly treatment of the elderly. Almost a 300-fold increase in hST recombinant protein levels was detected in chloroplast-transformed tobacco plants compared to nuclear-transformed plants (Staub et al., 2000). Thus, targeting antigenic protein into plastids should be more thoroughly explored as a likely approach to enhance accumulation of antigenic proteins in transgenic plants.

## REFERENCES

- Arakawa, T.; Chong, D. K. X.; Langridge, W. H. R. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nature Biotechnol.* 16:292–297; 1998.
- Arakawa, T.; Yu, J.; Langridge, W. H. R. Synthesis of a cholera toxin B subunit-rotavirus NSP4 fusion protein in potato. *Plant Cell Rep.* 20:343–348; 2001.
- Brennan, F. R.; Bellaby, T.; Helliwell, S. M.; Jones, T. D.; Kamstrup, S.; Dalsgaard, K.; Flock, J.-I.; Hamilton, W. D. O. Chimeric plant virus particles administered nasally or orally induce systemic and mucosal immune responses in mice. *J. Virol.* 73:930–938; 1999a.
- Brennan, F. R.; Jones, T. D.; Gilleland, L. B.; Bellaby, T.; Xu, F.; North, P. C.; Thompson, A.; Staczek, J.; Lin, T.; Johnson, J. E.; Hamilton, W. D. O.; Gilleland, H. E. *Pseudomonas aeruginosa* outer-membrane protein F epitopes are highly immunogenic in mice when expressed on a plant virus. *Microbiology* 145:211–220; 1999b.
- Buetow, D. E., Korban, S. S. Transgenic plants producing viral and bacterial antigens. *AgBiotechNet 2*, ABN 045, www.agbiotechnet.com; 2000.
- Carrillo, C.; Wigdorovitz, A.; Oliveros, J. C.; Zamorano, P. I.; Sadir, A. M.; Gómez, N.; Salinas, J.; Escribano, J. M.; Borca, M. V. Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. *J. Virol.* 72:1688–1690; 1998.
- Castañón, S.; Marín, M. S.; Martín-Aonso, J. M.; Boga, J. A.; Casais, R.; Humara, J. M.; Ordás, R. J.; Parra, F. Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. *J. Virol.* 73:4452–4455; 1999.
- Dalsgaard, K.; Uttenthal, Å.; Jones, T. D.; Xu, F.; Merryweather, A.; Hamilton, W. D. O.; Langeveld, J. P. M.; Boshuizen, R. S.; Kamstrup, S.; Lomonosoff, G. P.; Porta, C.; Vela, C.; Casal, J. I.; Meloen, R. H.; Rodgers, P. B. Plant-derived vaccine protects target animals against a viral disease. *Nature Biotechnol.* 15:248–252; 1997.
- Daniell, H.; Streatfield, J.; Wycoff, K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci.* 6:219–226; 2001.
- Dertzbaugh, M. T.; Elson, C. O. Comparative effectiveness of the cholera toxin B subunits and alkaline phosphatase as carrier for oral vaccines. *Infect. Immunol.* 61:48–55; 1993a.
- Dertzbaugh, M. T.; Elson, C. O. Reduction in oral immunogenicity of cholera toxin B subunits by N-terminal peptide addition. *Infect. Immunol.* 61:384–390; 1993b.
- Giddings, G.; Allison, G.; Brooks, D.; Carter, A. Transgenic plants as factories for biopharmaceuticals. *Nature Biotechnol.* 18:1151–1155; 2000.
- Gil, F.; Brun, A.; Wigdorovitz, A.; Catala, R.; Martínez-Torrecuadrada, J. L.; Casal, I.; Salinas, J.; Borca, M. V.; Escribano, J. M. High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS Lett.* 488:13–17; 2001.
- Gómez, N.; Carrillo, C.; Salinas, J.; Parra, F.; Borca, M. V.; Escribano, J. M. Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. *Virology* 249:352–358; 1998.
- Gómez, N.; Wigdorovitz, A.; Castañón, S.; Gil, F.; Ordás, R. J.; Borca, M. V.; Escribano, J. M. Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. *Arch. Virol.* 145:1725–1732; 2000.
- Guda, C.; Lee, S.-B.; Daniell, H. Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. *Plant Cell Rep.* 19:257–262; 2000.
- Haq, T. A.; Mason, H. S.; Clements, J. D.; Arntzen, C. J. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268:714–716; 1995.
- Huang, Z.; Dry, I.; Webster, D.; Strugnell, R.; Wesselingh, S. Plant-derived measles virus hemagglutinin protein induces neutralizing antibodies in mice. *Vaccine* 19:2163–2171; 2001.
- Kapusta, J.; Modelska, A.; Figlerowicz, M.; Pniowski, T.; Letellier, M.; Lisowa, O.; Yusinpv, V.; Koprowski, H.; Plucieniczak, A.; Legocki, A. N. A plant-derived vaccine against hepatitis B virus. *FASEB J.* 13:1796–1799; 1999.
- Kota, M.; Daniell, H.; Varma, S.; Garczynski, F.; Gould, F.; Moar, W. J. Overexpression of the *Bacillus thuringiensis* Cry2A protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. *Proc. Natl Acad. Sci. USA* 96:1840–1845; 1999.
- Lauterslager, T. G. M.; Florackb, D. E. A.; van der Wal, T. J.; Molthoffb, J. W.; Langeveld, J. P. M.; Bosch, D.; Boersma, W. J. A.; Hilgers, L.A.Th. Oral immunization of naive and primed animals with transgenic potato tubers expressing LT-B. *Vaccine* 19:2749–2755; 2001.
- Ma, J.K.-C.; Hiatt, A.; Hein, M.; Vine, N. D.; Wang, F.; Stabila, P.; van Dolleweerd, C.; Mostov, K.; Lehner, T. Generation and assembly of secretory antibodies in plants. *Science* 268:716–719; 1995.
- Mason, H. S.; Ball, J. M.; Shi, J. J.; Jiang, X.; Extes, M. K.; Arntzen, C. J. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl Acad. Sci. USA* 93:5335–5340; 1996.
- Mason, H. S.; Haq, T. A.; Clements, J. D.; Arntzen, C. J. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 16:1336–1343; 1998.
- Mason, H. S.; Lam, D. M. K.; Arntzen, C. J. Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl Acad. Sci. USA* 89:11745–11749; 1992.
- McGarvey, P. B.; Hammond, J.; Dienelt, M. M.; Hooper, D. C.; Fu, Z. F.; Dietzschold, B.; Koprowski, H.; Michaels, F. H. Expression of the rabies virus glycoprotein in transgenic tomatoes. *Biotechnology* 13:1484–1487; 1995.
- Modelska, A.; Dietzschold, B.; Sleysh, N.; Fu, Z. F.; Steplewski, K.; Hooper, D. C.; Koprowski, H.; Yusibov, V. Immunization against rabies with plant-derived antigen. *Proc. Natl Acad. Sci. USA* 95:2481–2485; 1998.
- Mor, T. S.; Sternfeld, M.; Soreq, H.; Arntzen, C. J.; Mason, H. S. Expression of recombinant human acetylcholinesterase in transgenic tomato plants. *Biotechnol. Bioengng* 75:259–266; 2001.
- Nemchinov, L. G.; Liang, T. J.; Rifaat, M. M.; Mazyad, H. M.; Hadidi, A.; Keith, J. M. Development of a plant-derived subunit vaccine candidate against hepatitis C virus. *Arch. Virol.* 145:2557–2573; 2000.
- Richter, L. J.; Thanaval, Y.; Arntzen, C. J.; Mason, H. S. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nature Biotechnol.* 18:1167–1171; 2000.
- Sandhu, J. S.; Krasnyanski, S. F.; Domier, L. L.; Korban, S. S.; Osadjan, M. D.; Buetow, D. E. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. *Transgenic Res.* 9:127–135; 2000.
- Sandhu, J. S.; Osadjan, M. D.; Krasnyanski, S. F.; Domier, L. L.; Korban, S. S.; Buetow, D. E. Enhanced expression of the human respiratory

- syncytial virus-F gene in apple leaf protoplasts. *Plant Cell Rep.* 18:394–397; 1999.
- Staub, J. M.; Garcia, B.; Graves, J.; Hajdukiewicz, P. T. J.; Hunter, P.; Nehra, N.; Paradkar, V.; Schlittler, M.; Carroll, J. A.; Spatola, L.; Ward, D.; Ye, G.; Russell, D. A. High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnol.* 18:333–338; 2000.
- Stöger, E.; Vaquero, C.; Torres, E.; Sack, M.; Nicholson, L.; Drossard, J.; Williams, S.; Keen, D.; Perrin, Y.; Christou, P.; Fischer, R. Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol. Biol.* 42:583–590; 2000.
- Streatfield, S. J.; Jilka, J. M.; Hood, E. E.; Turner, D. D.; Bailey, M. R.; Mayor, J. M.; Woodard, S. L.; Beifuss, K. K.; Horn, M. E.; Delaney, D. E.; Tizard, I. R.; Howard, J. A. Plant-based vaccines: unique advantages. *Vaccine* 19:2742–2748; 2001.
- Tackaberry, E. S.; Dudani, A. K.; Prior, F.; Tocchi, M.; Sardana, R.; Altosaar, I.; Ganz, P. R. Development of biopharmaceuticals in plant expression systems: cloning, expression and immunological reactivity of human cytomegalovirus glycoprotein B (UL55) in seeds of transgenic tobacco. *Vaccine* 17:3020–3029; 1999.
- Tuboly, T.; Yu, W.; Bailey, A.; Degrandis, S.; Du, S.; Erickson, L.; Nagy, E. Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants. *Vaccine* 18:2023–2028; 2000.
- Vaucheret, H.; Beclin, C.; Fagard, M. Post-transcriptional gene silencing in plants. *J. Cell Sci.* 114:3083–3091; 2001.
- Yu, J.; Arakawa, T.; Langridge, W. H. R. Assembly of cholera toxin-antigen fusion proteins in transgenic potato. *Transgenics* 40:1–10; 2000.
- Yu, J.; Langridge, W. H. R. A plant-based multicomponent vaccine protects mice from enteric diseases. *Nature Biotechnol.* 19:548–552; 2001.
- Yusibov, V.; Koprowski, H. Plants as vectors for biomedical products. *J. Med. Food* 1:5–12; 1998.
- Yusibov, V.; Modelska, A.; Steplewski, K.; Agadjanyan, M.; Weiner, D.; Hooper, C.; Koprowski, H. Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1. *Proc. Natl Acad. Sci. USA* 94:5784–5788; 1997.
- Zeitlin, L.; Olmsted, S. S.; Moench, T. R.; Co, M. S.; Martinell, B. J.; Paradkar, V. M.; Russell, D. R.; Queen, C.; Cone, R. A.; Whaley, K. J. A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nature Biotechnol.* 16:1361–1364; 1998.