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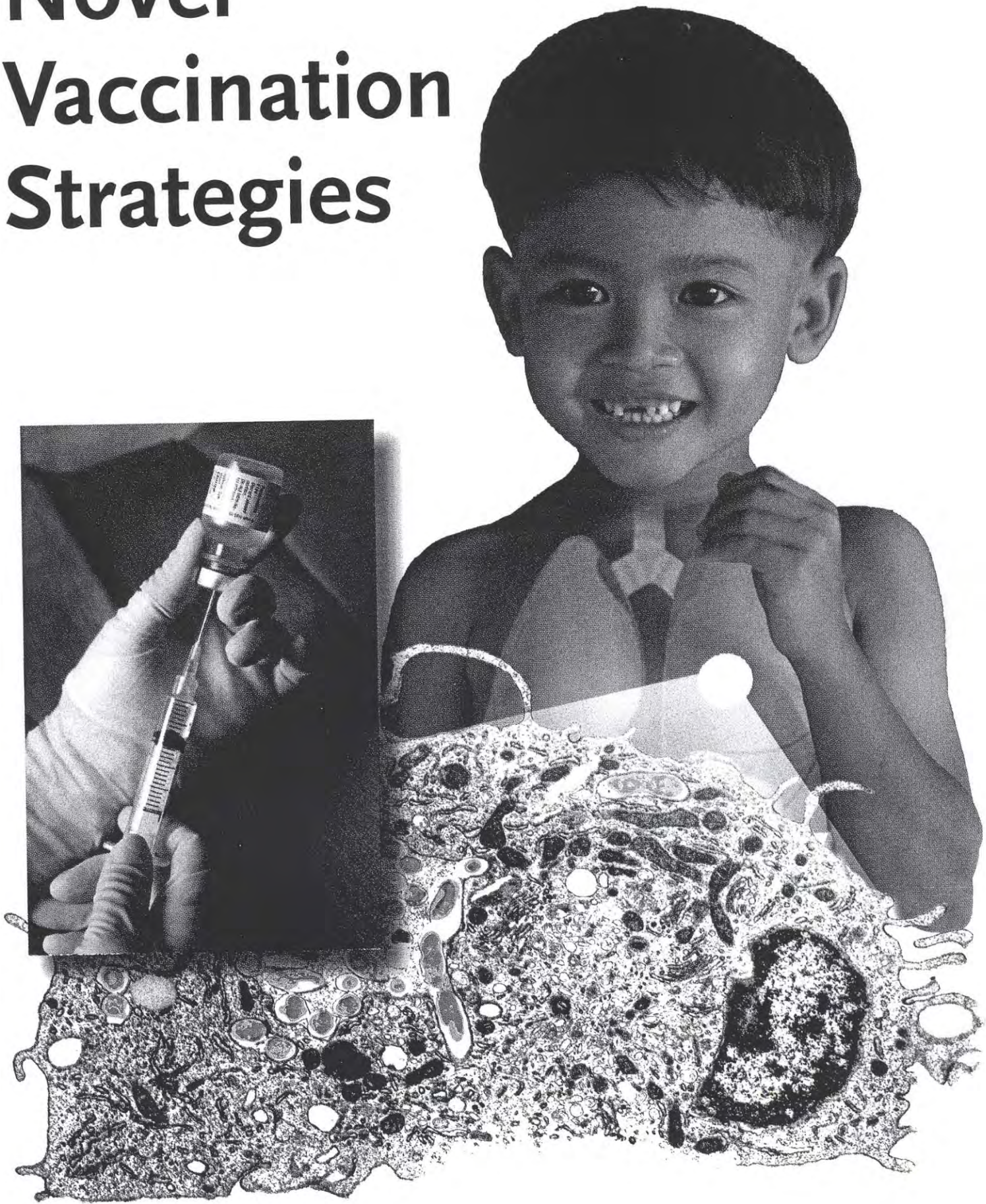

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18
Plant-based Oral Vaccines

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18.1
Introduction

Humanity have been using plants directly or indirectly as major sources of medicines for thousands of years. Many metabolic compounds, uniquely produced by plants for protection against their own pests and diseases, also have important therapeutic or preventive effects against human diseases. Such plant medicinal extracts used by our ancestors were mostly orally or topically administered.

The turn of the 21st century witnesses an unprecedented rapid accumulation of knowledge, especially on the frontiers of human science. The advancement of genomic information and genetic modification technology enables scientists to understand the mechanisms of plant metabolite production more precisely; this enables the extraction and utilization of the metabolites as medicines more effectively. In addition, it is now possible to insert bacterial, human, or other nonplant genes into crop plants and use the plants as biological factories for pharmaceutical production. In this review, we briefly describe the mucosal immunization strategy and its application in prevention of infectious diseases. We present advantages of plant-based vaccine production systems with emphasis on potential use for mucosal immunization of humans and animals. We also review the current achievements on plant-produced antigens and their efficacies in animal test systems. The relative advantages and disadvantages of various plant tissues and organs that are used for vaccine delivery or production are also discussed. Finally, we summarize important factors essential for a successful plant-based antigen-production system, using maize as a case study.

18.2
Mucosal Immunization

The first vaccination is attributed to Edward Jenner in 1796, when he used the heterologous agent vaccinia virus (cowpox) to protect James Phipps from variola (smallpox). The second major development is attributed to Louis Pasteur's serendipitous discovery that infectious agents could be attenuated to lose pathogenicity, yet still be

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used to protect against an infectious agent, beginning with fowl cholera. Pasteur's discovery during the 'Golden Age of Microbiology' (during which infectious agents for many known diseases were identified) gave birth to the search for protective vaccines [1]. Development of vaccines during most of the 20th century focused on parenteral (injectable) vaccines, with the notable exception of the oral polio vaccine. Attenuated live vaccines, like the oral polio vaccine, were successful because the attenuated organism could replicate to supply adequate immune stimulation, which was lifelong. However, the possibility of disease due to reversion of attenuated organisms or by attenuated organisms in immunocompromised individuals is a major concern and has limited their use.

The reasons for the rapid development of parenteral vaccines are numerous and reviewed in [1] and [2]. Killed (inactivated) or subunit vaccines are safer, yet need to be administered at a much higher concentration and repeatedly to induce an adequate immune response without adjuvants. Overall, however, adjuvants for parenteral killed vaccines were developed more rapidly than those for oral vaccines.

18.2.1 Vaccination Strategies for Infectious Diseases

The degree of protection afforded by a vaccine depends on the nature of the infectious process, as well as the components and route of administration of the vaccine. For example, *Clostridium tetani* grows within tissues and induces spastic paralysis by production of tetanus toxin. The best vaccination strategy is to stimulate systemic B-cell antibody production of a type that can diffuse into tissues and neutralize the toxin (IgGs). *Vibrio cholera* also causes disease by production of a toxin. However, that toxin affects gut epithelial cells from the lumen side of the gut and requires antibodies that can neutralize toxins at the mucosal surface (IgA). To develop appropriate vaccines for viral diseases, it is also important to produce a vaccine that neutralizes the virus at the level of infection. For example, rabies virus is often transmitted by the bite of a rabid animal directly into tissues and must be neutralized as it passes from one infected neuron to the next on its way toward the brain; in this case IgG is required. The best vaccine for an organism like polio, which enters the body via the gastrointestinal tract, is one that induces mucosal antibodies to neutralize the virus before it can adhere to the mucosa (IgA). However, polio is an interesting example, because high concentrations of systemic antibodies (in sera) induced by inactivated polio vaccine (IPV) can prevent paralytic polio. IPV, however, does not prevent wild polio virus from replicating in the gut and being shed to infect nonimmunized individuals. Thus, induction of IgG alone is not sufficient to control this disease in areas where wild polio virus still exists [1].

There is a need for vaccines that can elicit adequate immune responses systemically, mucosally, or both. Numerous organisms cause infections by breaching mucosal surfaces such as the gut, lungs, nasal/sinus surface, or vaginal tract, which could be prevented by neutralization at the mucosal level. Examples of diseases caused by such organisms are found in animals and humans. Organisms that are particularly problematic at this time in history include rotavirus, respiratory syncytial virus

(RSV), Norwalk virus, porcine reproductive and respiratory syndrome (PRRS) virus, the newly identified sudden acute respiratory syndrome (SARS) virus, and diarrhea-causing toxins from *Vibrio cholera* and *Escherichia coli*, which cause cholera and travelers' diarrhea, respectively. In all these instances a robust specific IgA antibody response at the appropriate mucosal surface may be important for neutralization of the infectious agent and prevention of disease pathology. Because most parenteral vaccines induce a primarily systemic IgG antibody response, vaccination strategies that induce an IgA response on the mucosal surfaces are currently an area of very active research [3].

18.2.2 Mucosal Immunization vs. Parenteral Immunization

To obtain a robust, specific antibody response with high affinity, it is necessary to stimulate T-helper cells. T-helper cells respond to the protein portion of the antigen when it is presented by antigen-presenting cells [4]. Upon activation, the T-helper cells can differentiate into a T-helper 1 (TH1) phenotype or a T-helper 2 (TH2) phenotype. The TH1 and TH2 cells produce distinct partially overlapping arrays of cytokines, which result in activation of different immune cells and mechanisms. Both T-helper phenotypes can help B cells to produce antibody, but the antibody induced is of different isotypes with different functions. TH1 cells induce B cells to make IgG2a, along with activating macrophages to clear intracellular organisms and activating T-cytotoxic cells to more efficiently kill virus-infected cells. TH1 activation induces an inflammatory response by activating macrophages, which are capable of producing proinflammatory cytokines (IL-1, IL-6, TNF-α) as well as IL-12, which can enhance TH1 activation. TH2 cells induce B cells to produce IgM, IgG1, IgA, and IgE, as well as activate eosinophil and mast cell production. In addition, TH2 cells produce IL-10 and IL-4, which are considered anti-inflammatory cytokines because they can suppress macrophage-induced inflammatory responses [4].

Activation of a TH1 or TH2 phenotype is partly determined by the presence of distinct cytokine microenvironments. The development of TH1 cells proceeds in an environment containing IL-12 and IFN-γ, while the presence of IL-4 and IL-10 can induce TH2 cell differentiation. Often, activation and differentiation of both TH1 and TH2 phenotypes occurs, with one type predominating [4].

Interestingly, immunization at mucosal surfaces most frequently induces a TH2 type response, due to the presence of IL-4 and IL-10 in the mucosal microenvironment. Use of different adjuvants can also induce T-helper cell differentiation towards TH1 or TH2 phenotype [15].

Taken together, mucosal immunization can result in activation of TH2 cells and production of secretory IgA, which is needed for neutralization of viruses, toxins, and bacterial adhesins that attack at the mucosal surfaces. In addition, some immune responses that result in pathogenic inflammatory processes may benefit from mucosal immunization strategies. For example, oral immunization of non-obese diabetic mice with an insulin-CT conjugate produced anti-insulin antibodies (type IgG1), reduced insulitis (inflammation of pancreatic islets), and delayed the onset of diabetes [5].

At times, a TH1 response is necessary to resolve disease. Lepromatous leprosy, the severe form of leprosy, is a result of insufficient TH1 response or activation of macrophages. Tuberculoid leprosy, the milder form of the disease, is related to activation of TH1 cells and results in activation of infected macrophages to clear the infectious agent, *Mycobacterium leprae.*

18.2.3 Mucosal Immunization and Adjuvants

Orally delivered mucosal vaccines must overcome three identified hurdles [6]: (1) effective delivery of antigen to the mucosal immune system (they often must pass through the acidic, enzymatically active digestive system); (2) the need for very large amounts of antigen or an effective adjuvant, and (3) production of protective immunity at the desired site (mucosal, systemic, or both).

An effective delivery system for oral administration of antigen must get past the digestive enzymes in saliva and the stomach. Oral immunizations of many mouse models use a combination of very high antigen concentrations and sodium bicarbonate to neutralize the acid proteases of the stomach [7]. The practical application of sodium bicarbonate 30 min prior to administering an oral antigen to humans is limited to research studies, because it complicates the ease of application sought in oral vaccines. Alternatively, production of the antigen in a live lactic acid bacteria or encapsulation of antigens in microspheres of synthetic polymer or water-soluble alginate or chitosan [8–10] can also protect the antigen.

To reduce the need for large amounts of antigen, effective and safe adjuvants for mucosal systems are needed. Only in the last decade of the 20th century have immunologists identified possible mucosal adjuvants, in the form of cholera toxin (CT) from *Vibrio cholera* and heat-labile toxin (LT) from enterotoxogenic strains of *E. coli.* However, although the whole toxins are powerful adjuvants, they also are toxic if used in their entirety. Use of the binding subunit (B) of cholera toxin (CT-B) and *E. coli* heat-labile toxin (LT-B) reveal that LT-B is a better adjuvant [11, 12]. However, alteration of whole LT to reduce or abrogate the toxicity of the A subunit appears to enhance its adjuvanticity [12, 13]. More interesting than the amount of adjuvanticity associated with various LT derivatives is that different derivatives appear to drive the immune system to either a TH1- or TH2-dominant immune response [14–16], the implications of which are addressed above. In addition, excessive adjuvanticity may be undesirable [17]. Whole CT is a powerful adjuvant, but when it is administered with peanuts or ovalbumin, it can induce allergic Type 1 hypersensitivity [18, 19].

Targeting the immune response to the appropriate mucosal site when using oral vaccines appears to be straightforward, because immunization at any mucosal site yields cross protection of all mucosal sites, as well as robust systemic immunity. Antibodies to antigens administered orally with an adjuvant can be found in intestinal excretions, lung washes, and nasal washes, as well as systemically [14]. In addition, researchers have used intranasal delivery of antigens to induce antibody responses in intestinal and respiratory mucosa, as well as systemically [20]. It appears that intranasal administration of antigen requires less antigen per dose, but also re-

quires coadministration of an adjuvant for a robust immune response. However, questions have arisen as to the safety of using adjuvants near the olfactory bulb and central nervous system during intranasal administration [17].

18.3
Plant-derived Edible Vaccines

The term 'plant-derived edible vaccines' refers to antigens produced in edible parts of a plant, such as fruits, leaves, roots, or tubers. Vaccines expressed in nonedible plant tissues may also be orally administered after processing. The term 'edible vaccine' may have created the misconception that individuals would be able to grow and administer their own vaccines without the supervision of pharmaceutical and health professionals. Ultimately, for practical applications, plant-expressed vaccines will require some form of processing to produce uniform doses for administration by oral and other routes. These products will be regulated, as all pharmaceuticals are, by government agencies such as the U.S. Food and Drug Administration (FDA), and their administration will require supervision by health care professionals. Also, not all plant-derived vaccine antigens are edible; they may be effective only when administered by the appropriate method (topically; intramuscularly, i.m.; intraperitoneally, i.p.; or subcutaneously, s.c.).

18.3.1
Advantages of the Plant-based System

Producing pharmaceuticals in plants has many potential advantages, which have been reviewed extensively [21–25]. Safety is a key attraction, since the production of recombinant proteins in plants minimizes potential human or animal pathogen and toxin contamination, such as occurs when a recombinant protein is produced in microbial or animal cultures. The potential for cost reduction is also enormous. This is especially significant for oral vaccines, because relatively large amounts are required. Plants are potentially more economical than industrial fermentation or bioreactor facilities [26], and the amounts of protein produced by plants are comparable to those produced by industrial approaches [27]. Scaling up production does not require huge investments up-front in setting up facilities, unlike the conventional systems. Often, technology already exists for harvesting and processing plant and plant products on a large scale. Purification and its associated costs may not be needed when the recombinant protein is produced in plant tissue that can be used as food or feeds (edible vaccine). The potential for oral administration and the convenient storage when the vaccines are produced in seeds and tubers could dramatically reduce the costs associated with syringes and needles and the requirement for cold storage. Elimination of needles in most vaccination programs will also cut back the risk associated with reuse of needles in some developing countries. Seed and tubers are convenient storage organs for relatively simple, cheap antigen storage. Plants offer enough flexibility that the recombinant proteins can be targeted to intracellular com-

partments where they are more stable. Alternatively, they can be expressed directly in those compartments or in organelles such as chloroplasts [23].

From a biological perspective, the plant's protein-production system can perform post-transcriptional modifications, such as glycosylation, which are essential for the biological activity of some proteins. This is certainly an advantage over the use of microorganisms such as bacteria and fungal systems, in which problems of differences in codon usage, metabolic pathways, protein processing, and formation of inclusion bodies often arise.

18.3.2 Transient and Stable Systems for Production of Plant-derived Proteins

Proteins can be derived from plants by two major approaches: transient expression using modified viruses or stable transformation of the nuclear or plastid genome. In transient expression, DNA encoding the protein of interest is introduced into the plant cell, where it is recognized by the transcription machinery and expressed. Stable transformation, on the other hand, involves introduction into the plant cell of a foreign gene and its stable integration into the plant genome. Both methods have their advantages and disadvantages, which have been reviewed extensively [28]. Viral vectors are usually more appropriate for expressing products in leaf tissue of high biomass producing plants, such as tobacco, ultimately for extraction and purification. Two major viral expression systems have been engineered: the tobacco mosaic virus (TMV)-based system for tobacco and the cowpea mosaic (CPMV) system for cowpeas. The expression of foreign protein typically occurs 2–4 weeks after inoculation, and production of antigen is rapid as the engineered virus spreads and multiplies throughout the plant [29]. Transient expression usually achieves substantially higher levels of protein than does stable transformation, but it requires inoculation of large numbers of plants with the recombinant virus. With viral vectors, the inserted gene is often lost over time, which is important for containment of the transgene.

Stable transformation offers multigenerational stable expression, compared to the use of viral vectors. The transgenes are incorporated into the plant genomes (in the nucleus or chloroplasts) and can be inherited by next generations. Expression of recombinant proteins by this method usually results in moderate to low expression, but higher levels of expression have been attained by using a number of regulatory sequence elements, such as strong promoters [30], optimized codon usage for plant expression [31], translation-enhancing leader sequences at the 5′ end, polyadenylation signals at the 3′ end [32], and microsomal retention sequences [33]. It is also possible to produce transplastomic plants with transgenes integrated in the chloroplast genome [23, 34] or into chloroplast-derived chromoplasts [35] to express high levels of foreign proteins. The ability in some species to sexually cross transgenic lines adds the flexibility of being able to express multiple proteins in the same plants (antigen stacking). However, stable transformation requires efficient transformation techniques and meticulous selection and breeding of the high-expressing transgenic lines.

18.3.3
Choice of Plants and Plant Tissues

Several practical issues must be considered in the production of foreign proteins in plants, including selection of appropriate crop plants and plant tissues in which to produce and deliver the desired amount of antigenic protein. The choice of plant usually depends on its capacity for transformation or infectivity with genetically engineered viruses, and to date, scientists have often made this decision based on the system with which they are most competent. Some plants, such as tobacco, potato, and tomato, are more amenable to tissue culture and transformation, and others, such as corn, soybean, and wheat, are relatively more recalcitrant to *in vitro* manipulation. The desired method of administering the recombinant protein also plays an important role.

Tobacco is most easily cultured and transformed, and thus has been used as a test system for many antigens. Tobacco produces large volumes of green tissue, and with several crops harvested per year by cutting the foliage, annual yields can be in excess of 100 metric tones (MT) per hectare [23]. However, tobacco is not palatable, and toxic alkaloids in the leaf tissue are not only incompatible with oral delivery but also necessitate costly protein purification prior to administration. Alfalfa is a palatable crop with a high leaf protein content and high annual yields of green leaf tissue. In this crop, pharmaceuticals could be administered in fresh or dried leaf or as leaf extract. Several proteins have been produced in alfalfa, including foot-and-mouth virus (FMDV) antigens [36]. Lettuce is a salad crop consumed raw, in which antigen production has been reported [37]. However, the inherently low protein content of its leaves presents a problem for practical protein production.

When the idea of edible vaccines was first articulated, people envisioned that a crop like bananas, which are highly palatable raw, would be the ideal system for edible vaccine production. Bananas are also cultivated in many parts of the world, including the developing countries where low-cost vaccines are most needed. However, inefficient transformation, inadequate information on gene expression, fruit-specific promoters, and the difficulty and expense of cultivation in the greenhouse has hampered pharmaceutical production in this crop. Tomato fruits are edible raw, and the transformation system, industrial greenhouse culture, and processing are well established, making this crop an attractive choice. However, the inherently low protein content may result in poor yields of recombinant protein, and the acid nature of the fruit may be a drawback especially for children's vaccines (H. Mason, personal communication).

Potato is another preferred crop because, not only is it convenient to transform, it also provides an edible tissue for use in immunological studies and for convenient storage of the protein. Some of the most comprehensive antigen production and immunological studies, including human clinical trials, were carried out with transgenic potato-derived antigens such as LT-B [31], CT-B [5], HBsAg [38], and Norwalk virus capsid proteins [39]. However, potato is not highly palatable and requires cooking, which may denature the antigens. Arakawa et al. [5] showed that cooking potatoes reduced the G_{M1}-binding capacity of CT-B by about 50%.

Cereals and seed legumes offer an attractive option for edible vaccines, because production of antigens in seeds makes for convenient administration of edible vaccines and simplified preparation of antibodies. These crops are particularly suitable for production of edible vaccines for humans and livestock, because many are natural components of food and livestock feeds. Yields of cereal grains such as wheat, rice, and maize are less abundant (3, 6, and 12 metric tonnes per hectare, respectively) than that of the green tissues of tobacco and alfalfa [23]. However, high-yield seed production in grains greatly simplifies the scale-up process. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized into protein bodies, specialized vacuoles in mature seeds. These provide a stable environment devoid of significant amounts of enzyme activity prior to germination. Promoters play an important role in tissue-specific expression. Seed-specific promoters are preferred for oral administration, simplified protein extraction, and where the presence of the antigen in growing tissues is detrimental to plant vigor [31] (R. Chikwamba, unpublished). Compared to proteins extracted from green tissues such as tobacco leaves, proteins extracted from seeds contain less contamination by plant secondary metabolites, which are undesirable for purification. Production of recombinant proteins in seeds has the added advantage of long-term storability of functional proteins in grain. Stoger et al. [40] reported no significant decrease in the levels of scFV antibody after storage of the transgenic rice seeds at room temperature for 6 months.

18.4
Plant-expression Systems for Antigen Production

Antigen production has been reported in many plant species. Table 18.1 is a summary of selected key antigens that have been produced in plants and tested in animal systems. The work presented in these reports demonstrated that plants, especially food crops, can be used to produce functional antigens in specific tissues. When such plant-derived antigens were administered directly by feeding or by injection of extracts in animals, specific antibodies against the antigen could be detected in immunized animals. In some instances, partial protection against a disease or toxin was achieved. The work of Tacket et al. [41] is notable in that it represents the first trial of a plant-produced vaccine in humans. The data on antibody responses was encouraging, comparing favorably with the responses obtained after administration of ETEC cells. Other human trials followed with the Norwalk virus capsid protein and the HBsAg (Table 18.1), and results published to date indicate a strong potential for plant-derived vaccines.

These pioneering studies have demonstrated the feasibility of using plants as a future production and delivery system for oral vaccines. However, for most of the antigens produced to date, several problems have been encountered. The common ones include poor gene expression in desired tissues, unstable gene expression over generations, and poor immunogenicity upon oral administration. To qualify as an efficient production system, the quantity of plant tissue constituting a vaccine dose must be of a practical size for consumption, making it critical to achieve high expres-

Tab. 18.1 Selected antigens produced in plants and tested in animal and human systems.

Pathogen	*Antigen*	*Plant species*	*Test system*	*Antigen administration*	*Observed responses*	*Ref.*
Human Pathogens						
Enteretoxigenic *E. coli* (ETEC)	LT-B	potato	mice	oral	Serum and mucosal Abs, partial protection	31
		potato	humans	oral	Serum and mucosal Abs	41
		maize	mice	oral	Serum and mucosal Abs, partial protection	98
		maize	mice	oral	Serum and mucosal Abs, partial protection	46
		tomato	mice (dam & pup)	oral & passive	Serum IgG in both dam and pup	114
Vibrio cholerae	CT-B	potato	mice	oral	Serum and mucosal Abs, partial protection; booster responses in previously immunized mice	115
Hepatitis B	HBsAg	lettuce	humans (naïve)	oral	Serum Abs	37
		potato	mice	oral	Serum and mucosal Abs	38
		potato	humans (immunized)	oral	Boosting Ab response	*)
Norwalk virus	NVCP	potato	mice	oral	Serum and mucosal Abs	78
		potato	humans	oral	Increased IgA and IgG Ab-secreting cells	39
Rotavirus	CT-B/NSP4 fusion, CT-A/CF1 fusion	potato	mice	oral & passive	Serum and mucosal Abs, increased IL-2, IFN-γ, protection of passively immunized pups	116
Livestock Pathogens						
Transmissible gastroenteritis virus (TGEV)	full length and N-terminus of S glycoprotein	arabidopsis	mice	ip or oral	Elicit neutralizing Abs	117
		potato	mice	ip or oral	Elicit neutralizing Abs	118
	S glycoprotein	tobacco	pigs	ip	Elicit neutralizing Abs	30
		corn	pigs	oral	Serum and mucosal Abs, partial protection	98, 106
Foot-and-mouth disease virus (FMDV)	VP1 capsid protein peptide	alfalfa	pigs	ip or oral	Serum and mucosal Abs, partial protection	36
		potato leaf	mice	ip	Serum and mucosal Abs, partial protection	119
	VP1 epitope fused with *gus* gene	alfalfa	mice	ip	Serum and mucosal Abs, complete protection	120
Canine parovirus (CPV)	linear antigenic peptide from VP2 capsid protein	arabidopsis	mice	ip or oral	Serum IgG Ab	121

*) H. Mason et al., personal communication

sion. Transgenic lines should therefore have high expression levels of the gene of interest in appropriate tissues. In addition, the recombinant proteins produced in the plant should be structurally and functionally similar to their counterparts produced in bacterial, yeast, or mammalian systems. Moreover, the transgenic plants should be fertile and transmit and express the transgene predictably over generations. Several strategies have been adopted to address these issues. In general, improvement of protein production in plants can be achieved at three levels transcription, post-transcription, and post-translation.

18.4.1 Transcriptional Level

18.4.1.1 Choice of Promoters

Promoters are available for tissue- or organ-specific, temporal, and/or induced expression. Promoter elements can be constitutive or tissue-specific, allowing developmental control of expression of the novel protein. Strong promoters are often used to achieve high gene expression. Promoters that work well in dicot plants do not necessarily work equally well in monocot plants and vice versa. Some of the promoters used for transgene expression in corn include the maize ubiquitin promoter [42], the cauliflower mosaic virus 35S RNA (CaMV 35S) promoter [43], and the maize seed-specific 27kDa γ zein promoter [44]. Both maize ubiqutin promoter and CaMV 35S promoter (in its tandem duplicated form) are considered strong constitutive promoters for transgene expression in maize. Transgenes driven by these promoters are expressed in most maize tissues, such as leaves, roots, pollen, and silks, and can be detected at almost all stages of plant development. In corn seed, the gene expressions driven by these promoters are strongly localized in embryos and aleurone layers (Chikwamba, unpublished). Maize 27-kDa γ zein promoter, on the other hand, is a seed-specific promoter. The transgene expression can be detected only in corn seed and predominantly in seed endosperm. Both constitutive and seed-specific promoters have been used successfully in production of nonplant proteins. Hood et al. [45] achieved high avidin expression by using the constitutive maize ubiquitin promoter. However, expression of a foreign protein with a strong constitutive promoter can have a detrimental effect on cell processes and plant growth. Mason and coworkers [31] noticed stunting of transgenic potato plants expressing a high level of the *Escherichia coli* heat-labile B subunit (LT-B) under the control of the enhanced CaMV 35S promoter. A similar effect was also observed by our group. When the same gene was introduced into maize plants, the developmental characteristics of the transgenic plants, such as height and fertility, especially in those plants showing high LT-B expression, were significantly compromised [46].

18.4.1.2 Transcriptional Gene Silencing

Transcriptional gene silencing (TGS) refers to transgene shutdown due to methylation of the promoter [47–50]. In plants, multiple copies of a transgene, especially of those with partial sequence homology to a gene already present in the plant cells, often cause methylation of its promoter and consequently, TGS. It is important, there-

fore, to avoid multicopy integration of a transgene into a plant genome during transformation. Two major methods have been widely used for plant nuclear transformation: *Agrobacterium tumefaciens* (a soil bacterium with natural ability to deliver DNA segments into the plant cell) [51] and the biolistic gun [52]. Although *Agrobacterium* can transform most dicot plants, the biolistic gun method has been effective in transforming more-recalcitrant cereal and legumes crops [53]. However, the biolistic method often introduces a greater number of DNA segments into the plant cell than the *Agrobacterium* method. The higher copy number of transgene integration can result in lower gene expression, due to transgene silencing [54]. Unfortunately, there is currently no efficient way of predicting gene expression from a given transgenic plant line other than by screening a large number of putative candidates. The general rule is to use *Agrobacterium*-mediated transformation if the plant of interest is amenable to this method or to select low copy number transgenic events resulting from the biolistic method. Use of a low quantity of DNA in coating gold particles for bombardment in the biolistic transformation also helps to achieve low copy number transgenic events (L. Marcell, personal communication). Conducting effective molecular and bioassay analysis on a large number of transgenic lines early in the transformation process is critical to the overall success of obtaining best transgenic lines.

18.4.2 Post-transcriptional Level

18.4.2.1 Introns

Many plant genes naturally contain intron sequences that positively affect gene expression by stabilizing the mRNA transcript and allowing its effective translation. In monocot plants such as corn, intron sequences placed between the promoter and a transgene may increase its level of expression [55–58]. The maize ubiquitin and Adh2 genes are examples. Callis et al. [55] showed that inclusion of introns in the 5′ untranslated region (UTR) may correlate with up to a 100-fold increase in protein accumulation. However, reverse genetics studies have also shown that introns naturally occurring within genes can be lost without loss of gene function [59]. The maize zein proteins are the most abundant proteins in maize seed [44]. However, the gene encoding for the 27-kDa γ zein does not contain an intron. When an intron from the maize *ADH* gene was placed between the γ zein promoter and the *gus* marker gene, no enhancement in GUS activity was detected compared to the construct in which no intron was included (Chikwamba, unpublished).

18.4.2.2 mRNA Stability and 3′ Terminator

In eukaryotic cells, one of the most important forms of control of gene expression is regulation of mRNA stability [60]. Appropriate mRNA 3′-end regions can strongly influence the level of transgene expression in plant cells [61]. In addition, the presence of instability elements within the mRNA and inadequate 3′-end processing of the mRNA in the nucleus can decrease the mRNA half life and consequently decrease mRNA levels [61, 62]. Premature polyadenylation within transgenes also results in lower transgenic mRNA levels [63]. No consensus sequences functioning as stabiliz-

ing determinants have been recognized to be responsible for the long half-life of an extremely stable transcript in plant systems. On the other hand, the presence of adenylate/uridylate-rich elements (AUUUA) in transcripts indicates that they are selectively targeted for rapid decay [64]. Reporter transcripts containing 11 repeats of the AUUUA motif in their 3′ untranslated regions are degraded more rapidly in stably transformed tobacco cells and accumulate to a lower level in transgenic tobacco plants than those of the control construct [65].

Recently, it was recognized that gene silencing also occurs at the mRNA level: post-transcriptional gene silencing (PTGS), also called 'cosuppression'. Introduction of transgenes or viral genes into plants can cause silencing of an endogenous gene having a sequence homologous to the introduced gene [66–69]. In PTGS, the transcript of the silenced gene is synthesized but does not accumulate because it is rapidly degraded. To avoid such a silencing effect on a transgene, it is important that no unnecessary homologous sequences are introduced into the plant. As well, low-copy-number transgene integration will likely give rise to better gene expression (H. Shou, personal communication).

18.4.2.3 An Optimal Start Context and 5′-end Enhancer for Translation

The process of translation is composed of three phases: initiation, elongation, and termination. Initiation is considered to be the rate-limiting step in the translation of most mRNAs [70]. It is also the phase most often subject to regulation. Most higher plant mRNAs are capped, have AU-rich leaders that reduce the potential for secondary structure formation, are less than 200 bp in length, and begin translation at the first AUG codon [71]. This means that, in the construction of transgenes, the presence of AUG codons upstream of the authentic initiation codon should be avoided. Extensive surveys of more than 5000 plant genes for their AUG context sequences indicated that 80% of the sequences had purines present at the –3 and +4 positions, the two most influential positions for efficient translational initiation [72]. It is also interesting that the context of the AUG codon in dicot mRNAs is aaA(A/C)aAUGGCu, which is similar to the higher-plant consensus caA(A/C)aAUGGCg, and monocot mRNAs have c(a/c)(A/G)(A/C)cAUGGCG as a consensus that exhibits an overall similarity to the vertebrate consensus [72].

The 5′-end untranslated region can also serve as a site for regulatory sequences that actively control the rate of translational initiation. Most of those examined and used in plants have come from viral mRNAs, e.g., the Ω leader (68 bp) of tobacco mosaic virus [73], AMV leader (36 bp) of alfalfa mosaic virus RNA 4 [74], and TEV leader (144 bp) of tobacco etch virus [75]. The translation-enhancing effect of the Ω leader on transgenes seemed more profound in dicots than in monocots [76]. Mason et al. [77, 78] demonstrated enhanced expression of antigens such as hepatitis B surface antigen (HBsAg) and Norwalk virus capsid protein (rNV) in transgenic tobacco and potato when the TEV leader was included in the constructs.

18.4.2.4 Codon Usage

Most amino acids are specified by more than one codon. For example, the codons GCU, GCC, GCA, and GCG all code for the amino acid alanine. However, genes of

different organisms use these codons with different frequencies. Only a subset of the total number of codons is used by each species. The pattern of codon usage in plants is different from that of other organisms. As well, codon usage in dicots is different from that of monocots [79]. The most important difference in codon usage between dicots and monocots is the G+C content in the third position of a codon [80]. For example, the average %(G+C) in the first, second, and third positions of the codon in maize are 57%, 43%, and 61%, respectively, but in tobacco they are 51%, 40%, and 39%, respectively (http://www.kazusa.or.jp/codon). Altering the composition of the heterologous cDNA to suit the plant's pattern can increase the rate of translation [21]. When the codon composition of a bacterial gene was modified to suit the preferred codon usage in tobacco, the expression level of the transgene was greatly enhanced [81]. This approach has been applied to other plant species, such as tomato, potato, rice, cotton, eggplant, and maize [82–89]. Mason et al. [31] synthesized the LT-B gene with a codon bias optimized for expression in potato and maize. They observed that the synthetic LT-B gene achieved higher LT-B expression in transgenic potatoes than did the unoptimized gene. The same gene was also expressed with success in maize [46].

18.4.3 Post-translational Level and Beyond

18.4.3.1 Targeting and Retention Signals

Subcellular localization is important for biological activity. Proper folding and stability of plant-synthesized proteins is critical and depends on the cellular environment where the protein is expressed and stored. Sometimes it is not desirable to target a protein to an intracellular compartment, because that may complicate extraction. On the other hand, expression of heterologous proteins in the cytoplasm could also be problematic, due to rapid degradation of foreign proteins by plant defense mechanisms, or because the accumulated protein may be toxic to plant cells. In plants, the default pathway for proteins transported through the ER is secretion, hence the proteins were localized in the extracellular space. The expectation was that higher levels of foreign protein would be obtained if the newly synthesized protein were targeted to the extracellular compartment. Alternatively, the protein could be sequestered into a compartment or organelle that may allow higher levels of protein accumulation.

Signal peptides have been used to influence the levels of protein accumulation and its spatial distribution within transgenic plant tissue. The barley α-amylase signal peptide was used to target avidin [45] and aprotinin [90] to the extracellular matrix of maize cells. Yields of foreign proteins have reached up to 2% (for avidin) and 0.069% (for aprotinin) of the aqueous soluble extracted protein from dry maize seed. Avidin targeted to the cytosol was completely toxic to engineered maize cells [45].

Targeting recombinant antibodies to the secretory pathway could significantly increase antibody yield compared to targeting to the cytosol [91]. It was observed that, although targeting proteins to the intercellular space beneath the cell wall increased expression levels, their retention in the lumen of the endoplasmic reticulum could result in 10- to 100-fold higher yields of recombinant antibody single chain Fv (scFv) in potato tubers, as well as tobacco leaves and seeds [91–93].

The SEKDEL (Ser-Glu-Lys-Asp-Glu-Leu) or KDEL (Lys-Asp-Glu-Leu) amino acid motifs have been used to retain the foreign proteins in the ER. The SEKDEL amino acid motif binds to the SEKDEL receptor in the ER [94]. Haq et al. [33] showed that the SEKDEL motif resulted in significantly higher LT-B accumulation compared with the LT-B gene without SEKDEL. These authors proposed that the cellular compartmentation of the SEKDEL protein could have facilitated oligomerization of LT-B monomers into pentamers detectable by ganglioside-dependent ELISA.

In maize, the effect of SEKDEL on protein levels was seen in conjunction with certain promoters. Our early work [95] showed that, when a constitutive promoter (CaMV 35S) was used to drive the LT-B gene, the inclusion of the SEKDEL signal sequence did not enhance the LT-B level in maize plants (callus tissue or seed). However, when the sequence encoding SEKDEL was included in a construct in which the LT-B gene was driven by the 27-kDa γ zein promoter (a seed-specific promoter), LT-B protein levels were significantly increased, up to 13 fold compared to the highest LT-B expression from constructs carrying no SEKDEL.

18.4.3.2 Stability of Gene Expression and Transmission of the Transgene

Differences in gene expression between transgenic events have been reported, perhaps due to different gene copy numbers and to the relative position of transgene insertion in the genome of the transgenic plants [66, 68, 96]. Variability in gene expression that was not attributable to any obvious genetic or environmental factors has also been observed. Molecular mechanisms causing unstable expression of foreign genes are not clearly understood, but the presence of multiple copies of the transgene and large variations in the amount of steady-state mRNA among individuals from the same transgenic event suggest that repeat-induced transgene silencing could be involved in regulation of foreign gene expression in transgenic plants [90]. Only those events expressing the desired levels of gene expression should be pursued. Variability in gene expression between generations has been observed [45, 90, 95]. The phenomenon of gene silencing in transgenic plants occurs in later generations of highly expressing transgenic lines. In our LT-B corn study, we monitored LT-B levels under greenhouse and field conditions over three generations. Significant variability in gene expression was observed between transgenic events and between plants within the same event. A maximum of 0.3% LT-B in TAEP (total aqueous extractable proteins) was measured in R_3 seed of a transgenic line carrying a CaMV 35S promoter/LT-B construct. In R_3 seed of a transgenic line carrying the γ zein promoter/LT-B construct, up to 3.7% LT-B in TAEP could be detected [95].

18.5 Maize as Production and Delivery System

Maize has been demonstrated to be an effective expression system for functional proteins of prokaryotic [46, 90, 97, 98], viral [98], and eukaryotic [45, 99] origin. The commercialization of β-glucosidase [97], aprotinin [90], and avidin [45] has demonstrated the viability of this expression system.

There are several benefits to producing antigens for use as edible vaccines in maize. Maize is a major food and feed crop worldwide, which is well tolerated by both humans and animals. In addition, maize yields are high and its seed is a natural protein storage site, which can be harnessed as a novel-protein production factory for direct use as feed and food. According to Hood and coworkers [27], maize can be used to produce foreign proteins at rates of more than 2 kilograms per acre at a cost of a few cents per milligram. Breeding techniques can be used in this crop to enhance foreign protein expression [45, 95, 97, 98] and to stack several antigens in the same crop. Antigens produced in corn can be conveniently stored and transported in dry grain. Production of an efficacious maize-based oral vaccine will provide effective and less expensive control of some important gut pathogens, for which mucosal immunity is important for protection. Moreover, the infrastructure for large-scale grain production and seed processing is in place for the crop in the U.S. The production of oral vaccines in maize grain or silage presents an opportunity to add value to unprocessed maize and to increase income for farmers. Finally, the genetic transformation methods are well established, allowing introduction of transgenes with reasonable efficiency.

We discuss here the production of the B subunit of the enterotoxigenic *E. coli* (ETEC) heat-labile enterotoxin (LT-B), and highlight some findings in the expression of a vaccine against transmissible gastroenteritis virus (TGEV) in transgenic maize seed.

LT-B is part of the heat-labile toxin (LT) produced by enterotoxigenic strains of *E. coli*, a leading cause of diarrhea in developing countries [100]. About 66% of enterotoxigenic strains of *E. coli* produce LT, and in half of these LT is the only toxin produced [101]. The bacterium is ingested in contaminated food or water and colonizes the gut, where it secretes toxins, including LT. LT is an 84-kDa polymeric protein composed of 2 major noncovalently associated, immunologically distinct regions or domains designated LT-A and LT-B. The A region (27 kDa) has the toxic enzymatic activity responsible for watery diarrhea [100]. LT-B is 55 kDa and consists of five noncovalently bound 11.6-kDa B subunits. These nontoxic B subunits are responsible for binding the protein to receptors on the surface of intestinal epithelial cells. Pentameric LT-B is responsible for binding the toxin to the host cell receptor, G_{M1} (galactosyl-N-acetylgalactosaminyl-sialyl galactosyl glucosyl ceramide), which is commonly found on the surface of eukaryotic cells. LT-B is a strong oral immunogen that could potentially be used as a component of a vaccine against ETEC diarrhea or as an adjuvant to enhance the effectiveness of coadministered vaccines [102].

Early work on LT-B expression in plants was reported in transgenic potato and tobacco plants [33]. In subsequent work a synthetic gene with codon usage optimized for expression in potato and maize was introduced into potato. Codon optimization resulted in increased expression of LT-B in potatoes [31]. Mice orally immunized with five 50-μg doses of LT-B over a 3-week period showed higher titers of anti-LT-B serum IgG and fecal IgA antibodies than in an earlier experiment [33]. These mice were partially protected from challenge with a 25-μg dose of LT. This work paved the way for subsequent work on LT-B expression in corn. To date, LT-B produced in potato and maize has been shown to be immunogenic in mice [31, 46, 98] and humans [41].

Antigens that are produced in maize seed can be directed to specific compartments within the seed. Expression can be directed to the endosperm by using endosperm-specific promoters or to embryo (germ) by using constitutive promoters as described in section 18.4.1.1. Endosperm and germ compose 83% and 11% of a corn kernel, respectively [103]. The major chemical components of the endosperm fraction are starch (88%) and protein (8%); the germ contains fat (33%), protein (18%), and starch (8%). The advantages of vaccine expression in germ are that it is rich in soluble proteins that are stable during storage, and it can be separated from other seed tissues to concentrate proteins and reduce dose size. Expression of vaccine in endosperm, on the other hand, may allow higher yields and ease of protein extraction, especially if the protein of interest can be separated together with the starch fraction. In practice, production of vaccine antigens in either tissue allows for processing of more palatable whole-corn snacks for humans or for production of unprocessed whole corn meal for livestock.

18.5.1 Antigen Production in Endosperm Tissue of Maize Seed

By using a codon-optimized synthetic LT-B gene [31], we have produced transgenic maize plants with LT-B expressed in either endosperm or germ of maize seed [46, 95]. We examined both seed-specific and constitutive expression using the maize 27-kDa γ zein and the CaMV 35S promoters, respectively, and also examined the effects of the SEKDEL endoplasmic reticulum retention motif. Ganglioside-dependent ELISA was used to determine the level of LT-B expression, because this technique detects only the correctly assembled pentameric form of LT-B which binds to gangliosides [100]. LT-B was analyzed in callus or ground whole kernel meal in a sodium phosphate buffer [46]. Constitutive expression resulted in levels of expression estimated at 0.01% LT-B in the total aqueous-extractable protein (TAEP) in R_1 seed and 0.3% in field-grown R_3 kernels of the best-performing line. The maize 27-kDa γ zein promoter, which directs endosperm-specific gene expression in maize kernels, showed higher expression and is discussed here in some detail.

Use of the maize 27-kDa γ zein promoter resulted in functional (ganglioside-binding) LT-B expression that was estimated as 0.07% in R_1 kernels of the selected highest-expressing transgenic event, which continued to increase in the R_2 and R_3 generations. Average individual ear expression was estimated to be about 3.7% LT-B in total soluble protein. Inclusion of both SEKDEL and the γ zein promoter resulted in a substantial increase in LT-B levels. Such an effect was not observed when this ER retention signal was combined with LT-B under the regulation of the constitutive CaMV35S promoter [95]. Significant variability of LT-B expression was noted between the different transgenic events, but perhaps most importantly, between plants within the same transgenic event. This variability could have important implications for the production of lines with high, stable, predictable levels of an antigenic vaccine in transgenic corn [95].

In our experiments, accurate quantification of LT-B in corn meal depended on the fineness to which the meal was ground, the length of time the meal was incubated

in extraction buffer, the presence of detergents such as Triton-X100 in the extraction buffer, and suitable dilution of the seed extracts for the ELISA procedure. The presence of a high concentration of Triton in seed extracts inhibited the ELISA reaction and thus could lead to underestimation of the LT-B expression level (R. Chikwamba, unpublished).

An intricate association was observed between LT-B and starch, which was shown by the persistence of LT-B in starch samples subjected to digestion by proteolytic enzymes. Digestion of whole kernel meal showed that the LT-B associated with maize tissues was 10 times more resistant to peptic degradation than recombinant LT-B mixed with an equal amount of nontransgenic corn meal [122].

Feeding experiments were conducted to determine the immunogenicity of the corn-derived LT-B. BALB/C mice were given four 1-g doses delivering 10 μg LT-B per dose in one gram of corn pellet on days 0, 3, 7, and 21. For positive controls, we used 10 μg of soluble recombinant LT-B from bacteria (John Clements) mixed with 1 g of nontransgenic corn; and for negative controls, we fed mice 1 g of nontransgenic corn. Analysis of sera and feces showed the induction of anti-LT-B and anti-CT-B serum and mucosal antibodies, which increased over the immunization period. The corn-derived LT-B was more immunogenic than an equivalent amount of soluble recombinant LT-B (positive control group). The orally immunized mice were then challenged with 25 μg of LT or CT toxins. The mice that received transgenic or recombinant LT-B showed less fluid accumulation in the gut than the nonimmunized controls fed with nontransgenic corn. Mice immunized with transgenic corn had the least fluid accumulation, showing that the transgenic corn was a more effective than recombinant LT-B from bacteria. We speculated from these observations that the natural bioencapsulation of LT-B in transgenic maize resulted in slow release of LT-B and perhaps prolonged exposure of the immune system to LT-B than with the recombinant (soluble) LT-B. Similar observations were made with yeast-derived recombinant and potato-encapsulated HBsAg [38]. We concluded from this work that LT-B generated in maize endosperm was a competent immunogen, and further studies will determine the potency of this antigen in humans.

18.5.2
Antigen Production in Embryo (Germ) Tissue of Maize Seed

Streatfield and coworkers [98, 104] also reported the production of synthetic LT-B in transgenic maize. They used an unspecified maize constitutive promoter to express a synthetic LT-B gene with a barley α-amylase signal peptide in maize. The bulk of the LT-B expression was primarily in the germ of transgenic kernels [98]. The level of LT-B expression reported was up to 9.2% LT-B in TAEP for an individual R_3 kernel. They used breeding techniques such as elite inbred lines to enhance the performance of LT-B-producing maize, using the transgenic lines as the pollen source. Their estimates of LT-B expression were based on total (as compared to functional) LT-B, which was quantified by using a polyclonal antibody as capture in a sandwich ELISA; however, in the study described in section 18.5.1, we used G_{M1} gangliosides to capture the multimeric form of LT-B, which is functional. Streatfield and cowor-

kers observed up to a 40% difference in the amount of LT-B measured, depending on whether a polyclonal antibody or G_{M1} gangliosides were used as captures in sandwich ELISA assays. Polyclonal antibody resulted in higher estimates when used in conjunction with a biotinylated antibody to detect the captured LT-B. Oral delivery of this LT-B to BALB/c mice resulted in the induction of protective oral and systemic immune responses.

One of the key findings of the work by Streatfield and coworkers [104] was that LT-B in transgenic maize kernel matrix was very heat resistant, withstanding temperatures up 170 °C, thus allowing heat extrusion to be used during processing. This is desirable for the industrial processing of maize expressing antigens into suitable formulations that can be practically administered.

Streatfield and coworkers [98, 104] also reported the production of another edible vaccine against the swine transmissible gastroenteritis virus (TGEV), which causes a severe diarrheal disease with high mortality in young piglets. This disease is economically important in commercial swine operations. TGEV is a multisubunit positive-strand RNA virus, encoding the membrane (M), nucleocapsid (N), and the spike (S) protein [105]. The S protein is a large surface glycoprotein that is involved in virus neutralization. The S protein was expressed in transgenic maize kernels. Expression levels reported were substantial, allowing oral delivery of 2 mg of the S protein in 50 g of transgenic corn. Oral administration of the maize expressing the S protein to piglets resulted in induction of a protective immune response in young piglets and subsequent protection from clinical levels of the virus [106].

Dosage regimes are critical for the quality of immune responses elicited upon oral administration of plant-based vaccines. In many studies, a low yield of antigen expressed in transgenic plants resulted in poor immune responses after oral administration. In other studies, repeated dosages were necessary. Using the S protein corn or the commercial vaccine, Lamphear and coworkers (2002) [106] studied different dosage regimes (4, 8, or 16 consecutive days) in oral immunization. They reported that with challenge to TGEV the animals fed on the S protein-producing corn for a 4-day regimen showed no morbidity, but 50% of animals fed nontransgenic corn displayed symptoms. The animals fed the transgenic corn for 8 and 16 consecutive days showed 20% and 36% morbidity, respectively, with challenge, indicating that the 4-day regimen was more effective in protecting the pigs. The frequency of dosage is therefore critical for obtaining effective protection against this pathogen. Piglets immunized with the commercial vaccine showed 9% morbidity with challenge compared to no morbidity in the animals immunized with the corn-derived vaccine on the 4-day dosage regime. These data suggest that the corn-derived S protein was somewhat more effective than the currently available conventional vaccine. This study represents one of the most comprehensive studies done with a corn-based edible vaccine, demonstrating its clinical efficaciousness in a practical situation.

18.5.3
Pharmaceutical Crop Production and Containment

Because maize is one of the most important crops globally, many issues arise regarding its use as a source of biopharmaceuticals. So far, no biopharmaceutical crops have been approved for commercial use, although nearly 40 plant-made pharmaceuticals and industrial products are nearing commercialization. Major concerns, especially relating to vaccine-producing maize plants, include whether the antigens will inadvertently enter the food chain and what preventive measures are in place. In the U.S., there are two types of growing systems for maize: commodity grain production and identity preservation. The first system is used for producing most maize grain in the United States, and the second system is used for specialty maizes, such as starch- or ethanol-producing maize. No specific regulations or restrictions cover either production system, except that in the identity-preservation system grains must be segregated from commodity maize during transportation and storage. For pharmaceutical-producing maize plants, a more stringent containment production system is required to prevent possible contamination from both directions.

One major concern about contamination is the potential for pollen drifting to neighboring fields. However, maize pollen is heavy compared with that of other species, and the majority of shed pollen falls within several feet of the parent plant. In addition, the pollen has a relatively short half life. Its viability decreases drastically within minutes (less than 20 min) after it is shed [107]. Several measures can be taken to prevent pollen contamination by a vaccine-producing corn plant. First, any APHIS (Animal and Plant Health Inspection Service, USA)-approved pharmaceutical maize field release currently requires a distance segregation of 1 mile (1.6 km) from the nearest other maize plants. This physical isolation is eight times greater than the distance required for producing foundation corn seed or in the identity-preservation production system. Second, the confinement strategies for transgenic maize produced under permit include conditions that confine its pollen so that it cannot pollinate surrounding maize. APHIS currently rules that maize may be grown within a half mile (0.8 km) of the test site if the test site maize is control-pollinated (using detasseling or bagging procedures). Surrounding maize must also be temporally isolated by planting it no fewer than 28 days before or after the regulated maize being field tested.

In addition to these physical and temporal isolations, it is also possible to produce vaccines in maize varieties that are male-sterile. Several cytoplasmic male-sterile varieties producing nearly 0% variable pollen can be used for this purpose [108]. Transgenic male-sterile corn will grow only silk, the female flower, which will be pollinated by a nontransgenic maize pollen to set seed. Half the seed obtained from this breeding approach will contain the proteins of interest.

18.6 Concluding Remarks

Plant-derived edible vaccines have enormous potential in producing safe, efficacious, inexpensive vaccines benefiting mankind, especially in the developing world. Maize seed can potentially become one of the most productive biofactories for pharmaceuticals. The physiological yield potential of maize under nonlimiting conditions has been estimated to be 31.4 to 81.5 tons ha^{-1} [109]. In reality, average maize yields in the U.S. are around 8 tons ha^{-1}, according to the U.S. National Corn Growers Association (http://www.ncga.com/03world/main/consumption.htm). Using the γ zein promoter, we have obtained transgenic maize plants with LT-B levels up to 350 μg g^{-1} of dry kernel. This expression level is therefore more than what is required to induce a protective immune response in experimental mice. Mason et al. [31] suggested that up to 1.1 mg per dose would be required to induce a protective immune response in humans, and this dosage requirement could be met with 3 g of dry maize meal from these transgenic kernels. Thus, from a 1-ha maize field with an average yield of 8 tons ha^{-1}, one can harvest about 2.8 kg of LT-B protein (350 μg × 8 × 10^6).

Although edible plant vaccines have shown tremendous pharmaceutical promise, several hurdles remain to be overcome. In the maize work discussed above, experimental results have demonstrated that biologically functional antigens can be produced in plants, can be conveniently used to orally immunize animals, and protected some of the animals from challenge with toxins or disease pathogens. Although functionally similar, it is yet to determine whether these maize-derived antigens are glycosylated differently than those from other systems. The biosynthesis of the glycan moiety is different in plant and mammalian systems [110, 111]. This difference could present obstacles for plant-derived pharmaceuticals. For example, lack of proper glycosylation could lead to the plant-derived subunit vaccines being less immunogenic and less efficacious than those produced in mammalian systems. In addition, inappropriate glycosylation by the plant system could make the plant-derived proteins allergenic to mammals upon immunization. Research focusing in this area is just getting under way. Limited biochemical and immunological studies have indicated that plant-derived antibodies have a greater diversity of glycan structures than the same products made in other expression systems [112, 113]. It is possible that plant-specific N-glycosylation could represent a limitation for the use of certain recombinant glycoproteins of human origin produced in transgenic plants. But the good news is that one may not have to be concerned about allergenicity of such products if they are produced in edible tissues of a well-tolerated crop plant, such as maize.

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References

1. Atkinson W, Wolfe CS. Epidemiology and prevention of vaccine-preventable diseases. 7th ed. ed. Atlanta, GA, USA: Dept. of Health & Human Services, Public Health Service, Centers for Disease Control and Prevention, 2002.
2. Hilleman MR. Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. *Vaccine* 2000; **18**:1436–47.
3. Bouvet JP, Decroix N, Pamonsinlapatham P. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol* 2002; **23**:209–13.
4. Santana MA, Rosenstein Y. What it takes to become an effector T cell: The process, the cells involved, and the mechanisms. *J Cell Physiol* 2003; **195**:392–401.
5. Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nat Biotechnol* 1998; **16**:934–38.
6. Cripps AW, Kyd JM, Foxwell AR. Vaccines and mucosal immunisation. *Vaccine* 2001; **19**:2513–15.
7. Xu-Amano J, Kiyono H, Jackson RJ, Staats HF, Fujihashi K, Burrows PD, Elson CO, Pillai S, McGhee JR. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J Exp Med* 1993; **178**:1309–20.
8. Morris W, Steinhoff MC, Russell PK. Potential of polymer microencapsulation technology for vaccine innovation. *Vaccine* 1994; **12**:5–11.
9. Illum L. Chitosan and its use as a pharmaceutical excipient. *Pharm Res* 1998; **15**:1326–31.
10. Seo JY, Seong SY, Ahn BY, Kwon IC, Chung H, Jeong SY. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. *Infect Immun* 2002; **70**:1143–49.
11. Millar DG, Hirst TR, Snider DP. Escherichia coli heat-labile enterotoxin B subunit is a more potent mucosal adjuvant than its vlosely related homologue, the B subunit of cholera toxin. *Infect Immun* 2001; **69**:3476–82.
12. Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, Mills KH, Rappuoli R, Del Giudice G. Mucosal vaccines: non toxic derivatives of LTand CTas mucosal adjuvants. *Vaccine* 2001; **19**:2534–41.
13. Bowman CC, Clements JD. Differential biological and adjuvant activities of cholera toxin and Escherichia coli heat-labile enterotoxin hybrids. *Infect Immun* 2001; **69**:1528–35.
14. Lu X, Clements JD, Katz JM. Mutant Escherichia coli heat-labile enterotoxin [LT(R192G)] enhances protective humoral and cellular immune responses to orally administered inactivated influenza vaccine. *Vaccine* 2002; **20**:1019–29.
15. McNeela EA, O'Connor D, Jabbal-Gill I, Illum L, Davis SS, Pizza M, Peppoloni S, Rappuoli R, Mills KH. A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM(197)) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery. *Vaccine* 2000; **19**:1188–98.
16. Ryan EJ, McNeela E, Pizza M, Rappuoli R, O'Neill L, Mills KH. Modulation of innate and acquired immune responses by Escherichia coli heat-labile toxin: distinct pro- and anti-inflammatory effects of the nontoxic AB complex and the enzyme activity. *J Immunol* 2000; **165**:5750–59.
17. Del Giudice G, Podda A, Rappuoli R. What are the limits of adjuvanticity? *Vaccine* 2002; **20**:S38-S41.
18. Snider DP, Marshall JS, Perdue MH, Liang H. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin. *J Immunol* 1994; **153**:647–57.

19. Li XM, Serebrisky D, Lee SY, Huang CK, Bardina L, Schofield BH, Stanley JS, Burks AW, Bannon GA, Sampson HA. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J Allergy Clin Immunol* 2000; **106**:150–58.
20. Constant SL, Lee KS, Bottomly K. Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2-type differentiation. *Eur J Immunol* 2000; **30**:840–47.
21. Kusnadi AR, Nikolov ZL, Howard JA. Production of recombinant proteins in transgenic plants: Practical considerations. *Biotechnology and Bioengineering* 1997; **56**:473–484.
22. Tacket CO, Mason HS. A review of oral vaccination with transgenic vegetables. *Microbes Infect* 1999; **1**:777–83.
23. Daniell H, Streatfield SJ, Wycoff K. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci* 2001; **6**:219–26.
24. Koprowski H, Yusibov V. The green revolution: plants as heterologous expression vectors. *Vaccine* 2001; **19**:2735–41.
25. Mason HS, Warzecha H, Mor T, Arntzen CJ. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. *Trends Mol Med* 2002; **8**:324–29.
26. Pen J, Sijmons PC, van Ooijen AJJ, Hoekema A. Protein production in transgenic crops: Analysis of plant molecular farming. In: Hiatt A, ed. Transgenic plants: fundamentals and applications. New York: Elsevier, 1993:239–241.
27. Hood EE, Kusnadi A, Nikolov Z, Howard JA. Molecular farming of industrial proteins from transgenic maize. *Adv Exp Med Biol* 1999; **464**:127–47.
28. Palmer KE, Arntzen CJ, Lomonossoff GP. Antigen Delivery Systems III. Transgenic Plants and Recombinant Plant Viruses. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, Bienenstock J, eds. Mucosal Immunology. 2nd edition ed. San Deigo, CA, USA: Academic Press, **1999**:793–807.
29. Cramer CL, Boothe JG, Oishi KK. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. *Curr Top Microbiol Immunol* 1999; **240**:95–118.
30. 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* 2000; **18**:2023–28.
31. Mason HS, Haq TA, Clements JD, Arntzen CJ. Edible vaccine protects mice against Escherichia coli heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 1998; **16**:1336–43.
32. Richter LJ, Thanavala Y, Arntzen CJ, Mason HS. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat Biotechnol* 2000; **18**:1167–71.
33. Haq TA, Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 1995; **268**:714–16.
34. Tregoning JS, Nixon P, Kuroda H, Svab Z, Clare S, Bowe F, Fairweather N, Ytterberg J, van Wijk KJ, Dougan G, Maliga P. Expression of tetanus toxin Fragment C in tobacco chloroplasts. *Nucleic Acids Res* 2003; **31**:1174–79.
35. Ruf S, Hermann M, Berger IJ, Carrer H, Bock R. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat Biotechnol* 2001; **19**:870–75.
36. Wigdorovitz A, Carrillo C, Dus Santos MJ, Trono K, Peralta A, Gomez MC, Rios RD, Franzone PM, Sadir AM, Escribano JM, Borca MV. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. *Virology* 1999; **255**:347–53.
37. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, Yusibov V, Koprowski H, Plucienniczak A, Legocki AB. A plant-derived edible vaccine against hepatitis B virus. *Faseb J* 1999; **13**:1796–99.
38. Kong Q, Richter L, Yang YF, Arnt-

ZEN CJ, MASON HS, THANAVALA Y. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc Natl Acad Sci U S A* 2001; **98**:11539–44.

39. TACKET CO, MASON HS, LOSONSKY G, ESTES MK, LEVINE MM, ARNTZEN CJ. Human immune responses to a novel norwalk virus vaccine delivered in transgenic potatoes. *J Infect Dis* 2000; **182**:302–05.

40. STOGER 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* 2000; **42**:583–90.

41. TACKET CO, MASON HS, LOSONSKY G, CLEMENTS JD, LEVINE MM, ARNTZEN CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat Med* 1998; **4**:607–09.

42. CHRISTENSEN AH, QUAIL PH. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res* 1996; **5**:213–18.

43. ODELL JT, NAGY F, CHUA NH. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. *Nature* 1985; **313**:810–12.

44. MARKS MD, LINDELL JS, LARKINS BA. Quantitative analysis of the accumulation of Zein mRNA during maize endosperm development. *J Biol Chem* 1985; **260**:16445–50.

45. HOOD EE, WITCHER DR, MADDOCK S, MEYER T, BASZCZYNSKI C, BAILEY M, FLYNN P, REGISTER J, MARSHALL L, BOND D, KULISEK E, KUSNADI A, EVANGELISTA R, NIKOLOV Z, WOOGE C, MEHIGH RJ, HERNAN R, KAPPEL WK, RITLAND D, LI CP, HOWARD JA. Commerical production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Molecular Breeding* 1997; **3**:291–306.

46. CHIKWAMBA R, CUNNICK J, HATHAWAY D, MCMURRAY J, MASON H, WANG K. A functional antigen in a practical crop: LT-B producing maize protects mice against Escherichia coli heat labile enterotoxin (LT) and cholera toxin (CT). *Transgenic Res* 2002; **11**:479–93.

47. WOLFFE AP, MATZKE MA. Epigenetics: regulation through repression. *Science* 1999; **286**:481–86.

48. STAM M, VITERBO A, MOL JN, KOOTER JM. Position-dependent methylation and transcriptional silencing of transgenes in inverted T-DNA repeats: implications for posttranscriptional silencing of homologous host genes in plants. *Mol Cell Biol* 1998; **18**:6165–77.

49. PARK YD, PAPP I, MOSCONE EA, IGLESIAS VA, VAUCHERET H, MATZKE AJ, MATZKE MA. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J* 1996; **9**:183–94.

50. MATZKE AJ, MATZKE MA. Position effects and epigenetic silencing of plant transgenes. *Curr Opin Plant Biol* 1998; **1**:142–48.

51. KOMARI T, KUBO T. Methods of genetic transformation: Agrobacterium tumefaciens. In: Vasil IK, ed. Molecular Improvement of Cereal Crops. Great Britain: Kluwer Academic Publishers, 1999:43–82.

52. SANFORD JC. Turning point article The development of the biolistic process. *In Vitro Cell Dev Biol* 2000; **36**:303–308.

53. BIRCH RG. Application of gene transfer to crop improvement. In: O'Brien L, Henry RJ, eds. Transgenic cereals. St. Paul, Minnesota, USA: American Association of Cereal Chemists, 2000:267–276.

54. DAI S, ZHENG P, MARMEY P, ZHANG S, TIAN W, CHEN S, BEACHY RN, FAUQUET C. Comparative analysis of transgenic rice plants obtained by Agrobacterium-mediated transformation and particle bombardment. *Molecular Breeding* 2001; **7**:25–33.

55. CALLIS J, FROMM M, WALBOT V. Introns increase gene expression in cultured maize cells. *Genes Dev* 1987; **1**:1183–1200.

56. MASCARENHAS D, METTLER IJ, PIERCE DA, LOWE HW. Intron-mediated en-

hancement of heterologous gene expression in maize. *Plant Mol Biol* 1990; **15**:913–20.

57. Luehrsen KR, Walbot V. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. *Mol Gen Genet* 1991; **225**:81–93.

58. Clancy M, Hannah LC. Splicing of the maize Sh1 first intron is essential for enhancement of gene expression, and a T-rich motif increases expression without affecting splicing. *Plant Physiol* 2002; **130**:918–29.

59. Rethmeier N, Seurinck J, Van Montagu M, Cornelissen M. Intron-mediated enhancement of transgene expression in maize is a nuclear, gene-dependent process. *Plant J* 1997; **12**:895–99.

60. Gutierrez RA, MacIntosh GC, Green PJ. Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. *Trends Plant Sci* 1999; **4**:429–438.

61. Ingelbrecht IL, Herman LM, Dekeyser RA, Van Montagu MC, Depicker AG. Different 3′ end regions strongly influence the level of gene expression in plant cells. *Plant Cell* 1989; **1**:671–80.

62. Mogen BD, MacDonald MH, Leggewie G, Hunt AG. Several distinct types of sequence elements are required for efficient mRNA 3′ end formation in a pea rbcS gene. *Mol Cell Biol* 1992; **12**:5406–14.

63. De Rocher EJ, Vargo-Gogola TC, Diehn SH, Green PJ. Direct evidence for rapid degradation of Bacillus thuringiensis toxin mRNA as a cause of poor expression in plants. *Plant Physiol* 1998; **117**:1445–1461.

64. Chen CY, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* 1995; **20**:465–70.

65. Ohme-Takagi M, Taylor CB, Newman TC, Green PJ. The effect of sequences with high AU content on mRNA stability in tobacco. *Proc Natl Acad Sci U S A* 1993; **90**:11811–15.

66. Kooter JM, Matzke MA, Meyer P. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci* 1999; **4**:340–347.

67. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in post-transcriptional gene silencing in plants. *Science* 1999; **286**:950–52.

68. Fagard M, Vaucheret H. (Trans)gene siliencing in plants: How many mechanisms? *Annu Rev Plant Physiol Plant Mol Biol* 2000; **51**:167–94.

69. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001; **2**:110–19.

70. Jagus R, Anderson WF, Safer B. The regulation of initiation of mammalian protein synthesis. *Prog Nucleic Acid Res Mol Biol* 1981; **25**:127–85.

71. Gallie DR. The role of post-transcriptional control in transgenic gene design. In: Owen MRL, Pen J, eds. Transgenic plants: a production system for induscrtrial and pharmaceutical proteins. Chichester; New York: J. Wiley, 1996:50–74.

72. Joshi CP, Zhou H, Huang X, Chiang VL. Context sequences of translation initiation codon in plants. *Plant Mol Biol* 1997; **35**:993–1001.

73. Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TM. The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts in vitro and in vivo. *Nucleic Acids Res* 1987; **15**:3257–73.

74. Jobling SA, Gehrke L. Enhanced translation of chimaeric messenger RNAs containing a plant viral untranslated leader sequence. *Nature* 1987; **325**:622–25.

75. Carrington JC, Freed DD. Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. *J Virol* 1990; **64**:1590–97.

76. Gallie DR, Lucas WJ, Walbot V. Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. *Plant Cell* 1989; **1**:301–311.

77. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. *Proc Natl Acad Sci U S A* 1992; **89**:11745–49.

78. Mason HS, Ball JM, Shi JJ, Jiang X,

Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc Natl Acad Sci U S A* 1996; **93**:5335–40.

79. Gowri G, Campbell WH. Complementary DNA Clones for Corn Leaf Nadh Nitrate Reductase and Chloroplast Nad-Ppositive Glyceraldehyde-3-Phosphate Dehydrogenase Characterization of the Clones and Analysis of the Expression of the Genes in Leaves as Influenced by Nitrate in the Light and Dark. *Plant Physiology* 1989; **90**:792–98.

80. Murray EE, Lotzer J, Eberle M. Codon usage in plant genes. *Nucleic Acids Res* 1989; **17**:477–98.

81. Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA. Modification of the coding sequence enhances plant expression of insect control protein genes. *Proc Natl Acad Sci U S A* 1991; **88**:3324–28.

82. Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT, Fischhoff DA. Insect resistant cotton plants. *Biotechnology* (N Y) 1990; 8:939–43.

83. Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE. The reconstruction and expression of a Bacillus thuringiensis cryIIIA gene in protoplasts and potato plants. *Plant Mol Biol* 1993; **21**:1131–45.

84. Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K. Insect resistant rice generated by introduction of a modified delta- endotoxin gene of Bacillus thuringiensis. *Biotechnology* (N Y) 1993; **11**:1151–55.

85. Koziel GM, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright MS, Evola SV. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from Bacillus thuringiensis. *Bio Technology* 1993; **11**:194–200.

86. van der Salm T, Bosch D, Honee G, Feng L, Munsterman E, Bakker P, Stiekema WJ, Visser B. Insect resistance of transgenic plants that express modified Bacillus thuringiensis cryIA(b) and cryIC genes: a resistance management strategy. *Plant Mol Biol* 1994; **26**:51–59.

87. Armstrong CL, Parker GB, Pershing JC, Brown SM, Sanders PR, Duncan DR, Stone T, Dean DA, Deboer DL, Hart J, Howe AR, Morrish FM, Pajeau ME, Petersen WL, Reich BJ, Rodriguez R, Santino CG, Sato SJ, Schuler W, Sims SR, Stehling S, Tarochione LJ, Fromm ME. Field evaluation of European corn borer control in progeny of 173 transgenic corn events expressing an insecticidal protein from Bacillus thuringiensis. *Crop Science* 1995; **35**:550–557.

88. Jansens S, Cornelissne M, De CR, Reynaerts A, Peferoen M. Phthorimaea operculella (Lepidoptera: Gelechiidae) resistance in potato by expression of the Bacillus thuringiensis CryIA(b) insecticidal crystal protein. *Journal of Economic Entomology* 1995; 88:1469–1476.

89. Iannacone R, Grieco PD, Cellini F. Specific sequence modifications of a cry3B endotoxin gene result in high levels of expression and insect resistance. *Plant Mol Biol* 1997; **34**:485–96.

90. Zhong GY, Peterson D, Delaney DE, Bailey M, Witcher DR, Register JC, III, Bond D, Li CP, Marshall L, Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA. Commercial production of aprotinin in transgenic maize seeds. *Molecular Breeding* 1999; **5**:345–56.

91. Conrad U, Fiedler U. Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity. *Plant Mol Biol* 1998; **38**:101–09.

92. Schouten A, Roosien J, van Engelen FA, de Jong GA, Borst-Vrenssen AW, Zilverentant JF, Bosch D, Stiekema WJ, Gommers FJ, Schots A, Bakker J. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory

pathway in transgenic tobacco. *Plant Mol Biol* 1996; **30**:781–93.

93. Fiedler U, Phillips J, Artsaenko O, Conrad U. Optimization of scFv antibody production in transgenic plants. *Immunotechnology* 1997; **3**:205–16.

94. Munro S, Pelham HR. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 1987; **48**:899–907.

95. Chikwamba R, McMurray J, Shou H, Frame B, Pegg SE, Scott P, Mason H, Wang K. Expression of a synthetic E. coli heat-labile enterotoxin B subunit (LT-B) in maize. *Mol. Breeding* 2002; **10**:253–265.

96. Matzke MA, Matzke A. How and Why Do Plants Inactivate Homologous (Trans)genes? *Plant Physiol* 1995; **107**:679–685.

97. Witcher DR, Hood EE, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh R, Kappel W, Register J, Howard JA. Commercial production of beta-glucuronidase (GUS): A method system for the production of proteins in plants. *Molecular Breeding* 1998; **4**:301–312.

98. Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA. Plant-based vaccines: unique advantages. *Vaccine* 2001; **19**:2742–48.

99. Yang SH, Moran DL, Jia HW, Bicar EH, Lee M, Scott MP. Expression of a synthetic porcine alpha-lactalbumin gene in the kernels of transgenic maize. *Transgenic Res* 2002; **11**:11–20.

100. Spangler BD. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. *Microbiol Rev* 1992; **56**:622–47.

101. Svennerholm AM, Holmgren J. Oral vaccines against cholera and enterotoxigenic Escherichia coli diarrhea. *Adv Exp Med Biol* 1995; **8**:1623–28.

102. Dickinson BL, Clements JD. Use of Escherichia coli heat-labile enterotoxin as an oral adjuvant. In: Kiyono H, Ogra PL, McGhee JR, eds. Mucosal Vaccines: Academic Press, 1996:73–87.

103. Johnson LA. Corn: The Major Cereal of the Amricas. In: Kulp K, Ponte Jr. JG, eds. Handbook of Cereal Science and Technology. 2nd edition ed. New York, NY, USA: Marcel Dekker Inc., 2000:31–80.

104. Streatfield SJ, Mayor JM, Barker DK, Brooks C, Lamphear BJ, Woodard SL, Beifuss KK, Vicuna DV, Massey LA, Horn ME, Delaney DE, Nikolov ZL, Hood EE, Jilka JM, Howard JA. Development of an edible subunit vaccine in corn against enterotoxigenic strains of Escherichia coli. *In Vitro Cell. Dev. Biol. – Plant* 2002; **38**:11–17.

105. Jimenez G, Correa I, Melgosa MP, Bullido MJ, Enjuanes L. Critical epitopes in transmissible gastroenteritis virus neutralization. *J Virol* 1986; **60**:131–39.

106. Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA. Delivery of subunit vaccines in maize seed. *J Control Release* 2002; **85**:169–80.

107. Neuffer MG. Growing maize for genetic studies. In: Freeling M, Walbot V, eds. The Maize Handbook. New York, NY, USA: Springer, 1994:197–209.

108. Gabay-Laughnan S, Laughnan JR. Male sterility and restorer genes in maize. In: Freeling M, Walbot V, eds. The Maize Handbook. New York, NY, USA: Springer, 1994:418–423.

109. Tollenaar M. What is the current upper limit of corn productivity? Conference on Physiology, Biochemistry and Chemistry Associated with Maximum Yield Corn. St. Louis, Missouri, USA: Foundation for Agronomic Research and Patash and Phosphate Institute, 1985.

110. Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Laine AC, Gomord V, Faye L. N-glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol Biol* 1998; **38**:31–48.

111. Jenkins N, Parekh RB, James DC. Getting the glycosylation right: implications for the biotechnology industry. *Nat Biotechnol* 1996; **14**:975–81.

112. Ma JK, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB,

Lehner T. Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat Med* 1998; 4:601–06.

113. Samyn-Petit B, Gruber V, Flahaut C, Wajda-Dubos JP, Farrer S, Pons A, Desmaizieres G, Slomianny MC, Theisen M, Delannoy P. N-glycosylation potential of maize: the human lactoferrin used as a model. *Glycoconj J* 2001; **18**:519–27.

114. Walmsley AM, Kirk DD, Mason HS. Passive immunization of mice pups through oral immunization of dams with a plant-derived vaccine. *Immunol Lett* 2003; **86**:71–76.

115. Arakawa T, Chong DK, Langridge WH. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat Biotechnol* 1998; **16**:292–97.

116. Yu J, Langridge WH. A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat Biotechnol* 2001; **19**:548–52.

117. Gomez N, Carrillo C, Salinas J, Parra F, Borca MV, Escribano JM. Expression of immunogenic glycoprotein S polypeptides from transmissible gastroenteritis coronavirus in transgenic plants. *Virology* 1998; **249**:352–58.

118. Gomez N, Wigdorovitz A, Castanon S, Gil F, Ordas R, Borca MV, Escribano JM. Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus. *Arch Virol* 2000; **145**:1725–32.

119. Carrillo C, Wigdorovitz A, Trono K, Dus Santos MJ, Castanon S, Sadir AM, Ordas R, Escribano JM, Borca MV. Induction of a virus-specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants. *Viral Immunol* 2001; **14**:49–57.

120. Dus Santos MJ, Wigdorovitz A, Trono K, Rios RD, Franzone PM, Gil F, Moreno J, Carrillo C, Escribano JM, Borca MV. A novel methodology to develop a foot and mouth disease virus (FMDV) peptide-based vaccine in transgenic plants. *Vaccine* 2002; **20**:1141–47.

121. Gil F, Brun A, Wigdorovitz A, Catala R, Martinez-Torrecuadrada JL, Casal I, Salinas J, Borca MV, Escribano JM. High-yield expression of a viral peptide vaccine in transgenic plants. *FEBS Lett* 2001; **488**:13–17.

122. Chikwamba, R.K., Scott, M.P., Mejia, L.B., Mason, H.S., Wang, K. Localization of a bacterial protein in starch granules of transgenic maize kernels. *Proc. Natl. Acad. Sci. (USA)* 2003, **100**: 11127–11132.