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Chapter 65

Mucosal Vaccines from Plant Biotechnology

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Chapter Outline

INTRODUCTION

Plant biotechnology has experienced exponential development during the past 30 years, facilitated in large part by the capacity of a clever soil bacterium (*Agrobacterium tumefaciens*) to transfer DNA segments into plant cells (Gelvin, 2003). This powerful function, harnessed by clever plant biologists during the 1980s, now enables routine delivery of recombinant DNA into plant cells for, among other things, production of recombinant proteins in plants. The use of plants for expression of subunit vaccine antigens, first pursued in the early 1990s (Curtiss and Cardineau, 1990; Mason et al., 1992), was a natural outgrowth of the emerging technology, coming soon after the groundbreaking demonstration that immunoglobulins could be produced in transgenic tobacco (Hiatt et al., 1989). Interested readers are referred to recent review articles that describe advances in the use of plants for production and delivery of vaccine antigens (Mason and Herbst-Kralovetz, 2012; Pelosi et al.,

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2012; Rybicki, 2009, 2010; Streatfield, 2006; Tacket, 2007; Yuki and Kiyono, 2009; Yusibov et al., 2011; Thanavala et al., 2006; Yusibov and Rabindran, 2008).

Unfortunately, commercial development of plant-derived vaccine products has been very slow, perhaps due to a very conservative pharmaceutical industry that is hesitant to try new technologies. A hypothesis that drove much of the early work on plant-derived vaccines held that cheap and orally delivered "edible" subunit vaccines could provide protection against infectious diseases in developing countries with limited medical infrastructures. Thus, ingestion of plant material would stimulate protective immunity, mediated by uptake and processing of antigens by the gut-associated lymphoid tissue (GALT) (Streatfield, 2006; Thanavala et al., 2006). A number of hurdles can interfere with the effectiveness of this approach, including poor stability of proteins in the gut, substantial variability of antigen content in plant tissues, and regulatory concerns for the containment of transgenic crop plants and their use exclusively as pharmaceutical products. These and other issues are discussed in greater detail in other papers (Mason and Herbst-Kralovetz, 2012; Rybicki, 2009; Rybicki et al., 2013). Such challenges notwithstanding, most workers and watchers of the field continue to see great value in the use of plants for production of vaccine antigens. Importantly, compelling data on the protective efficacy of orally delivered plant-derived vaccine antigens continue to accumulate, as discussed below in detail in the section on animal vaccines.

The approaches for plant-based protein expression include stably integrated transgenes into chromosomes in either the nucleus or the chloroplast, and transient expression using vectors that may use plant virus replication elements to amplify the transgene. We present only a very brief discussion here, and interested readers are referred to excellent recent review articles (Rybicki, 2010; Thuenemann et al., 2013). Nuclear genes are inherited in a Mendelian fashion, whereas chloroplast genes are passed through progeny only from the maternal parent, since pollen cells are devoid of chloroplasts. Tissue-specific promoters allow foreign proteins to accumulate in seeds (Nochi et al., 2007; Streatfield, 2006; Yuki and Kiyono, 2009; and see discussion of MucoRice below). Proteins that accumulate in seeds are very stable to temperature extremes and can be stored for months to years with little loss of protein activity.

During the past decade, the use of rapid transient expression in plants (rather than the slow production of plants with stably integrated transgenes) has increased greatly and represents an ever-growing proportion of plant-based protein expression work. The development of viral vectors has enabled very fast and very high levels of protein expression in plants (Thuenemann et al., 2013; Gleba et al., 2007; Yusibov et al., 2006). *Nicotiana benthamiana*, a tobacco relative (Goodin et al., 2008), is the typical plant host, due to its ease of culture and effective support of plant virus replication. Protein expression levels at 1–2 mg per kilogram of leaf mass are often ≥10-fold higher than those obtained with stably transgenic plants, and indicate the potential for purification of plant-produced proteins at costs that are economically competitive with microbial fermentation systems. Moreover, culture of *N. benthamiana* can be readily scaled up to massive levels (Pogue et al., 2010). Kentucky Bioprocessing, Inc. (Owensboro, KY, http://www.kbpllc.com/) has developed a large-scale system for plant growth, infiltration with *Agrobacterium*, and purification of recombinant proteins (Figure 1). The convenience of transient plant-based expression enables rapid response to emerging viruses such as influenza and norovirus, as described by Charles Arntzen in his address to the 238th National Meeting of the American Chemical Society (Dylewski, 2009). These developments make plant-based expression and purification of vaccine antigens, including virus-like particles (VLP) (Huang et al., 2009; Santi et al., 2008), for mucosal delivery commercially viable.

We can also utilize purified antigens for intranasal delivery, which requires more concentrated antigen at

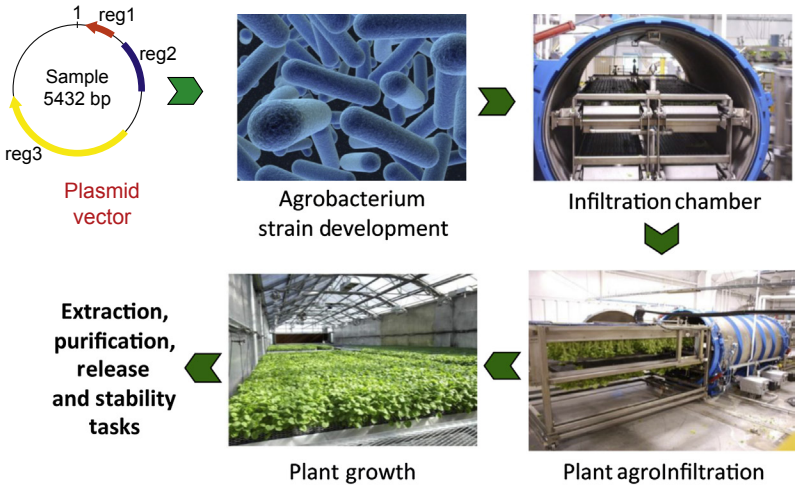

FIGURE 1 Large-scale process for transient expression of recombinant proteins at Kentucky Bioprocessing (Owensboro, KY). A plant expression vector, often a viral replicon, is constructed and mobilized into *Agrobacterium tumefaciens*. The *Agrobacterium* cultures are used for massive infiltration of whole *Nicotiana benthamiana* plants in a large vacuum chamber. The plants are then cultured for 4–10 days, depending on the recombinant protein and expression vector, before they are harvested and extracted to purify the recombinant protein.

lower doses than oral delivery. Nasal immunization induced antigen-specific mucosal IgA responses in salivary glands, respiratory tract, intestines, and reproductive tracts in mammals (Harandi et al., 2003; Imaoka et al., 1998; Kozlowski et al., 1997; Rudin et al., 1999; Staats et al., 1997). Moreover, mucosal immunization can prime both mucosal and systemic immune responses (Kunkel and Butcher, 2002). Thus, intranasal delivery of plant-derived vaccine antigens, especially VLP, is a promising strategy, as we discuss further below.

In this chapter, we will describe the progress in selected areas with mucosal vaccines in human and animal hosts. We focus on studies that involved humans or have shown strong indication of efficacy in preclinical studies.

MUCOSAL VACCINES FOR HUMANS

Norovirus Vaccine and Mucosal Adjuvants

Noroviruses are causal agents of epidemic gastroenteritis (Herbst-Kralovetz et al., 2010), sometimes called "stomach flu," and when occurring on ocean liners, "cruise ship virus" (Dylewski, 2009). Other than foundering or loss of power on the ship, a norovirus epidemic with projectile vomiting and persistent diarrhea must be one of the most disappointing vacations imaginable. There is yet no vaccine available for norovirus, but much work during the past 20 years has focused on production and immunogenicity testing of norovirus VLP (NVLP) (Herbst-Kralovetz et al., 2010). The capsid protein (NVCP) assembles VLP when expressed in insect cells (Jiang et al., 1992) or plants (Mason et al., 1996), and the VLP are substantially acid stable and thus amenable to oral delivery.

Preclinical studies of transgenic tobacco and potato-derived NVLP delivered orally to mice showed efficient production of antigen-specific serum IgG and fecal IgA (Mason et al., 1996). A clinical trial tested feeding human volunteers 150 g raw potato tuber tissue containing ~500 μg NVCP in two or three doses over a period of 3 weeks indicated promising results (Tacket et al., 1998). Nearly all of the subjects who ingested NVCP potato developed antigen-specific serum IgG and/or IgA responses, but the titers were modest compared to those obtained in a later study with 250 μg/dose of purified insect cell-derived NVLP (Tacket et al., 2003). A difficulty with the raw potato vehicle was the variable amounts of NVCP among different tuber samples and limited 50–60% efficiency of VLP assembly. Thus, purified NVLP is the preferred material for oral vaccine delivery.

Transgenic tomato fruit expressing NVCP proved to be a more tractable system (Zhang et al., 2006). Freeze-dried tissues of transgenic tomato fruit and potato tuber were fed to mice, with tomato shown to be a superior vehicle. Four doses of 0.4 g dried tomato (64 μg NVCP, 40 μg NVLP) caused seroconversion in 80% of mice, and double those amounts resulted in 100%. Furthermore, excellent levels of anti-NVCP fecal IgA were induced in all seroconverted mice. Conversely, similar doses of freeze-dried potato produced substantially poorer results, probably due to oxidation of phenolic compounds that are abundant in potato tuber. Since tomato fruit is low in phenolics, and high in the antioxidant ascorbic acid, such oxidation effects were minimal. Although the tomato delivery strategy seemed "ripe" for further development, the winds of change were blowing and bringing faster and more robust transient expression systems.

When it became apparent that NVLP from transient expression in leaves could be purified in consistently high yield and quality (Huang et al., 2009; Santi et al., 2008), the stable transgenic systems were largely left behind. Description of a process to produce NVLP under "current good manufacturing practice" (cGMP) conditions (Lai and Chen, 2012) paved the way for future clinical studies that are likely to occur soon (Charles Arntzen, personal communication). Meanwhile, several interesting studies investigated intranasal (IN) delivery of plant-derived NVLP in mice, and showed strong effects of Toll-like receptor agonists as mucosal adjuvants.

CpG oligodeoxynucleotides (CpG ODN) are known to interact with TLR9 to stimulate maturation and proliferation of multiple cell types of the immune system, and provide strong vaccine adjuvant effects (Abe et al., 2006). When 25 μg purified plant-derived NVLP was delivered IN to mice in two doses given 3 weeks apart, much stronger serum and mucosal antibody responses were obtained when 10 μg CpG ODN was codelivered with NVLP (Mason and Herbst-Kralovetz, 2012). The mucosal anti-NVLP IgA responses measured at multiple epithelial sites, including intestinal and vaginal secretions, were strongly enhanced by the adjuvant effect. Moreover, IN delivery of 25 μg NVLP without adjuvant was vastly superior to oral delivery of 100 μg NVLP for production of antigen-specific serum IgG. These data make a compelling case that IN delivery of NVLP could make an efficacious vaccine, and support the case that IN delivery of other VLP vaccines against sexually transmitted diseases (Balmelli et al., 1998) is a viable strategy.

Herbst-Kralovetz and coworkers studied the effects of IN immunization of mice with NVLP and other adjuvants, including TLR7 agonists (Velasquez et al., 2010). They tested resiquimod (R848), a TLR7/8 agonist that is FDA-approved for clinical trials, and a related midazoquinoline-based TLR7 agonist, gardiquimod, compared with cholera toxin (CT), a strong but risky mucosal adjuvant that is an experimental "gold standard." In experiments similar to those described above with CpG adjuvant, gardiquimod provoked anti-NVCP serum IgG and mucosal IgA responses (intestinal, respiratory, and reproductive mucosa) on par with CT and superior to R848. Thus, a TLR7 agonist produces excellent mucosal immune responses at intestinal and vaginal sites distal from the nasal delivery site.

Herbst-Kralovetz and coworkers also studied murabutide as a vaccine adjuvant for plant-derived NVLP delivered IN in mice (Jackson and Herbst-Kralovetz, 2012). Murabutide is a synthetic immunomodulator derived from muramyl dipeptide (MDP), the smallest bioactive unit of bacterial peptidoglycan. Unlike MDP, murabutide has no pyrogenic activity and is thus safer. Murabutide is recognized by the nucleotide binding oligomerization domain-containing protein 2 receptor on mammalian cells, and was approved for testing in several clinical trials as an injectable vaccine adjuvant. Jackson and Herbst-Kralovetz (2012) showed that it is a potent mucosal adjuvant as well, inducing serum and mucosal anti-NVLP antibodies after IN delivery. Murabutide was tested at 25, 100, and 250 μg/dose delivered with 25 μg NVLP/dose, and showed adjuvant activity at all doses, with 100 μg being optimal. Moreover, its mucosal adjuvant activity was comparable with gardiquimod (TLR7 agonist) and CT, both of which suffer some safety concerns. Thus, a murabutide adjuvanted IN-delivered NVLP is an excellent candidate for clinical evaluation.

Further studies with plant-derived NVLP tested the adjuvant effect of a gelling polysaccharide derived from the plant *Aloe vera* (Velasquez et al., 2011). The product is called GelSite®, manufactured under cGMP by Nanotherapeutics (http://www.nanotherapeutics.com/products_pipeline_gelvac.php) and is a high-molecular-weight anionic polysaccharide (sodium polygalacturonate). The preparation is liquid but can be processed to make a dry powder formulation called GelVac™, and creates a gel whether in liquid or powder form upon contact with body fluids at the site of administration. The GelVac dry formulation with NVLP delivered IN to mice produced anti-NVLP serum and mucosal antibodies at titers higher than the liquid formulation. The latter could be improved by addition of gardiquimod adjuvant, but was still similar to GelVac. Presumably, the mucosal adjuvant activity of the polygalacturonate derives from a depot effect, which prevents draining or ciliary clearance of the IN-delivered material away from the lymphoid effector sites in the nasal mucosa.

Charles Arntzen is collaborating with Advanced Bionutrition (ABN) (http://www.advancedbionutrition.com/default.aspx) to evaluate norovirus oral vaccine formulations, with a goal of entering human clinical trials. The oral delivery formulation is supported by US Patent No. 7,998,502, which makes use of bioadhesive materials in order to enhance the efficiency of oral delivery.

Human Immunodeficiency Virus Vaccines

Human immunodeficiency virus (HIV) is now a well-known and feared infectious agent that targets $CD4^+$ lymphoctyes and ultimately results in loss of adaptive immune system functions. After monumental attempts over the past 30 years by researchers worldwide to develop a vaccine, the results to date are modest, at best. A number of studies have used plants for recombinant expression of HIV antigens. A consensus of opinion among plant-based vaccinologists holds that mucosal immune responses are important, if not crucial, to protective efficacy, and thus often focus on delivery of antigens at mucosal sites. We will review selected works here, and readers are directed to more extensive review articles (Meyers et al., 2008; Yusibov et al., 2011) for further information.

Many plant-based efforts at HIV vaccines have utilized VLP as a means to increase the antigen density and provide a repeating antigen array that can more effectively engage B-cell receptors. An interesting early example is the use of potato virus X (PVX) to produce chimeric plant virus particles that display the highly conserved "ELDKWA" epitope from the HIV glycoprotein gp41 (Marusic et al., 2001). The epitope coding sequence was fused to the N-terminus of the PVX coat protein, and the recombinant virus used to infect *N. benthamiana* plants, resulting in robust production of chimeric virus particles. Purified virus was used to immunize mice either intraperitonially (IP) or mucosally IN without adjuvant, resulting in serum IgG and IgA specific for ELDKWA. The authors also used a mouse model for human immunity: severe combined immunodeficient mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID), immunized with human autologous monocyte-derived dendritic cells pulsed with chimeric ELDKWA virus particles. Serum from both normal and hu-PBL-SCID mice neutralized HIV-1, demonstrating the potential for protection against HIV infection. The authors suggest that the PVX chimeric particle system could also be used for T-cell epitopes. However, it is interesting to note that even low-titer antibodies could protect nonhuman primates against repeated low-dose mucosal exposure to SHIV (Hessell et al., 2009), which suggests that neutralizing antibodies alone may be at least partially protective.

Mor and his colleagues have also pursued the HIV gp41 and peptides derived from it. They fused the ELDKWA epitope to cholera toxin B subunit (CTB) in order to achieve targeting to the mucosal epithelium (Matoba et al., 2004). Mice immunized IN with CTB- ELDKWA produced in *Escherichia coli* developed serum and mucosal (fecal) antibodies that inhibited transcytosis of HIV-1 in vitro. The authors demonstrated expression of the fusion protein in *N. benthamiana*, but did not test the immunogenicity of that product. Later, Mor and colleagues created stable transgenic plant lines that expressed a CTB fusion that contained a longer segment of the gp41 membrane proximal region, called CTB-MPR(649-684) (Matoba et al., 2009). They performed extensive characterization of the purified fusion protein, including glycan analysis, as they found asparagine-linked glycosylation of a consensus site in the CTB sequence. The glycan, not found in bacterially expressed CTB, did not interfere with ganglioside binding

or display of the HIV epitope. Mice immunized IN with 35 μg of liposome-conjugated CTB-MPR(649-684) and 1 μg cholera toxin for 5 consecutive weeks and boosted IP with 3 μg of CTB-MPR(649-684) at week 9 produced substantial serum IgG and vaginal IgA specific for MPR(649-684). Thus, one can expect a need to balance the advantage of mucosal targeting afforded by CTB with its overpowering immunogenicity.

Mor and colleagues recently turned to a different strategy for plant-based HIV vaccine: a VLP comprising gag p55 and gp41 (Kessans et al., in press; Figure 2). They created stably transgenic *N. benthamiana* expressing HIV gag p55 and showed that gag VLP accumulated in the plants. The p55 was not processed and remained as mostly 55 kDa protein. They then used an ICON TMV-based replicon to express "deconstructed gp41" (dgp41), comprising the membrane proximal external region (MPER), transmembrane domain, and cytoplasmic tail, in transgenic gag plants. The result was accumulation in leaves of VLP containing both gag p55 and dgp41. The evidence for VLP formation included electron micrographs showing particles forming at the plasma membrane, in endosomes, and in the extracellular space. Moreover, both antigens cosedimented in rate zonal density gradients. These workers immunized mice with particle preparations and with CTB-MPR, priming IP with one antigen (weeks 0, 2, and 4) and boosting IP with the same or with the other at weeks 8 and 10 (Figure 3). Mice primed and boosted with VLP showed strong anti-gag serum IgG, with all mice responding by week 6. Anti-gp41 responses were less impressive, but seven of eight mice seroconverted by week 12 after VLP prime and boost. All mice primed with either VLP or CTB-MPR and boosted with the other antigen seroconverted for gp41 by week 10. All immunizations were adjuvanted with Ribi adjuvant (Sigma) to a final concentration of 2% oil, which suggests that a good mucosal adjuvant will be needed if the VLP are used for IN delivery.

Another VLP approach for plant-based HIV vaccines used membrane particles generated by fusions with hepatitis B surface antigen (HBsAg). HBsAg expressed in plant cells inserts into the ER membrane, which invaginates to form particles within the ER lumen (Smith et al., 2003). Transgenic potato tuber expressing HBsAg was orally immunogenic in mice (Richter et al., 2000) and humans (Thanavala et al., 2005; and see below). Thus, HBsAg fusions seem a reasonable strategy. One fusion (TBI-HBsAg) encodes an artificial polypeptide composed of several immunogenic T- and B-cell epitopes of HIV-1 env and gag proteins fused to the N-terminus of HBsAg small protein (Shchelkunov et al., 2006). These workers generated stable transgenic tomato plants, and freeze-dried the fruits for oral delivery to mice. Tomato powder was suspended in water and gavaged into mice at a dose of 30 ng antigen in 1 mL, three times over a 4-week period. The mice were then boosted at day 42 using plasmid DNA encoding TBI-HBsAg. Over the time course, anti-HBsAg antibodies rapidly accumulated in serum and fecal samples, which increased after boosting with the DNA vaccine. Anti-HIV responses were more modest, but measureable in both serum and feces, although no endpoint titers were performed. Nonetheless, the results looked promising.

Sala and colleagues aimed to elicit cellular immune responses against HIV-1, using a polyepitope comprising eight HLA-A*0201 restricted class I epitopes identified in five major HIV-1 proteins (Gag p17 and p24, protease, reverse transcriptase, and integrase), fused to the N-terminus of HBsAg small protein and the C-terminus of the HBsAg preS2 domain (Greco et al., 2007; Guetard et al., 2008). They generated stably transgenic tobacco plants expressing the fusion protein "HIV-1/HBV" and fed HSB humanized transgenic mice, in which HLA-A2.1 restricted epitopes are presented by the human class I MHC allele on a mouse knockout background. Ten days before ingesting the freeze-dried transgenic tobacco (eight doses over 2 weeks), the mice were primed with a plasmid for expression of the same fusion protein. The treatment produced anti-HIV-1-specific $CD8^+$ T-cell activation in mesenteric lymph nodes, indicating the potential to develop specific cytotoxic T-cell-mediated immunity. However, the authors also found significant activation of regulatory T cells in the same animals, suggesting the potential for development of

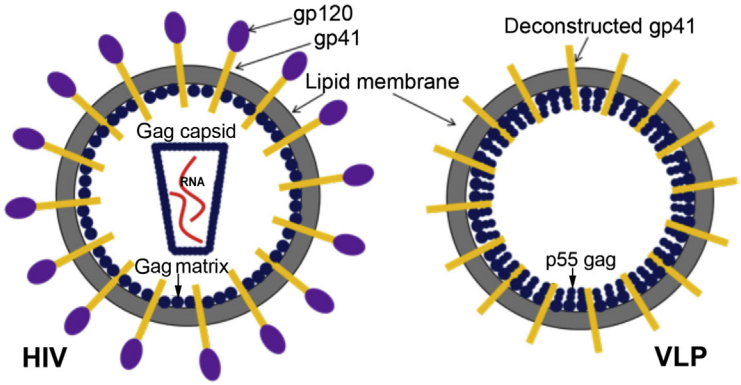

FIGURE 2 Models of HIV virion and deconstructed gp41 virus-like particles (VLP). The HIV virion (left) contains a core of gag capsid enclosing the RNA genome segments, gag matrix protein associated with a lipid membrane (derived from host cell plasma membrane), and envelope glycoproteins gp120/gp41. Kessans et al. (2013) describe a VLP (right) made in plants by coexpression of gag p55 and "deconstructed" gp41, which lacks the gp120 component, thus exposing the membrane proximal external epitopes that are conserved and capable of inducing HIV-neutralizing antibodies.

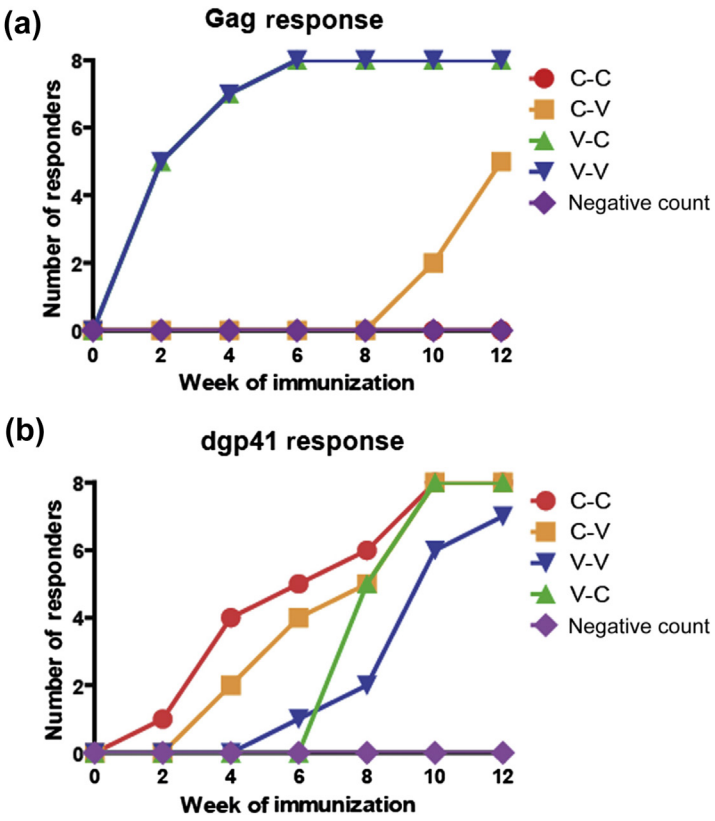

FIGURE 3 Seroconversion of mice immunized with various combinations of HIV-derived CTB-MPR (C) and VLP (V). Groups of eight mice were primed IP with either CTB-MPR or VLP at weeks 0, 2, and 4, and subsequently boosted IP with either CTB-MPR or VLP at weeks 8 and 10. The number of mice in each group that seroconverted with antibody responses to gag protein (a) or gp41 (b) are plotted vs. the week number.

tolerance. These conflicting results indicate that one must be very careful when interpreting results of feeding studies.

Hepatitis B Vaccines

Hepatitis B virus (HBV) infection may lead to liver cancer, with a million deaths per year worldwide. Because HBV can be transmitted by sexual contact and births to infected mothers, mucosal immunity would enhance the systemic protection provided by the injectable yeast-derived (BsAg vaccine (McAleer et al., 1984)). A large volume of work over the past 20 years has demonstrated expression of HBsAg in a variety of plant systems including potato, lettuce, tomato, tomatillo, corn, and banana (Streatfield, 2005a). A recent review article provides a very good survey of this field (Shchelkunov and Shchelkunova, 2010).

HBsAg expression in plants was first demonstrated in transgenic tobacco (Mason et al., 1992), and then transgenic potato tubers (Richter et al., 2000), which were used to demonstrate oral immunogenicity in mice (Kong et al., 2001). The antibody titers without cholera toxin adjuvant were modest, and a "subimmunogenic" priming dose with yeast-derived HBsAg was required to enable demonstration of a boosting effect by fed HBsAg potatoes. A subsequent clinical trial showed that 19 of 33 previously vaccinated subjects who ate 100 g raw potato doses (~850 µg HBsAg) had substantial boosting of serum anti-HBsAg titers (Thanavala et al., 2005). These data indicate that oral delivery of HBsAg could be used as a booster vaccination, since some of the subjects experienced the boosting effect 10–15 years after their intramuscular vaccinations. A smaller study showed that two naïve human subjects were seroconverted after eating transgenic lettuce leaves that contained submicrogram quantities of HBsAg (Kapusta et al., 1999). Although neither of these studies used an adjuvant (unless one considers the possibility that compounds found in either potato or lettuce could function so), an interesting study of oral immunogenicity in mice of alum-adsorbed yeast-derived vaccine at low doses over longer intervals showed efficient seroconversion (Kapusta et al., 2010). The suggestion from that study was that low doses and longer intervals would yield better results for oral delivery of HBsAg.

Immune tolerance is always a potential concern when antigens are delivered orally, and needs further study. In humanized transgenic mice (HLA-A2.1 and HLADR1 alleles), oral delivery of different doses of transgenic HBsAg tobacco resulted in increasing levels of regulatory T cells (Tregs, associated with tolerance) with increasing HBsAg dose (Kostrzak et al., in press). Serum and fecal IgG and IgA correlated inversely with antigen dose, suggesting that oral tolerance occurred at higher doses. However, the relatively poor antibody responses made the correlations somewhat tenuous, and Tregs increased substantially in mice immunized with wild-type tobacco. Nonetheless, the

differences in Tregs in HBsAg tobacco-immunized and in wild-type tobacco-immunized mice were statistically significant. Consistent with these findings, Pniewski and coworkers (Pniewski et al., 2011) immunized mice with low doses of freeze-dried transgenic lettuce containing 0.1 μg HBsAg without adjuvant at a 60-day interval between prime and boost immunizations, and observed mucosal and humoral anti-HBsAg antibodies. These data coupled with those described above (Kapusta et al., 2010; Kostrzak et al., in press) argue for lower doses in order to avoid development of Treg-mediated tolerance.

Different findings were obtained by feeding mice HBsAg transgenic potatoes at different doses (0.1–30 μg/dose) with 10 μg CT adjuvant, three times weekly followed by a parenteral boost of 0.5 μg HBsAg at week 8 (Youm et al., 2010). In this study, anti-HBsAg antibodies in both serum and fecal samples increased in direct relation to the oral dose. The difference in these and the results discussed above (Kapusta et al., 2010; Kostrzak et al., in press) may relate to the use of CT adjuvant in the oral doses and the parenteral boosting after oral dosing, both of which could suppress Treg activation.

In summary, much work using oral delivery of plant-derived HBsAg shows substantial potential for vaccine development. It seems likely that for human use, a purified preparation will be preferred, in order to accurately adjust the dosage to optimal levels, and to avoid possible and variable interfering effects due to other components in crude samples. In this regard, high-level expression will facilitate purification, and the use of a viral replicon system will benefit these pursuits (Huang et al., 2008).

Rice-Based Oral Vaccine (MucoRice)

Attractiveness of Oral Vaccine for the Healthy Life

The mucosal immune system is capable of simultaneously executing two opposing immune responses including active (e.g., antigen-specific neutralizing SIgA antibodies) and quiescent ones (e.g., oral tolerance; Chapter 42). For example, oral immunization of protein antigens with an appropriate mucosal delivery vehicle and/or mucosal adjuvant has been shown to induce antigen-specific SIgA and serum IgG antibody responses, leading to a double layer of protection against mucosally invading pathogens. On the other hand, injectable vaccines, which are currently and most commonly used in the field, are designed to induce protective immunity in the systemic compartment, but are not aimed at inducing mucosal immunity. Since most pathogens related to emerging and reemerging infectious diseases (e.g., *Vibrio cholera*, rotavirus, HIV, influenza virus, and enterotoxigenic *E. coli*) infect the host at the mucosal surface of respiratory or digestive tracts, mucosal vaccination will most likely improve the efficacy of protective immunity offered by the current parenterally administered vaccines and could potentially prevent invasion of the mucosal surface by pathogens. We recognize that cooking the rice (or other edible plant material) is very likely to denature the vaccine antigens, thus rendering them poorly immunogenic. In order to overcome this challenge, Kiyono and coworkers are investigating the oral delivery of rice flour derived from transgenic seeds. A clinical examination of the safety of powdered MucoRice-CTB (dissolved in water) in healthy volunteers showed no adverse reactions (Kiyono et al., unpublished data). A phase I clinical trial is being planned for 2015.

Development of Rice-Based Oral Vaccines from Edible Grains

To overcome critical concerns of the production, storage, and delivery of vaccines, an adaptation of the transgenic rice system has been used for the development of rice-based vaccines (or MucoRice system; Figure 4). The MucoRice system has recently attracted interest as an alternative approach for the development of oral vaccines (Nochi et al., 2007, 2009; Tokuhara et al., 2010; Yuki and Kiyono, 2009; Yuki et al., 2012).

Rice seeds are edible tissues and capable of producing and storing relatively large amounts of transgenic recombinant proteins (Boothe et al., 2010; Streatfield et al., 2001). Recombinant proteins expressed and accumulated in rice seeds are stable for prolonged periods (Daniell et al., 2001b). Another advantage of rice plants is the presence of two protein storage vacuoles called protein bodies (PB) in the starchy endosperm of seeds, which provide a greater space for the accumulation of recombinant proteins (Katsube et al., 1999). PB-I and PB-II are distinguished by differences in shape, density, and protein composition (Yamagata and Tanaka, 1986). The main storage proteins for PB-I are the alcohol-soluble prolamins (e.g., 13K prolamin) and the water-soluble glutelins (e.g., glutelin B1), which accumulate preferentially in PB-II (Katsube et al., 1999; Yamagata and Tanaka, 1986). Due to their water solubility, the proteins in PB-II are more vulnerable to digestion in the gastrointestinal tract than the proteins stored in PB-I. The PB system is thought to be a great advantage of rice for its applicability to oral vaccine development, since an effective oral vaccine would benefit from a built-in safeguard against digestion, particularly against the harsh acidic environment in the stomach. Further, the unique nature of protein storage systems of PB-I and PB-II could be used as a natural way to provide either slow or immediate release of vaccine antigens in the gut. These novel characteristics of the rice seed protein expression and storage system can be used for the development of oral vaccine.

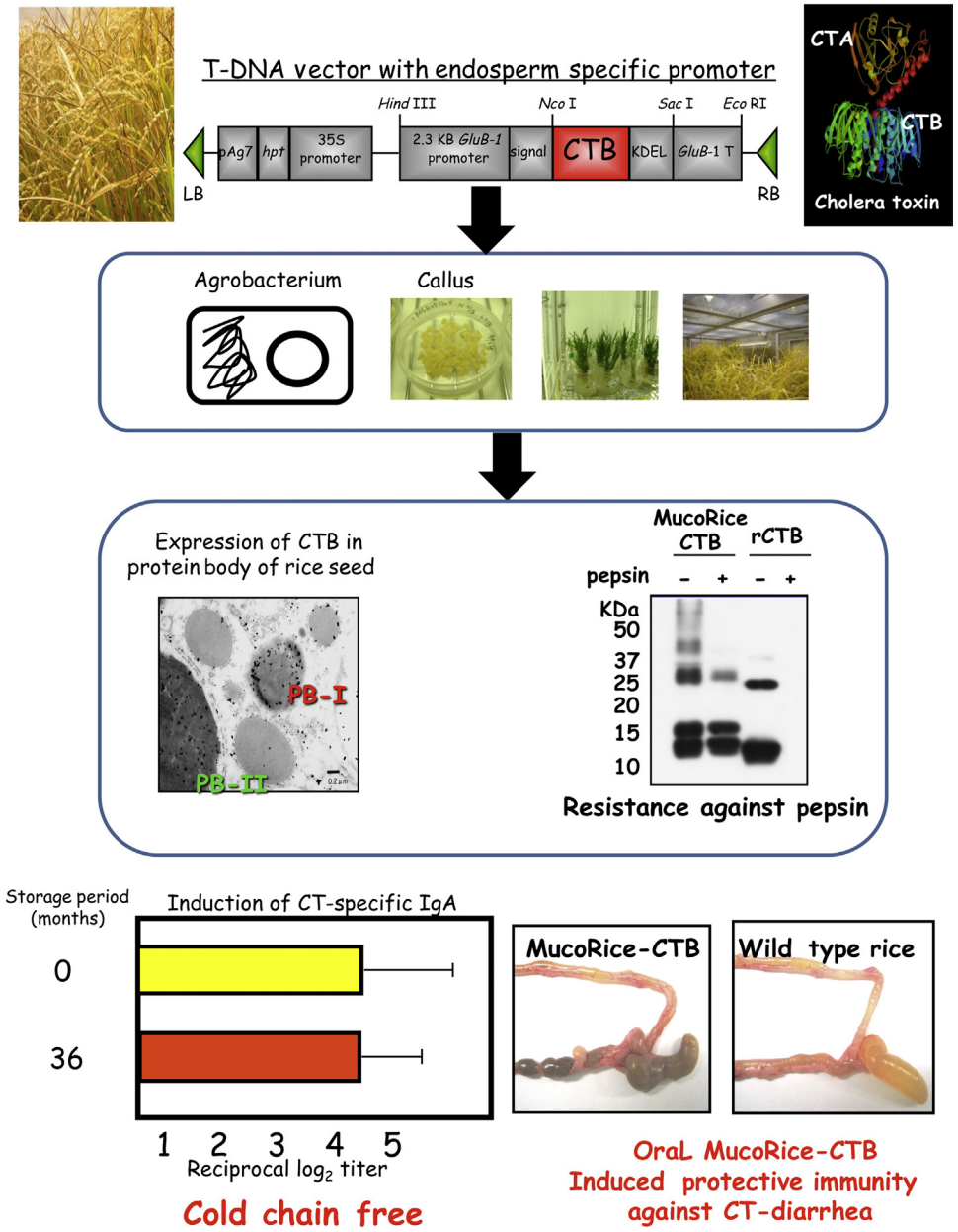

FIGURE 4 Rice-based oral vaccine induces protective immunity against diarrheal diseases. The MucoRice system using the rice protein body (PB)-specific promoter containing the B subunit of cholera toxin (CTB) resulted in the generation of rice seeds expressing CTB. The CTB expressed in the rice seeds was protected from digestive enzyme and stable over a prolonged time (e.g., 36 months) without refrigeration storage. Oral vaccination of mice with MucoRice-CTB resulted in the induction of protective immunity (e.g., CTB-specific SIgA antibodies) against CT-induced diarrhea. Lower right shows intestines from mice after treatment with cholera toxin (CT).

Rice-Based Oral Vaccine for Infectious Diseases

For the proof of concept of MucoRice vaccines, the B subunit of cholera toxin (CTB), an epithelial GM1 ganglioside receptor-binding molecule, was purposely selected as a prototype antigen to demonstrate: (1) the capacity of the rice-based mucosal vaccine to induce systemic as well as mucosal immunity, and (2) to showcase the practicality of using the rice transgenic expression system (Figure 4). Using the transgenic rice system, the accumulation of CTB was achieved at approximately 2% of total seed protein (0.15% of seed weight), which was higher than the other stable transgeneic plant expression systems (e.g., potato tubers and tobacco leaves) (Arakawa et al., 1998; Daniell et al., 2001a; Nochi et al., 2007). Transgenic rice is stable in the harsh environment of the gastrointestinal tract because

rice seed possess protein storage organelles (PB) that can be used as natural antigen-containing capsules. To examine the ability of CTB in rice PB (or MucoRice-CTB) to withstand digestive enzymes in the intestinal tract, total seed proteins were subjected to pepsin treatment in vitro. Most of the CTB accumulated in PB-I of MucoRice seeds remained intact after pepsin treatment, whereas most of the antigen accumulated in PB-II was digested by pepsin under the same conditions (Nochi et al., 2007). It was further shown that the vaccine antigens expressed in the rice seed PB system are stable at room temperature for several years without loss of immunogenicity (Nochi et al., 2007; Tokuhara et al., 2010). The MucoRice system is thus qualified as a suitable system for the expression, accumulation, and storage of vaccine antigens.

To examine the ability of MucoRice-CTB to induce antigen-specific immune responses, MucoRice-CTB was delivered to the intestinal immune system in mice. When the murine intestinal tract containing Peyer's patches (PP) was exposed to MucoRice-CTB, the rice-based vaccine was taken up by professional antigen-sampling M cells located in the follicle-associated epithelium of PP. Successful oral vaccination of mice with MucoRice-CTB thus induced antigen-specific mucosal IgA and serum IgG antibody responses that showed toxin-neutralizing activity and thus prevented toxin-induced diarrhea in the murine model (Nochi et al., 2007; Yuki et al., 2009; Tokuhara et al., 2010). Furthermore, the oral MucoRice-CTB vaccine induced cholera toxin-neutralizing antibodies in nonhuman primates (Nochi et al., 2009). The MucoRice system has further advanced to the expression of the nontoxic form of the entire CT toxin consisting of the mutant A and B subunits (Yuki et al., 2009). To demonstrate the versatility of the MucoRice system, the other toxin-related antigen, a nontoxic fragment of the C-terminal half of the heavy chain from botulinum neurotoxin type A (BoHc), was expressed and produced by the seeds of MucoRice and induced neutralizing antigen-specific antibody responses in mice orally immunized with MucoRice-BoHc (Yuki et al., 2012).

In another adaptation of the transgenic rice seed system, researchers showed the expression of the major outer membrane protein (MOMP) of *Chlamydophila psittaci*, a causative agent for avian chlamydosis, with or without genetic fusion to the B subunit of *E. coli* heat-labile enterotoxin (LTB) (Zhang et al., 2009, 2013). Oral immunization of experimental animals with the rice expressing MOMP or MOMP-LTB resulted in the induction of antigen-specific protective immunity. Further, the rice-based vaccine development was extended to the expression of virus-associated antigens. A neutralizing epitope of porcine epidemic diarrhea virus, the capsid precursor polypeptide of foot-and mouth-disease virus, and the glycoprotein of Newcastle disease virus have been expressed in the rice seeds and showed oral immunogenicity for the induction of virus antigen-specific immune responses (Huy et al., 2012; Wang et al., 2012; Yang et al., 2007b) These findings clearly demonstrate the usefulness and attractiveness of the rice transgenic system for the development of cold chain-, syringe, and needle-free vaccines.

Seed-Based Oral Immunotherapy for the Control of Allergy

The concept of oral tolerance (or systemic unresponsiveness to orally exposed antigen) has been considered as an attractive and logical strategy for the development of immunotherapy for the prevention and/or treatment of allergic diseases. The cellular and molecular mechanisms of oral tolerance have not yet been precisely clarified; however, it is well known that prolonged oral administration of allergens can induce a state of systemic unresponsiveness to the mucosally exposed allergens, perhaps via the generation of regulatory T cells in the GALT. For the purpose of continuous delivery of allergen via an oral route for the induction of oral tolerance, the transgenic rice system has been adopted for the generation of rice seeds expressing allergenic proteins (Takagi et al., 2005). To minimize undesired anaphylactic reactions that might be elicited during the oral desensitization process using allergens, the transgenic rice plants expressing T-cell epitope peptides from pollen allergens of Japanese cedar (e.g., Cry j I and Cry j II) were generated. Oral administration to mice of the transgenic rice seeds expressing the Cry j I and Cry j II T-cell peptides prior to systemic challenge with total cedar pollen allergens resulted in the induction of oral tolerance with the inhibition of allergen-specific allergy-inducible T helper 2 (Th2) responses and their supported allergen-specific IgE responses (Takagi et al., 2005). It was also important to note that the tolerance induced by the rice expressing the T-cell peptides of Cry j I and Cry j II resulted in the inhibition of pollen-induced clinical symptoms of nasal sneezing in a murine inhalation allergic model. These data supported a possibility for the adaptation of transgenic rice seeds expressing allergen-specific T-cell epitopes for oral delivery and induction of oral tolerance against pollen allergen-specific responses.

The transgenic rice system for the expression of allergens was extended to allergic asthma caused by house dust mites (HDM) (Suzuki et al., 2011; Yang et al., 2008). A major HDM allergen, Der p1, and Der p1-associated immunodominant human and mouse T-cell epitopes have been expressed and accumulated in the rice PB system and used for the induction of oral tolerance. Oral administration of mice with the rice seed expressing these HDM antigens resulted in the inhibition of allergen-specific pathological responses including bronchial hyperresponsiveness (Suzuki et al., 2011). These evidences suggest that the rice

transgenic system can be a useful tool for the development of allergen-specific mucosal immunotherapy.

PLANT-BASED MUCOSAL ANIMAL VACCINES

Over 75% of newly emerging human diseases of the past 10 years have been zoonotic, i.e., of animal origin, and some seasonal diseases, such as influenza, can infect both humans and livestock. In addition to this direct link between animal and human health, veterinary diseases indirectly affect public health and well-being through their potentially devastating impact on food production and trade. There is a growing need for the development of safe, effective, and affordable veterinary vaccines.

Plant-based production platforms can help to meet some of the specific requirements for the development of successful and economically viable animal vaccines: The cost of production in the case of veterinary vaccines must be very low in order to allow the vaccine to find broad use in the farming community, particularly in developing countries where these vaccines are often most needed (Rybicki et al., 2013). Production in transgenic plants is scalable and relatively inexpensive. Veterinary vaccines will ideally be stable and not require cold chain storage and transport; expression of vaccine antigens in plant storage organs such as seeds and tubers provides a protective storage vehicle. An easy route of administration will allow the vaccine to be used more broadly in settings where trained veterinary professionals are not at hand and for administration to large populations: Production of an edible vaccine in a fodder crop or reconstituted in feed pellets allows for easy administration by feeding. Most importantly, any vaccine must be safe and effective at protecting the immunized animal from the target disease: Plant-production systems have been used to express protective vaccine candidates, without the risk of contamination with animal pathogens. Since the transmission routes of many animal pathogens are fecal-oral, respiratory, or through direct contact resulting in infection via mucosal surfaces, vaccines targeting these diseases must elicit a strong mucosal immune response in order to be efficacious.

Plants particularly lend themselves to the development of oral vaccine candidates for stimulating mucosal immunity. Since the report of the first potential plant-based animal vaccine in 1993, a chimeric plant-virus particle displaying an epitope of foot-and-mouth disease virus (FMDV) VP1 (Usha et al., 1993), many groups have successfully used plants as a production platform for the development of veterinary vaccines (comprehensively reviewed by Streatfield (2005b), Floss et al. (2007), Hammond and Nemchinov (2009), Rybicki (2010), Rybicki et al. (2013)). In 2006, the United States Department of Agriculture (USDA) licensed the first plant-produced vaccine, a veterinary vaccine against Newcastle disease virus in chickens to Dow AgroSciences (Vermij, 2006), setting a precedence for regulatory approval of plant-made vaccines. Here we will review some of the recent developments in the field of plant-produced mucosal animal vaccines. Particularly, we will focus on vaccines for four target animal groups: bovine/ovine, porcine, poultry, and aquaculture.

Bovine and Ovine Vaccines

Early efforts in the development of plant-made veterinary vaccines have focused on FMDV (Carrillo et al., 1998; Usha et al., 1993). Foot-and-mouth disease is to this day one of the most devastating diseases affecting farmed animals and wild ruminants worldwide, causing economic losses associated with a high morbidity rate and containment procedures involving culling and movement restrictions. Current vaccines are based on inactivated virus, with safety risks associated with its production. In fact, a vaccine manufacturing facility and associated research laboratories were linked to the 2007 outbreak of FMDV in the United Kingdom (Cressey, 2007). Efforts are therefore focused on developing an inherently safe subunit vaccine.

All plant-produced FMD vaccine candidates are based on all or parts of the polyprotein precursor P1, the products of which (VP1, VP2, VP3, and VP4) form the FMDV viral capsid. Various strategies have been followed to express P1 or epitopes thereof, ranging from display of VP1 epitopes on chimeric plant virus particles (Usha et al., 1993; Yang et al., 2007a; Zhang et al., 2010), expression of a hybrid protein containing FMDV B- and T-cell epitopes (Andrianova et al., 2011), fusion of VP1 to reporter proteins (Lentz et al., 2010), and expression of full-length P1 to allow formation of FMDV-like particles (Dus Santos et al., 2005). Chimeric VLPs have been produced by virus inoculation of *Chenopodium* species with an epitope of VP1 displayed on tobacco necrosis virus A (TNV-A) (Zhang et al., 2010) and bamboo mosaic virus (BaMV) (Yang et al., 2007a). Similar yields of 0.33 mg/g fresh weight tissue (FWT) and 0.5 mg/g FWT were achieved, respectively. The efficacy of the BaMV-VP1 preparation with adjuvant was tested by intramuscular (i.m.) and subcutaneous injection of swine, followed by challenge with FMDV. The results indicated stimulation of high levels of anti-VP1 IgG and neutralizing antibodies, production of IFN-γ, and full protection against challenge, even at a single dose of 1 mg BaMV-VP1 (Yang et al., 2007a). Testing of the TNV-VP1 preparation was performed in mice via the intranasal route without adjuvant. While animals developed high titers of serum IgG and IgA as well as fecal and vaginal IgA, much of the immune response was raised against the carrier TNV, with lower levels of anti-VP1 IgG and IgA detected (Zhang et al., 2010). No challenge experiment was performed. Nevertheless, the results indicate that a mucosal immune response was induced.

Following another strategy, the full precursor polypeptide P1 of FMDV has been expressed in transgenic alfalfa (Dus Santos et al., 2005), rice leaves (Wang et al., 2012), and transiently in leaves of *N. benthamiana* (Thuenemann et al., 2013). The strategy of Dus Santos et al. was to express P1 together with the 3C protease, which cleaves this polyprotein during natural FMDV replication and assembly, with the aim of producing virus-like particles. Relatively low expression levels of 0.005–0.01% total soluble protein (TSP) were achieved in transgenic alfalfa leaves; however, IP immunization of mice with crude leaf extract (total of four injections with adjuvant) induced an FMDV-specific immune response and successfully protected mice against challenge (Dus Santos et al., 2005). Production of FMDV VLPs was also attempted by transient coexpression of a modified FMDV VP1 with protease cleavage sites recognized by cowpea mosaic virus 24K protease, and the protease itself (Thuenemann et al., 2013). Though expression and processing of P1 was detected, no VLP formation was observed. Recently, Wang et al. demonstrated the oral immunogenicity in mice of P1 expressed in transgenic rice leaves at up to 0.13% TSP (Wang et al., 2012). Serum IgG and mucosal IgA were detected after the first 2 weeks of a 4-week feeding regimen of five weekly doses of 10 μg P1 antigen. However, only 20–40% of animals showed virus clearance after challenge. One reason for the low efficacy of orally delivered FMDV vaccine from plants may be the low expression levels achieved.

This low expression level was addressed by the transplastomic expression of FMDV VP1 cotranslationally fused to the β-glucuronidase (β-GUS) reporter (Lentz et al., 2010). Expression levels of up to 51% TSP were achieved and the fusion protein was able to induce serum IgG production after IP injection in mice. Further investigations are necessary to determine whether the described FMDV vaccine candidates are efficacious in the target animals, cattle and sheep. Another fusion protein consisting of B- and T-cell epitopes of FMDV VP1, VP4, 2C, and 3D has been expressed transiently in *N. benthamiana* at levels of 0.5–1% TSP (Andrianova et al., 2011). Immunogenicity studies in guinea pigs showed the formation of neutralizing antibodies (NA) and protection from challenge with FMDV.

Recently, a VLP-based strategy has been employed for the production of a bovine papillomavirus (BPV) vaccine candidate by transient expression in *N. benthamiana* (Love et al., 2012). Bovine papillomavirus is tumorigenic in cattle and horses and traditional vaccines cannot be produced because BPV cannot be grown in culture. Virus-like particles of BPV were produced by expression of BPV L1 protein, and yields of 183 μg/g FWT were obtained after purification. Immunogenicity of the particles was shown by parenteral administration in rabbits.

A group in India has shown the potential of plants as a production platform for oral rinderpest virus (RPV) and peste des petits ruminants virus (PPRV) by performing immunogenicity studies in the target animals. Both viruses are members of the Morbillivirus genus of the *Paramyxoviridae* and can cause severe diseases in cattle and small ruminants, respectively. Khandelwal et al. have expressed the hemagglutinin (H) protein of RPV and hemagglutinin-neuraminidase (HN) protein of PPRV in transgenic peanut leaves. Cattle were fed with 5–7.5 g of peanut leaves expressing RPV H protein at a level of 0.5% TSP. Single doses administered once a week for 3 weeks elicited a humoral anti-RPV response and NA were detected (Khandelwal et al., 2003). Later immunogenicity tests in mice revealed that oral vaccination induced serum IgG and IgA (Khandelwal et al., 2004). In the case of PPRV HN protein, sheep were fed in a similar way with 5 g of transgenic peanut leaves once a week for 5 weeks. High levels of NA were reported after the first week of feeding and a cell-mediated response was detected (Khandelwal et al., 2011). These results demonstrate that plant-produced edible vaccines can elicit strong immune responses in large animals. However, in the case of rinderpest a successful Global Rinderpest Eradication Programme started in the 1990s has resulted in the World Organization for Animal Health (OIE) declaring the world free of rinderpest in 2011, therefore eliminating the need for an edible RPV vaccine.

In contrast, an increasingly economically important disease of cattle is bovine viral diarrhea, caused by bovine viral diarrhea virus (BVDV). The disease is mainly associated with fertility problems in infected cattle and can lead to abortion and malformations of calves. In a quest to develop a safer alternative to the commercially available inactivated vaccine, recent progress has been made by two groups in Argentina using plant expression systems. Dus Santos and Wigdorovitz reported the development of transgenic alfalfa plants expressing BVDV structural protein E2, which encodes the main antigenic sites (Dus Santos and Wigdorovitz, 2005). The same group has further developed this system by fusing a truncated version of E2 (tE2) to an APCH molecule, which targets antigen presenting cells (Aguirreburualde et al., 2013). Expression levels were relatively low at 1 μg/g FWT; however i.m. immunization of cattle with plant extract containing 3 μg APCH-tE2, followed by a booster shot, was able to afford protection from challenge with BVDV, with no virus detectable in these animals. Another group achieved higher expression levels of 20 μg/g FWT by transient expression of truncated E2 (not as a fusion) in *Nicotiana tabacum* (Nelson et al., 2012). Guinea pigs were immunized subcutaneously with 20 μg of E2 antigen that was shown to stimulate production of NA in six out of seven animals. It will be interesting to see whether these vaccine candidates can stimulate mucosal responses in cattle when applied directly to the mucosa.

The research groups in Argentina have also investigated the plant-based production of bovine rotavirus

(BRV), which is the leading cause of diarrhea in neonatal calves in that country. Calves are susceptible to the disease within the first 3 weeks after birth; therefore induction of lactogenic immunity is a preferred method of protection. In 2004, Wigdorovitz et al. reported on the efficacy of a plant-produced fusion protein of *eBRV4*, a previously identified peptide capable of eliciting a protective response, to β-GUS (Wigdorovitz et al., 2004). This fusion protein was expressed in transgenic alfalfa at levels up to 0.09% TSP. When tested in a suckling mouse model, pups born to dams fed weekly for 2 months with 6 μg *eBRV4* were protected against BRV challenge. The same group recently produced BRV VP8*, the major immunogenicity determinant, in transplastomic *N. tabacum* at levels of 600 μg/g FWT (150 μg/g FWT soluble fraction) and tested this antigen in the suckling mouse model (Lentz et al., 2011). Dams were IP immunized with 2 μg VP8* and boosted once. Dams showed serum IgG and NA responses. Pups were challenged 5 days after birth and were 80–100% protected compared to control pups, 100% of which developed diarrhea. These results indicate that a plant-based vaccine conferring lactogenic immunity to BRV in calves may be possible. Some early successes in the production of vaccine candidates in plants were achieved with the development of recombinant rabies subunit vaccines in tomato, spinach, and tobacco (McGarvey et al., 1995; Modelska et al., 1998; Yusibov et al., 2002). Oral immunogenicity and parenteral protective efficacy of these vaccines were shown in mice.

Rabies is a zoonotic disease affecting mammals worldwide, spread by bites of infected animals. In developing countries, rabies represents a particular health threat due to widespread infection in the stray dog population. In Latin America, another key reservoir of the rabies virus is the bat population that can spread the disease to livestock. Recently, Loza-Rubio et al. have demonstrated the oral efficacy of a maize-based rabies vaccine in sheep (Loza-Rubio et al., 2012). Sheep in different groups were fed with a single dose of ground maize kernels containing 0.5–2.0 mg of rabies virus G protein (25 μg/g seeds). All doses were shown to elicit NA, with the 2.0-mg dose producing NA titers comparable to those elicited by a commercial inactivated rabies vaccine. After challenge with a lethal dose of rabies virus, all vaccinated groups achieved survival rates of at least 50%, with the highest dose of 2.0 mg recombinant G-protein achieving the same survival rate as the inactivated vaccine (83%). These results show that a plant-produced subunit vaccine can be effective at protecting large livestock animals against rabies.

In addition to these virus vaccines, plants have also been investigated as vaccine production platforms to protect livestock from bacterial pathogens. *Mannheimia haemolytica* is one of the main causal agents of pneumonic pasteurellosis in cattle, a disease with worldwide economic impact. Current treatment involves the use of antibiotics, but prevention with vaccines would be preferable. Lee et al. have expressed the GS60 outer membrane lipoprotein of *M. haemolytica* in transgenic alfalfa at levels of 0.02% TSP (Lee et al., 2008). An immunogenicity study in rabbits was performed to compare oral to i.m. application. Whereas injection efficiently induced anti-GS60 antibody production, oral administration only elicited an immune response in one of six animals. This may be due in part to the low expression levels achieved. Further investigations in cattle should be undertaken.

Porcine Vaccines

Bacterial and parasitic diseases, in addition to several viral diseases, have also been targeted by researchers investigating plants as production platforms for porcine vaccines. In 2012, researchers reported the oral efficacy of plant-produced vaccines against porcine cysticercosis (Betancourt et al., 2012) and *Toxoplasma gondii* (Yácono et al., 2012), as well as the production of a mucosal immunogen against postweaning diarrhea (Kolotilin et al., 2012). Porcine cysticercosis, or pig tapeworm, is caused by the parasite *Taenia solium*. A vaccine consisting of three peptides has been produced in transgenic papaya callus (S3Pvac-papaya) (Hernandez et al., 2007). Rabbits received oral administration of 20 mg of callus, boosted on day 7, and were challenged (with *Taenia pisiformis*, closely related to *T. solium*) on day 15. Upon sacrifice of the animals, researchers found a 90% reduction in hepatic lesions and 94% reduction in cysticerci recovered, thereby showing efficient protection of rabbits (Betancourt et al., 2012).

Another economically important parasite of pigs, as well as humans and other warm-blooded animals and livestock, is *T. gondii*, which causes toxoplasmosis and infects its host through gut mucosa. The *T. gondii* GR4 antigen was expressed in transplastomic *N. tabacum* at levels of 6 μg/g FWT, and suspensions of lyophilized leaf extract were used to orally immunize mice by gavage (Yácono et al., 2012). Mice were orally challenged and later sacrificed to enable brain examination. The experiment showed induction of both systemic and mucosal immune responses and a 59% reduction in brain cyst load compared to that of a control.

In addition to these parasitic diseases, the bacterial postweaning diarrhea caused by enterotoxic *E. coli* (ETEC) has recently been targeted. Kolotilin et al. have expressed ETEC F4 fimbrial adhesin (FaeG) to high levels of 11.3% TSP in transplastomic tobacco, and up to 20% TSP by transient chloroplast-targeted expression in *N. benthamiana* (Kolotilin et al., 2012). The authors showed that the antigen could survive extended incubation in simulated gastric and intestinal fluids, indicating its stability in the gastrointestinal tract and suitability as an oral immunogen. Porcine edema disease in also caused by *E. coli* and a shiga toxin B subunit has recently been expressed in transgenic lettuce (Matsui et al., 2011). Oral immunogenicity experiments in swine are currently in progress.

Most of the porcine diseases currently targeted by plant-based vaccines are caused by viruses. One of the earliest examples was the production of glycoprotein S of porcine transmissible gastroenteritis virus (TGEV) in *Arabidopsis thaliana* and its immunogenicity in mice following i.m. injection (Gómez et al., 1998). This host plant is not compatible with large-scale production of antigens; therefore other groups have expressed the S protein in transgenic *N. tabacum* and maize (Tuboly et al., 2000; Lamphear et al., 2002). Maize-produced TGEV S protein (13 mg/kg seed) was shown to induce NA after oral immunization of sows, and NA were also detected in milk after farrowing (Lamphear et al., 2004), showing the potential of lactogenic immunity in piglets. Furthermore, passive immunization of piglets was achieved by oral application of a plant-produced small immune protein (SIP) targeting TGEV (Monger et al., 2006). Transient expression was used to produce SIP at levels of 2% TSP in cowpea, and animals receiving daily oral doses of resuspended lyophilized plant extract showed a reduction of TGEV virus titer in gut and lung as well as reduced pulmonary lesions compared with the control group.

Several groups have recently investigated the efficacy of plant-produced subunit vaccines against porcine reproductive and respiratory syndrome (PRRS). This disease, first recognized in 1987, has become one of the most important disease of farmed pigs in North America and Europe, and is becoming a problem in other parts of the world, particularly Asia. Current commercial vaccines targeting the causative agent, porcine reproductive and respiratory syndrome virus (PRRSV), either have limited efficacy (killed virus vaccines) or carry inherent risks of reversion (modified-live virus vaccines); therefore an alternative safe, efficacious vaccine is desperately needed. A group in Taiwan first expressed PRRSV envelope glycoprotein GP5, the major target of neutralizing antibodies, as well as an LTB-GP5 fusion protein in transgenic *N. tabacum* (Chia et al., 2010, 2011). Oral immunogenicity and efficacy studies in pigs demonstrated induction of serum IgG and salivary IgA after the second administration, as well as a reduced viral load in organs and reduced clinical disease signs after intranasal challenge with PRRSV. However, yields in these studies were very low at 108–155 ng/g FWT. Improved yields were achieved by expression of GP5 in transgenic potato at up to 4.7 μg/g FWT in leaves and 1.2 μg/g FWT in tubers, with immunogenicity confirmed by intragastric immunization of mice (Chen and Liu, 2011). Other groups have recently reported expression and immunogenicity of the nucleocapsid protein (N) produced in transgenic soybean (Vimolmangkang et al., 2012) and the matrix protein (M) produced in transgenic maize callus (Hu et al., 2012). Production of antigen in soybean and maize has the benefit of easy storage without cold chain; however, further experiments will be needed to show the efficacy of these vaccine candidates.

Vaccines against other viral diseases relevant to the swine industry have been investigated for production in plants with limited success. One example is that of classical swine fever virus (CSFV) which is related to BVDV in cattle and can cause severe acute illness in pigs resulting in fever, loss of appetite, diarrhea, and a high mortality rate. Strategies for vaccine production in plants have focused on CSFV glycoprotein E2 either as a subunit vaccine (He et al., 2007; Legocki et al., 2005) or as an epitope displayed on a chimeric VLP of potato virus X (Marconi et al., 2006). Of these studies, only one was able to show induction of serum IgG and fecal IgA after oral immunization of mice (Legocki et al., 2005). Another example is porcine epidemic diarrhea virus (PEDV), a coronavirus related to TGEV but causing less severe symptoms. In 2003, a subunit vaccine candidate based on the neutralizing epitope of the TGEV spike protein (COE) was expressed in transgenic *N. tabacum* and shown to stimulate production of specific serum IgG and fecal IgA when administered orally to mice (Bae et al., 2003). Similar approaches have involved the rice-based expression fusion proteins of COE to LTB (Oszvald et al., 2007), and COE to the M cell-targeting ligand Co1 (Huy et al., 2012). The latter COE-Co1 showed weak induction of serum IgG and fecal IgG when administered to mice by gavage. Challenge experiments with PEDV and testing in the target animal will need to be performed to determine efficacy of these vaccine candidates.

Avian Vaccines

There has been a recent surge of interest in plants as a production platform for poultry vaccines. This may in part be due to the USDA approval of a plant cell culture-based vaccine for Newcastle disease virus (NDV) in chickens in 2006 (Vermij, 2006). Though the vaccine has not been marketed, this was a significant milestone for the regulatory approval of plant-produced vaccines and pharmaceuticals in general. In addition to NDV, targets of recently developed plant-based poultry vaccine candidates include infectious bursal disease virus (IBDV), avian reovirus, *C. psittaci*, and the causal agent of chicken coccidiosis.

Infectious bursal disease is an economically important disease worldwide, which can cause either subclinical infections leading to long-lasting immunosuppression, or acute clinical infection that can have a high mortality rate. The main immunogenicity determinant IBDV VP2 has been expressed in *A. thaliana* and rice (Wu et al., 2004, 2007). Feeding of chickens with 5 g of transgenic rice grains on four occasions induced production of IgG and NA and after challenge with IBDV afforded better protection against formation of bursal lesions than a live-attenuated commercial vaccine administered intranasally. Recently, a different strategy has seen the presentation of a VP2 antigen on bamboo mosaic virus particles, and chimeric VLPs were shown

to induce IBDV-specific serum IgG after i.m. injection in chicken (Chen et al., 2012). These results indicate that a plant-based mucosal vaccine against IBDV may provide a good alternative to commercial vaccines.

Chlamydophila psittaci is the causal agent of avian chlamydiosis, a zoonotic disease that causes respiratory symptoms in birds as well as humans. Transgenic rice seed has been used for the expression of *C. psittaci* MOMP as well as fusions of MOMP to LTB (Zhang et al., 2008). Though expression levels were very low (approaching 0.01% TSP), MOMP-specific humoral and mucosal immune responses were detected after oral application of plant-produced MOMP in mice (Zhang et al., 2009, 2013). In addition to serum IgG and fecal IgA, the induction IFN-γ and other cytokines was reported. Despite these positive results, only approximately 50% of animals were protected against challenge with aerosol-contained *C. psittaci*.

Chicken coccidiosis is caused by protozoans of the *Eimeria* genus that can cause various species-dependent symptoms associated with damaged intestinal mucosa. The widespread use of anticoccidial drugs has led to the development of resistance; therefore safe, effective, and low-cost vaccines are needed to control the disease. Recently, Sathish et al. have reported the plant-based transient expression of *Eimeria tenella* microneme-1 and microneme-2 by vacuum infiltration of *N. tabacum* (Sathish et al., 2011, 2012). Chickens were immunized by i.m. injection and challenged by oral inoculation with sporulated oocycsts. Researchers found induction of serum IgG, a 60% reduction in oocyst shedding, and significantly higher weight gain compared to unvaccinated challenged animals. It will be interesting to see whether administration of these vaccine candidates via mucosal routes further enhances the protective efficacy.

Recent progress has also been made in the development of plant-made natural and recombinant adjuvants and immune stimulators for use in the poultry sector. Natural ginseng stem-and-leaf saponins, a traditional Chinese herbal medicine, have been found to enhance the immunogenicity of a commercial mucosal NDV vaccine when administered orally with water for 7 days before vaccination (Zhai et al., 2011). Another group has shown the safety and immunogenicity of a plant-produced mutant *E. coli* heat labile holotoxin (LTA-K63/LTB) for use as an adjuvant in the poultry industry (Miller et al., 2012). The combination of these adjuvants and stimulators with the plant-made poultry vaccine candidates described above may help in the formulation of more efficacious vaccines in this sector.

Aquaculture Vaccines

A relatively new area of application for plant-produced vaccines is in the aquaculture industry. Farming of fish and other aquatic organisms is gaining increasing importance as many seas become overfished. Intensive fish farming brings increased risks of disease that must be dealt with in an efficient and cost-effective manner. In-feed delivery of vaccines is one potential method of protecting fish stock without labor-intensive application.

In 2006, a publication provided the first indication that oral immunization of fish with plant-produced antigens could work. Companjen et al. used transgenic potato plants to express two model fusion protein antigens: *E. coli* heat-labile toxin B (LTB) fused to either GFP (LTB-GFP) or epitopes of influenza HA and canine parvovirus VP2 (LTB-p) (Companjen et al., 2006). Oral administration by incorporation of LTB-GFP potato into food pellets induced an anti-GFP antibody response in carp. Anal intubation of carp with extract from LTB-p potato induced an antiparvovirus response after the first administration. These results provide a good indication that oral immunization of fish is possible using plant-produced antigen. However, further studies using antigens derived from relevant fish pathogens, as well as challenge experiments, will need to be performed.

Recently, a group in Thailand reported the protection of shrimp from infection with white spot syndrome virus (WSSV) by i.m. coinjection of WSSV with a viral binding protein produced in *A. thaliana* suspension cultures (Thagun et al., 2012). Shrimp do not possess a developed immune system as such; therefore this cannot be classified as a vaccine. However, it does indicate the growing acceptance of plant-produced pharmaceuticals for use in aquaculture.

CONCLUSIONS

The use of plants for production of recombinant proteins has substantially evolved over the past 25 years. The first plant-based vaccines were expressed in stably transgenic plants, with the idea to conveniently deliver "edible vaccines" by ingestion of the antigen-containing plant material. These systems provided a proof of concept that oral delivery of vaccines in crude plant material could stimulate antigen-specific serum and mucosal antibodies. Several clinical trials have shown the potential of plant-derived orally delivered vaccines in humans using raw potato tubers as a convenient model system. Difficulties with implementation of the potato tuber system led to the use of transgenic grains such as rice and corn (maize). "MucoRice" expressing CT-B emerged as a strong candidate for clinical trials, providing a stable and robust vehicle for oral antigen delivery. Moreover, transgenic grains have strong potential for use as edible vaccines for agricultural animals (swine, poultry), and several studies support the idea.

Some challenging issues exist with stably transgenic plants, including relatively low expression levels and regulatory issues related to the use of human food crops as hosts. Thus, many recent studies used transient expression with plant viral vectors to achieve rapid, high-level expression

in a nonfood plant, *N. benthamiana*, followed by purification of the antigenic proteins. Delivery of purified proteins by oral or intranasal routes can result in robust stimulation of both systemic and mucosal immune responses. A few studies examined the potential of novel adjuvant, mucoadhesive, or immune-modulating molecules for mucosal delivery with very promising results.

In conclusion, the use of plants for production and mucosal delivery of vaccine antigens has grown tremendously in research laboratories around the world. The acceptance of plant-based technologies by the vaccine industry may yet be some years away, but is likely to occur when further safety and efficacy studies prove their worth.

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