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MINI-REVIEW

Recent advances and safety issues of transgenic plant-derived vaccines

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Abstract Transgenic plant-derived vaccines comprise a new type of bioreactor that combines plant genetic engineering technology with an organism's immunological response. This combination can be considered as a bioreactor that is produced by introducing foreign genes into plants that elicit special immunogenicity when introduced into animals or human beings. In comparison with traditional vaccines, plant vaccines have some significant advantages, such as low cost, greater safety, and greater effectiveness. In a number of recent studies, antigen-specific proteins have been successfully expressed in various plant tissues and have even been tested in animals and human beings. Therefore, edible vaccines of transgenic plants have a bright future. This review begins with a discussion of the immune mechanism and expression systems for transgenic plant vaccines. Then, current advances in different transgenic plant vaccines will be analyzed, including vaccines against pathogenic viruses, bacteria, and eukaryotic parasites. In view of the low expression levels for antigens in plants, high-level expression strategies of foreign protein in transgenic plants are recommended. Finally, the existing safety problems in transgenic plant vaccines were put forward will be discussed along with a number of appropriate solutions that will hopefully lead to future clinical application of edible plant vaccines.

Keywords Transgenic · Plant · Vaccine · Safety

Introduction

Infectious diseases have become grave threats to human and animal health. However, the use of existing vaccines is an effective way of preventing the occurrence of these diseases. With the development of biotechnology, the type of vaccines has developed from the bacterial vaccine to subunit vaccine or DNA vaccine, but these products are difficult to

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market because of high production costs. Transgenic plant-based vaccines have some advantages over traditional injected vaccines, such as a wide-range possible expressed epitopes, large biomass of cultivation, low cost, and ease of transportation and preservation. Above all, vaccination can be directly carried out orally, rather than parenterally, and often without extraction and purification. The existence of the plant cell wall obviously has a sustained-release effect for the delivery of the antigen. Therefore, in recent years, the research and application of genetically modified (GM) plant vaccines has been a hot topic in vaccine research. This paper reviews current advances in different transgenic plant vaccines, such as vaccines against pathogenic viruses, bacteria, and eukaryotic parasites, as well as the existing safety problems in products obtained from transgenic plant vaccines.

Immune response to transgenic plant vaccines

Research on genetically modified plant vaccines has progressed for over 20 years. Curtiss and Cardineau 1990 first successfully expressed *Streptococcus* surface protein A (SPaA) gene in tobacco seeds, and the SPaA expressed in transgenic tobacco plants was shown to produce an immune reaction. Afterwards, Mason et al. (1992) produced Hepatitis B vaccine in transgenic tobacco, which demonstrated that proteins expressed in transgenic plants cannot only maintain their natural conformation, but they also retain their antigenic determinants by stimulating the immune response of B and T cells. Thus, the concept of an edible vaccine was presented for the first time. The idea of edible vaccine obtained from plants has received a great deal of attention from both academia and private enterprise. Kapusta et al. (1999) expressed HBsAg in transgenic lettuce and observed a preliminary immune effect via oral administration given to volunteers, which showed that edible vaccines from transgenic plants could induce potent specific immune responses in human beings. It has been demonstrated on the one hand that transgenic plant vaccine administered by an oral route could induce the body to produce both humoral immune and cellular immune responses, which ultimately can give rise to immunity (Rigano et al. 2006; Li and Xi 2004). On the other hand, both the stomach and intestinal tract have an independent mucosal immune system, which we consider in the following in a description of the mechanisms of activation of the immune system when plant virus vaccines are administered orally.

After human or animals eat transgenic plants (such as potatos or tomatoes) expressing the oral vaccine, the released antigens are identified and swallowed by ruffled cells (M cells) in intestinal mucosa associated with lymphoid tissue (such as Peyer's patch), and then are transferred to lower follicular tissue. After that, its antigen-presenting cells (APC cells) produce the antigen on the surface of APC cell and subsequently activate B lymphocytes with the help of Th cells. The activated B lymphocytes, which participate in mucosal immunity, are transferred to the mesentery lymph node and finally develop into plasma cells. The secreted IgA in plasma cells reaches membrane cavities through the epithelium and combines with membrane secretion to form secretory IgA (SIgA). Once they have been transferred to the membrane cavity, the SIgA will combine with specific pathogens (such as pathogenic microorganism toxin), and thereby produce certain immune protection. Subsequently, B lymphocytes activated in the mucosa, secrete serum type-specific antibodies into the blood, and generate certain immune protection. The cytokines in Th cells of the mucosal immune system also can activate cytotoxic T lymphocyte (CTL) and elicit a strong cellular immune response. Thus, transgenic plant vaccines can induce mucosal immunity and also cause humoral and cellular immunity. Studies have shown that mucosal immunity can produce immune protection against both gastrointestinal tract and non-gastrointestinal tract infections (Pearay et al. 2001; Zhu and Zhang 2006).

Plant expression system in plant-based vaccines

Exogenous target genes can be stably expressed and correctly assembled in plant cells via plant expression systems, which is key to successful transgenic plant vaccine production. Different methods of obtaining transgenic plant vaccines are presented in Fig 1. The current plant expression system has the following three principal aspects, including genome expression, chloroplast expression, and plant viral expression system.

Plant cell genome expression system

The basic characteristic of the plant expression system is that it is easy to operate. Recombinant protein products expressed in plant seeds or tubers are easy to preserve and are especially well-suited for the production of polyvalent vaccines and poly-recombinant antibodies. Guerrero-Andrade et al. (2006) transformed the Newcastle disease virus fusion (F) protein

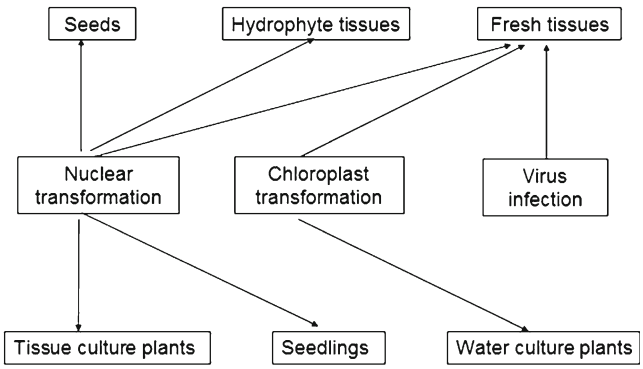

Fig. 1 Different methods of acquiring transgenic plants vaccine

gene into the maize, driven by the maize ubiquitin promoter. Fed with kernels containing the F protein, antibody production was induced in chickens. An exogenous target gene was integrated into the plant genome and transgenic seeds could be bred by traditional breeding technology, which means that they are easily stored, and ready for replanting. Plants are sufficiently versatile that multiple foreign genes may be stored in the same plant through the hybridization of different transgenic plants.

Chloroplast expression system

By gene gun bombardment, foreign genes are introduced to the plant chloroplasts and are likely to be integrated into chloroplast chromosomes. Although the chloroplast transformation system has only been applied to certain plants, the ability to introduce multiple copies of a gene would improve foreign protein expression in plants (Daniell et al. 2002). Moreover, the rigorous maternal inheritance pattern (namely a hereditary phenomenon controlled by the chromosomes outside the nucleus) of chloroplast genes may avoid genetic contamination during plant pollination and eliminate gene silencing in the process of transformation.

Plant viral expression system

This expression system is based on infection of a plant by a plant virus, which is competent to independently replicate, transcribe, and translate so as to produce many copies of a recombinant protein introduced into the plant viral genome. The advantages of this expression system lie in rapid research methods, higher yields, and smaller demands on the planting area. By improving plant virus expression systems, exogenous genes cannot only be expressed as soluble protein and distributed in the cytoplasm of the host plant cell, but they also can fuse with plant virus capsid protein genes and constantly be expressed when virus particles replicate and translate viral proteins in the infected state. The native cottontail rabbit papillomavirus (CRPV) L1 capsid protein gene was expressed transgenically via *Agrobacterium tumefaciens* transformation and transiently via a tobacco mosaic virus (TMV) vector in *Nicotiana* spp. L1 protein was detected in concentrated plant extracts at concentrations up to 0.4 mg/kg in TMV-infected plants (Kohl et al. 2006). A recombinant plant virus (CPMV-PARVO1) was inactivated by UV treatment to remove the possibility of the recombinant plant virus replication in a plant host after the manufacture of the VP2 capsid protein vaccine of canine parvovirus. The result showed that the inactivated canine parvovirus (CPV) was able to protect dogs from a lethal challenge with CPV, and all of the immunized dogs elicited high titers of peptide-specific antibody, which neutralized CPV in vitro (Langeveld et al. 2001). Yang et al. (2007) successfully expressed foot-and-mouth disease VP1 protein by Bamboo Mosaic virus carrier. In addition, other plant viruses are used for expression vectors, such as Tomato bushy stunt virus (TBSV) and Alfalfa mosaic virus (ALMV) (Yang et al. 2007).

Recent advances in transgenic plant vaccines

Over the past 10 years, since heavy-chain and light-chain immunoglobulins were successfully expressed in tobacco, and both of them were then assembled into a functioning antibody, plants have gradually become viable expression systems for the production of various types of antibodies. The number of articles published every year on transgenic plant vaccines in various databases (such as ScienceDirect, Springerlink, Wiley InterScience and Web of science) is shown in Fig 2. There were 835 articles in Elsevier, 841 in Springerlink, 808 in Wiley, and 313 in Web Of Science in 2011. Figure 2 shows that from 2000 to 2011, an increasing number of vaccines have been successfully expressed in various transgenic plants. At present, there are dozens of plants used for animal and human vaccines. The main immunogenic genes include: HBsAg gene, *Escherichia coli* heat-sensitive bowel toxin B subunit (LT-B) gene, rabies

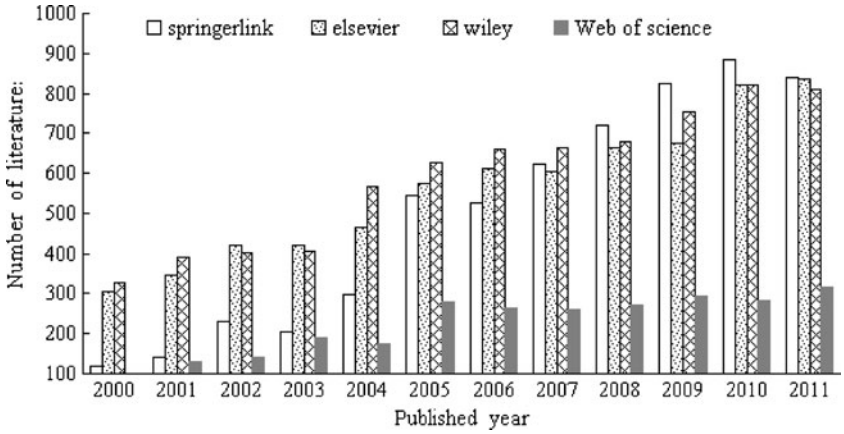

Fig. 2 Number of published articles on transgenic plant vaccines in recent decades

virus glycoprotein (G protein) gene, foot-and-mouth disease VP1 gene, rotavirus gene, and so on.

Recent research has concentrated on the application of transgenic plant vaccines in humans and animals. Reported transgenic plant vaccines can be divided into the following four types: against bacteria, against viruses, against parasites, and immunocontraceptive vaccines.

Bacterial vaccine

The bacterial subunit vaccine products with great potential are the heat labile toxin (LT) of enterotoxigenic *E. coli* B subunit vaccine (LT-B) and the cholera toxin (CT) of *Vibrio cholerae* B subunit vaccine (CT-B). Recent applications of the two kinds of vaccines are shown in Table 1.

Enterotoxigenic strains of *E. coli may* produce a heat-labile holotoxin (LT), which will cause diarrhea. Karaman et al. (2006) engineered corn seeds to produce LT-B, the non-toxic subunit of LT, to serve as a plant-derived vaccine to traveler's diarrhea and as an adjuvant for co-administered proteins. Specific IgA and IgG antibodies were detectable in mice fed with transgenic corn. Tae-Jin Kang et al. (2003) reported a feasibility study for producing the nontoxic LT-B via chloroplast transformation of tobacco. The amount of LT-B protein detected in transplastomic tobacco leaf was approximately 2.5 % of the total soluble plant protein, approximately 250-fold higher than in plants generated via nuclear transformation.

Cholera is a highly epidemic diarrheal disease that continues to devastate many developing countries with poor socio-economic conditions, where the sanitation and public hygiene systems are rudimentary (Kaper et al. 1995). Cholera is caused by *V. cholerae* and disease severity is mediated by the potent action of the cholera toxin (CT), which stimulates the secretion of water and electrolytes into the intestine (Field et al. 1989). The CT has been reported as representative of adjuvants that can induce mucosal immunity efficiently (Elson 1989; McGhee et al. 1992). The generation of non-toxic CT derivatives that retain adjuvant activity could provide a safe alternative for the evaluation of these toxins as mucosal adjuvants in humans and animals. CT is composed of distinct A and B subunits. The pentameric B subunit (CT-B) contains five identical polypeptides, shows good immunogenicity, targets the glycosphingolipid receptors on eukaryotic cell surfaces, so consequently CT-B subunit is a well-characterized antigen against cholera. Jiang et al. (2007) developed transgenic tomato-expressing CT-B protein, especially in the ripening tomato fruit under the control of the tomato fruit-specific E8 promoter. Gavage of ripe transgenic tomato fruits induced both serum and mucosal CT-B-specific antibodies in mice. Kim et al. (2009) fused CT-B to an endoplasmic reticulum (ER) retention signal (SEKDEL) and expressed the protein in carrot roots. The produced sCT-B in transgenic carrot roots demonstrated strong affinity for GM1-ganglioside, suggesting that the sCT-B conserved the antigenic sites for binding and proper folding of the pentameric sCT-B structure. The expression level of sCT-B comprised approximately 0.48 % of total soluble protein (TSP) in root of transgenic carrot. A synthetic CTB was fused with a synthetic neutralizing epitope gene of the porcine epidemic diarrhea virus (sCTB–sCOE), and the sCTB–sCOE fusion gene was introduced into a plant expression vector under the control of the ubiquitin promoter. This plant expression vector was transformed into lettuce (*Lactuca sativa* L.) by the *Agrobacterium*-mediated transformation. The expression level of CTB–COE fusion proteins reached 0.0065 % of the total soluble protein in transgenic lettuce leaf tissues (Huy et al. 2011).

Viral vaccine

The most common applications of viral antigens in transgenic plants and current advances of research on the various viral vaccines are summarized in Table 2.

Foot-and-mouth disease virus vaccine Foot-and-mouth disease (FMD) affects all domesticated cloven-hoofed animals (such as cows, pigs, and sheep) as well as wild ruminants. The causative foot-and-mouth disease virus (FMDV), an aphthovirus of the Picornaviridae family, is highly contagious. Thus, FMD is the most significant constraint to international trade in live animals and animal products today (Grubman and Baxt 2004). The FMDV structural protein VP1 plays a key role in the immunogenicity of the virion, which can elicit the production of specific antibodies against a major immunogenic site located between amino acids 140 and 160 (Meleon et al. 1979; Brown 1992).

The VP1 protein has been overexpressed in *Arabidopsis thaliana*, alfalfa, potato, and so on. Wigdorovitz et al. (1999) reported the development of transgenic plants of alfalfa expressing the structural protein VP1 of FMDV. Mice parenterally immunized by using leaf extracts from the transgenic plants developed a virus-specific immune response. Subsequently, Dus Santos et al. (2002) produced transgenic plants of alfalfa expressing the immunogenic site between amino acid residues 135–160 of structural protein VP1 (VP135–160) and the fused βGUS protein. After the FMDV epitope expressed in plants was highly immunogenic in mice, a strong anti-FMDV antibody response against a synthetic peptide representing the region VP135–160 was detectable. In 2005, Dus Santos et al. obtained the production of transgenic alfalfa plants containing the genes encoding the polyprotein P1 and the protease 3C of FMDV. Parenterally immunized mice developed a strong antibody response and were completely protected when challenged with the virulent virus. The transgenic tomato plants were

Table 1 Advances of bacterial vaccine expressed in transgenic plants

Pathogen	Antigen	Production system	Expression	Efficacy	References
Heat-labile toxin	LT-B[a]	Tobacco leaf; potato	0.001 %TSP/ 0.0002 % FW	Immunogenic by oral delivery to mice	(Haq et al. 1995)
Heat-labile toxin	LT-B	Tobacco leaf	0.095 % of TSP	None tested	Wang et al. (2001)
Heat-labile toxin	LT-B	Potato tuber	0.2 % TSP/ 0.001 % FW	Immunogenic and protective by oral delivery to mice	Mason et al. (1998)
Heat-labile toxin	LT-B	Potato tuber	None tested	Immunogenic and protective by oral delivery to humans	Tacket et al. (1998)
Heat-labile toxin	LT-B	Potato tuber	None tested	Immunogenic and protective by oral delivery to nice	Lauterslager et al. (2001)
Heat-labile toxin	LT-B	Maize seed	1.8%TSP	None tested	Streatfield et al. (2001); Streatfield et al. (2002), (2003)
Heat-labile toxin	LT-B	Maize seed	0.07~3.7 % TSP	Immunogenic and protective by oral delivery to mice	Chikwamba et al. (2002a, b);
Heat-labile toxin	LT-B	Maize seed	None tested	Immunogenic and protective by oral delivery to mice and swine	Lamphear et al. (2002)
Heat-labile toxin	LT-B	Chloroplasts of tobacco	2.5 % TSP	None tested	Kang et al. (2003)
Heat-labile toxin	LT-B	Tobacco mosaic virus-infected *Nicotiana benthamiana* plants	75 μg/g FW	Immunogenic by oral delivery to mice	Wagner et al. (2004)
Heat-labile toxin	LT-B	Transgenic corn	None tested	LT-B expressed in transgenic corn was well tolerated and immunogenic in humans.	Tacket et al. (2004)
Heat-labile toxin	LT-B	Tobacco	3.3 % of TSP	None tested	Kang et al. (2005)
Heat-labile toxin	LT-B	Corn seeds	None tested	Systemic and mucosal antibody responses to LT-B in young and aged mice, and recall responses to oral administration and injection of LT-B in aged mice.	Karaman et al. (2006)
Heat-labile toxin	LT-B	Siberian ginseng	0.36 % of TSP	None tested	Kang et al. (Kang et al. 2006a, b, c)
Heat-labile toxin	LT-B	Carrot	0.3 % of TSP	None tested	Rosales-Mendoza et al. (2007a)
Heat-labile toxin	LT-B	Soybean seed	2.4 % of the total seed protein	Induced mice both systemic IgG and IgA, and mucosal IgA antibody responses	Tomas et al. (2007)
Heat-labile toxin	LT-B	Lettuce leaf	1.0–2.0 % of TSP	None tested;	Kim et al.(2007)
Heat-labile toxin	LT-B	Lettuce leaf	0.05 % of TSP	Balb/c mice elicited specific and significant antibody responses in both serum and intestinal tissues	Martı´nez-Gonza´lez et al. (2011)

Table 1 (continued)

Pathogen	Antigen	Production system	Expression	Efficacy	References
Heat-labile toxin; Chlamydophila psittaci	A DNA fragment encoding the MOMP gene of Chlamydophila psittaci was fused to LT-B gene (LTB-MOMP)	Rice leaves	0.0033e0.0054 % of TSP	None tested	Zhang et al., (2008)
Heat-labile toxin	LT-B	Carrot	None tested	Balb/c mice elicited significant serum and intestinal antibody responses	Rosales-Mendoza et al. (2008)
Heat-labile toxin	The *Escherichia coli* heat-labile enterotoxin B subunit	Watercress	1.3 % of TSP	None tested	Loc et al.(2011a)
Heat-labile toxin	*Escherichia coli* heat-labile enterotoxin B subunit	*Peperomia pellucida*	0.75 % of TSP	None tested	Loc et al. (2010)
NDV Newcastle disease virus	Synthetic LT-B and Hemagglutinin–Neuraminidase-Neutralizing Epitope Fusion Protein	Tobacco chloroplasts	0.5 % of TSP	None tested	Sim et al. (2009)
Heat-labile toxin	An *Escherichia coli* antigenic fusion protein comprising LT-B and the heat stable toxin	Tobacco plants	2.3 % of TSP	Oral immunization of mice led to the induction of both serum and mucosal LTB-ST specific antibodies.	Rosales-Mendoza et al. (2007b)
Enteropathogenic *Escherichia coli* (EPEC)	Bundle-forming pilus structural subunit A	Tobacco leaf	8 % TSP/1 % FW	Immunogenic by oral delivery to ice	da Silva et al. (2002)
Cholera toxin	CT-B[b]	Potato tuber	0.3 % TSP/0.002 % FW	Immunogenic and protective by oral delivery to mice	Arakawa et al. (1997) Arakawa et al. (1998)
Cholera toxin	CT-B	Tobacco leaf	4 % TSP/0.5 % FW	No published data	Daniell et al. (2001)
Cholera toxin	CT-B	Tomato leaf and fruit	0.04 % TSP/0.005 % FW	No published data	Jani et al. (2002)
Cholera toxin; rotavirus enterotoxin	CT-B fused to rotavirus enterotoxin NSP4	Potato tuber	0.0003 % FW	Immunogenic and protective (passive immunity) by oral delivery to mice	Yu and Langridge, (2001)
Cholera toxin	CT-B	Tomato fruit	0.081 % of TSP	Gavage of mice induced both serum and mucosal CTB specific antibodies.	Jiang et al. (2007)
Cholera toxin	CT-B	Tomato fruit	0.9 % of TSP	No published data	Loc et al. (2011b)
Cholera toxin	CT-B fused to an endoplasmic reticulum retention signal (SEKDEL)	TCarrot (*Daucus carota L.*)	0.48 % of TSP	None tested	Kim et al. (2009)
Cholera toxin; porcine epidemic diarrhea virus	A synthetic CT-B was fused with a synthetic neutralizing epitope gene of the porcine epidemic diarrhea virus (sCTB–sCOE),	Lettuce (*Lactuca sativa* L.)	0.0065 % of TSP	Induce efficient immune responses against porcine epidemic diarrhea virus infection by administration	Huy et al. (2011)

Table 1 (continued)

Pathogen	Antigen	Production system	Expression	Efficacy	References
Cholera toxin	A cDNA encoding the simian–human immunodeWciency virus (SHIV 89.6p) Tat regulatory element protein was fused to the cterminus of CT-B (*ctx*B-*tat*)	Potato leaf	0.005–0.007 % of TSP or 4.6 mg per 100 g potato tuber tissue	None tested	Kim et al. (2004a)
Cholera toxin; anthrax	A DNA encoding the 27-kDa domain I of anthrax lethal factor protein (LF), was linked to the carboxyl terminus of CT-B (CTB–LF).	Potato leaf	0.002 % of TSP	None tested	Kim et al. (2004b)
Cholera toxin; simian immunodeficiency virus	A deoxyribonucleic acid (DNA) fragment encoding CT-B was linked 5' to the simian immunodeficiency virus (SIVmac) Gag p27 capsid gene (CTB–Gag)	Potato leaf	0.016–0.022 % of TSP	None tested	Kim et al. (2004c)
Cholera toxin	CT-B	Tobacco plants	1.5 % of TSP	None tested	Kang et al. (2004)
Cholera toxin; simian rotavirus	A gene encoding VP7 of simian rotavirus SA11 was fused to the carboxyl terminus of CT-B	Potato leaf	0.01 % of the total soluble tuber protein	None tested	Choi et al. (2005)
Cholera toxin	CT-B	Lettuce plants	0.24 % of TSP	None tested	Kim et al. (2006)
Cholera toxin	A synthetic CT-B	Tobacco plant	1.8 % of TSP	None tested	Kang et al. (2006)
Cholera toxin	CT-B	Maize	0.0197 % of TSP	Anti-CTB IgG and anti-CTB IgA were detected in the sera and fecal samples of the orally immunized mice	Karaman et al. (2012)
Cholera toxin	Fusion gene of CT-B and the B chain of human insulin	Tobacco plants	0.11 % of TSP	None tested	Li et al. (2006a)
Cholera toxin	Fusing CT-B with allergen-specific T-cell epitopes	Rice seed	None tested	Mice suppressed allergen-specific IgE responses and pollen-induced clinical symptoms	Hidenori et al. (2008)
Cholera toxin; *Vibrio cholerae*	Accessory colonization factor subunit A (ACFA) of *Vibrio cholerae* and ACFA fused to CT-B	Tomato	0.25 % and 0.08 % of TSP	None tested	Sharma et al. (2008a)
Cholera toxin	Synthetic CT-B gene	Rice endosperm	2.1 % of TSP	None tested	Oszvald et al. (2008)
Cholera toxin; *Vibrio cholerae*	Toxin co-regulated pilus subunit A (TCPA) of *Vibrio cholerae* and its immunogenic epitopes fused to CT-B	Tomato	0.17 and 0.096 % of TSP	None tested	Sharma et al. (2008b)

Table 1 (continued)

Pathogen	Antigen	Production system	Expression	Efficacy	References
Cholera toxin; dengue virus	Cholera toxin B subunit-domain III of dengue virus envelope glycoprotein E fusion protein	Tobacco plant	0.019 % of TSP	None tested	Kim et al. (2010)
Enterotoxin ricin; insulin	A fusion protein (INS–RTB) (the non-toxic B chain of the plant AB enterotoxin ricin (RTB) and linked to the coding region of the proinsulin gene (INS)	Potato plants	None tested	None tested	Carter et al. (2010)
Cholera toxin; Rabies	Rabies glycoprotein fused with CT-B	Tobacco plants	0.4 % of TSP	None tested	Roy et al. (2010)

[a] LT-B—heat labile toxin (LT) of enterotoxigenic *E. coli* (ETEC) B subunit

[b] CT-B—Cholera toxin B subunit

also used to express the structural polyprotein P1-2A and protease 3C from FMDV. Guinea pigs immunized intramuscularly with foliar extracts from P1-2A3C-transgenic tomato plants were found to develop a virus-specific antibody response against FMDV (Pan et al. 2008). A single-chain variable antibody fragment (scFv) recognizing FMDV coat protein VP1 was expressed in transgenic tobacco plants. This scFv–ELP fusion accumulated up to 0.8 % of total soluble leaf protein in transgenic tobacco (Joensuu et al. 2009).

Hepatitis B virus vaccine Hepatitis B virus (HBV) infection may lead to liver cirrhosis or hepatocellular carcinoma (Michel 2002). As a retrovirus, HBV is one of the smallest known eukaryotic DNA viruses. HBV negative strand has four ORFs, known as the C, P, S, and X gene, which respectively encode the four major viral proteins—core antigen (HBeAg/ HBcAg), HBV DNA polymerase (HBV DNA P), surface antigen (HBsAg), and X antigen (HBxAg). HBsAg, which is an outer membrane protein encoded by S zone of the HBV genome, is mainly related to the HBV vaccine.

Richter et al. (2000) developed transgenic potatoes expressing 1.1 $\mu g g^{-1}$ HBsAg fresh tubers. By being fed three weekly doses of transgenic potato tubers plus 10 mg cholera toxin (CT), mice produced a primary serum antibody response that peaked at 73 $mIU ml^{-1}$ 3 weeks after the last dose. In addition, transgenic potato plants have been obtained by expressing HBsAg gene under the control of the double promoters of both 35S RNA of cauliflower mosaic virus (CaMV 35S) and the promoter of the patatin gene of potato tubers (Shulga et al. 2004). Huang et al. (2005) also constructed pMHB and transformed tobacco plants (*Nicotiana benthamiana*). The transgenic tobacco leaf extracts (with 1 μg of HBsAg) were administered to mice by intraperitoneal injection at weeks 0, 1, and 2. The serum anti-HBsAg antibody titers gradually increased and at the 12th week the immunized animals had an average titer of 1,165 mIU/ml.

Research has been reported on tomato transformation and regeneration containing HBsAg gene (Ma et al. 2002; Carolina and Francisco 2004; Wang and Li 2008). Our research group successfully trans-ferred HBsAg gene into cherry tomato (*Lycopersivon esculentum* Mill.) and peanut (a higher-expressing level of 2.41 μg/g FW) by *Agrobacterium*- mediated transformation (Zhu et al. 2006; Zhang et al. 2005; Guan et al. 2010).

Besides the plants noted above, an increasing number of transgenic plant systems are in the process of development, such as banana (*Musa acuminate*) (Sunil-Kumar et al. 2005; Elkholy et al. 2009), kelp (*Thallus laminariae*) (Jiang et al. 2002), cherry tomatillo (*Physalis ixocarpa* Brot) (Gao et al. 2003), carrot (Deineko et al. 2009), and soybean (Smith et al. 2002). Kapusta et al. (1999) took the lead in expressing

Table 2 Advances of viral vaccine expressed in transgenic plants

Disease	Pathogen	Antigen (protein or peptide types expressed)	Production system	Expression	Efficacy	Number of references
Food and mouth disease	FMDV[a]	Structural protein VP1	*Arabidopsis thaliana*	None tested	Mice elicited specific antibody responses to synthetic peptides representing amino acid residues 135 to 160 of VP1	Carrillo et al. (1998)
Food and mouth disease	FMDV	Structural protein VP1	Alfalfa plants	None tested	Mice elicited a specific antibody response to a synthetic peptide representing amino acid residues 135–160 of VP1	Wigdorovitz (1999)
Food and mouth disease	FMDV	Structural protein VP1	Potatoes plants	None tested	The male BALB/c mice presented a FMDV VP1 specific antibody response and protection against the experimental challenge	Carrillo et al. (2001)
Food and mouth disease	FMDV	A highly immunogenic epitope from FMDV fused to glucuronidase (*gus* A) reporter gene	Alfalfa plants	None tested	Mice elicited a strong anti-FMDV antibody response against a synthetic peptide representing the region VP135–160	Dus Santos et al. (2002)
Food and mouth disease	FMDV	Dominant epitopes of FMDV serotype O	Tobacco plant	None tested	Most guinea pigs were protected against FMDV challenge; Most of the suckling mice were also protected against FMDV challenge; indicating that antibodies produced in guinea pigs immunized with the TMVF11/TMVF14 mixture specifically neutralized FMDV	Wu et al. (2003)
Food and mouth disease	FMDV	Containing the genes encoding the polyprotein P1 and the protease 3C of FMDV	Alfalfa plants	None tested	Parenterally immunized mice developed a strong antibody response and were completely protected when challenged with the virulent virus	Dus Santos et al. (2005)
Food and mouth disease	FMDV	The epitope of t FMDV fused with a hepatitis B core protein	Tobacco (*Nicotiana tabacum*) plants	None tested	Mice, immunized intraperitoneally with a soluble crude extract of transgenic tobacco leaves, were found to produce specific antibody responses to both HBcAg and FMDV VP1	Huang et al. (2005a)

Table 2 (continued)

Disease	Pathogen	Antigen (protein or peptide types expressed)	Production system	Expression	Efficacy	Number of references
Food and mouth disease	FMDV	Containing FMDV VP1 gene and the selective marker *aadA* gene	Tobacco chloroplast	2–3 % of TSP	None tested	Li et al. (2006c)
Food and mouth disease	FMDV; Cholera toxin	pBI121CTBVP1, (FMDV protein VP1 fused with CT- B subunit)	Potato (*Solanum tuberosum*)	None tested	None tested	He et al. (2007)
Food and mouth disease	FMDV	Structural protein VP1 of FMDV	*Stylosanthes guianensis* cv. Reyan II.	0.1–0.5 % of TSP	Mice developed a virus-specific immune response to the structural VP1 and intact FMDV particles	Wang et al. 2008a)
Food and mouth disease	FMDV	P1-2A3C, the structural polyprotein, P1-2A, and protease, 3C, from FMDV	Tomato plants	None tested	Guinea pigs developed a virus-specific antibody response against FMDV. Vaccinated guinea pigs were fully protected against a challenge infection	Pan et al. (2008)
Food and mouth disease	FMDV	A single chain variable antibody fragment (scFv) recognizing FMDV coat protein VP1	Tobacco plants	0.8 % of TSP	None tested	Joensuu et al. (2009)
Hepatitis B	HBV[b]	HBsAg (S protein)	Tobacco (*Nicotiana tabacum cv. Samsun*)	16.5 ng/g FW	None tested	Mason et al. (1992)
Hepatitis B	HBV	HBsAg	Tobacco (*Nicotiana tabacum cv. Samsun*)	None tested	Mice elicited B- and T-cell responses	Thanavala et al. (1995)
Hepatitis B	HBV	pre-S2+S gene (M protein)	*Nicotiana benthamiana* leaves	None tested	M protein provoked stronger serum antibody responses against HBsAg in mice and humans	Huang et al. (2005b)
Hepatitis B	HBV	HBsAg	Lupin (*Lupinus luteus L.*)	11–150 ng/g FW	Mice developed significant levels of HBsAg-specific antibodies	Kapusta et al.(1999)
		S protein	Potato tubers	16 ug/g FW	A primary immune response was induced in mice fed transgenic tubes which could be greatly boosted by intraperitoneal delivery of a single subimmunogenic dose of commercial HBsAg vaccine. And mice elicited B-cell and T-cell responses	Richter et al. (2000)
Hepatitis B	HBV	S protein	Potato	200–400 ng/g FW	None tested	Shulga et al.(2004)
Hepatitis B	HBV	S protein	Tomato	40–160 ng/gFW	None tested	Ma et al.(2002)
Hepatitis B	HBV	S protein	Tomato	None tested	None tested	Carolina and Francisco (2004)
Hepatitis B	HBV	S protein	Tomato	None tested	None tested	Wang and Li (2008)
Hepatitis B	HBV	S protein	Cherry tomato	None tested	None tested	Hao et al. (2007)
Hepatitis B	HBV	S protein	Peanut *Arachis hypogaea*	2.41 ug/gFW	None tested	Zhu et al.(2006)

Table 2 (continued)

Disease	Pathogen	Antigen (protein or peptide types expressed)	Production system	Expression	Efficacy	Number of references
Hepatitis B	HBV	S protein	Banana	38 ng/g FW (pEFEHBS) 19.92 ng/g FW (pHER)	None tested	Sunil-Kumar et al. (2005)
Hepatitis B	HBV	S protein	Banana	None tested	None tested	Elkholy et al. (2009)
Hepatitis B	HBV	S protein	Kelf	6.67 ug/gFW	None tested	Jiang et al. (2002)
Hepatitis B	HBV	S protein	Cherry tomatillo	2–10 ug/gFW	Elicited an immediate high level recall antibody response	Gao et al. (2003)
Hepatitis B	HBV	S protein	Carrot	None tested	None tested	Zhao et al. (2002)
Hepatitis B	HBV	HBsAg (S protein)	Lettuce (*Lactuca sativa* L.)	1-1.5 ng/g FW	Human volunteers developed specific serum-IgG response to plant-produced protein	Kapusta et al.(1999)
Hepatitis B	HBV	preS2–S protein	Carrot	10 ng/g FW	None tested	Deineko et al. (2009)
Hepatitis B	HBV	L protein	Apple	11.18 ng/gFW	None tested	Lou et al. (2005)
Porcine viral diarrhea disease	PEDV[c]	Neutralizing epitope of Korean PEDV (K-COE)	Tobacco plants	0.1 % of TSP	None tested	Kang et al. (2005b)
Porcine viral diarrhea disease	PEDV	Neutralizing epitope of Korean PEDV (K-COE)	Potato plants	0.1 % of TSP	None tested	Kim et al. (2005)
Porcine viral diarrhea disease	PEDV	The synthetic neutralizing epitope gene of PEDV	No-*nicotine tobacco* plants	2.1 % of TSP	None tested	Kang et al. (2005a, b)
Porcine viral diarrhea disease	PEDV	Spike gene of PEDV	Sweet potato plants	None tested	None tested	Yang et al. (2005)
Porcine viral diarrhea disease	PEDV	A fusion protein consisting of the synthetic LTB and a synthetic neutralizing epitope of PEDV	Tobacco plants	None tested	None tested	Kang et al. (2006a, b, c)
Porcine viral diarrhea disease	PEDV	A fusion protein consisting of LTB and a synthetic core-neutralizing epitope (COE) of PEDV	Rice endosperm	None tested	None tested	Oszvald et al. (2007)
Porcine viral diarrhea disease	PEDV	A fusion gene encoding the synthetic enterotoxigenic LTB genetically fused with a synthetic neutralizing epitope of PEDV (sLTB-sCOE)	Lettuce cells (*Lactuca sativa*)	0.026~0.048 % of TSP	None tested	Huy et al. (2009)
Porcine viral diarrhea disease	Rotavirus	Capsid protein (VP6)	*Nicotiana benthamiana* plants	None tested	None tested	O'Brien et al. (2000)
Porcine viral diarrhea disease	Bovine group A rotavirus	Capsid protein VP6	Potato plants	None tested	Adult BALB/c mice after immunization showed the anti-VP6 response.	Matsumura et al. (2002)
Viral diarrhea disease	Murine rotavirus	Murine rotavirus gene six encoding the 41 kDa group specific capsid structural protein VP6	Potato (*Solanum tuberosum*)	0.01 % of TSP	Oral immunization of CD-1 mice generated measurable titers of both anti-VP6 serum IgG and intestinal IgA antibodies	Yu and Langridge (2003)

Table 2 (continued)

Disease	Pathogen	Antigen (protein or peptide types expressed)	Production system	Expression	Efficacy	Number of references
Rabies	Rabies virus	A gene for the rabies virus glycoprotein (G-protein)	Tomato plants	None tested	None tested	McGarvey et al. (1995)
Rabies	Rabies virus and HIV	Recombinant virus gene of rabies virus and HIV	Tobacco	None tested	Both antigens elicited specific virus-neutralizing antibodies in immunized mice	Yusibov et al. (1997)
Rabies	Rabies virus	Antigenic determinants from rabies virus glycoprotein (G protein) and nucleoprotein (N protein)	*Nicotiana tabacum* cv. Samsun NN plants; *Nicotiana benthamiana* and spinach (*Spinacia oleracea*) plants	None tested	Mice immunized with recombinant virus were protected against challenge infection	Yusibov et al. (2002)
Rabies	Rabies virus	A synthetic gene coding for the surface glycoprotein (G protein) of rabies virus	Tobacco	0.38 % of TSP	Mice immunized intraperitoneally elicited high level of immune response. The plant-derived G protein induced complete protective immunity in mice against rabies virus	Ashraf et al. (2005)
Rabies	Rabies virus	A human anti-rabies virus monoclonal antibody	A *Nicotiana tabacum* cv. Xanthi cell culture	None tested	None tested	Girard et al. (2006)
Rabies	Rabies virus	A full-length nucleoprotein gene of rabies virus	Tomato; *Nicotiana benthamiana*	1–5 % of TSP in tomato and 45 % in N. benthamiana.	Mice were immunized both intraperitoneally (i.p.) and orally with tomato protein extracts containing the N protein induced the production of antibodies. The antibody titer of mice immunized i.p., was at least four times higher than that of mice immunized orally.	Perea Arango et al. (2008)
Rabies; acute diarrhea	Rabies virus and cholera toxin	Rabies glycoprotein fused with B subunit of cholera toxin	Tobacco	0.4 % of TSP	None tested	Roy et al. (2010)
SARS	SARS virus	A partial spike (S) protein of SARS-CoV	Tobacco plant cytosol and chloroplasts	None tested	None tested	Li (2006b)
AIDS	HIV	Tat protein of HIV-1 vaccine	Spinach	None tested	Mice having previously received oral Tat developed higher antibody titers to Tat than did the controls	Karasev (2005)
Newcastle disease	Newcastle disease virus	Fusion (F) protein of NDV	Maize	None tested	Feeding chickens with kernels containing the F protein induced the production of antibodies, which conferred protection against a viral challenge	Guerrero-Andrade et al. (2006)

Table 2 (continued)

Disease	Pathogen	Antigen (protein or peptide types expressed)	Production system	Expression	Efficacy	Number of references
Epidemic gastroenteritis	Norwalk virus	Norwalk virus capsid protein	Tomato	None tested	Four doses of 0.4 g freeze-dried tomato fruit containing 64 μg rNV (40 μg VLPs) induced NV-specific serum IgG and mucosal IgA in≥80 % of mice, while doses of 0.8 g elicited systemic and mucosal antibody responses in all mice	Zhang (2006)
Epidemic gastroenteritis	Norwalk virus	The capsid protein of Norwalk virus	Tobacco and potato	None tested	Mice developed serum IgG specific for rNV	Mason et al. (1996)
Tuberculosis (TB)	Bacterium Mycobacterium tuberculosis	Tuberculosis (TB) ESAT6 antigen	Tobacco	800 mg/kg of fresh leaves	None tested	Dorokhov et al. (2007)
Avian influenza	Pathogenic avian influenza A (H5N1) virus	Hemagglutinin (HA)	Barley grains	None tested	None tested	Bruchmüller et al. (2007)
Colorectal cancer	–	Colorectal cancer antigen EpCAM (pGA733)	*Beta vulgaris* var. *cicla* (Swiss chard) plants	None tested	Recombinant plant-derived antigen induced a humoral immune response in BALB/c mice	Brodzik et al. (2008)
Encephalitis	Encephalitis type B virus	Heavy-china variable domain of mAb against Encephalitis Type B virus	Tobacco	None tested	None tested	Wu and Xiong (1997)

[a] FMDV—Food and mouth disease virus

[b] HBV—hepatitis B virus

[c] PEDV—porcine epidemic diarrhea virus

HBsAg in lupin (*Lupinus luteus*) and lettuce (*L. sativa*) callus. These investigators found that the specific antibodies were produced in mice fed with the transformed callus. The HBV large-surface antigen gene PRS-S1S2S has also been expressed in transgenic apples (Lou et al. 2005).

Porcine viral diarrhea disease vaccine Porcine viral diarrhea is a general term for some acute infectious diseases, which produce a water-like diarrhea and are caused by porcine transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), or porcine rotavirus (RTV).

Porcine transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets, which provokes high mortality rates in affected farms. Protective immunity against this disease has to be developed in pregnant sows in order to confer passive protection to the piglets through colostrum and milk. Neutralizing antibodies against the virus are directed mainly to glycoprotein S, and relevant epitopes in neutralization have been mapped into the N-terminal domain of this protein (Garwes et al. 1978; Jiménez et al. 1986; Correa et al. 1988). Four major antigenic sites have been described in the globular part of glycoprotein S (gS), of which site A is considered to be immunodominant (De Diego et al. 1992, 1994; Sánchez et al. 1990). Transgenic potato plants were created to express the N-terminal domain of the glycoprotein S (N-gS) from TGEV. Extracts from transgenic potato tubers were inoculated intraperitoneally into mice, and the vaccinated mice developed serum IgG specific for TGEV (Gómez et al. 2000). In addition, Tuboly et al. (2000) generated three transgenic tobacco plant lines expressing the spike (S) protein of TGEV. The results showed that antigens from all these lines induced TGEV-specific immune responses in pigs, and the resultant antibody titers for all three constructs were similar.

Porcine epidemic diarrhea virus (PEDV) is an infectious, highly contagious virus of swine, which belongs to the *Coronaviridae* family (Pensaert and de Bouck 1978). PEDV destroys villus enterocytes and causes villus atrophy within the jejunum and ileum. It leads to enteritis in swine of all ages, and thus is often fatal in neonatal piglets (de Bouck and Pensaert 1980). Bae et al. (2003) developed transgenic tobacco plants that expressed the antigen protein corresponding to the neutralizing epitope of PEDV spike protein. Feeding the transgenic plants to mice induced both systemic and mucosal immune responses against the antigen. By using a tobacco mosaic virus (TMV)-based vector, the coding sequence of a core neutralizing epitope of PEDV (COE) gene was optimized based on the modification of codon usage in tobacco plant genes. The recombinant COE protein was up to 5.0 % of the total soluble protein in the leaves of tobacco plants infected with the TMV-based vector containing synthetic COE gene (Kang et al. 2004a). A fusion gene encoding the synthetic LT-B subunit genetically fused with a synthetic neutralizing epitope of porcine epidemic diarrhea virus (sLTB-sCOE) was introduced into lettuce cells (*L. sativa*) by *Agrobacterium*-mediated transformation methods. LTB-COE fusion protein comprised about 0.026–0.048 % of the total soluble protein in the transgenic lettuce leaf tissues (Huy et al. 2009).

Rotavirus is the leading cause of viral gastroenteritis in young children and animals worldwide. Rotavirus particles contain 11 dsDNA genomic segments surrounded by three concentric capsid protein layers: an inner layer VP2, an intermediate layer VP6, and an outer layer made up of capsid proteins VP7 and VP4 (Estes and Cohen 1989). Capsid protein VP6 is the major structural protein of rotavirus and makes up about 50 % of virion mass (Hsu et al. 1997). VP6 has been expressed in *N. benthamiana* plants either independently or as a fusion with the potato virus X coat protein. These plant-produced VP6 retained the ability to form trimers (O'Brien et al. 2000). Matsumura et al. (2002) reported expression of the major capsid protein VP6 of bovine group A rotavirus (GAR) in transgenic potato plants. Adult BALB/c mice were immunized intraperitoneally with concentrated transgenic potato extracts emulsified in Freund's adjuvant. Sera collected after immunization showed the anti-VP6 response. Subsequently, murine rotavirus gene six encoding VP6 was stably inserted into the *Solanum tuberosum* genome. The amount of VP6 protein in transgenic potato leaf and tuber was approximately 0.01 % of total soluble protein. Oral immunization of CD-1 mice generated measurable titers of both anti-VP6 serum IgG and intestinal IgA antibodies (Yu and Langridge 2003).

Rabies virus vaccine Rabies virus, a rhabdovirus of the genus *Lyssavirus*, remains a significant threat to human and animal health throughout much of the world (Meslin et al. 1994). In the design of a rabies vaccine, representation of both the rabies virus glycoprotein (G protein) and nucleoprotein (N protein) antigens is desirable. The G protein is the major antigen responsible for the induction of protective immunity, while the N protein triggers rabies virus-specific T cells, facilitating the production of neutralizing antibodies and other immune mechanisms (Cox et al. 1977; Tollis et al. 1991).

A chimeric peptide containing antigenic determinants from rabies virus glycoprotein and nucleoprotein was cloned and expressed in tobacco and spinach plants. Mice immunized with recombinant virus were protected against challenge infection. Three of five human volunteers responded against the peptide antigen after ingesting infected spinach leaves (Yusibov et al. 2002). A *Nicotiana tabacum* cv. Xanthi cell culture was initiated from a transgenic plant expressing a human anti-rabies virus monoclonal antibody. Quantification of antibody production in plant cell suspension culture revealed 30 $\mu g g^{-1}$ of cell dry weight for the highest-producing culture (0.5 mg/L), three times higher than from the original transgenic plant (Girard et al. 2006). Perea Arango et al. (2008) expressed a full-length

nucleoprotein gene of rabies virus in transgenic tomato plants and also transiently expressed it in *N. benthamiana* plants by agroinfiltration. In both cases, the nucleoprotein was expressed at high levels, 1–5 % of total soluble protein in tomato and 45 % in *N. benthamiana*. In addition, Roy et al. (2010) reported the expression of a chimeric protein comprising the synthetic CT-B fused at its C-terminal with rabies surface glycoprotein (G protein) in tobacco plants. The approximately 80.3 kDa fusion polypeptide expressed at 0.4 % of the total soluble protein in leaves of the selected transgenic lines.

Other viral diseases In recent years, there were other recombinant virus vaccines expressed in the transgenic plants, including: Tat protein of HIV-1 vaccine produced in spinach (Karasev et al. 2005), expression of the Newcastle disease virus (NDV) fusion protein in transgenic maize (Guerrero-Andrade et al. 2006), accumulation of recombinant SARS-CoV spike protein in tobacco plant cytosol and chloroplasts (Li et al. 2006b), Norwalk virus capsid protein expressed in tomato plant (Zhang et al. 2006), transgenic tobacco and potato (Mason et al. 1996), superexpression of tuberculosis antigens in tobacco leaves (Dorokhov et al. 2007), expression of influenza A (H5N1) vaccine in barley grains for oral bird immunization (Bruchmüller et al. 2007), and expression of EpCAM antigen in *Beta vulgaris* var. *cicla* (Swiss chard) plants (Brodzik et al. 2008).

Malaria vaccine

A gene encoding the C-terminal region of a major surface antigen of *Plasmodium falciparum*, referred to as PfMSP119, a major vaccine candidate for malaria, was cloned and transformed in tobacco leaves (Ghosh et al. 2002). Wang et al. (2008b) induced protective immunity against malaria infection in transgenic tobacco plants using *Plasmodium yoelii* merozoite surface protein 4/5 (PyMSP4/5) in a mouse model of malaria infection. PyMSP4/5 protein from the codon-optimized construct accumulated to 0.25 % of total soluble protein. In addition, tobacco-produced PyMSP4/5 protein was able to induce antigen-specific antibodies in mice.

The plant expression vector for the truncated fragments of SAG1 gene (t2SAG1) of *Toxoplasma gondii* was constructed and transformed into tomato plants by *Agrobacterium tumefaciens* under control of the both constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter and the tomato fruit-specific E8 promoter (Zhou et al. 2004). In addition, there are relevant studies on the expression of a His-tagged truncated version of *T. gondii* dense granule 4 protein ($Gra4_{163\text{-}345}$) in tobacco leaves (Ferraro et al. 2008), the protective antigenic gene FH3 of *Fasciola hepatica* in alfalfa plants (Li et al. 2003), and the ferritin gene of *Schistosoma japonicum* in rape plants (Yuan et al. 2008).

Immunocontraceptive vaccine

Fertility control may provide a non-lethal, more humane alternative for population control of vertebrate animals. A long-term strategy for it is to adopt immunocontraception, namely, that vaccination is used to induce antibodies against reproductive self-antigens, resulting in reduced fertility (Ferro 2002). In most cases, the zona pellucida (ZP) has been used as the vaccine antigen, which is composed of three sulfated glycoproteins; it can function in the fertilization of the oocyte by providing a substrate for sperm binding (Bleil and Wassarman 1980a; Munro and Pelham 1987). One of the three glycoproteins, ZP3, is the primary binding site for the sperm and has been investigated as a target for immune contraception (Bleil and Wassarman 1980b; Sacco 1979).

Marsupial-specific reproductive antigens expressed at high levels in edible transgenic plant tissue might provide a kind of safe, effective, and cheap oral delivery bait for immunocontraceptive control. As proof of concept, female possums vaccinated with immunocontraceptive antigens showed reduced fertility, and possums fed with potato-expressed LT-B had mucosal and systemic immune responses to the antigen. This demonstrated that immunocontraception was effective in possums and oral delivery in edible plant material might be possible (Polkinghorne et al. 2005).

High-level expression strategies of foreign protein in transgenic plants

In the early literature, expression levels of recombinant antigens in transformed plants were not high. Therefore, the main problem of transgenic plant vaccines was a high-level expression of foreign genes in the plants. Many scientists have launched research programs in order to improve the antigen expression level and to make plant vaccines produce effective immunological function with some significant achievements that will be described in the following.

Promoter optimization

The choice of a suitable plant promoter is an important way to enhance exogenous gene expression. At present, constitutive promoters are usually used in plant genetic engineering; for example, CaMV 35S promoter is used for dicotyledonous plants, and the actin promoter of rice and ubiquitin promoter of maize are used for monocotyledonous plants. However, constitutive promoters may cause a constant and continuous expression of target genes in plant tissues, leading to an excessive consumption of material and energy within the cell. Thus, constitutive expression may result

in the inability to effectively regulate the expression of target genes both temporally and spatially. The consequence may be to interrupt the intrinsic metabolic balance in the plant, which is not conducive to the enhancement of the yield and the quality of the transgenic plant expression of target genes. Instead, exogenous genes should be expressed in particular periods and specific tissues or organs, rather than with a continuously high-expression level in receptor plants, in order to maximize production of plant-based vaccines or other medically relevant proteins. Therefore, the use of inducible or tissue-specific promoters may effectively improve the expression level of foreign proteins in a particular period or in specific organs. For example, tomato plants were transformed with the gene encoding CT-B along with an endoplasmic reticulum retention signal (SEKDEL) under the control of the CaMV 35S promoter via *Agrobacterium*-mediated transformation. Both tomato leaves and fruits expressed CTB at levels up to 0.02 % and 0.04 % of total soluble protein, respectively (Jani et al. 2002), but under the control of the tomato fruit-specific E8 promoter, transgenic tomato plants expressing CTB protein were developed, with the highest amount of CTB protein being 0.081 % of total soluble protein (Jiang et al. 2007).

Codon optimization

The difference in codon usage in plants, animals, and microbes is an issue that must be considered in any heterologous protein expression system. In order to optimize expression, the codons of foreign genes should be analyzed and replaced with appropriate codons preferred by plants. Codon optimization can significantly improve the expression level of the exogenous gene in plants. Mason et al. (1998) constructed a plant-optimized synthetic gene encoding LT-B for use in transgenic plants as an edible vaccine against enterotoxigenic *E. coli*. Expression of the synthetic LT-B gene in potato plants under the control of a constitutive promoter yielded increased accumulation (ranging from 5-to 40-fold) of LT-B in leaves and tubers, as compared to the bacterial LT-B gene. Dus Santos et al. (2005) transformed the genes encoding the polyprotein P1 and the protease 3C of FMDV by the codon preferred by *A. thaliana* into alfalfa plants. The highest accumulation of VP1 protein was found in transformed leaves at 0.5–1 mg/g fresh weight. Other research has shown that plant-optimized coding sequences can greatly enhance the expression of other bacterial genes in plants (Perlak et al. 1991; Adang et al. 1993).

Signal peptide utilization

The synthetic peptide in the cytoplasm and endoplasmic reticulum can further be located on different areas of the cell by the decision of a peptide signal sequence. The endoplasmic reticulum in plant cells provides an exogenous protein with a relatively stable environment for protein maturation. For this reason, the addition of an ER-retention signal at the C terminus has been suggested to enhance the accumulation of recombinant proteins in transgenic plants (Munro and Pelham 1987; Napier et al. 1998; Wandelt et al. 1992). Stoger et al. (2000) reported that protein accumulation was irrespective of the mRNA level, and suggested that the addition of KDEL at C terminus resulted in the accumulation of antibodies in plants. For example, the G protein of the rabies virus, which contained an endoplasmic reticulum retention signal in its C terminus, expressed in transgenic tobacco plant lines at 0.38 % of the total soluble leaf protein (Ashraf et al. 2005).

Addition of immune adjuvants

LT-B and CT-B have been shown to be effective carriers and adjuvants for genetically linked foreign proteins (Bagdasarian et al. 1999; Arakawa et al. 1998a; b). They can bind to ceramide-galactose sugar receptor molecules such as GM1 ganglioside (Holmgren et al. 1975). The performances of LTB carrier and adjuvant in animals immunized with the LTB-antigen fusion proteins are contingent on pentamer assembly. They can make their toxicity subunits (A) closely combine on the cell surface of intestinal mucosa so that fusion protein more easily react with gastrointestinal mucosa, thus stimulating mucosa to produce IgA and serum IgG and strengthening the immunity effect (Kim et al. 2007). Therefore, LTB and CTB can be used as immune adjuvants by fusing with antigenic determinant gene to enhance hapten immunogenicity.

Adoption of the chloroplast transformation system

Due to the high copy number of the chloroplast genome, chloroplast transformation can greatly improve the level of recombinant protein in transgenic plants. Integration of an unmodified CTB-coding sequence into chloroplast genomes (up to 10,000 copies per cell) resulted in the accumulation of up to 4.1 % of total soluble tobacco leaf protein as functional oligomers (410-fold higher expression levels than that of the unmodified LTB gene expressed via the nuclear genome) (Daniell et al. 2001). In order to resist the fungal pathogen *Colletotrichum*, that is so destructive to tobacco, the antimicrobial peptide MSI-99, an analog of magainin 2, was expressed via the chloroplast genome to obtain high levels of expression in transgenic tobacco (*Nicotiana tabacum* var. Petit Havana) plants (DeGray et al. 2001). The chloroplasts were estimated to express MSI-99 at 21.5 % to 43 % percent of total soluble protein (TSP). At present, a variety of vaccines (which can protect against Cholera, tetanus, anthrax, amebic disease, rotavirus, and so on) have had a successful expression in the plant chloroplasts (Verma and Daniell 2007). However, without the endoplasmic reticulum and golgi apparatus, the

synthesis of protein in chloroplasts cannot be modified by glycosylation. Thus, chloroplasts are suitable only for expressing bacterium antigens and not for glycoproteins.

Safety issues and solutions for transgenic plant vaccines

The good qualities of GM plants are produced according to human needs, and the subsequently developed transgenic plants may create enormous economic and social benefits for mankind. However, as new species, once they are released to the natural environment, GM plants may bring mankind tremendous potential or real harm. In this regard, we have had profound lessons of history, such as the famous British Pusztai events, Cornell University butterflies event (Losey et al. 1999), Canada "superweeds" event (Orson 2001), Mexico corn event (Dalton 2001) and China Bt insect-resistant cotton event. All of these events are wake-up calls for the whole human race in all fields of research and application of GM organisms. Recently, safety problems in the environment and the role of transgenic plant vaccines in the human diet have become the focus of controversy.

Possible environmental safety problems

The effects of transgenic plants to non-objective organisms The genes obtained by transformation technology will not only be spread to nature, possibly enter into the wild group through genetic drift, change genetic traits of the wild population, and then possibly cause adverse effects. Therefore, the release of genetically modified organisms into nature may contaminate the natural gene pool, break original ecological balance, and produce some unsuspected impact on the ecological environment (Ma et al. 2001). Meanwhile, beside pest and germ toxigenicity, insect or disease resistance by transgenic plants released into the environment may have direct or indirect negative effects on many beneficial organisms in the environment and even cause the death of some beneficial organisms (Zhuang and Cao 2008).

New environmental problems

Many genetically modified varieties contain the genes extracted from *Bacillus*, which may produce a kind of protein toxic to pests. If the anti-pest GM crop is planted for a long time in large areas, pests are likely to develop resistance and then pass it on to their offspring. So transgenic plants no longer have insect-resistance ability, the original pesticides will also no longer be effective, and eventually more intractable super pests will produce (Chen and Wang 2002). Meanwhile, transgenic plants released into the environment may generate gene transfer through pollination and allow some genes (such as genes of insect resistance, disease resistance, herbicide or environmental stresses tolerant) to become dominant over the related wild species or weeds (World Health Organization. 2004). Once obtaining adversity-resistant character of transgenic organisms, these weeds will become "superweeds", severely endanger normal survival and growth of other creatures, and eventually disrupt the balance of the ecosystem.

The impacts on biodiversity Hilbeck et al. (1998) fed transgenic *Bacillus thuringiensis* variety *kurstaki* (Berliner) corn plants to the European corn borer (lepidopterous target pest) and *Spodoptera littoralis* (Boisduval) (lepidopterous nontarget pest for *B. thuringiensis*). Mean total immature mortality for chrysopid larvae raised on *B. thuringiensis*-fed prey was 62 % compared with 37 % when raised on *B. thuringiensis*-free prey. Some biologists think that in order to keep its own stability and purity, nature's species strictly regulated the change of genetic material and genetic drift is limited to between the same species or allied species. A GM organism is obtained through an artificial method of mutual transfer between animals, plants, microbes and even human genes, so it breaks through the traditional concept of kingdom. GM organisms open a new door and span the intrinsic barrier among the species, thereby presenting many features and advantages that native species do not possess. If such species are released into the environment, the competitive relationship between species will change. The original natural ecological balance will be destroyed, the intrinsic complete food chain in ecological systems will also be damaged, and eventually species extinction and loss of biodiversity are all possibilities that must be considered (Yang et al. 2002).

Possible dietary safety problems

Toxicity Food safety is a necessary prerequisite for diet, so the safety of bioengineered food products is currently of the greatest concern to people. The nutrient component changes and the appearance of anti-nutritive factors in some GM organism bodies (such as the changes of protease inhibitors or lipoxidase) are likely to produce some adverse effects on human health. Moreover, people know little about gene activity and worry that the sudden change of genes will lead to the production of some toxic substances (such as solanine and cucurbitacin). Furthermore, the transformed genes are not the ones of the original parent animals and plants, some of which even come from other creatures of different genera (including all sorts of bacteria, viruses, and organisms). Therefore, the safety of exogenous gene expression product is in doubt.

Irritability The allergic substances may increase, and new allergens will also appear in the food supply due to gene transformation. Food anaphylaxis involves various abnormal reactions of human immune system to a certain protein. It has been discovered that peanut protein in soybean (*Glycine max*), Cry 9C poisonous protein in corn and 2S albumin in Brazil nuts may induce some people or animals' allergic reaction (Nordlee et al. 1996).

Antibiotic resistance At present, most of the selected vectors in genetic engineering are antibiotic-resistant marker genes. The antibiotic resistance is introduced into the food chain through gene transfer. Therefore, it may produce drug-resistant bacteria or viruses within humans or animals, induce people to develop resistance to particular kinds of antibiotics, and eventually affect the effectiveness of antibiotic treatment (Cheng 2002). In 2002, a human DNA residual test of GM food was demonstrated in Britain. Seven volunteers, who underwent operations for colonic tissues excision, ate hamburgers with GM soybean. In another instance, a transgenic DNA residue was detectable inside small intestine intestinal flora (Chen and Yuan 2008). For transgenic soybean with marker antibiotics, it is thought that the antibiotic-containing food makes intestinal or oral bacteria produce a kind of resistance to antibiotics, but this has not been established.

Resolution of safety issues in transgenic organisms

Choice of biosafety marker genes Marker genes in transgenic plants mainly include selected genes (enrichment of transformed cells, many of which are antibiotic-resistance genes) and reporter genes (easily detected expression products, such as green fluorescent protein (GFP) gene). In order to avoid some of the potential dangers of traditional gene selection (such as genetic drift, the development of immune tolerance in the human body, and damage to the ecological balance), marker genes should be eliminated or a positive screening system should be developed. The knock-out of marker genes has the drawbacks of low efficiency and complex operation; the most effective method at present is to screen for biological safety by using non-resistance marker genes. Although the transformed cell cannot be killed, the positive screening method may make the cell have a specific metabolic advantage or exploit a specific material by introducing specific genes, so as to develop vigorous growth and to achieve the screening purpose. The safe marker genes for the positive selection system are found to include mainly as follow: the genes coding enzymes that catalyze special sugars (pmi and xylA), the genes coding enzymes that interfere with amino acid metabolism (ak and dapA), green fluorescent protein (GFP) gene, beta–Glucuronidase (GUS) gene, ribitol operon(rtl) gene and chlorophyllous biosynthesis enzyme gene (hemL).

Reduction of unexpected side effects and enhancement of genetic stability Due to the characteristics of transgenic technology, transformed plants can easily produce unexpected side effects. The integration of foreign DNA can also affect chromosome structures in the host plant so as to increase the mutation rate in the plant. The molecular detection methods used to detect such negative side effects are mainly proteomic analysis technology (such as two-dimensional electrophoresis) and gene expression analysis technology (such as gene chip technology), both of which have international general "real identity" principle as a guide to analyze the safety of transgenic plants. It is much more difficult to analyze chromosomal mutation in transgenic plants. However, it is remarkable that although mutation induced by change in chromosomal structure is an unfavorable factor in the commercialization of plant genetic engineering, it will provide the material basis for research on the transcription mechanisms of foreign genes and the influence of foreign genes on the genome of receptor plants.

The technique of the exogenous gene removal According to a report in the Beijing Agricultural Journal, after 6 years of hard work, the laboratory research team led by Chinese scientist Li Yi made a great breakthrough in the research on the potential threats of transgenic plants to ecological environment and human health (Chui and Li 2007). They developed a technique to remove an exogenous gene and to insert the special gene in the target plants, which was controlled by a DNA regulatory fragment promoter. According to the intention of researchers, the special gene could spontaneously remove the exogenous gene at a specific time or a specific location in the plant. The pollen, sedes, and fruit of transgenic plants no longer contain foreign genes by using this method. Thus, a new method has been developed that permits non-transformed plants to be produced by using transgenic plants. This technique is expected to thoroughly resolve safety problems of genetically modified plants for application in food and medicine.

Other strategies In addition to the above methods, there are other strategies, such as to improve the security management system of genetically modified organisms, to execute area-delimited management of production, to strengthen import–export control of transgenic agricultural products and to raise the level of safety consciousness of all people to transgenic foods.

Summary and perspectives

Transgenic plant vaccines have significant advantages compared with traditional vaccines. Future research should focus on producing safe, reliable, and efficient plant vaccines as

pharmaceutical products. The low level of current expression systems is one of the major limitations to application of transgenic plants vaccines. However, recent publications show that expression levels can be improved by the chloroplast transformation, plant breeding, or food processing technology. Moreover, it is also been indicated that the application of carrier protein and auxiliary proteins or adjuvants may enhance the ability of the immune system to recognize antigens (Rigano and Walmsley 2005). This research has enabled the production of cheap and safe plant vaccines that can be administered orally to prevent the spread of disease. However, there is still a long way to go to industrialize transgenic plant vaccines. Especially some European countries continue to resist the use of transgenic plants, which poses difficulties for the development of transgenic plant vaccines. Consequently, much vaccine research is still in the experimental stage. Therefore, in order to promote the rapid development of transgenic plant vaccines, technological innovation should be intensified, and at the same time, the public should be informed about the advantages of transgenic plants to remove the fear. In 2006, the United States Department of Agriculture (USDA) approved the commercialization of transgenic plants vaccine against Newcastle disease. Although the vaccine has not yet reached the market, there is no doubt that this is a successful landmark example for transgenic plant vaccines. It is hoped that this example will play a significant role in promoting the development of other transgenic plant vaccines. With the establishment and optimization of GM technologies and corresponding means of detection, transgenic plant vaccines and antibodies have the prospect for vast applications in industrialized production.

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