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## Research review paper

## Plants as bioreactors for the production of vaccine antigens

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## ABSTRACT

Plants have been identified as promising expression systems for commercial production of vaccine antigens. In phase I clinical trials several plant-derived vaccine antigens have been found to be safe and induce sufficiently high immune response. Thus, transgenic plants, including edible plant parts are suggested as excellent alternatives for the production of vaccines and economic scale-up through cultivation. Improved understanding of plant molecular biology and consequent refinement in the genetic engineering techniques have led to designing approaches for high level expression of vaccine antigens in plants. During the last decade, several efficient plant-based expression systems have been examined and more than 100 recombinant proteins including plant-derived vaccine antigens have been expressed in different plant tissues. Estimates suggest that it may become possible to obtain antigen sufficient for vaccinating millions of individuals from one acre crop by expressing the antigen in seeds of an edible legume, like peanut or soybean. In the near future, a plethora of protein products, developed through 'naturalized bioreactors' may reach market. Efforts for further improvements in these technologies need to be directed mainly towards validation and applicability of plant-based standardized mucosal and edible vaccines, regulatory pharmacology, formulations and the development of commercially viable GLP protocols. This article reviews the current status of developments in the area of use of plants for the development of vaccine antigens.

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## 1. Introduction

Infectious diseases account for more than 45% of total deaths in developing countries (Arntzen, 2005). Vaccination is the most effective means to prevent infectious diseases. More than 30 million children in the world are not immunized against treatable or preventable diseases ([www.care.org/campaigns/childrenpoverty/facts.asp](http://www.care.org/campaigns/childrenpoverty/facts.asp)) because the currently used approaches to vaccine production are technologically complex and expensive. Specialized requirements of packaging, cold chain and mode of delivery add to the cost. This makes vaccination unaffordable to a large proportion of population in developing and poor economies. Currently used mammalian cell line based vaccine manufacturing requires large investment and expertise. These factors limit their scale up and thus, global availability. These problems are also equally important for the vaccination of farm animals (Floss et al., 2007). A fresh thinking is required to make vaccine development, manufacture and delivery simpler and affordable.

Advances in molecular biology techniques during the 1980s, helped in the development of new strategies for the production of subunit vaccines. These comprised of proteins derived from pathogenic viruses, bacteria or parasites. Although mammals, their tissues and cell lines are currently utilized for commercial production of vaccines, these systems are expensive and their scale up is not easy (Larrick and Thomas, 2001; Houdebine, 2009). Toxins, infectious agents and other noxious compounds get carried in animal cell based processes and are often difficult to remove. Such production systems are prone to microbial contamination which sometimes escapes detection even in purified vaccines. Expression of recombinant antigen proteins in *E.coli* is often not feasible because of lack of a variety of post translational modifications and folding requirements. Some of the mammalian-type post translational processing and modifications in protein happen in yeast and insect cell lines. However, immunologically significant differences in the pattern of post translational modifications limit their deployment in the expression of vaccine antigens (Streatfield and Howard, 2003a,b; Chen et al., 2005; Houdebine, 2009). As a major alternative, plants are emerging as a promising system to express and manufacture a wide range of functionally active proteins of high value to health industry.

Various plant biotechnological techniques, such as, modern breeding methods, clonal propagation, somatic hybridization, protoplast/cell suspension culture, hairy root culture and genetic transformation can play a vital role in establishing the use of plants as “surrogate production organisms”. One or more immunoprotective antigens of pathogens can be produced in plants by the expression of gene(s) encoding the protein(s). In recent years, plant-based novel production systems aimed at developing “edible” or “oral” vaccines have also been discussed (Ma et al., 2003, 2005a,b; Koprowski, 2005; Lal et al., 2007; Mishra et al., 2008; Houdebine, 2009). Compared to traditional vaccines, edible vaccines offer simplicity of use, lower cost, convenient storage, economic delivery and mucosal immune response.

The original concept of edible vaccines implied that transgenic fruit or vegetable expressing an antigen from a virus or bacteria can be eaten raw without any previous processing, and act as a vaccine for launching sufficiently protective immune response against a particular disease. Currently, it is widely accepted that this original concept was rather naive mainly because of two reasons. First, different fruits from the same plant express different levels of antigens and therefore it is crucial to make plant-derived vaccine by using pools of fruits with homogeneous antigen concentration (vaccine dose). In general, at least a minimum processing, pooling and freeze-drying of fruits from the same or different plants will be required before incorporation into formulations or capsules for oral vaccination. Second, it is important to ensure complete separation of fruits or vegetables intended for human or animal consumption from fruits or vegetables intended for pharmaceu-

tical uses like vaccines. Hence, the approach to “edible vaccines” has been replaced by “plant-derived vaccine antigens”. Antigen expression in plant tissue opens an important alternative to meet the global demand for cheaper, safer and quality vaccines. Compartmentalization of vaccine antigens in plant cell, tissue or organ can provide it a protective covering. This can facilitate transportation without expensive infrastructure to the manufacturing site where dose standardization and packaging may be undertaken. Such edible encapsulation protects the antigen through the mucosal and gut systems to allow its absorption.

Considering the cost of protein purification as comparable, savings in the upstream components make the production cost of a commercially important protein in plants substantially less than other systems. Genetically modified plants can be grown in large area. Large biomass can be obtained easily without high investment (Kusnadi et al., 1998; Evangelista et al., 1998; Chen et al., 2005; Yonekura-Sakakibara and Saito, 2006). The cost of goods sold (COGS) for bulk production of recombinant protein in plants has been estimated to be 1/10th to 1/50th of bacterial fermentation (Hood et al., 2002). Therefore it is economically sound to use transgenic plants for antigen production. A variety of gene expression and protein localization systems now available for plants allows stable accumulation of the recombinant proteins in target plant tissue.

This review focuses on the methods and applications of plant, tissue and cell culture based expression strategies and their use as bioreactors for large scale production of pharmaceutically important proteins. Recent advances in stable, transient and other cell/tissue culture based plant transformation methods, their prospects and limitations in the development and commercialization of therapeutic proteins are discussed.

## 2. Selection of plant system

Successful development of vaccine antigens against human and animal pathogen(s) in plants requires selection of one or more immunoprotective antigens and designing of genes and promoters that would express the antigen(s) at a high level in target plant tissue. Genetic transformation methods are then utilized for introducing the gene in the target plant species (Fig. 1). A plant ideal for oral vaccine production should have the following features: (i) amenability to transformation, (ii) expression in edible tissue that can be consumed uncooked since vaccine antigens are heat sensitive, (iii) targeted tissue to be rich in protein because vaccine protein will only be a small percentage of the total protein, (iv) targeted tissue should not produce toxic molecules and (v) would allow correct folding of the antigen protein and desired post translational modifications.

Earlier studies used tobacco (*Nicotiana tabacum*) as a model plant for the production of antigens lacked some of the above features. However, recently, plants with high food value have been chosen as expression systems. Many of them can be eaten raw, eliminating the need for processing and purification. The expression of vaccine antigens in plants such as apple, banana, tomato and guava (fruits), peanut, corn, soybean and chickpea (seeds), cabbage, lettuce, potato and spinach (vegetables) has been reported (Reviewed by Stoger et al., 2005; Kumar et al., 2007; Floss et al., 2007).

## 3. Selection of plant tissue

Expression of antigen in edible tissue offers a convenient and inexpensive source to deliver a vaccine. Expression of commercially important proteins in leaf tissue is not a good strategy (Stevens et al., 2000; Stoger et al., 2005; Benchabane et al., 2008) on account of the following reasons: (i) overall protein content in leaf tissue is low; (ii) leaves have high protease activity; (iii) the presence of pigments and phenolics makes purification of recombinant protein from leaves, cumbersome and expensive; (iv) implementation of good

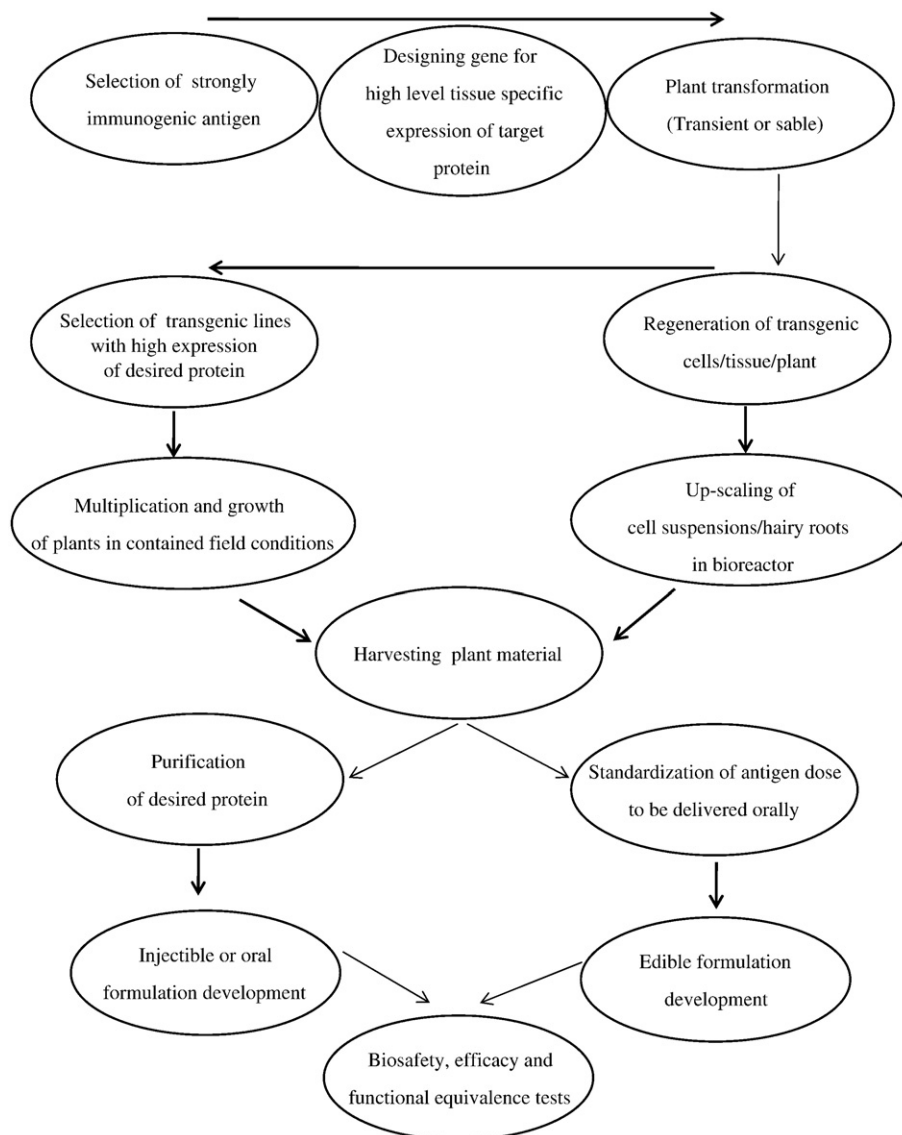


Fig. 1. Steps in the production of plant-derived vaccine antigens.

manufacturing practices becomes more difficult due to the need for handling large volume and biomass.

The expression levels of plant-derived bio-pharmaceuticals need to be increased before commercial production can be accomplished (Daniell et al., 2001b; Chen et al., 2005) on economically competitive basis. The expression levels of the recombinant proteins in the transgenic plants are also influenced by environmental factors. High expression levels could be best achieved in cell suspension, hairy root cultures (*in vitro*) and seeds (*in vivo*). Seed tissue represents potentially a very promising target for producing pharmaceutically important proteins for extraction at commercial level. The recombinant seeds also offer the possibility of direct use as an edible vaccine. Single chain antibodies expressed in seeds of rice and wheat showed high biological activities and remained stable for several years (Stoger et al., 2005). Thus, the proteins expressed in seeds are highly stable. Long term storage and easy transportability of seeds is possible due to very low moisture content of mature seeds. Rich mix of chaperones and disulfide isomerases present in the developing seed facilitate correct protein folding (Muntz, 1998). Other tissues like hairy root and cell suspension cultures could be useful target tissues to express recombinant proteins (Kumar et al., 2005b, 2006; Benchabane et al., 2008), though the establishment and running costs of such *in vitro* systems are higher.

#### 4. Selection of promoter

One of the most important aspects in molecular bio-farming is the selection of promoter to achieve high level expression of the antigen coding gene. The choice of promoters affects transgene transcription, resulting in changes not only in concentration, but also in the stage, tissue and cell specificity of its expression. Cauliflower mosaic virus 35S (*CaMV35S*) promoter has widely been used because of its strong and constitutive expression. High level protein expression is essential to develop economically competitive plant-based process for cultivation of the transgenic variety within confined fields with controlled environmental and contained biosafety conditions. However, expression in tissues other than the target organ to be used for preparation of the vaccine antigen is an avoidable drain on plant resources. In this respect, highly expressing and yet tightly controlled promoters are desirable for bio-farming proteins from plants (Fischer et al., 2004; Chen et al., 2005; Stoger et al., 2005). Expression of a protein with *CaMV35S* promoter does not permit regulated gene expression. Though *CaMV35S* promoter expresses genes at a relatively high level in leaves and roots, low level of total protein (2–5% on fresh weight basis) in these tissues gives a poor expression system, not suitable for bio-farming. Using *CaMV35S* promoter, expression levels of about 0.2% of total soluble protein (TSP) have been reported in leaves. Using a two

component expression systems (Chaturvedi et al., 2007), our laboratory has reported protein expression to about 1% of total protein in tobacco seeds. However, by developing a seed specific expression system the expression of vaccine antigen at 1% of total protein can give more than hundred fold higher total yield of the target antigen per unit area as compared to the expression in leaf (Table 1). Levels of expression, as high as 20% of TSP, have been reported by expressing transgenes in chloroplasts. One acre of chloroplast transgenic tobacco plants have been estimated to produce up to 360 million doses of anthrax vaccine antigens (Koya et al., 2005). This can allow fairly high harvest of protein from leaf biomass. However, due to bacteria-like post translational processing, chloroplasts may not be suitable for expressing a large number of mammalian antigens. For example, glycosylation of proteins often determines solubility, stability and immunogenicity of antigens and chloroplasts do not have the glycosylation machinery. Table 1 gives theoretical calculations to approximately reflect the economics of expressing vaccine antigen in chloroplasts, leaves and seeds, taking peanut (groundnut) as a model plant. Assuming that 5 µg antigen may comprise one dose of a vaccine, it should be possible to vaccinate 468 million individuals from one acre produce, using seed-based expression system! An amazingly impressive possibility, worth a serious attempt, even if the calculations needs tens of fold correction to account for the processing and purification losses. Absence of pigments and phenolics in seeds make protein purification easier as compared to that from the leaves. Various seed specific promoters, mainly derived from seed storage protein genes, have been employed to restrict recombinant protein expression to different parts of the seed (Perrin et al., 2000; De Jaeger et al., 2002; Philip et al., 2001). The trans-protein accumulation in protein body of seeds facilitates stability of protein against cytoplasmic proteolytic degradation. Soybean seed specific *glycinin* and endosperm specific *glutelin* (*GluB-1*) promoters were employed for LTB expression in soybean cotyledon protein bodies (Moravec et al., 2007) and CTB expression in rice seed protein bodies (Nochi et al., 2007), respectively.

## 5. Role of adjuvant

Adjuvants are important for enhancing the immune response to antigens. The addition of adjuvants to vaccines sustains and directs the immunogenicity and modulates appropriate immune responses. This reduces the amount of antigen required and improves the efficacy

**Table 1**  
Estimated economics of scale for expression of vaccine antigens in leaves, seeds and chloroplasts.

Economics of deploying different plant expression systems for producing a vaccine antigen in peanut			
(i) Total leaf biomass per plant			25 g
(ii) Total protein (%) in leaf by fresh weight			3%
(iii) Total protein in leaf biomass per plant			750 mg
(iv) Total seed biomass per plant			60 g
(v) Total protein (%) in peanut seed			26%
(vi) Total protein in seed produced per plant			15.6 g
(vii) Number of plants per acre (4000 M <sup>2</sup> )			15,000
(viii) Protein required per dose vaccine			5 µg
	I	II	III
	(Antigen yield from leaves using <i>CaMV35S</i> promoter)	(Antigen yield from seeds using seed specific promoter)	(Antigen yield from leaves using chloroplast specific promoter)
a. Level of target protein expression in leaf	0.2% TSP	1% TSP	20% TSP
b. Target protein in plant	1.5 mg	156 mg	150 mg
c. Target protein per acre	22.5 g	2.34 kg	2.25 kg
d. Antigen doses per acre	4.5 million	468 million	450 million

of vaccines. Traditional live vaccines do not require the addition of adjuvant. While the modern recombinant vaccines, particularly the highly purified or synthetic antigens require adjuvants to induce a protective and long-lasting immune response. Although aluminum salts are the most commonly used adjuvant for human vaccines, they are weak and have complex mechanisms that favor induction of antibodies rather than cellular immunity. New forms of vaccine adjuvants that have been proposed for various vaccines feature oil-based emulsions; bacterial products (*Vibrio cholerae* toxin B subunit, *E. coli* heat labile enterotoxin B subunit or CpG nucleotides); viral products (virus-like particles); plant products (saponin derivatives); biodegradable particles (liposomes); molecular adjuvants; and synthetic adjuvants (Lambrecht et al., 2009; Reed et al., 2009).

Adjuvant mechanisms include depot effects, recruitment of innate immunity, specific targeting mechanisms, and carrier functions that hold the antigen in an appropriate conformation. The safety of proposed adjuvants is a primary consideration. Therefore it is often necessary to devise methods to reduce or eliminate the reactogenic effects of an adjuvant while preserving the efficacy. The most effective use of adjuvants for certain types of vaccines, particularly for stimulating mucosal immunity, may be to combine the adjuvant with a particular mode of delivery, such as oral, intranasal, or transcutaneous immunization. Carriers that carry and combine both the adjuvant and the antigen in a single formulation can serve as the basis for creation of important formulations for improved vaccines.

The enhancement of immunogenicity of antigenic protein is an important aspect if vaccine antigens, expressed at a modest level in edible plant part have to succeed in mounting sufficiently high immune response after passage through the mucosal and gut route. General immune-stimulators (adjuvants) and better targeting to the immune system might compensate in part for low delivery of antigen. One of the targeting strategies involves linking antigens to molecules that bind well to immune system components such as M cells in the intestinal lining. M cells take up antigens that enter the small intestine and pass them to other cells of the immune system. If white blood cells (helper T lymphocytes) recognize the fragments as foreign, they induce B lymphocytes (B cells) to secrete neutralizing antibodies and initiate other strategies against the perceived enemy. The *V. cholerae* toxin B subunit (CTB) and *E. coli* heat labile enterotoxin B subunit (LTB) are potent mucosal immunogens and adjuvants. They both bind directly to the GM1-ganglioside receptor molecules on M cells by fusing antigens from other pathogens to any of these subunits (Cuatrecasas, 1973). This ushers foreign antigen into the M cells. By fusing antigens to this subunit, it is possible to improve uptake of antigens by M cells and enhance immune responses. The carrier molecules also serve to modulate immune response against watery diarrhea (Yasuda et al., 2003). Expression of cholera toxin B sub-unit fused to rabies glycoprotein antigen has been reported by us in peanut using seed specific expression system (Tiwari, 2008). The transgenic seeds showed a high expression of the functional fusion protein (unpublished data). Work is in progress to examine if the transgenic peanut seeds give active protection against rabies virus and *V. cholerae*.

## 6. Expression of vaccine antigens in plants

Many therapeutic proteins can be expressed in stable or transient state in whole plants, plant tissues or cell suspension cultures. A comparison of the three approaches and their significance is presented in Table 2. Some of the major reports published on the expression of antigenic proteins by different methods are summarized as below.

### 6.1. Stable expression in transgenic plants

Stable expression of a candidate antigen does not interfere with subsequent propagation of plants either by vegetative or sexual

**Table 2**  
Comparison of three expression systems for recombinant protein production in plants.

Features	Stable transformation	Transient transformation	Stable cell line based expression
Requirement	Optimized transformation	Virus/ <i>A.tumefaciens</i> susceptible species	Optimized cell culture
Expression	Whole plant or tissue specific	Only leaf	Cell suspension or hairy roots
Expression level (Bulk)	High*	Medium	Medium
Consumption	Edible/Purified	Edible/Purified	Purified
Time required for Production	Long	Short	Short
Cost of infrastructure	Low	Low	High
Cost of final product	Low**	Medium	High
Transgene silencing***	Yes	Yes	Yes
Multiplication	Slow	NA****	Fast
Horizontal gene transfer	Yes	No	No

High\*—if expressed in storage tissue.

Low\*\*—if expressed in edible tissue.

Transgene silencing\*\*\*—could be reversed by using viral suppressors like P19 (Voinnet et al., 2003; Alvarez et al., 2008).

NA\*\*\*\*—not applicable.

methods. Another transgene can be introduced in previously transformed plants to generate multi-component vaccines. Judicious choice of regulatory elements facilitates tissue specific expression of foreign antigens in leaves, fruits, tubers and seeds. A variety of tissues have been targeted for the expression of recombinant proteins in various plant species. A number of published reports on stable expression of antigen(s) are summarized in Table 3. In some of them, protection against the targeted disease has been reported in animal models. Some of the distinct success stories are as follows.

#### 6.1.1. Cholera toxin B subunit (CTB) of *V. cholerae*

Cholera is an acutely dehydrating, watery diarrhoeal disease caused by *V. cholerae*. It remains a threat in developing countries where access to safe drinking water and proper sanitation is not available. The structure of cholera toxin (CT) is typical of the A–B subunit group of toxins in which B subunit serves to bind the holotoxin to the eukaryotic cell receptor and A subunit possesses a specific enzymatic function that acts intracellularly. The mature B subunit contains 103 amino acids with a subunit mass of 11.6 kDa. The interaction of CTB with receptors is perhaps the most extensively studied and well characterized function of the toxin (Cuatrecasas, 1973; Fishman, 1982; Dalziel et al., 1984; Fukuta et al., 1988; Cai and Yang, 2003; Dawson, 2005). CTB is responsible for inducing both mucosal and serum immunity. Since the cholera toxin is internalized by the receptors present on mucosal lining, the CTB was one of the early toxins selected for testing the concept of edible vaccines. Further, CTB being a bacterial protein is not glycosylated in native form. Hence, its feasibility for developing vaccine antigen has been examined by expressing the gene in plants both by transformation in to chloroplastic and nuclear genome. In some cases, CTB fusions with target antigens have been used as a potent mucosal immunogen and adjuvant because of its high binding affinity for the GM1-ganglioside receptor in mucosal epithelium.

Daniell et al. (2001a) reported integration of the CTB gene into tobacco chloroplast genome. Functional oligomers of CTB were formed in chloroplast, showing expression level as high as 4.1% of the TSP. Nuclear transformation of tobacco was reported using *Agrobacterium tumefaciens* carrying a gene encoding the CTB engineered with a sequence specific for an endoplasmic reticulum localization signal (SEKDEL) under the control of *CaMV35S* promoter (Jani et al., 2004). The tobacco leaves expressed CTB protein at 0.02% of TSP. The

leaf expressed protein when given to mice by intradermal route behaved like native CTB with regard to effects on T-cell proliferation and cytokine level. We reported CTB expression in tobacco leaves to 0.58% of TSP. The fusion of ubiquitin at N-terminal end increases the level further to 0.91% of TSP (Mishra et al., 2006). Li et al. (2006b) fused CTB with three tandem repeat sequences of human insulin-B chain (InsB<sub>3</sub>) and expressed by *CaMV35S* promoter in tobacco leaves. The fusion protein accounted for 0.11% of the TSP. Sharma et al. (2008) expressed toxin co-regulated pilus subunit A (TCPA) of *V. cholerae* or its immunogenic epitopes (P4 or P6) fused with CTB in transgenic tomato (*Solanum lycopersicum*) plants. The recombinant proteins TCPA, CTB-P4 and CTB-P6 accumulated up to 0.12, 0.17 and 0.096% of TSP, respectively, in tomato fruits. An efficient fusion vaccine that could provide both anti-toxin as well as anticolonization immunity against cholera infection was anticipated, though immunoprotection studies have not yet been published. Jiang et al. (2007) demonstrated CTB expression up to 0.081% of TSP in tomato fruits by using *E8* fruit specific promoter. Transgenic tomato fruits were orally fed to mice which produced high IgG and IgA response in serum and faeces, respectively and provided protection against cholera. He et al. (2008) expressed CTB under the control of *E8* promoter in tomato and tobacco plants. They observed that *E8* promoter tightly regulated expression of CTB only in ripened tomato fruit but not in tobacco tissues. They concluded that certain DNA binding protein(s) (*E4/E8*-binding protein) may have increased during fruit ripening in tomato to increase expression. Nochi et al. (2007) developed rice based oral vaccine for cholera. They expressed CTB under the control of endosperm specific *GluB-1* promoter and showed that each seed stored an average of 30 µg CTB. The experiments on oral feeding of transgenic rice seeds to mice induced CTB specific serum IgG and mucosal IgA antibodies with neutralizing activity. Rice-expressed CTB was stable and maintained immunogenicity at room temperature for more than 1.5 years. Therefore, the development of vaccine in edible plant organ is a cost effective and safe strategy. We reported (Mishra et al., 2006) that the CTB expressed in tobacco leaves was glycosylated. The plant expressed glycosylated CTB bound to the receptors with a higher affinity than the native bacterial protein. From the higher molecular mass of plant-expressed CTB published in earlier works, we interpreted that the bacterial protein expressed in those cases was also likely to be glycosylated. The results suggest the need for pharmacokinetic and biosafety studies on vaccine antigens expressed in plant systems.

#### 6.1.2. Heat labile enterotoxin B subunit (LTB) of *E. coli*

Heat labile enterotoxic *E. coli* (ETEC) protein structure and function is nearly similar to that of the cholera toxin (Sixma et al., 1991). ETEC infection and colonization in the small intestine cause acute diarrhea. The toxin is made of two subunits, one is heat labile toxin A (LTA), which is a toxic subunit of 27 kDa and the other is heat labile toxin B (LTB) which exists as a non-toxic doughnut-shaped pentamer of 11.6 kDa essential for binding to GM1 gangliosides on intestinal epithelial cells (Tsuji et al., 1985). LTB is a potent mucosal immunogen and is commonly used as an adjuvant to stimulate antibody response, when co-expressed with other antigens. LTB and its genetic fusions with other proteins have been successfully expressed in tobacco, potato, maize, tomato, *Arabidopsis thaliana*, soybean and carrot (Haq et al., 1995; Mason et al., 1998; Chikwamba et al., 2002; Walmsley et al., 2003; Rigano et al., 2004; Moravec et al., 2007; Rosales-Mendoza, 2008). Mason et al. (1998) demonstrated the oral immunogenicity in potato tubers against LT. They expressed synthetic and plant based codon optimized LTB gene in potato plants under the control of *CaMV35S* promoter. The raw tubers fed to mice stimulated strong serum and mucosal antibody response against LTB and provided protection against oral challenge with LT. Chikwamba et al. (2002) expressed LTB in maize kernels using a seed endosperm specific (*gamma zein*) promoter. They demonstrated that the maize-

**Table 3**  
Stable expression of vaccine antigens in plants.

Plant/tissue	Promoter	Pathogen/causing agent	Disease	Antigenic protein	Reference
Tobacco/leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	Cholera toxin B subunit (CTB)	Hein et al. (1996)
Potato/tuber, leaf	<i>Mannopine synthase</i>	<i>V. cholerae</i>	Cholera	CTB	Arakawa et al. (1997)
Potato/tuber, leaf	<i>Mannopine synthase</i>	<i>V. cholerae</i>	Cholera	CTB	Arakawa et al. (1998a)
Potato/tuber, leaf	<i>Mannopine synthase</i>	<i>V. cholerae</i> and IDDM	Cholera and Diabetes	CTB-INS (Insulin)	Arakawa et al. (1998b)
Potato/tuber, callus	<i>Mannopine synthase</i>	<i>V. cholerae</i> and rotavirus	Cholera and Gastroenteritis	CTB-Rotavirus enterotoxin protein (NSP4)	Arakawa et al. (2001)
Tobacco/leaf (chloroplast)	Plastid rRNA operon (Prn)	<i>V. cholerae</i>	Cholera	CTB	Daniell et al. (2001a)
Potato tuber	<i>Mannopine synthase</i>	<i>V. cholerae</i> , rotavirus and <i>E. coli</i>	Cholera, gastroenteritis and diarrhea	CTB-NSP4/CTA2, CFA/1	Yu and Langridge (2001)
Tomato/fruit, leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	CTB	Jani et al. (2002)
Potato/tuber	<i>Mannopine synthase</i>	<i>V. cholerae</i> and rotavirus	Cholera and gastroenteritis	CTB-NSP4	Kim and Langridge (2003)
Tobacco/leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	CTB	Jani et al. (2004)
Tobacco/leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	CTB-InsB3	Li et al. (2006b)
Tobacco/leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	CTB	Mishra et al. (2006)
Tobacco/hairy root	<i>CaMV35S</i>	<i>V. cholerae</i> and <i>Erysipelothrix rhusiopathiae</i>	Cholera and erysipelas	CTB-Surface protective antigen (SpaA)	Ko et al. (2006)
lettuce/leaf	<i>CaMV35S</i>	<i>V. cholerae</i>	Cholera	sCTB-KDEL	Kim et al. (2006)
Tobacco/leaf	<i>psbA</i>	<i>V. cholerae</i> and Canine parvovirus (CPV)	Cholera and Haemorrhagic gastroenteritis and myocarditis	CTB-2L21	Molina et al. (2004)
Tomato/fruit	Fruit specific <i>E8</i>	<i>V. cholerae</i>	Cholera	CTB	Jiang et al. (2007)
Rice/seed	Endosperm specific <i>GluB-1</i>	<i>V. cholerae</i>	Cholera	CTB	Nochi et al. (2007)
Peanut/seed	Seed specific <i>legumin</i>	<i>V. cholera</i> and Rabies	Cholera and Rabies	CTB-Rabies glycoprotein (RGP)	Tiwari (2008)
Tomato/fruit and tobacco/leaf, flower, seed	Fruit specific <i>E8</i> and <i>CaMV35S</i>	<i>V. cholera</i> and Hepatitis B virus (HBV)	Cholera and Hepatitis	CTB and HBsAg	He et al. (2008)
Tomato/fruit	<i>CaMV35S</i> with leader peptide from alfalfa mosaic virus ( <i>CaMV35SE2L</i> )	<i>V. cholerae</i>	Cholera	CTB-P4/CTB-P6/ TCPA	Sharma et al. (2008)
Rice/seed	Wheat endosperm specific Bx17 HMW	<i>V. cholerae</i>	Cholera	CTB	Oszvald et al. (2008)
Potato/tuber, tobacco/leaf	<i>CaMV35S</i>	<i>E. coli</i>	Diarrhea	Heat labile toxin B subunit (LTB)	Haq et al. (1995)
Potato/leaf, tuber	<i>CaMV35S</i>	<i>E. coli</i>	Diarrhea	LTB	Mason et al. (1998)
Potato/tuber	Tuber-specific <i>patatin</i>	<i>E. coli</i>	Diarrhea	LTB	Lauterslager et al. (2001)
Maize/seed	<i>CaMV35S</i>	<i>E. coli</i> and Swine transmissible gastroenteritis corona virus (TGEV)	Diarrhea and Swine transmissible gastroenteritis (TGE)	LTB and TGEV glycoprotein S	Streatfield et al., 2001; Lamphear et al., 2002
Maize/seed	Endosperm specific <i>gamma zein</i>	<i>E. coli</i>	Diarrhea	LTB	Chikwamba et al. (2002)
Tobacco/leaf	<i>CaMV35 S</i>	Enteropathogenic <i>E. coli</i> (EPEC)	Diarrhea	Bundle-forming pilus structural subunit A (BfpA)	da Silva et al. (2002)
Tomato/fruit, leaf	<i>CaMV35S</i>	<i>E. coli</i>	Diarrhea	LTB-Mouse zona pellucida 3 epitope (ZP3)	Walmsley et al. (2003)
<i>Arabidopsis thaliana</i> /leaf	<i>CaMV35S</i>	<i>E. coli</i> - <i>M. tuberculosis</i> , <i>M. Bovis</i>	Diarrhea and Tuberculosis	LTB-Early secretory antigenic target-6 (ESAT-6)	Rigano et al. (2004)
Tobacco/leaf	<i>CaMV35S</i>	<i>E. coli</i>	Diarrhea	LTB-SEKDEL	Kang et al. (2005)
Siberian ginseng/somatic embryos	<i>CaMV35S</i> and <i>Ubiquitin</i>	<i>E. coli</i>	Diarrhea	LTB	Kang et al. (2006b)
Soybean/seed	Seed specific <i>glycinin</i>	<i>E. coli</i>	Diarrhea	LTB	Moravec et al. (2007)
Carrot/leaf, root	<i>CaMV35S</i>	<i>E. coli</i>	Diarrhea	LTB	Rosales-Mendoza et al. (2008)
Tobacco/chloroplast	plastid 16S rRNA gene promoter (Prn)	Enterotoxigenic <i>E. coli</i> (ETEC) strains	Diarrhea	LTB-Heat stable toxin (ST)	Rosales-Mendoza et al. (2009)
Potato/tuber	<i>CaMV35S</i>	Porcine epidemic diarrhea virus (PEDV)	Diarrhea	Neutralizing epitope of PEDV (COE)	Kim et al. (2005)
Tobacco/leaf	<i>CaMV35S</i>	Porcine epidemic diarrhea virus (PEDV)	Diarrhea	Neutralizing epitope of PEDV (CO-26K)	Kang et al. (2006a)

Table 3 (continued)

Plant/tissue	Promoter	Pathogen/causing agent	Disease	Antigenic protein	Reference
Tobacco/leaf	<i>CaMV35S</i>	Hepatitis B virus (HBV)	Hepatitis	Hepatitis B surface antigen (HBsAg)	Mason et al. (1992)
Tobacco/leaf	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg	Thanavala et al. (1995)
Potato/tuber	<i>CaMV35S</i>	HBV	Hepatitis	HBsAgM	Ehsani et al. (1997)
Lupin/callus, lettuce/leaf	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg	Kapusta et al. (1999)
Potato/tuber, potato/leaf	Tuber specific <i>patatin</i> and <i>CaMV35S</i>	HBV	Hepatitis	HBsAg and HBsAg-VSP $\alpha$ S/VSP $\alpha$ L	Richter et al. (2000)
Potato/tuber	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg	Kong et al. (2001)
Tobacco NT1 and soybean W82 cell suspension cultures	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg	Smith et al. (2002a,b)
Cherry tomatillo/leaf, stem, fruit	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg	Gao et al. (2003)
Tobacco/NT-1 cell line culture	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg-VSP $\alpha$ S	Sojikul et al. (2003)
Potato/tuber	Tuber specific <i>patatin</i> and <i>CaMV35S</i>	HBV	Hepatitis	HBsAg S and preS2 antigens	Joung et al. (2004)
Tobacco/leaf and tomato/leaf, fruit	<i>CaMV35S</i>	HBV	Hepatitis and Gastroenteritis	HBsAgM/S and NVCP	Huang et al. (2005)
Banana/fruit, leaf	<i>ubq3</i> and <i>Ethylene forming enzyme (EFE)</i>	HBV	Hepatitis	HBsAg	Kumar et al. (2005a)
Tobacco/cell line suspension culture	<i>ubq3</i> and <i>EFE</i>	HBV	Hepatitis	HBsAg	Kumar et al. (2005b)
Potato/tuber, hairy root	<i>EFE</i>	HBV	Hepatitis	HBsAg	Kumar et al. (2006)
Tomato/fruit	<i>CaMV35S</i>	HBV	Hepatitis	HBsAg-ENV,GAG epitopes of HIV-1	Shchelkunov et al. (2006)
Potato/tuber	<i>CaMV35S</i>	HBV	Hepatitis	HBsAgM	Youma et al. (2007)
Tomato/fruit	Fruit specific <i>2A11</i>	HBV	Hepatitis	PRS-S1S2S	Lou et al. (2007)
Potato/tuber, tobacco/leaf	Tuber specific <i>patatin</i> and <i>CaMV35S</i>	Norwalk virus (NV)	Gastroenteritis	Norwalk virus capsid protein (NVCP)	Mason et al. (1996)
Potato/ leaf, tuber	<i>CaMV35S</i>	Bovine group A rotavirus (GAR)	severe viral diarrhea in humans and animals	Major capsid protein VP6	Matsumura et al. (2002)
Potato/tuber, leaf	<i>CaMV35S</i>	Rotavirus	Gastroenteritis	capsid of rotavirus glycoprotein VP7	Wu et al. (2003)
Alfalfa/leaf	<i>CaMV35S</i>	Bovine rotavirus (BRV)	Gastroenteritis in mammals	eBRV4	Wigdorovitz et al. (2004)
Alfalfa/leaf	<i>CaMV35S</i>	Rotavirus	Viral gastroenteritis	PBsVP6 human group A rotavirus	Dong et al. (2005)
Potato/tuber, leaf	<i>CaMV35S</i>	Rotavirus	Gastroenteritis	capsid of rotavirus glycoprotein VP7	Li et al. (2006a)
Potato/tuber, leaf	<i>CaMV35S</i>	human papillomaviruses (HPV)	Cervical cancer	HPV11 L1 major capsid protein	Warzecha et al. (2003)
Tobacco/leaf, potato/tuber, leaf	<i>CaMV35S</i>	human papillomaviruses (HPV)	Cervical cancer	HPV 16 Virus-Like Particles	Biemelt et al. (2003)
<i>A. thaliana</i> /leaf, tobacco/leaf	<i>CaMV35S</i>	HPV	Cervical cancer	HPV11 L1 major capsid protein	Kohl et al. (2007)
Tomato/leaf, fruit	<i>CaMV35S</i>	<i>Corynebacterium diphtheriae</i> , <i>Bordetella Pertussis</i> and <i>Clostridium tetani</i>	Diphtheria, Pertussis and Tetanus (DPT)	epitopes of the <i>C. diphtheriae</i> , <i>B. pertussis</i> and <i>C. tetani</i> exotoxins	Soria-Guerra et al. (2007)
Tomato/leaf, fruit	<i>CaMV35S</i>	Rabies virus	Rabies	RGP	McGarvey et al. (1995)
Tobacco/leaf	<i>CaMV35S</i>	Rabies virus	Rabies	RGP	Ashraf et al. (2005)
Tomato/leaf, fruit	<i>CaMV35S</i>	Rabies virus	Rabies	Rabies nucleoprotein (RNP)	Arango et al. (2008)
Rice/leaves, seeds	<i>ubiquitin</i> and seed specific <i>glutelin</i>	Newcastle disease virus (NDV)	Newcastle disease (ND)	NDV envelope fusion (F) glycoprotein	Yang et al. (2007)
Tobacco/leaf	<i>CaMV35S</i>	Measles virus (paramyxovirus)	Measles	MV-H (Measels virus hemagglutinin)	Huang et al. (2001)
Tobacco/leaf	<i>CaMV35S</i>	Measels virus	Measles	MV-H	Webster et al. (2002)
Carrot/leaf, root	<i>CaMV35S</i>	Measles virus	Measles	MV-H	Marquet-Blouin et al. (2003)
Tobacco/leaf	<i>CaMV35S</i>	Measels virus	Measels	MV-H	Webster et al. (2005)
Peanut/leaf	<i>CaMV35S</i>	Rinderpest virus (RPV)	Rinderpest	H protein of rinderpest virus	Khandelwal et al. (2003)
Collard/leaf, cauliflower/ floret of mature curd	<i>CaMV35S</i> and synthetic OCS3MAS.	Vaccinia virus, human SARS coronavirus	Smallpox and human SARS	vaccinia virus B5 coat protein and coronavirus spike glycoprotein epitope	Pogrebnyak et al. (2006)

(continued on next page)



**Table 3** (continued)

Plant/tissue	Promoter	Pathogen/causing agent	Disease	Antigenic protein	Reference
Arabidopsis/leaf	CaMV35S	Swine transmissible gastroenteritis corona virus (TGEV)	Transmissible gastroenteritis (TGE)	Glycoprotein S	Gómez et al. (1998)
Potato/tuber	CaMV35S	TGEV	TGE	Glycoprotein S	Gómez et al. (2000)
Tobacco/leaf	Synthetic super promoter	TGEV	TGE	Glycoprotein S	Tuboly et al. (2000)
Arabidopsis/leaf	CaMV35S	Foot and mouth disease virus (FMDV)	Foot and mouth disease	Structural protein VP1	Carrillo et al. (1998)
Potato/tuber, leaf	CaMV35S	FMDV	Foot and mouth disease	Structural protein VP1	Carrillo et al. (2001)
Alfalfa/leaf	CaMV35S	FMDV	Foot and mouth disease	Structural protein VP1	Dus Santos et al. (2002)
Potato/leaf, tuber	CaMV35S	Rabbit haemorrhagic disease virus (RHDV)	Rabbit hemorrhagic syndrome	Structural protein VP60	Castañón et al. (1999)
Potato/leaf, tuber	CaMV35S, sunflower Polyubiquitin and B33patatin	RHDV	Rabbit hemorrhagic syndrome	Structural protein VP60	Castañón et al. (2002)
Potato/tuber	CaMV35S	RHDV	Rabbit hemorrhagic syndrome	Structural protein VP60	Martín-Alonso et al. (2003)
Tobacco/leaf	CaMV35S	<i>Streptococcus mutans</i>	bacteraemia and infective endocarditis	Surface protein antigen A	Curtiss and Cardineau (1990)
Tobacco/leaf	CaMV35S	Vesicular stomatitis virus (VSVG)	Severe acute respiratory syndrome	Glycoprotein of VSVG	Galbraith et al. (1992)
Tobacco/seed	Seed specific <i>glutelin Gt3</i>	Human cytomegalovirus (HCMV)	Central nervous system disease	Glycoprotein B	Tackaberry et al. (1999)
Arabidopsis/leaf	CaMV35S	Canine parvovirus	Canine parvovirus disease	VP2 capsid protein of canine parvovirus (CPV)	Gil et al. (2001)
White clover/leaf	CaMV35S	<i>Mannheimia haemolytica</i> (bovine pneumonia pasteurellosis)	Bovine viral diarrhea	Leukotoxin (Lkt)	Lee et al. (2001)
Tobacco/leaf	CaMV35S	<i>Bacillus anthracis</i>	Anthrax	Protective antigen	Aziz et al. (2002)
Tobacco/leaf (chloroplast)	Plastid rRNA operon (Prn)	<i>Clostridium tetani</i>	Tetanus	Tetanus vaccine antigen (TetC)	Tregoning et al. (2003)
Tobacco, collard/leaf	<i>rbcS</i> and CaMV35S	Vaccinia virus	Smallpox	Vaccinia virus B5 coat protein	Golovkin et al. (2007)
Tomato/fruit	Fruit specific <i>E8</i>	Human immunodeficiency virus (HIV)	AIDS	Tat protein of HIV-1	Ramírez et al. (2007)
Potato/tuber	CaMV35S	Caused by neuro-degeneration	Alzheimer's	Human $\beta$ -amyloid (A $\beta$ )	Kim et al. (2003)
Tomato/leaf	CaMV35S	Caused by neuro-degeneration	Alzheimer's	Human $\beta$ -amyloid (A $\beta$ )	Youma et al. (2008)
Rice/seeds	Seed specific <i>glutelin A</i>	Caused by inflammation of joint cartilage	Arthritis	Type II collagen peptide	Hashizume et al. (2008)
Peanut/callus	CaMV35S	Bluetongue virus (BTV)	Bluetongue	VP2 gene that constitutes the outer capsid of the virus	Athmaram et al. (2006)
Papaya/Embryogenic clones (ETgpC)	CaMV35S	<i>Taenia solium</i>	Cysticercosis	Synthetic peptides KETc1, KETc12, KETc7	Hernández et al. (2007)
Tomato/leaf, fruit	CaMV35S	Organophosphate poisoning	Severe acute pancreatitis and myocardial injury	Human acetylcholinesterase (AChE)	Mor et al. (2001)
Tomato/fruit	CaMV35S and fruit specific <i>E8</i>	Respiratory syncytial virus (RSV)	Serious respiratory tract disease	RSV F protein	Sandhu et al. (2000)
Tobacco/leaf, lettuce/leaf	CaMV 35S	Severe Acute Respiratory Syndrome Coronavirus	Severe Acute Respiratory Syndrome	Partial spike (S) protein of SARS-CoV	Li et al. (2006c)
Tobacco/leaf	CaMV35S	Avian influenza virus H5/HA1 variant	Avian flu	H5/HA1 variant -HDEL	Spitsin et al. (2009)
Tomato/fruit	CaMV35S	<i>Yersinia pestis</i>	Pneumonic/bubonic plague	Antiphagocytic capsular envelope glycoprotein (F1) and low calcium response virulent antigen (V) fusion protein	Alvarez et al. (2006)
Tobacco/chloroplast	<i>psbA</i>	<i>Yersinia pestis</i>	Pneumonic/bubonic plague	F1-V fusion protein	Arlen et al. (2008)
Tobacco and Arabidopsis/leaf	CaMV35S	Human immunodeficiency virus (HIV-1) and hepatitis B virus (HBV)	AIDS and Hepatitis B	Recombinant HIV-1/HBV virus-like particles	Greco et al. (2007)

synthesized LTB had a similar affinity for GM1 gangliosides, as the bacterial-synthesized LTB. Mice feeding experiments showed that the maize-synthesized LTB stimulated a protective immune response against LT and its closely related cholera toxin (CT). Rigano et al. (2004) reported the expression of a fusion protein consisting of the LTB and a 6 kDa tuberculosis antigen in transgenic *A. thaliana*. Both

components of the fusion protein were expressed under the control of CaMV35S promoter and detected using GM1-ELISA. Thus, the fusion protein retained its native form. Moravec et al. (2007) reported high level accumulation (2.4% of the total seed protein) of LTB in transgenic soybean seed. Seed specific *glycinin* promoter was used to target protein in soybean seeds. The endoplasmic reticulum (ER) retention

sequence (KDEL) attached at the 3' end of the transgene increases the stability of active heterologous protein. Soybean-based LTB is assembled correctly into pentamers that were bound to GM1, and were highly immunogenic when used in a prime-boost immunization strategy. Recently Rosales-Mendoza et al. (2008) expressed LTB encoded synthetic gene driven by the *CaMV35S* promoter in tap roots of adult carrot plants. Due to high homology between LT and CT, ingestion of transformed carrot material induces LTB-specific intestinal and systemic antibodies in mice and provided protection against oral LT as well as CT challenge.

### 6.1.3. Surface antigen (HBsAg) of Hepatitis B virus

Hepatitis B virus (HBV) is a major cause of acute and chronic hepatitis. HBV DNA is found in most of the newborns from hepatitis B surface antigen (HBsAg)-positive mothers. The current HBV vaccine is a biotechnological product that falls in the category of “subunit vaccines” and is made from yeast cells grown by fermentation. However, intramuscular administration of the vaccine causes some pain, thus it is not widely accepted, especially for children.

The HBV is a double stranded DNA virus in the *Hepadnaviridae* family. The HBV genome includes four genes: *pol*, *env*, *precore*, and *X* which encode the viral DNA polymerase, envelope protein, precore protein (which is processed to viral capsid), and protein X, respectively. The most recent vaccine is based on the cloned copies of the *env* gene in yeast (Kapusta et al., 1999). The *env* gene codes for three related proteins: (i) S protein; (ii) pre-S1 protein (iii) pre-S2 protein. The analysis of blood from hepatitis B virus carriers reveals the presence of 22 nm particles consisting of a viral envelope surface (S) protein. The S antigenic protein plays an important role in the process of virus infection and the induction of a defensive host response. First report on the development of hepatitis vaccine in plants was published by Mason et al. (1992) where tobacco plants were genetically engineered by introducing HBsAg under the control of a *CaMV35S* constitutive promoter. However, low expression levels of only upto 0.01% of total leaf soluble protein were reported. The HBsAg has been expressed in lupin (*Lupinus luteus* L.) callus and lettuce (*Lactuca sativa* L.) leaves (Kapusta et al., 1999) also. Rather low HBsAg expression at levels of 150 ng/g fresh weight in lupin callus and 5.5 ng/g fresh weight in lettuce leaves were obtained, yet the mice fed with transgenic lupin tissue developed significant level of hepatitis B virus-specific antibodies. Human volunteers, fed with transgenic lettuce plants expressing hepatitis B virus surface antigen also developed specific serum-IgG response. Shchelkunov et al. (2006) expressed a synthetic chimeric fusion gene encoding the immunogenic ENV and GAG epitopes of human immunodeficiency virus (HIV-1) and HBsAg under the control of *CaMV35S* promoter in tomato plants. Dried transgenic tomato fruits containing the chimeric antigen were fed to mice. Plant based oral vaccine against the two viruses elicited immune responses in the test animal. Recently, Qian et al. (2008) expressed SS1 gene that encodes a protein which consists of pre-S1 protein fusion with the truncated C-terminus of HBsAg protein. The expression of the fusion protein was controlled by seed specific *GluB-4* promoter in rice seeds and highest expression level reported was to 31.5 ng/g dry weight of rice grains. This recombinant SS1 protein induced immunological response against S and preS1 protein in mice. He et al. (2008) expressed Hepatitis B virus surface antigen middle protein gene (HBV S2+S/HBsAgM) under the control of *E8* promoter in tomato and tobacco plants. The *E8* promoter regulated expression of the antigen in ripened tomato fruit but not in tobacco leaves, flowers and seeds. Their results suggest that *E8* promoter might act in a species-specific fashion. Banana fruit has also been used for the expression of HBsAg. Since bananas are propagated through suckers and do not set seeds, this plant is a suitable candidate for gene containment with no segregation of the transgene. Kumar et al. (2005a) expressed HBsAg in banana but reported rather low level of expression at 1 ng/g fresh weight of fruits and up to 38 ng/g fresh

weight in leaves. The expression of HBsAg antigen in other plant based systems such as tobacco cell line suspension culture (Sojikul et al., 2003; Kumar et al., 2005b) and potato hairy roots culture (Kumar et al., 2006) have been discussed separately in this review. Major achievements in the expression, characterization and immunogenicity of HBsAg antigen in plant based systems have recently been reviewed by Kumar et al. (2007).

### 6.1.4. Other potential vaccines

Many other antigens have been designed for optimal expression in different tissues of plants. Although, most of these have not been tested for immune response, yet they possess a good prospect for use as vaccines against many infectious diseases. Some of the genes encoding various vaccine antigens against infectious diseases are as follows.

- Norwalk virus capsid protein (NVCP) and rotavirus outer capsid protein (VP7) against gastroenteritis;
- Human papillomavirus-like particles (HPV VLPs) L1 major capsid protein against cervical cancer;
- Diphtheria–pertussis–tetanus (DPT) exotoxin epitopes joined by peptide linkers against diphtheria, pertussis (whooping cough) and tetanus;
- Rabies glycoprotein and nucleoprotein against rabies virus;
- Measles hemagglutinin (H) glycoprotein against measles virus;
- Newcastle disease virus (NDV) F fusion protein against newcastle virus;
- Rinderpest surface hemagglutinin (H) glycoprotein against rinderpest virus (RPV);
- *B. anthracis* LF protein against anthrax;
- Antiphagocytic capsular envelope glycoprotein (F1) and low calcium response virulent antigen (V) against plague;
- A27L, L1R, A33R and B5R proteins of Variola virus against smallpox.

Some of the published reports related to stable antigen expression in different plant tissues are mentioned below.

Mason et al. (1996) transformed a gene encoding norwalk virus capsid protein (NVCP) under the control of *CaMV35S* and potato tuber specific *patatin* promoter into tobacco and potato, respectively. Tobacco leaves and potato tubers expressed 0.23% and 0.37% NVCP respectively out of the TSP. The capsid protein extracted from tobacco leaves and potato tubers were in the form of 38-nm Norwalk virus-like particles. The plant expressed Norwalk virus-like particle (rNV) showed antigenic properties similar to the gene expressed in recombinant baculovirus-infected insect cell lines. The potato tubers expressing rNV fed orally to mice, developed serum IgG specific antibody for rNV.

Rotavirus is an important cause of viral gastroenteritis in young children and animals. Li et al. (2006a) expressed outer capsid of rotavirus major glycoprotein VP7, over fifty generations to check the stability of antigen in potatoes. The oral delivery of antigen in mice detected that specific cytotoxic T lymphocytes provided long term immuno-protection against rotavirus infection. Humeral and mucosal responses were successfully induced in mice fed with the fiftieth generation transformed potato tubers. No significant differences were observed in serum IgG and fecal IgA between the mice fed with the first and fiftieth generation potatoes. This is the first report regarding stability of vaccines derived from plants that can be propagated for many generations.

Cervical cancer is linked to infection with human papillomaviruses (HPV). There is a great demand for the development of an HPV preventive vaccine as it is the third most common cancer among women worldwide. Human papilloma-virus-like particles (HPV VLPs) have shown considerable response as a vaccine for the prevention of cervical cancer and its precursor lesions. Warzecha et al. (2003) introduced HPV type 11 (HPV11) L1 major capsid protein coding sequence into potato plants. Transgenic potato was used to feed mice

which resulted in an activation of anti-VLP immune response that was qualitatively similar to that induced by VLP parental administration. This immune response in mice was enhanced significantly by subsequent oral booster with purified insect cell-derived VLPs. Kohl et al. (2007) expressed HPV11 L1 major capsid protein in transgenic *A. thaliana* (Yield up to 12 µg/g) and *N. tabacum* (yield up to 2 µg/g). Neutralizing monoclonal antibodies binding assay suggested that plant and insect cell-derived VLPs displayed similar antigenic properties.

The diphtheria–pertussis–tetanus (DPT) is commercially available vaccine and is utilized to immunize against diphtheria, pertussis (whooping cough) and tetanus. However neurological complications and the secondary effects like fever, drowsiness, learning disabilities and physical handicaps have been reported after children were vaccinated (Meszaros et al., 1996). Soria-Guerra et al. (2007) generated transgenic tomato plants expressing an optimized synthetic gene encoding a polypeptide with epitopes of the DPT exotoxins under the control of *CaMV35S* promoter. This was the first report of successful expression of a multi-component DPT subunit vaccine. However, challenge experiments were not reported.

Rabies, an acute contagious infection of the central nervous system, is caused by rabies virus, which enters the body through the bite of an infected animal. Dodet (2007) estimated that 55,000 human deaths occur per year from rabies worldwide. Most of these were in case of children. Highest incidence of rabies virus infections was found in developing countries. Effective anti-rabies vaccines are available in the market but they are very expensive. The rabies virus surface glycoprotein (G protein) and nucleoprotein (N protein) antigens have been used for expression in plants to induce immuno-protection. The G protein has been considered as the major antigen responsible for the induction of protective immunity. The N protein is recognized as the main immunomodulator which provides partial protection against rabies virus. McGarvey et al. (1995) expressed G protein coding gene under the control of *CaMV35S* promoter in tomato. A synthetic gene coding for the G protein of rabies virus was designed and expressed in tobacco leaves in our laboratory (Ashraf et al., 2005). The tobacco leaf-expressed G protein showed glycosylation. When given by intraperitoneal route, it provided immuno-protection against the virus challenge in mice. Arango et al. (2008) recently reported the expression of native N protein under the control of *CaMV35S* promoter in tomato plants. The expression level was 1–5% of total soluble fruit protein. However, only intraperitoneally immunized mice showed weak protection against viral challenge while the orally immunized mice were not protected. Oral delivery of inactivated rabies virus has been reported to give no immunoprotection in mice, apparently due to instability of virus in the gut. The results suggest that all vaccine antigens may not be sufficiently stable, when delivered through oral route. However, experiments involving the development of appropriate formulations for rapid absorption in buccal mucosa may find a solution to such problems.

Marquet-Blouin et al. (2003) developed transgenic carrot plants to express an immune-dominant antigen of the measles virus. The hemagglutinin (H) glycoprotein is the principal target of neutralizing and protective antibodies against measles. The recombinant protein seemed to have 8% lower molecular weight than the viral protein. Immunization of mice with leaf or root extracts induced high titers of IgG1 and IgG2a antibodies that cross-reacted strongly with the measles virus and neutralized the virus *in vitro*. Muller et al. (2003) generated vaccine-induced immunity against measles which was less robust than natural immunity. Waning of immunity in vaccines may eventually require revaccination of adults. Measles antigens expressed in plants have been shown to be both antigenic and immunogenic after invasive and oral vaccination.

In poultry production, the Newcastle disease (ND) can cause a high level of mortality (up to 100%). Newcastle disease virus (NDV) is one of the most grievous pathogens affecting all species of birds and can

cause a serious respiratory and neurological disease (Gallili and Ben-Nathan, 1998). Thus ND considered as one of the most destructive diseases for poultry, resulted in worldwide trade barriers and drastic economic losses in the commercial poultry industry. The traditional commercially available vaccine shows various problems such as high labour cost, reduced egg-laying and an increased susceptibility to microorganism infections. The development of plant-derived vaccine antigens that could be easily delivered with water or diet is highly desirable. Yang et al. (2007) expressed NDV envelope fusion (F) glycoprotein under the control of maize *glutelin* promoters into rice leaves and seeds. The mice immunized intraperitoneally with crude protein extracts elicited specific antibodies and developed immune response.

Rinderpest, an acute, highly contagious and often fatal disease of large and small ruminants is caused by rinderpest virus (RPV). The commercially available attenuated vaccine is heat-labile. It needs to be maintained in cold chain. Thus, in hot regions where rinderpest is endemic, failure of the vaccine is a common occurrence. Therefore, the development of plant based vaccine is desired strategy. Khandelwal et al. (2003) reported oral immunization of cattle with peanut leaves expressing hemagglutinin (H) protein of rinderpest virus. The *CaMV35S* expressed H protein at 0.2–1.3% of TSP. Development of plant-derived vaccine antigens based on transgenic forage crops, such as alfalfa and peanut (commonly used in the diet of domestic animals) is desirable.

*Bacillus anthracis* is the causative agent of anthrax considered to be one of the most potent bio-terror agents, as *B. Anthracis* spores can be transmitted by aerosolization. The stable vaccine antigen expression has been reported in transgenic tobacco, potato and tomato plants (Aziz et al., 2002, 2005; Kim et al., 2004; Watson et al., 2004; Koya et al., 2005). The tobacco-made vaccine antigen was biologically active as was demonstrated through its ability to lyse a macrophage cell line *in vitro* when combined with lethal factor (Aziz et al., 2002). Also, mice immunized with the tomato-expressed antigens generated lethal toxin neutralizing antibodies (Aziz et al., 2005). For the production of an edible anthrax vaccine, Kim et al. (2004) generated potato plants expressing detectable amount of cholera toxin B-subunit–anthrax LF conjugate fusion protein. Later, vaccine antigen was expressed in chloroplasts, in an effort to produce an anthrax vaccine in large quantities in transgenic tobacco leaves (Watson et al., 2004; Koya et al., 2005). Subcutaneous immunization of mice with partially purified *B. anthracis*-derived antigen with adjuvant yielded immunoglobulin G titers up to 1:320,000, and both groups of mice survived challenge with lethal doses of toxin.

Pneumonic plague is a highly contagious and mortal disease in different regions of the world which can be transmitted by aerosol from infected to naïve hosts. Two *Y. pestis* antigens are of interest for developing new vaccines against plague: the anti-phagocytic capsular envelope glycoprotein (F1) and the low calcium response virulent antigen (V). These two proteins were successfully and rapidly expressed separately or as a combined fusion protein in tomato fruits as a oral subunit vaccine. The vaccine elicited IgG1 in serum and mucosal IgA in fecal pellets (Alvarez et al., 2006). In a different study, the fusion protein F1-V was expressed in transgenic tobacco chloroplasts. The maximum expression levels of the fusion protein were observed in mature leaves and were as high as 14.8% of TSP (Arlen et al., 2008).

Variola virus is the causative agent of smallpox. Four immunogenic proteins A27L and L1R proteins specific to the intracellular mature virus (IMV) and the A33R and B5R proteins specific to the extracellular enveloped virus (EEV) are the best molecular candidates for the development of a smallpox vaccine. The antigen was produced in soluble and insoluble forms upon transient and stable plant transformation. A27L and the A33R protein are stably expressed by *Agrobacterium*-mediated transformation of the nuclear genome and biolistic transformation of the plastome. The integration of the A27L

gene into the chloroplast genome resulted in high-yield accumulation of the recombinant protein (Rigano et al., 2009). Production of the recombinant vaccinia virus B5 antigenic domain (B5) in tobacco leaves was reported by Golovkin et al. (2007). Intranasal administration of soluble B5 led to a rise of B5-specific immunoglobulins. Pogrebnyak et al. (2006) reported the use of vegetable plants—transgenic collard and cauliflower for the expression of antigens. They transformed collard plants with smallpox antigen vaccinia virus B5 coat protein driven by *CaMV35S* promoter. The expression levels of antigen in collards were not decreased after several months' growth in greenhouse conditions.

The transgenic cauliflower plants developed with human SARS coronavirus spike glycoprotein epitope driven by synthetic OCS3MAS promoter showed detectable amount of antigen in floret tissue of mature curd (Pogrebnyak et al., 2006). Although they did not perform virus challenge experiments, this report suggests successful use of vegetable crops for the expression of commercially useful antigens.

### 6.2. Tissue/cell suspension culture based expression

Transgenic plants require cultivation, harvesting, extraction and if required, purification of recombinant proteins to be used as vaccine antigens. Plants grown under field conditions often shows variable expression of recombinant protein(s). Hence, good manufacturing practices (GMP) and doze standardization may pose difficulties in the use of field grown plants as a source of plant-derived vaccine antigens. Further, linkage between farm and protein handling units may make the operational chain complex and add to costs. On the other hand, plant cell/tissue culture and hairy root culture could provide significant advantage in controlled production of therapeutic proteins. These processes are independent of seasonal variations and enable continuous supply of the product. The continuous secretion and recovery of foreign proteins from cellular and culture medium can minimize the time and cost of process standardization, improve protein recovery, make the process more easily reproducible and reduce protein degradation during handling. The targeting of recombinant proteins with appropriate signal peptides for extracellular secretion can mimic the natural process in plants. Some proteins can be recovered easily from the secretion fluid or culture media. The addition of protein stabilizing agents into the suspension culture medium can increase the accumulation of recombinant protein (Magnuson et al., 1996).

The expression of recombinant proteins in suspension and hairy root culture offers promising potential for exploitation as large bioreactors. The two tobacco plant cell lines, Bright Yellow-2 (BY-2) and *N. tabacum*-1 (NT-1) are utilized extensively for foreign protein production because of easy transformation and synchronous growth in liquid culture (Hellwig et al., 2004). Sojikul et al. (2003) expressed HBsAg protein attached with eukaryotic ER signal peptide from soybean vegetative storage protein VSP $\alpha$ S or ER retention signal (SEKDEL) at the N-terminal and C-terminal ends, respectively in tobacco NT-1 cell line culture. The expression of fusion protein was controlled by *CaMV35S* promoter. They suggested that VSP $\alpha$ S signal peptide directly targeted trans-protein in ER and remained uncleaved. This enhanced VSP $\alpha$ S-HBsAg fusion accumulation and stability in tobacco plant cells. The SEKDEL did not show any significant effect on total HBsAg accumulation. Kumar et al. (2005b) reported HBsAg expression in NT-1 tobacco cell line suspension culture. The results suggested that HBsAg could be expressed in plant cell cultures in the required form and elicited antigen-specific immune response in mice. However, there is a need to enhance and optimize the expression levels and maintain the stability of plant cell cultures for constant and stable expression in long-term cultures.

Continuous rhizosecretion of recombinant proteins is another promising strategy. Root biomass can be significantly increased by hairy root formation using *Agrobacterium rhizogenes*. The secreted

proteins can be recovered easily from the hydroponic medium and used as simple source material for protein enrichment and purification (Komarnytsky et al., 2004). Potato hairy roots were utilized for the expression of HBsAg (Richter et al., 2000; Kumar et al., 2006). These offer several advantages for the production of HBsAg, including the availability of efficient genetic transformation, short regeneration time, availability of tissue specific promoters and genetic stability. The rhizosecretion has also been exploited recently for heterologous expression of human alkaline phosphatase (Gaume et al., 2003) and immunoglobulin G antibodies (Komarnytsky et al., 2006).

### 6.3. Transient transformation for rapid expression

Genetically-engineered plant viruses and *A. tumefaciens* can be employed to produce vaccine antigens in transiently infected plants, tissues and plant cells. The natural virus infection of a plant begins with the entry of infectious material into the cells, followed by amplification and accumulation of the virus, and then spread through the entire plant. As plant viruses do not get incorporated into the plant genome, therefore virus vectors only provide transient expression of foreign proteins. The plant viruses have advantage of achieving high level expression of recombinant protein in less time. Moreover, the viral infections are easily transmissible by mechanical inoculation and spread rapidly from plant to plant, making it possible to infect large number of plants in less time. Many reports have been published on the virus based transient expression of antigen(s). The expressions of antigen(s) in plants and/or protection reported in specific cases are given in Table 4. Some of the important points are summarized below.

Haynes et al. (1986) provided a concept that an antigen epitope gene could be expressed in the plant systems by linking it with a self assembling viral peptide. The self-assembly of such chimeric molecule into virus like particle could result in an immunogen containing the foreign antigenic epitope repeated many times on its surface. They performed experiments in *E.coli* expression system using tobacco mosaic virus (TMV) coat protein gene fused with polio virus. A similar approach of using TMV coat proteins as carriers has been extended to transient expression of foreign genes in intact plants (Dawson et al., 1986). The TMV coat protein gene was among the first plant virus proteins to be used as a carrier molecule for antigenic epitopes (Hamamoto et al., 1993). There are several reviews on the TMV, cowpea mosaic virus (CPMV) and alfalfa mosaic virus (AIMV) where, plant virus-based transient expression vectors have been discussed to produce recombinant antigens in plants (Pogue et al., 2002; Lacomme et al., 2001; Arntzen, 2005; Liu et al., 2005; Grill et al., 2005). The major limitation of the use of TMV coat protein is that not more than 25 amino acid long peptide can be used in fusion with the TMV coat protein. This is a significant limitation for the production of various molecules of biomedical importance (Koprowski and Yusibov, 2001). However genetically engineered AIMV and CPMV have overcome the limitation of long peptide expression. The CPMV was used for the first time to carry peptides fused to the surface of virus coat protein (Usha et al., 1993). Modelska et al. (1998) and Yusibov et al. (2002) reported genetically engineered AIMV for the expression of two rabies virus epitopes. Mice were immunized intraperitoneally or orally with virus-infected spinach leaves. They detected mucosal immune response after oral induction with a plant-virus-derived vaccinogen. Moreover, the AIMV coat protein can be expressed from a heterologous virus vector such as TMV and particle assembly occurs independently of the carrier virus. Yusibov et al. (1997) reported that mice immunized with recombinant AIMV particles containing either rabies or HIV peptides showed neutralizing antibodies against rabies and HIV, respectively. An attempt has also been made to develop plant-derived contraceptive from a recombinant plant virus (Fitchen et al., 1995). The *zona pellucida* ZP3 protein of mammalian oocyte has been a target for immune contraception. An epitope of 13 amino acids from murine ZP3 was expressed in plant as a fusion protein with TMV capsid protein.

**Table 4**  
Transient expression of vaccine antigens in plants.

Plant/tissue	Vector	Pathogen	Disease	Antigenic protein	Reference
Tobacco/leaf	Tobacco mosaic virus (TMV)	B cell lymphoma	Non-Hodgkin's lymphoma	Single chain Fv fragment of immunoglobulin (ScFv)	McCornick et al. (1999)
Tobacco/leaf	Potato virus X (PVX)	B cell lymphoma	Lymphoma	(ScFv40)	Hendy et al. (1999)
Tobacco/NT1 cell lines	pGFP210 contained CaMV35S-Green fluorescent protein	human papilloma viruses (HPV)	Cervical cancer	HPV11 L1 major capsid protein	Warzecha et al. (2003)
Tobacco/leaf	PVX	Growth factor receptor (cancer diagnosis 7 therapy)	Lung Cancer	scFv-HER2 (Human epidermal growth factor receptor-2)	Galeffi et al. (2005)
Tobacco/leaf	pBID4 (Agro infiltration)	Human papilloma virus type 16 (HPV)	Cervical cancer	Fusions of E7 and the E7 mutant E7GGG to <i>Clostridium thermocellum</i> $\beta$ -1,3-1,4- glucanase (LicKM) {LicKM-E7, and LicKM-E7GGG}	Massa et al. (2007)
Tobacco/leaf, <i>Brassica rapa</i> /leaf	L2ACPE7 (Agro infiltration)	Human papilloma virus type 16 (HPV)	Cervical cancer	HPV16 epitopes	Hoffmeisterova et al. (2008)
Cowpea/leaf	Cowpea mosaic virus (CPMV)	Foot and mouth disease virus (FMDV)	Foot and mouth disease (FMD)	Structural protein VP1	Usha et al. (1993)
Tobacco/leaf	TMV	FMDV	FMD	Structural protein VP1	Wigdorovitz et al. (1999a)
Alfalfa/leaf	TMV	FMDV	FMD	Structural protein VP1	Wigdorovitz et al. (1999b)
<i>Chenopodium quinoa</i> and tobacco/leaf	Bamboo mosaic virus (BaMV)	FMDV	FMD	Structural protein VP1	Yang et al. (2007)
Tobacco/leaf	Self assembling virus like particle (MagnICON viral vector)	Hepatitis B virus (HBV)	Hepatitis B	Hepatitis B surface Antigen (HBsAg)	Mason et al. (1992)
Tobacco/leaf	Self assembling virus like particle (MagnICON viral vector)	HBV	Hepatitis B	HBsAg	Thanavala et al. (1995)
Tobacco/leaf	TMV	Murine hepatitis virus	Hepatitis B	Peptide of glycoprotein S	Koo et al. (1999)
Tobacco/leaf	TMV	Hepatitis C virus	Hepatitis C	Hypervariable region 1 of envelop protein 2 fused to cholera toxin B subunit	Nemchinov et al. (2000)
Tobacco/leaf	pGFP (Agro infiltration)	HBV	Hepatitis B	HBsAg	Huang and Mason (2004)
Tobacco/leaf	pHB117 and pMHB	HBV	Hepatitis B	HBsAgS / M	Huang et al. (2005)
Tomato/fruit	pHER, pHBS and pEFE (agro infiltration)	HBV	Hepatitis B	HBsAg	Srinivas et al. (2008)
Apple/leaf, protoplast	CaMV35S	Respiratory syncytial virus (RSV)	Serious respiratory tract disease	RSV F protein	Sandhu et al. (1999)
Tobacco/leaf	Alfalfa mosaic virus (AIMV)	RSV	Serious respiratory tract disease	RSV G protein	Belanger et al. (2000)
Tobacco/leaf	AIMV	RSV	Lower respiratory tract illness	RSV G protein	Yusibov et al. (2005)
Tobacco/leaf	Self assembling virus like particle	Influenza virus	Acute respiratory illness (Flu)	Heamagglutinin Epitope fused with tobacco mosaic virus coat protein	Porta et al. (1994)
Cowpea/leaf	CPMV	Human rhinovirus type 14	AIDS	VP1 protein	Porta et al. (1994)
Tobacco/leaf	TMV	HIV-1 and Influenza	AIDS and Acute respiratory illness (Flu)	HIV-1 gp epitope and Influenza heamagglutinin	Sugiyama et al. (1995)
Cowpea/leaf	CPMV	Human immunodeficiency virus type 1	AIDS and neurological injury	HIV transmembrane protein gp41	McLain et al. (1996)
Tobacco/leaf	AIMV-CP/TMV-CP	Human immunodeficiency virus type 1	AIDS	V3 loop of gp120 protein	Yusibov et al. (1997)
Tobacco/leaf	Tomato bushy stunt virus	Human immunodeficiency virus type 1	AIDS	V3 loop of gp120 protein	Joelson et al. (1997)
Cowpea/leaf	CPMV	Human immunodeficiency virus type 1	AIDS and neurological injury	HIV transmembrane protein gp41	Durrani et al. (1998)
Cowpea/leaf	CPMV	Human immunodeficiency virus type 1	AIDS and neurological injury	HIV transmembrane protein gp41	McInerney et al. (1999)
Tobacco/leaf	Tomato bushy stunt virus	Human immunodeficiency virus type 1	AIDS	Nucleocapsid protein p24	Zhang et al. (2000, 2002)
Tobacco/leaf	PVX	Human immunodeficiency virus type 1	AIDS	HIV transmembrane protein gp41	Marusic et al. (2001)
Spinach/leaf	TMV	Human immunodeficiency virus type 1	AIDS	Tat protein of HIV-1	Karasev et al. (2005)

Table 4 (continued)

Plant/tissue	Vector	Pathogen	Disease	Antigenic protein	Reference
Tobacco/leaf	pTRAc (Agro infiltration)	HIV-1	AIDS	HIV-1Pr55Gag, Gag(p17/p24), p24	Meyers et al. (2008)
Tobacco/leaf	AIMV-CP/TMV-CP	Rabies virus	Rabies	Chimeric peptides of rabies glycoprotein (RGP) and rabies nucleoprotein (RNP)	Yusibov et al. (1997)
Tobacco, spinach/leaf	TMV	Rabies virus	Rabies	Chimeric peptides RGP and RNP	Modelska et al. (1998)
Tobacco, spinach/leaf	AIMV	Rabies virus	Rabies	Chimeric peptides of RGP and RNP	Yusibov et al. (2002)
Tobacco/leaf	pICH10990 (Agro infiltration)	Rabies virus	Rabies	RNP	Arango et al. (2008)
Tobacco/leaf	TMV	Porcine epidemic diarrhea virus	Acute enteritis	PEDV-COE (Core neutralizing epitope of Porcine epidemic diarrhea virus)	Kang et al. (2004)
Tobacco/leaf	Plum pox potyvirus	Canine parvovirus	Myocarditis and Fatal enteritis	Capsid protein VP2	Fernandez-Fernandez et al. (1998)
Tobacco/leaf	PVX	Rotavirus	Gastroenteritis	Inner capsid protein	O'Brien et al. (2000)
Tobacco/leaf	PVX	Human papillomavirus type 16	Cutaneous warts /epithelial neoplasia	E7 oncoprotein	Franconi et al. (2002)
Cowpea/leaf	CPMV	Canine parvovirus	Myocarditis and fatal enteritis	Capsid protein VP2	Langeveld et al. (2001)
Cowpea/leaf	CPMV	Canine parvovirus	Myocarditis and fatal enteritis	Capsid protein VP2	Nicholas et al. (2002)
Cowpea/leaf	CPMV	Mink enteritis virus	Acute interstitial pneumonia	Capsid protein VP2	Dalgaard et al. (1997)
Cowpea/leaf	CPMV	<i>Staphylococcus aureus</i>	Folliculitis pneumonia, septicemia, osteomyelitis and food poisoning	D2 peptide of fibronectin-binding protein (FnBP)	Brennan et al. (1999a)
Cowpea/leaf	CPMV	<i>Pseudomonas aeruginosa</i>	Systemic and nosocomial infections	Outer-membrane protein F	Brennan et al. (1999b,c)
Cowpea/leaf	CPMV	<i>Pseudomonas aeruginosa</i>	Systemic and nosocomial infections	Outer-membrane protein F	Gilleland et al. (2000)
Tobacco/leaf	TMV	<i>Pseudomonas aeruginosa</i>	Systemic and nosocomial infections	Outer-membrane protein F	Staczek et al. (2000)
Tobacco/leaf	PVX	Human papillomavirus type 16	Cutaneous warts / epithelial neoplasia	UT1, neonatal meningitis and gastroenteritisPotato virus X	Franconi et al. (2002)
Tobacco/leaf	CPMVand PVX	<i>Staphylococcus aureus</i>	Suppurative and nosocomial infections	D2 peptide of fibronectin-binding protein (FnBP)	Brennan et al. (1999d)
Tobacco/leaf	pCaSF1-V110 (Agro infiltration)	<i>Yersinia pestis</i>	Pneumonic/Bubonic Plague	F1-V fusion protein	Alvarez et al. (2006)
Tobacco/leaf	pICH18360 (Agro infiltration)	<i>Yersinia pestis</i>	Pneumonic/Bubonic Plague	F1-V fusion protein	Santi et al. (2006)
Tobacco/leaf	pCaSF1-V110 (Agro infiltration)	<i>Yersinia pestis</i>	Pneumonic/Bubonic Plague	F1, V and F1-V fusion protein	Mett et al. (2007)
Tobacco/leaf	PVX based agro infiltration	<i>Mycobacterium tuberculosis</i>	Tuberculosis	ESAT-6 protein	Zelada et al. (2006)
Tobacco/leaf	TMV based agro infiltration	<i>Mycobacterium tuberculosis</i>	Tuberculosis	Ag85B, ESAT-6 and ESAT-6: Ag85B fusion	Dorokhov et al. (2007)
Tobacco, collar/leaf	pICH115999 (Agro infiltration)	Vaccinia virus	Smallpox	Vaccinia virus B5 coat protein	Golovkin et al. (2007)
Tobacco/leaf	pICH115999 (Agro infiltration)	Vaccinia virus	Smallpox	Vaccinia virus B5 coat protein	Portocarrero et al. (2008)
Tobacco/leaf	TMV	<i>Plasmodium falciparum</i>	Malaria	Peptides of circumsporozoite protein	Turpen et al. (1995)
Tobacco/leaf	Plum pox potyvirus	Rabbit haemorrhagic disease virus (RHDV)	Viral haemorrhagic disease (VHD)	Structural protein VP60	Fernandez-Fernandez et al. (2001)
Tobacco/leaf	TMV based agro infiltration	<i>Bacillus anthracis</i>	Anthrax	LicKM-LFD1	Chichester et al. (2007)
Tobacco/leaf	pBID4 (agro infiltration)	Avian influenza virus H5N1	Avian flu	Influenza virus haemagglutinin antigen (HA)	Shoji et al. (2009)
Tobacco/leaf	pICH115999 (Agro infiltration)	Avian influenza virus H5/HA1 variant	Avian flu	H5/HA1 variant-HDEL	Spitsin et al. (2009)
Tobacco/leaf	pLKT60 (Agro infiltration)	Shipping fever	Bovine pneumonic pasteurellosis	<i>Manheimia haemolytica</i> A1 leukotoxin 50 fusion protein	Lee et al. (2001)

The recombinant virus accumulated high level of the antigenic protein in the infected leaves of tobacco plant. It was observed that mice immunized with the recombinant virus produced antibodies against ZP3. Therefore, foreign antigens expressing on the surface of virus like particles could be isolated from plant tissues, purified partially and then used for immunogenicity experiments. The antigenic epitopes from several human and animal pathogens have been produced in

plants using coat proteins from plant viruses as carriers. However, the limitation of this system is that it is applicable only to virus susceptible plant varieties.

In virus recalcitrant plant species, *A. tumefaciens* can be used for achieving transient expression as another approach to quick and inexpensive large-scale production of recombinant proteins in plant systems (Table 4). Foreign gene expression occurs during several days

after agro infiltration without integration of recombinant DNA into plant genome. This system was first introduced in tobacco but can apply to any leafy species and has been developed commercially in alfalfa by the Canadian Biotechnology Company, Medicago Inc., Quebec, Canada (Yusibov and Rabindran, 2008). A novel system of agro infiltration known as Magniffection (Gleba et al., 2005), which combines the use of plant viruses and *Agrobacterium* binary plasmids, has been used to express plague antigens in plants (Santi et al., 2006). The use of F1 and V antigens and the derived protein fusion F1-V produced by transient expression in *N. benthamiana* using a deconstructed tobacco mosaic virus-based system has shown great potential as a protective anti-plague vaccine in animal studies. The plant-derived purified antigens, administered subcutaneously to guinea pigs, generated systemic immune responses and provided protection against an aerosol challenge with virulent *Y. pestis* (Santi et al., 2006). Using a similar transient system, Mett et al. (2007) expressed the antigens F1 and V as fusions with lichenase. When administered to *Cynomolgus Macaques*, the purified plant-produced antigens stimulated strong immune responses and provided complete protection against lethal challenge with *Y. pestis* (Mett et al., 2007). Chichester et al. (2007) described a candidate subunit vaccine against *B. anthracis* consisting of domain 4 of antigen (PAD4) and domain 1 of LF (LFD1). Each domain was fused to lichenase, a thermostable enzyme from *Clostridium thermocellum*, and transiently expressed in *Nicotiana benthamiana*. Immunization of mice with this candidate vaccine resulted in high titers of lethal toxin neutralizing antibodies. For protection against poxvirus infections, Giulini et al. (2007) produced A27L, A33R, L1R and B5R immunogenic proteins through transient expression in tobacco protoplasts. The extracellular virion membrane protein B5 was transiently expressed using the “magniffection” procedure. The authors analyzed the impact of purity of plant-based B5 subunit vaccine preparation on specific antibody responses in mice, and demonstrated the efficiency of mucosal administration of plant-derived smallpox vaccine in obtaining a potent immune response (Portocarrero et al., 2008). Tuberculosis (TB) antigen ESAT6 was transiently expressed in *N. benthamiana* leaves for the creation of prophylactic and therapeutic vaccine against TB through PVX based agro infiltration (Zeladaa et al., 2006). Other antigens (Ag85B, ESAT6, and ESAT6:Ag85B fusion) have been expressed using agroinjection method (Dorokhov et al., 2007). The level of Ag85B antigen accumulation was reported at about 800 mg/kg of fresh leaves. Deletion of transmembrane domains from Ag85B caused a dramatic increase in its intracellular stability.

## 7. Clinical trials

Plant-derived vaccine antigens generate target-specific systemic and mucosal immune responses. Several plant-derived vaccine antigens show efficacy not only in mouse model, but also in higher animals such as ferrets or non-human primates (Yusibov and Rabindran, 2008). To date, the results of at least eleven clinical trials involving oral delivery of plant-derived vaccine antigens have been published and several are ready to get clearance for phase II trials (Basaran and Rodriguez-Cerezo, 2008; Yusibov and Rabindran, 2008). Plant-derived vaccine antigens for diarrhea, gastroenteritis, swine gastroenteritis, traveler's disease, hepatitis B and rabies are presently in phase I clinical trials. The data obtained from phase I trials conclude that plant-derived vaccine antigens are safe and induce sufficiently high immune response in healthy subjects.

The first phase I human clinical trial approved by US Food and Drug Administration was performed in 1997 on transgenic plant-derived LTB antigen. Human volunteers fed on potato tubers (Arizona State University, USA) or corn seeds (Prodigene, USA) genetically engineered against diarrhea-causing *E. coli*, showed the appearance of anti-LTB antibodies in both mucus and serum (Tacket et al., 1998; Tacket, 2005). Transgenic potato tubers carrying a gene for Norwalk virus capsid

protein (NVCP) have undergone phase I clinical trials. The human volunteers fed on potato-derived vaccine antigen at Boyce Thompson Institute for Plant Research, USA were found to developed anti-NVCP antibodies in serum (IgG, IgM) and stool (IgA) (Tacket et al., 2000; Tacket, 2005). There were two different reports of clinical trials of HBsAg antigen expressed in potato (Arizona State University, USA and Roswell Park Cancer Institute, USA) and lettuce (Thomas Jefferson University). The phase I clinical trial examined oral delivery of raw potato to volunteers that had previously been primed by injection with a licensed hepatitis B subunit vaccine. The potato HBsAg vaccine antigen was reported to boost anti-HBsAg antibodies in serum (Thanavala et al., 2005). A small scale clinical trial with transgenic lettuce leaves expressing HBsAg antigen has also been conducted (Kapusta et al., 1999). Two of the three volunteers who ingested transgenic lettuce leaves triggered specific serum-IgG response at levels considered to be protective. A rabies vaccine antigen made in spinach by using plant viral vectors is also in phase I trials (Thomas Jefferson University, USA). Antigenic peptides of the anti-rabies glycoprotein and nucleoprotein fused to AIMV coat protein expressed in spinach leaves were given to two groups of human volunteers (Yusibov et al., 2002). One group consisted individuals who had previously been vaccinated with licensed rabies vaccine and the second composed of rabies-naïve individuals. The significant elevations of rabies-specific serum IgG levels were observed in three of five previously immunized volunteers. In the naïve individuals belong to non-vaccinated group, low but significant elevations in rabies-specific serum IgG levels were seen in five of the nine volunteers. Increased serum rabies-specific IgA antibodies were also detected in three of the five individuals. The HBsAg expressed in transgenic potato at Arizona State University, USA and the Non-Hodgkin's lymphoma antigen expressed in tobacco at Large Scale Biotechnology, USA have been submitted for phase II clinical trials (Basaran and Rodriguez-Cerezo, 2008).

ProdiGene (USA) has conducted clinical trials on pigs using corn-derived edible vaccine antigen for transmissible gastroenteritis virus (Lamphear et al., 2004). They found neutralizing antibodies in piglets. This vaccine antigen was found to be effective in boosting lactogenic immunity. Dow Agro, USA based company received the first regulatory approval from U.S. Agriculture Department's Center for Veterinary Biologics, and also met the requirements of FDA for a plant cell culture-derived veterinary vaccine antigen that protects poultry from Newcastle disease ([www.thepoultrysite.com](http://www.thepoultrysite.com)).

A few plant-derived pharmaceutical proteins for therapeutic applications have also reached phase II clinical trials. The CaroRX antibody expressed in transgenic tobacco meant to give protection against dental caries has been submitted for phase II clinical trials by Planet Biotechnology Company. Another antibody Avicidin expressed in transgenic corn (Monsanto Protein Technology, USA) for protection against colorectal cancer had to be withdrawn from phase II trial stage due to non specific effects of the antibody. Gastric lipase, an enzyme produced from transgenic corn and used to treat cystic fibrosis, is in phase II clinical trials (Meristem Therapeutics, France). Human intrinsic factor, to be used against vitamin B12 deficiency and produced in transgenic *A. thaliana* plants has been taken up for phase II clinical trials by Cobento Biotechnology, Denmark.

Eleven plant-derived non-pharmaceutical proteins (avidin, trypsin,  $\beta$ -glucuronidase, aprotinin, lactoferrin, lysozyme, thyroid-stimulating hormone receptor, Hantaan and Puumala viral antigens, peroxidase, laccase and cellulase) have been developed and marketed (Basaran and Rodriguez-Cerezo, 2008). In 2004, the first plant-derived recombinant protein product (bovine-sequence trypsin; trade-name—TrypZean) developed in corn plant (ProdiGene, USA) was commercialized. The current status of plant-derived protein products suggests huge potential of transgenic plant technology in diagnostic and therapeutic industry. These are early indications that plant-derived antigens/antibodies will become an essential component in disease-prevention arsenal in near future.

## 8. Conclusions and future prospects

With the world's population at over 6.4 billion (<http://esa.un.org>), majority of the poor need affordable technological solutions to health. Protection from viral infections is currently the most difficult area to address through drug development. Progress in plant genetic engineering has opened novel opportunities to use plants as bioreactors for safe and cost effective production of vaccine antigens. As is clear from several examples cited in this review, the production of recombinant proteins in plant systems has a great potential. Recent developments in this area have significantly increased its utility and enabled various groups to explore the possibility of producing vaccine antigens from a variety of plants, which can be directly or indirectly used to develop commercial processes. Transgenic plants that can produce biologically active proteins or subunit oral vaccines and antibodies have been developed, though the applications of these technologies are at least a decade away. There are several technical and logistic problems which need to be addressed before plant-derived edible vaccine becomes a reality. Some of these are as follows.

- (a) Most inserted genes are expressed at very low level in plants. The development of efficient promoters that target the expression of therapeutic proteins in plant tissues is required. The selection of suitable plant tissue specific promoters, signal sequences, ER retention sequences, plant preferred codon usage, protein stabilization, post translational modification etc. will lead to increasing the accumulation of transproteins in plant tissues.
- (b) Transgenic fruits or leaves stored under ambient conditions may require immediate extraction and purification of the antigens. On the other hand, the expression in seeds allows storage at room temperature for a year and longer. Thus, seed based approaches need to be emphasized to rid of the dependence on cold chain.
- (c) Enhancing the stability of vaccine antigens expressed in plants can give a distinct advantage in utility of such plant based approaches. However, stable expression of the recombinant proteins needs to be tightly regulated to avoid passage of transgene into unwanted plants or consumer food stocks. Besides physical containment of transgenic crops, the chloroplast based genetic transformation and the use of vegetatively propagated varieties or triploid hybrids provide simpler natural containment strategies for grain and fruit-producing crops. Recently Liu et al. (2008) developed a novel chimeric *fpSC* promoter for complete containment of pollen- and seed-mediated gene flow. This has application in transgene containment of non-fruit and non-grain crops.
- (d) Development of tolerance to the antigen consumed orally as a result of suppression of systemic immunity is an area of concern. There is need to identify useful adjuvants that could enhance oral immunogenicity and develop formulations to improve stability and rapid absorption through mucosal tissue.
- (e) Standardization of dosage in case of edible vaccine is a major problem as plant to plant and generation to generation expression of the vaccine antigen may vary. The expression also depends on where and when the plants are grown. Therefore, a delivery scheme needs to be developed to ensure the delivery of required dosage level. Collaborative research between plant and medical scientists may help in resolving these and related issues. The plant-based edible vaccine technology might first be targeted to wild life and animals to gain experience. In fact, such an approach may benefit agriculture as billions of dollars are spent presently on vaccinating farm animals and poultry.
- (f) Much effort needs to be devoted towards examining issues related to commercialization of the concept of edible vaccines.

Strategies to increase yield potential of the concerned protein, glycosylation pattern in comparison to the mammalian protein, pharmacological efficacy and formulation development deserve high priority.

- (g) The development of stable transgenic plants may also be avoidable by adopting transient expression, plant cell culture and hairy root culture systems. The root exudates are devoid of pigments and tannins. The endoplasmic reticulum signal may be used for secretion of the biologically active proteins in medium. Such preparations are free of pathogenic contamination and can be used through oral route. These strategies are more conveniently amenable to GLP and GMP protocols.
- (h) Issues relating to the ethical, social, biosafety and environmental impact which directly/indirectly effect the deployment of genetically modified crops (de Melo-Martin and Meghani, 2008) are a concern. However, these will be met in course of time, through enhancing the awareness of merits and the development of regulatory and standard operating procedures.

The need to establish safety, efficacy and functional equivalence of the vaccine antigens should guide future development and research in plant based preventive and therapeutic technologies. Though several examples establishing the proof of concept have become available, development of plant-based edible vaccines has a long way to traverse, before it becomes successful like the oral polio vaccine. The present opportunity to develop vaccine antigens using plants as expression systems represents an example wherein, in spite of a major societal need, the progress in the development of edible or oral vaccines has been rather slow. This is because of inadequate networking among plant scientists, immunologists and experts in vaccine research. It requires more innovative thinking for the approach to work and needs larger investments. The public and non-profit organizations need to give leadership in form of greater investments to unlock the potential of plant-derived vaccines.

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