

Review

Utilizing Transgenic Plants as Bioproduction Systems for Vaccines

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Edible vaccine technology represents an alternative to fermentation based vaccine production system. Transgenic plants are used for the production of plant derived specific vaccines with native immunogenic properties stimulating both humoral and mucosal immune responses. Keeping in view the practical need of new technology for production and delivery of inexpensive vaccines, especially in developing world, plant derived edible vaccines is the best option in hand to combat infectious diseases. Plant derived vaccine is easy to administer, cost effective, readily acceptable, have increased safety, stability, versatility and efficacy. Several plant derived vaccines are under research, some are under clinical trials for commercial use. Like most biotechnology products, the IP situation for edible vaccines is complex as IP rights influence every stage of vaccine development.

Keywords: Transgenic plants, edible vaccines, chimeric viruses, bacterial diseases, viral diseases.

INTRODUCTION

Transgenic plants are the plants in which foreign genes of desired characters have to be inserted. Transgenic plant have been found to have many advantages like, development of high yielding varieties of crop plants and disease resistant, and are plants with improved tolerance to biotic and abiotic stress (Ahmad et al., 2008; 2010a; b; 2011; Ahmad and Umar, 2011; Ahmad and Prasad,

2012a; b; Sarwat et al., 2012). Apart from the above, transgenic plants have been employed for the production of vaccines for the treatment of various infectious diseases (Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011; Twyman et al., 2012). Infectious diseases are major cause of mortality and morbidity worldwide (Goldblatt and Ramsay, 2003)

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Abbreviations: IP, Intellectual Property; HBsAg, hepatitis B surface antigen; HIVgag, HIV Gag protein ; LT-B, heat labile enterotoxin B subunit; CT-B, cholera toxin B subunit; ETEC, entero toxigenic Escherichia coli; M cells, microfold cells; CaMV, Cauliflower mosaic virus; CpMV, Cow pea mosaic virus; TMV, Tobacco mosaic virus; CTB, Eholera toxin B subunit; PA, protective antigen; LF, pethal factor; HBV, hepatitis B virus; JEV, Japanese encephalitis virus

and one-third of the deaths are caused by the infectious agents. Vaccine is an immuno-biological substance, used for specific protection against both infectious and non-infectious diseases (reviewed by Ahmad et al., 2012; Twyman et al., 2012). Vaccine is responsible for the stimulation of protective antibody and other immune mechanisms. The vaccines can be made from live or killed inactivated organisms, extracted cellular fractions, toxoid or combination of these. Recent preparations are sub-unit vaccines and recombinant vaccines. The main limitation with vaccines is their dependence on cold chain system, which is used to store and transport the vaccine under strict controlled conditions (Park, 2005). Other limitations are risk of adverse reactions such as reactions inherent to inoculation, reactions due to faulty techniques etc (Goldblatt and Ramsay, 2003). Thus, for the implementation of a successful global vaccination strategy, a well designed subunit oral vaccine system should satisfy the following criteria (Chargelegue et al., 2005; Levine et al., 2006; Nochi et al., 2007): (a) Produce sufficient quantities of desired antigen; (b) preserve the expressed antigen for a long time at room temperature; (c) induce protective immunity; (d) be protected from enzymatic digestion in the gastrointestinal tract.

Therefore, in the 1990s, an International campaign was initiated to immunize all the world's children against six devastating diseases. The target was to reach 80% of infants and reduce the annual death toll from these infections by roughly three million. Still, 20% of infants are un-immunized by six vaccines against polio, measles, diphtheria, pertusis, tetanus and tuberculosis. In many developing countries, millions of children still die from infectious diseases due to immunizations being non-existent, unreliable or too costly (Ramsay et al., 1999). None will be entirely safe until every child has routine access to vaccines. Hence, there is an urgent need to search for vaccines which are easy to administer, easy to store, cost effective, easy to transport and possess readily acceptable delivery system. Hence, there is a lot of scope in developing plant derived vaccine (Streatfield et al., 2001; Ahmad et al., 2012). Now the question arises what is plant derived vaccine? Advances in transgenic research have made use of crop plants to serve as bioreactor for the production of recombinant molecules (Raskin et al., 2002; Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011). This means that transgenic plants are used to express antigen proteins induced by plant transgenic vectors and to produce certain special vaccines with high anti-disease ability (reviewed by Mei et al., 2006; Malabadi et al., 2012) (Figure 1). Plant derived vaccines significantly increase availability of vaccines in places where maintenance of cold chain system is difficult (Webster et al., 2002; Kant et al., 2011; Vianna et al., 2011; Yoshida et al., 2011; Sharma and Sood, 2011; Twyman et al., 2012). Important examples on the development of plant bio-reactors are shown in Table 1.

The immunogenicity and safety of plant derived vaccines was declared in phase I clinical studies (Tacket, 2009). During the last decade, different types of efficient plant-based expression systems have been studied and more than 100 different types of recombinant proteins including plant-derived vaccine antigens have been successfully expressed in different types of plant tissues (Tiwari et al., 2009; Rybicki, 2010; reviewed by Ahmad et al., 2012). Positive effects of edible vaccines include decrease in potential hazards such as toxic compounds, responses to allergy and risk of attenuated strains reverting to pathogenic strains associated with established production technologies that use bacteria, yeast and mammalian cells (Pelosi et al., 2012).

TRANSGENIC PLANTS FOR THE PRODUCTION OF PLANT DERIVED VACCINES

Through recombinant DNA technology, different level of antigen expression for each independent line has been observed in plants (Karg and Kallio, 2009; Shih and Doran, 2009; Wilken and Nikolov, 2012). In 1990 first edible vaccine, surface protein A from *Streptococcus mutans* was expressed in tobacco (Curtis and Cardineray, 1990). Plant derived vaccine in the form of seed or fruit can be easily stored and transported from one place to another without the worry of its degradation or damage. A large amount of plant derived vaccine can be easily produced by cultivation in fields with relatively few inputs. Autoimmune disorders like Type I diabetes, multiple sclerosis, rheumatoid arthritis etc., can also be suppressed by using plant derived vaccines (Prakash, 1996).

Plants are selected which expresses highest level of antigen and least number of adverse effects. Till date various types of antigens are successfully expressed in different plants (Mason and Arntzen, 1995; reviewed by Ahmad et al., 2012). With the development of plant genetic engineering, the expression system for transgenic plants are no longer limited to model plants, but extended to some orally or high protein content plants. Various plant platforms have been demonstrated for production of recombinant proteins in plants, including leafy crops, cereals and legume seeds, oil seeds, fruits, vegetables, higher plant tissue and cell cultures, hydroponic systems, algae and halobios (reviewed by Mei et al., 2006). Co-expression of adjuvant along with antigen has also been done in the same plant (Lal et al., 2007). The use of rice storage protein gene promoters to express transgenes in rice grain is well documented (Nicholson et al., 2005).

Furtado et al. (2008) compared use of storage protein gene promoter and non-storage gene promoter with regard to spatial and temporal control of expression from barely, wheat and rice. Storage protein promoter from barley and wheat directed the expression in endosperm but not in embryo; expression was leaky, as it was observed in seed maternal tissues, leaf and root tissues;

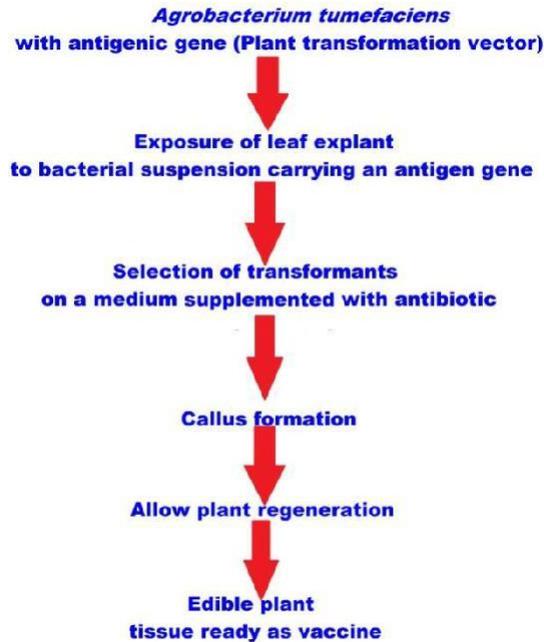


Figure 1. Strategy for the production of candidate vaccine antigen in plants

Table 1. Representative plant-based vaccines: under clinical development or in market.

S/N	Product	Plant Host	Expression system	Indication	Route of administration	Product stage of development	Reference
1	<i>E. coli</i> LT-B	Potato	Transgenic	Diarrhea	Oral	Phase 1	Tacket et al. (2007)
		Maize kernels	Transgenic			Phase 1	Tacket et al. (2009) Chikwamba et al. (2003)
2	Norwalk virus	Potato Tobacco	Transgenic	Diarrhea	Oral	N A	Tacket et al. (2000) Santi et al. (2008) Zhong et al. (2005)
		Tobacco (VLP's) Tomato fruit (Capsid protein)					
3	HBsAg IgG (hepatitis B virus)	Potato Banana Tobacco	Transgenic	Hepatitis B	Oral	Phase 1	Kong et al. (2001) Kumar et al. (2005) Kostrzak et al. (2009) Gao et al. (2003); Valdes et al. (2003)
		Cherry, tomato Tobacco					
4	Rabies virus GP/NP	Spinach	Transient (viral vector)	Rabies	Oral	Phase 1	Modelska et al. (1998) Roy et al. (2010)
		Tobacco					
5	Newcastle disease virus HN	Tobacco Cell Suspension	Transgenic	Newcastle disease	Subcutaneous	USDA approved (not marketed)	Yusibov et al. (2011) Gómez et al. (2008)
		Potato					
6	Personalized anti-idiotype scFVs	<i>Nicotiana benthamiana</i>	Transient (viral vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase 1	Yusibov et al. (2011)

Table 1. Contd.

7	Personalized anti-idiotype dcFVs	<i>Nicotiana benthamiana</i>	Transient (magnICON vectors)	Non-Hodgkin's lymphoma	Subcutaneous	Phase (ongoing)	1	Yusibov et al. (2011)
8	H5N1 influenza HA VLP	<i>Nicotiana benthamiana</i>	Transient (agrobacterial binary vector)	H5N1 "avian" influenza	Intramuscular	Phase (ongoing) Phase (Health Canada approved)	1 2	Yusibov et al. (2011)
9	H5N1 influenza HA1	<i>Nicotiana benthamiana</i>	Transient (launch vector)	H5N1 "avian" influenza	Intramuscular	Phase 1		Yusibov et al. (2011)
10	H1N1 influenza HAC1	<i>Nicotiana benthamiana</i>	Transient (launch vector)	H1N1 "swine" influenza	Intramuscular	Phase (ongoing)	1	Yusibov et al. (2011)
11	L1 capsid protein	Potato	Transient	Human Papilloma virus	Subcutaneous	NA		Biemelt et al. (2003)
		Tobacco	Transient (biolistic delivery system)		Oral Subcutaneous Intramuscular	NA		Warzecha et al. (2003) Kohl et al. (2006)
12	Protective antigen (PA)	Tobacco	Transient	Anthrax	Subcutaneous	NA		Aziz et al. (2002) Koya et al. (2005)
		Tobacco	Transgenic (Microprojectiles)		Subcutaneous	NA		Watson et al. (2004)
13	S protein	Tomato Tobacco	Transient Transient	SARS	Oral Oral	NA		Pogrebnyak et al. (2005)
14	MV-H protein	Tobacco	Transient	Measles Virus	Oral	NA		Webster et al. (2002)
		Lettuce	NA NA		Intraperitoneal intranasal	NA NA		Webster et al. (2005) Webster et al. (2005)
15	Spike protein	Maize	NA	Swine transmissible gastroenteritis virus	Oral	NA		Lamphear et al. (2004)
16	D2 peptide of fibronectin-binding protein (FnBP)	Cowpea	Transient	<i>Staphylococcus aureus</i>	Intranasal oral	NA		Brennan et al. (1999)
17	Intimin protein	Tobacco	Transient	<i>E. coli</i> 0157:H7	Oral	NA		Judge et al. (2004)
18	FaeG of K88 fimbrial antigen	Tobacco	Transient	Enterotoxigenic <i>E. coli</i> (Strain K88)	Intraperitoneal	NA		Huang et al. (2003)
19	Cry jI, Cry jII	Rice	NA	Japanese Cedar pollen allergens	Oral	NA		Takagi et al. (2005)
20	VP1	Alfalfa	NA	Foot and Mouth Disease Virus	Parenterally oral	NA		Wigdorovitz et al. (1999)
		Tobacco chloroplasts						Li et al. (2006)

Table 1. Contd.

21	F protein	Tomato		NA	Respirator y Syncytial Virus	Oral		NA	Sandhu et al. (2000)
22	SSA	Narrow Lupin	Leaf	NA	Sunflower seed albumin	oral		NA	Smart et al. (2003)
23	B5	Tobacco		NA	Influenza Virus	NA		NA	Shoji et al. (2008)
24	F1-V fusion protein	Tomato		Transient	Plague	Oral		NA	Alvarez et al. (2006)
25	2L2I peptide	Tobacco Chloroplast		NA	Canine Parvovirus	Parenteral route with booster	Oral	NA	Molina et al. (2005)
26	Envelope protein (E)	Tobacco Rice			Japanese encephalitis virus				Appaiahgari et al. (2009) Wang et al. (2009)
27	ESAT-6 antigen	Arabidopsis		NA	Tuberculosis	Oral		NA	Rigano et al. (2005)
28	VP6 HRV-VP7	Alfalfa Potato		NA	Rotavirus	Oral		NA	Yuan and Saif (2002) Yu-Zhang et al. (2003)

whereas, rice promoters directed the endosperm-specific expression in transgenic rice (Furtado et al., 2008). Alfalfa (*Medicago sativa*) is considered as a good bioreactor for production of recombinant proteins as it contains high levels of protein content and low levels of secondary metabolites (Dus Santos et al., 2002). Cereal crops can be the most suitable candidate and can be used to enhance the antigen concentration and help to reduce oral dose as they have ample amount of soluble protein in endosperm (Ahmad et al., 2012). Potato, tomato and carrot have been successfully reported to express vaccine candidates (Walmsley and Arntzen, 2000). Antigen genes encoding HBsAg, HIVgag and Rabies Capsid Proteins have been successfully trans-formed to tomato (Sala et al., 2003). High levels of recombinant protein expression were observed in proplastids of cultured carrot cells (Daniell et al., 2005). Oral delivery of the therapeutic proteins via edible carrot preserved the structural integrity of their target proteins as no cooking is needed (Muller et al., 2003). Other vegetable crops like lettuce (*Lactuca sativa*), celery cabbage (*Brassica rapa* var. *pekinensis*), cauliflower (*Brassica oleracea* var. *botrytis*) are under study for the production of vaccines. The only problem in these vegetables is low expression levels (Koprowski, 2005; Tacket and Mason, 1999). The earliest fruit used for the plant transgenic programme is banana (*Musa acuminata*) (Mason et al., 2002).

According to Trivedi and Nath (2004) papaya (*Carica papaya*) is another ideal plant species for vaccine production. Apart from fruit, vegetable and cereal crops scientists have used algae to produce metabolites and heterologous proteins for pharmaceuticals applications (Mayfield and Franklin, 2005). The species under study

are: *Chlamydomonas reinhardtii* (Sun et al., 2003), *Phaeodactylum tricornutum* (Zaslavskaja et al., 2000), *Amphidinium carterae*, *Symbiodinium microadriaticum* (ten Lohuis and Miller, 1998) and *Cylindrotheca fusiformis* (Fischer et al., 1999). Exciting progress has been made with the chloroplast based production of two particularly important classes of pharmaceuticals, vaccines and antibodies (Bock and Warzecha, 2010; Scotti et al., 2012). Extraordinarily high expression levels and the prospects of developing edible pharmaceuticals make transgenic chloroplasts a promising platform for the production of next-generation vaccines and antimicrobials (Waheed et al., 2012). During the past few years, several vaccine candidates have been produced successfully via plastid transformation, which emphasizes that transplastomic plants, as a second generation expression system, have great potential to fill gaps in conventional production platforms. A salient feature of plastids is that they combine characteristics of prokaryotic and eukaryotic expression systems, which is exemplified by the production of virus like particles and of bacterial antigens (reviewed by Bock and Warzecha, 2010). Successful expression of antigens in plants was carried out for *Escherichia coli*, heat labile enterotoxin B subunit (LT-B) in tobacco and potato (Hirst and Holmgren, 1987), *Rabies virus* G protein in tomato (Mc Garvery et al., 1995), *Hepatitis B virus* surface antigen in tobacco and potato (Thanavala et al., 1995), *Norwalk virus* capsid protein in tobacco and potato (Mason et al., 1996) and cholera toxin B subunit (CT-B) in potato (Arakawa, 1997).

Antigen expressed in plant or plant products can be administered orally or by intramuscular or by intravenous injection. Homogenized leaves, fruits or vegetables are used through oral route. Purified antigen containing plant

tissue can be delivered in a capsule or powder (pill) form. Capsule may be suitable because capsule coating can be modified in such a way that coating material dissolves in particular area of stomach, and vaccine can be released in a specific area of the body. Purified component can also be used by intramuscular and intravenous administration. Oral administration of plant derived vaccine induces both mucosal and systemic immunity. When antigen is administered orally, it induces more mucosal response than intramuscular or intravenous injections. So, more importance has been given to those antigens, which induce mucosal immune response to produce secretory Ig A at mucosal surfaces. Mucosal immunity is very effective in diarrhoeal diseases caused by rotavirus, *Norwalk virus*, *Vibrio cholerae*, enterotoxigenic *E. coli* (ETEC) and also in respiratory diseases such as pneumonia.

Second generation plant derived vaccines are known as multi component vaccines, provides protection against several pathogens. Both Enterotoxigenic *Escherichia coli* (ETEC) heat-labile enterotoxin (LT-B) and the capsid protein of *Norwalk virus* were successfully expressed in plants and induced immune response against both *E. coli* and *Norwalk virus* in mice (Huang et al., 2001).

ADVANTAGES OF EDIBLE VACCINES OVER INJECTED VACCINES

Edible vaccines have many advantages over the injected vaccines like:

1. Edible vaccines are cost effective, have low risk of contamination and no cost for transportation. Pharmaceutical companies spend million dollars for the production of vaccines and to preserve vaccines. Transgenic plants does not need cold chain storages.
2. Pharmaceutical companies need the hitech machines for the production of vaccines. In the case of edible vaccines production we need soil rich land instead of machines.
3. Long distance transportation is not required in the case of edible vaccines.
4. The cost of materials needed for field grown plants is lower compared to cell culture grown in bioreactors (Xu et al., 2011).
5. Edible vaccines have a low cost for medical equipment as well, because needles and syringes are not needed for delivery (Streatfield, 2006; Xu et al., 2011).
6. Medical professionals are not needed for oral delivery (Streatfield, 2006).
7. Transgenic plants have low contamination risks as compared to injected vaccines
8. Needles and syringes are responsible for spreading of second hand diseases (Nochi et al., 2007).
9. Oral delivery has efficiency to provoke a mucosal immune response, which produces cell mediated

responses (Streatfield, 2006).

Edible vaccines have multi-component ability that is possible due to the crossing of 2 plant lines (Lal et al., 2007). These vaccines with multi-component abilities are known as second generation edible vaccines as they allow for several antigens to approach M cells (microfold cells) simultaneously (Lal et al., 2007). The multi-component edible vaccines can prevent multiple diseases for example ETEC, cholera and rotavirus (Lal et al., 2007). Injected vaccines do not have this property, so there are less effective than edible vaccines (Ramessar et al., 2008a; b; Naqvi et al., 2011).

Chimeric viruses

Over-coat and epi-coat technology is used to produce chimeric viruses. Over-coat technology provides expression of entire protein, whereas epi-coat technology permits the plant to produce only the foreign proteins (<http://www.geocities.com/plantvaccines/transgenicplants.html>). Plant viruses redesigned to carry the desired genes and used to infect differently in different parts of the plant. *Alfalfa mosaic virus*, CaMV (*Cauliflower mosaic virus*), CpMV (*Cow pea mosaic virus*), TMV (*Tobacco mosaic virus*), *Tomato bushy stunt virus* and *Potato virus* are redesigned to express fragments of antigens on their surface. There are reports that they produce plant based chimeric virus such as foot and mouth disease virus; mint enteritis virus. Fragment of gp41 surface protein of HIV virus put into CpMV could evoke a strong neutralizing antibody response in mice (Moffat, 1995; Wang et al., 2012).

APPROACHES TO PRODUCE PLANT DERIVED VACCINES

Plants serve as an important source to produce cost-effective vaccine derivatives. Plant based production of vaccine candidates can help to reduce the economic burden on the developing countries and can be made easily available to every individual. Various models to produce vaccine candidates are described below.

Bacterial

Enterotoxigenic Escherichia coli (ETEC)

Enterotoxigenic *Escherichia coli* strains are a major cause of enteric diseases in live stock and humans. ETEC is attached to specific receptors on the surface of enterocytes in the intestinal lumen by fimbriae. ETEC produces a heat-stable enterotoxin (ST) which consists of five B subunits and one A subunit. B subunit binds to sugar residues of ganglioside Gm1 on the cells lining the

villi and crypts of the small intestine. Insertion of the B subunit into the host cell membrane forms a hydrophilic transmembrane channel through which the toxic A subunit can pass into the cytoplasm (Roy et al., 2010). Raw transgenic potato expressing LT-B were fed to 11 volunteers, out of which 10(91%) developed neutralizing antibodies and 6(55%) of individuals also showed mucosal response (Tacket et al., 1998). Different reports are there on synthetic heat-labile enterotoxin (LT-B) gene and their expression in plants such as potato, banana, tobacco and tomato; and all were tested in mice (Mason et al., 1998). Expression of *E. coli* fimbrial subunit protein in transgenic plants can be used to vaccinate against these diseases. Joensuu et al. (2006) evaluated transgenic plants to produce Fae G protein and adhesion of F4 fimbriae. Oakes et al. (2007) reported the edible transgenic soybean plant producing *E. coli* fimbrial subunit proteins. Tacket (2009) discussed early human studies of oral transgenic plant-derived vaccines against enterotoxigenic *Escherichia coli*. Genetic combination of gene coding for an LTB:ST protein in tobacco by *Agrobacterium* mediated transformation displays antigenic determinants from both LTB and ST. Presence of mucosal and systemic humoral responses in mice when dosed orally with transgenic tobacco leaves also confirmed that plant-derived LTB:ST can lead to immunogenicity development via oral route (Rosales-Mendoza et al., 2011).

Vibrio cholera

Cholera is due to contaminated food or water which triggers an acute intestinal infection by *V. cholera* (López-Gigosos et al., 2011). Enterotoxin such as cholera toxin (CT) was expressed in tobacco plant (Arakawa et al., 1998). Nochi et al. (2007), showed oral immunization with transgenic rice encoding the cholera toxin B subunit (CTB) which stimulates secretory Ig A, shows resistant to gastrointestinal digestion. Karaman et al. (2012) introduced synthetic gene encoding for CT-B by the control of a γ -zein promoter in maize seeds. CT-B levels were checked via ganglioside dependent ELISA. Anti-CTB IgG and anti-CTB IgA were found in the sera and fecal samples of the orally immunized mice protected against holotoxin challenge with CT.

Anthrax

Anthrax is a disease most commonly occur by inoculation of *B. anthracis* through the skin of infected animals, their products and inhalation of spores in dust or wool fibers. Virulence factors is a toxin complex, which consists of three proteins. The protective antigen (PA) binds the complex receptors on the macrophage surface. After proteolysis, oedema factor and lethal factor are released

which after endocytosis, blocks the adenyl cyclase pathway within the cell. The main effect of this toxin complex is to increase vascular permeability, which leads to a shock. Protective antigen was expressed in transgenic tobacco chloroplasts by inserting the pag A gene into the chloroplast genome. Cytotoxicity measurements in macrophage lysis assays showed that chloroplast-derived PA was equal in potency to PA produced in *B. anthracis*. Chloroplast-derived protective antigen provides cleaner and safer anthrax plant-derived-vaccine at a lower production cost (Koya et al., 2005). Koya et al. (2005) published for the first time the PA expression in plants from stable nuclear-transgenic tobacco. Aziz et al. (2002) also reported the expression of PA in leaves of stable nuclear-transgenic tomato plants. Expression of PA in tobacco or tomato was enhanced in combination with a second *B. anthracis* protein, lethal factor (LF), and showing cytolytic activity when applied to macrophage-like cell lines. Also, when tomato leaf material was injected into mice, antisera could be recovered with neutralizing activity to anthrax lethal toxin (LT), which is a combination of PA and LF.

Porphyromonas gingivalis

Periodontal diseases are caused by oral anaerobic bacterium *Porphyromonas gingivalis*. It is thought to be initiated by the binding of *P. gingivalis* fimbrial protein to saliva coated oral surfaces. Shin et al. (2009) has successfully transferred FIM A protein producing gene into potato tuber tissues and produced native FIM A protein in edible plant cells.

Viral

Norwalk virus

Calci viruses are a major cause of food and water associated outbreaks of diarrhoea and vomiting, affecting individuals of all age groups. A capsid protein of *Norwalk virus* was expressed in transgenic tobacco and potato plants. Potato tubers expressing *Norwalk virus* antigen were fed to mice, it developed serum IgG specific for *Norwalk virus* (Mason et al., 1996). According to Tacket et al. (2000) volunteers fed with transgenic potato expressing *Norwalk virus* antigen showed seroconversion.

Hepatitis B virus

It is estimated that 3 to 6% of the world population has been infected with Hepatitis B virus (HBV) and there are 300 to 400 million carriers in the world. India alone has over 40 million carriers. In the acute stage there are signs of inflammation in the portal triads: the infiltrate is mainly lymphocytic. In the liver parenchyma, single cells show

ballooning and form acidophilic (councilman) bodies as they die. In chronic hepatitis, damage extends out from the portal tracts, giving the piecemeal necrosis appearance. Some lobular inflammation is also seen. As the disease progresses fibrosis develops and eventually, cirrhosis. Hepatitis B virus replicates in the hepatocytes, reflected in the detection of viral DNA and HBs Ag in the nucleus and HBs Ag in the cytoplasm and at the hepatocytomembrane (Simmonds and Peutherer, 2003). Hepatitis B virus is carried in the blood and blood derived bodily fluids of infected persons and can be transferred through contact with a carrier's blood caused by unsafe injections or transfusions, sexual contact and tattooing. Long term protection against Hepatitis B virus is possible with vaccine. HBs Ag was expressed in transgenic potato plant and tested in mice for production of antibodies (Richter et al., 2000).

Pniewski et al. (2011) has shown the production of small surface antigen for HBV (S-HBsAg) in genetically modified glufosinate-resistant lettuce. They orally immunized mice by using lyophilized form of plant material and showed the presence of secretory IgA (S-IgA) and total serum antibodies. Li et al. (2011) also demonstrated the transformation of HBsAg (hepatitis B surface antigen) gene in to tomato mediated by *Agrobacterium tumefaciens*.

Lou (2007) has experimentally expressed hepatitis B virus large surface antigen in transgenic tomato plant. Transgenic lettuce plant carrying recombinant hepatitis B virus antigen HBs Ag was demonstrated in Brazil (Marcondes and Hansen, 2008). Tacket (2009) has discussed early human studies of oral transgenic plant-derived vaccines against hepatitis B virus. A phase I clinical trial with plant derived hepatitis B vaccine has boosted antigen-specific serum antibodies titer (Tacket, 2009).

Measles

Millions of people live in areas where measles are endemic and resources are scarce. Measles are transmitted from person to person by respiratory droplets. Measles is an acute febrile illness, the onset is flu-like with high fever, cough and conjunctivitis, red spots with a bluish-white centre on the buccal mucosa called Koplik's spots. Measles antigens expressed in plants have been shown to be antigenic and immunogenic both after invasive and oral vaccination (Marcondes and Hansen, 2008). Crude *Quillaja* saponin extracts stimulates measles' virus specific immune responses in mice, following oral immunization with plant based measles virus haemagglutinin protein (Pickering et al., 2006).

Webster et al. (2002) confirmed that the transgenic tobacco plants-derived MV-H protein vaccine, which when, modified to MV-H DNA vaccine, to prime-boost vaccination strategy demonstrated the MV hemagglutinin protein (MV-H) expression. Orally immunized mice with plant-

derived MV-H showed MV-specific IgG.

Japanese encephalitis

JE virus is a single stranded positive sense RNA virus belonging to family flaviviridae transmitted through a zoonotic cycle between mosquitoes, pigs and water birds. It causes encephalitis all over the world especially in Eastern and South-eastern Asia. JE affects some primary organs like thalamus, corpus striatum, brainstem and spinal cord. With the absence of specific antiviral therapy, it is managed mainly by its symptom and by supportive therapies along with preventive measurements (Misra and Kalita, 2010). Transgenic rice expressing the envelope protein of *Japanese encephalitis* virus (JEV), under control of a dual cauliflower mosaic virus (CaMV 35s) promoter, was generated. JEV specific neutralizing antibody was detected in mice after immunization of mice with protein extracts from transgenic rice plant by intraperitoneal or oral immunization (Wang et al., 2009). Appaiahgari et al. (2009) showed the expression of Japanese encephalitis viral envelope protein (E) in transgenic tobacco can produce immunogenic response in mammalian system.

Influenza virus H5N1

Shoji et al. (2009) described the production of hemagglutinin from A/Indonesia/05/05 strain of H5N1 influenza virus by transient expression in plants. The results indicate that immunization of ferrets with plant-derived hemagglutinin elicited serum hemagglutinin-inhibiting antibodies and protected the ferrets against challenge infection with a homologous virus. Plant derived vaccine may be the solution in the rapid, large scale production of influenza vaccine in the face of pandemic.

Kalthoff et al. (2010) showed the expression of full-length recombinant hemagglutinin (rHA0) of H5N1 in *Nicotiana benthamiana* with optimized expression levels. Their results showed to provide an immunogenic protection protect chicken against lethal challenge infection with heterologous HPAIV H5N1 of 96% homology to rHA0 by plant-expressed hemagglutinin. Jul-Larsen et al. (2012) demonstrated recombinant influenza haemagglutinin antigen (HAC1) that was derived from the 2009 pandemic H1N1 virus and expressed in tobacco plants. They showed that the tobacco derived recombinant HAC1 antigen is a promising vaccine candidate recognized by both B- and T cells.

Shoji et al. (2011) showed the advantages provided by the plant system for influenza vaccine antigen production is their independence from pathogenic viruses, and cost and time efficiency. They produced large-scale of recombinant hemagglutinin proteins from A/California/04/09 (H1N1) and A/Indonesia/05/05 (H5N1) strains of influenza virus in *N. benthamiana* plants, and

tested their immunogenicity (serum hemagglutination inhibition and virus neutralizing antibodies), and safety in animal models.

Madhun et al. (2011) produced influenza subunit antigen in transient plant expression systems as an alternative. A needle-free intranasal influenza vaccine is an attractive approach to be followed. Plant-derived influenza H5N1 (A/Anhui/1/05) antigen, alone or formulated with bis-(3', 5')-cyclic dimeric guanosine monophosphate (c-di-GMP) as adjuvant induces strong mucosal and systemic humoral immune responses. Search for safe and effective adjuvant to enhance H5N1 intranasal vaccine with extracts of mushroom mycelia was found to be good (Ichinolhe et al., 2010).

LIMITATIONS

Before the commercial production of plant derived vaccines, there is urgent need to consider the following points;

1. Searching for suitable plant which will give ideal antigen expression.
2. Identification of proper dosage (whether plant parts, plant products, pill, intramuscular or intravenous injection of purified antigen) can produce proper dose.
3. Verification of allergens in the plant and plant products.
4. Study the impact of plant derived vaccines on the environment and human health.
5. Genetically altered crops producing plant derived vaccine could get mixed with human food supply or animal feed, causing potential threat to public health.
6. Cross pollination and their problems.
7. Effects on insects and soil microbes.
8. Regulation of plant derived vaccines in the form of food, drug or agricultural product.
9. Cultivation of plant derived vaccines and their delivery in capsule or pill form.

Risks of plant derived vaccines

Plant derived vaccines pose serious risks to the public if they are not handled with care. Safety of transgenic plants includes many aspects like ecology, agronomy and molecular biology which focus on food and environmental safety (Ahmad et al., 2012). Environmental issues and biodiversity concern are raised because of the transgenic seeds or plants that escape into the wild. Moreover, plant derived vaccines cannot be distinguished from non-plant derived vaccines of the same plant. Plant derived vaccine tomato plant looks like a traditional tomato. There is always a risk of mis-administration.

Although, plant derived vaccine technology can save many lives in developing countries. At the same time, there is an urgent need to address proper commercia-

ization of plant derived vaccine technology and to prevent misuse of technology because it possesses great risk on environment and human health. Development of vaccine into a stable seed form or production in leaf is mostly favoured but its to spoilage to prevent loss/leaking out of antigen into environment is to be checked. The amount of plant which can be taken up as raw food is to be strictly monitored as over dose may cause toxic/allergic reactions. Most of the edible crops are destroyed by attack of insects and hence their effect on vaccine producing plant has to be evaluated. Even though plant derived vaccines have shown promising results but evaluation of their tolerance needs in-depth study (Ahmad et al., 2012).

CONCLUSION AND FUTURE PERSPECTIVE

Edible plant derived vaccine may lead to a future of safer and more effective immunization. They would overcome some of the difficulties associated with traditional vaccines like production, distribution and delivery and they can be incorporated in to the immunization plans. Edible vaccines have lot of advantages over injected vaccines like, well established cultivation, low cost of production, no need for "cold chain" delivery, rapid scale-up, simple distribution by seeds, ease of genetic manipulation, oral delivery and low health risks from human pathogen and toxin contamination, etc. Significant progress has been achieved in employing plants as vaccine expression system, for example vegetables, fruits, cereal crops, etc. Tobacco, tomato, maize, rice are leading production platforms for recombinant protein production. The basic advantage of using plants as vaccine production system is that plants being higher eukaryotes provide opportunities for unlimited production, the range and diversity of recombinant molecules namely peptides, polypeptides and complex multimeric proteins that cannot be made in microbial systems. Plant production system provides a wider flexibility in designing of new pharmaceutical proteins. Days are not too far when we eat delicious vegetables, fruits etc, to prevent ourselves from infectious diseases. Developing and under-developed countries will be benefited more by this edible vaccine production system because the methods in production are reasonably affordable and the vaccine products would be more openly accessible to the population.

One of the most important bottlenecks in edible vaccine technology is yield improvement, as this factor has a major impact on economic feasibility. Different strategies in hand which can lead to improved production of edible vaccines include the development of novel promoters, improvement in protein stability by protein engineering approach, targeted expression of protein of interest and last but not least the improvement in downstream process-ing. The potential concern of edible vaccine technology

is differential glycosylation of proteins in *in vitro* systems or in non-native species. Strategies should be devised to humanize the plant glycosylation machinery by inhibiting glycosylation enzymes. The use of plastids as vaccine production platform is quite promising to prevent transgene escape through pollens or seed dispersal and it needs an extensive research to improve expression levels and prevention of proteolysis in plastids.

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