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Review Article

Recent advances in plant derived vaccine antigens against human infectious diseases

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The use of plants for the production of vaccine is one of the important applications in the modern medicine. There are many advantages of using plants as the production system compared to traditional mammalian system. Many plant species have been exploited to accumulate vaccine antigens for human infectious diseases, and vaccine candidates are approaching the market. The transgenic plants are considered as cheap source and found alternative approach to fermentation for large-scale production of vaccine antigens. The autotrophic growth of plants requires only soil minerals, water, nitrogen, sunlight energy for the synthesis of vaccine antigens. Therefore, vaccine production by using plants is one of the cheap and efficient technologies. This review covers the recent advances of plant derived vaccine antigens for the prevention of human infectious diseases and focuses on the current methods.

Key words: Clinical trials, Infectious diseases, Immunization, Transgenic plants, Vaccine

The use of vaccines against many human infectious diseases is one of the direct applications of biotechnology in the modern medicine. The most cost-effective approach for prevention of many human infectious diseases is *via* vaccination. However, the high cost of vaccination makes it unaffordable for immunization of people living in developing countries (Davoodi-Semiromi *et al.* 2009, 2010; Bock and Warzecha, 2010; Gomez *et al.* 2010; Daniell *et al.* 2009; Rybicki, 2010; Malabadi *et al.* 2011). Currently, vaccine production is very expensive for many reasons, due to their complex production system, route of administration, expensive fermentation systems, prolonged purification steps, additional expenses associated with adjuvant, cold storage, transportation and sterile delivery (Davoodi-Semiromi *et al.* 2009, 2010; Rybicki, 2010; Daniell *et al.* 2009). Most vaccines in use today are based on killed or attenuated whole bacteria or viruses, which might cause a risk of infection by the microorganisms during the vaccination (Rybicki, 2010).

Exploiting plants as biological bioreactors for the production and delivery of subunit vaccines is an exciting and promising application of biotechnology (Korban, 2002; Hiatt *et al.* 1989; Hellwig *et al.* 2004; Vermij and Waltz, 2006; Malabadi, 2008; Tiwari *et al.* 2009; Rybicki, 2010; Malabadi *et al.* 2010; Malabadi *et al.* 2011). Plant derived green vaccines eliminate the expenses associated with fermenters, purification, adjuvant, cold chain logistics, storage/transportation, needle free-administration, and sterile delivery (Davoodi-Semiromi *et al*. 2009, 2010; Walmsley and Arntzen, 2003; Pascual, 2007; Malabadi, 2008; Daniell *et al*. 2009; Gomez *et al*. 2010). Safety is one of the important issue in any clinical trial, and plant derived vaccine antigens could offer higher safety because they are needle/syringe free. Alternately, green vaccines would also reduce the need for trained medical personnel to administer the vaccine (Vermij and Waltz, 2006; Lal *et al.* 2007; Pascual, 2007; Tiwari *et al.* 2009; Malabadi *et al*. 2011). Plant derived vaccine antigens are less contaminated with human and animal pathogenic microorganisms than those derived from animal cells, because plants are not hosts for human infectious agents (Giddings *et al*. 2000; Ma *et al*. 2004; Desai *et al*. 2010; Malabadi *et al.* 2010). One more advantage is that if the vaccine is delivered orally, the plant cell would protect the antigen from the acidic environment /enzymes of the stomach because of the bio-encapsulation of antigen by the plant cell walls (Daniell *et al.* 2009; Thanavala *et al.* 1995; Malabadi, 2008; Davoodi-Semiromi *et al*. 2009, 2010; Malabadi *et al.* 2011). Plant cells are able to perform complex posttranslational modification of recombinant proteins, such as glycosylation and disulfide bridging that are often essential for biological activity of many mammalian proteins, allowing for the retention of native biological activity (Lienard *et al*. 2007; Mason *et al*. 1992, 1996; Ma *et al.* 1997, 1998, 2004, 2005; Rybicki, 2010; Tremblay *et al*. 2010). Therefore, plants have been considered as an alternative production systems for subunit vaccines (Goldstein and Thomas, 2004; Haq *et al.* 1995; Malabadi, 2008; Hellwig *et al.* 2004; Chen and Liu, 2011; Phoolcharoen *et al*. 2011; Farrance *et al.* 2011; Mathew *et al*. 2011; Malabadi *et al.* 2011**)**. Plant growth requirements are very simple since plants uses light as its energy source than mammalian or insect cell culture growth, resulting in a more robust and inert system for the production of subunit vaccines in different plant systems (Trembley *et al.* 2010; Vermij and Waltz, 2006; Rybicki, 2010; Tiwari *et al*. 2009). The concept of using plants to produce vaccine antigens is 20 years old this year, and it would be used as an established technology (Rybicki, 2010). The plant- derived vaccine technology has proven its worth as a means of cheap, easily scalable production of materials (Arakawa *et al.* 1998; Ma *et al*. 1997, 1998, 2004, 2005; Tiwari *et al*. 2009; Hefferon, 2010; Daniell *et al.* 2009; Bock and Warzecha, 2010;Rybicki, 2010; Shoji *et al*. 2011; Chen and Liu, 2011; Phoolcharoen *et al*. 2011; Farrance *et al*. 2011; Penney *et al*. 2011; Mathew *et al*. 2011; Malabadi *et al*. 2011**)**. Recently a novel technique developed by Icon Genetics (Bayer Crop science, Germany), based on the Magnifection, has additional advantageous than the routine methods used for the production of subunit vaccines in plants. This system allows very fast production, high recombinant protein expression levels for example hepatitis B virus (HB core) with an accumulation level exceeding 7% of total soluble protein in tobacco (Gomez *et al*. 2010; Yusibov and Rabindran, 2008; Gleba *et al*. 2005;Huang *et al.* 2006). Therefore, plant-derived vaccines could be used as a powerful weapon to combat infectious diseases (Tacket *et al.* 1998, 2000; Sala *et al.* 2003). The reason for this is that the applications are based on established plant tissue culture protocols for many plants (Malabadi and Nataraja, 2002; Malabadi *et al.*

2004; Malabadi *et al.* 2005; Malabadi and van Staden, 2005a, 2005b, 2005c; Malabadi and Nataraja, 2006; Malabadi *et al*. 2011), gene cloning and plant transformation technology (Maliga, 2004; Daniell *et al*. 2009; Daniell *et al.* 2009; Bally *et al*. 2009; Tremblay *et al.* 2010; Malabadi and Nataraja, 2007a, 2007b; Malabadi *et al.* 2008a, 2008b, 2008c, 2008d), and that development requires relatively limited investment (Sala *et al*. 2003). Hence with the invention of modern plant genetic engineering techniques, unlimited number of transgenic plants can be produced under *in vitro* conditions. The seeds collected from these transgenic lines could be stored, and used as and when necessary for the molecular farming. Furthermore plant DNA is not known to interact with the animal DNA and plant viral recombinants do not invade mammalian cells (Sala *et al*. 2003). Among the existing plant based technologies, there is one more method, which uses the plant viruses as expression vectors (Desai *et al.* 2010). Virus infected plants have been used to produce several antigenic proteins. This method has more advantages as compared to other methods due to the rapid onset of expression, and the systemic spread of virus so that protein is produced in every cell (Desai *et al*. 2010). Plant systems also constitute a convenient oral delivery option, preventing the cost and inconvenience associated with purification and injections. Furthermore, the production of vaccine antigens in plants is economically advantageous and there is no risk of contamination by animal pathogens (Malabadi *et al*. 2011).

Immunogenicity of plant derived vaccines

Plant produced glycoproteins can lead to immunogenicity, affect its pharmacokinetic properties and certain regulatory issues (Ma *et al*. 2005; Sethurman and Stadheim, 2006; Lienard *et al.* 2007; Obembe *et al*. 2011; Desai *et al*. 2010). Recent advances made in genetic modification of glycosylating enzymes in plants have made it possible to add or remove desired sugars and make protein with desired glycosylation pattern similar to human (Desai *et al*. 2010; Malabadi *et al*. 2011). It has been suggested that plant-derived recombinant proteins or antibodies may have increased immunogenicity or allergenicity as compared to mammalian counterparts (Ma *et al*. 2005; Penney *et al*. 2011). This is well explained by the fact that a state of tolerance or energy has been gained by the daily consumption of plant glycol-proteins in our food (Ma *et al.* 2005). The antigenicity of plant glycans, or the immunogenicity of plant glycans is still a major concern in the context of plant proteins, there have been no published analyses in humans on the effect of immunizing with a mammalian protein bearing plant glycans (Ma *et al.* 2005). However, Large Scale Biology Corporation has examined 15 patients who were systemically immunized with glycosylated single chain antibodies produced in plant and did not find serious adverse reactions (Dr. L. Grill, personal communication) (Ma *et al*. 2004, 2005). In one of the human study of mucosal application of plant secretory antibody, no evidence for an immune response to the plant recombinant glycoprotein was detected after six applications of antibody (Ma *et al.* 1998; Ma *et al.* 2004; Ma *et al*. 2005; Desai *et al*. 2010). Plant glycan patterns may not represent a problem in terms of human health; they may affect conformational epitopes, or clearance of plant derived antibodies (Bakker *et al.* 2001; Ma *et al.* 2005). Comparison of plant and mammalian N-glycan biosynthesis indicates that 13 (1,4)-galactosyltransferase is the most important enzyme that is missing for conversion of typical plant N-glycans into mammalian-like N-glycans (Bakker *et al*. 2001; Ma *et al.* 2005). Expression of this key enzyme in transgenic tobacco resulted in 15% of proteins expressing terminal 13 (1, 4)-galactose residues. Back-crossing of 13 (1, 4)-

galactosyltransferase with tobacco plants expressing murine Ig heavy and light chains resulted in expression of antibody exhibiting partially galactosylated N-glycans (Bakker *et al.* 2001; Ma *et al*. 2004, Ma *et al*. 2005; Desai *et al*. 2010; Malabadi *et al*. 2011). Various methods have been adopted to modify the N-glycosylation pattern in plants. The most common approach is the fusion of the ER-retention signal KDEL and production of knock out mutant lines in *Arabidopsis* and a moss plant, *Physcomitrella patens*, which synthesize N-glycans lacking immunogenic xylose and fucose epitope (Koprevova *et al.* 2004; Kang *et al.* 2008; Obembe *et al*. 2011; Malabadi *et al*. 2011). Furthemore, synthesis of sialic acid in plant and sialyation of plant-expressed protein has also been reported (Castilho *et al*. 2010; Obembe *et al.* 2011). These studies have clearly indicated the potentialities of the plants for the production of glycosylated proteins with native biological activities for the development of vaccine antigens against human diseases. In one of the earlier studies, as reported by Palacpac and coworkers (1999), transgenic tobacco BY2 suspension-cultured cells were produced by the transfer of the full-length human galactosyl-transferase gene placed under the control of the cauliflower mosaic virus 35S promoter (Palacpac *et al*. 1999). The transformants were analyzed and confirmed that the highest level of enzymatic activity has glycans with galactose residues at the terminal non-reducing ends (Palacpac *et al*. 1999). This study has clearly reported the successful modification of the plant cell N-glycosylation pathway, which also led to the conclusion that plant glycan structures could be modified successfully (Palacpac *et al.* 1999; Hellwig *et al*. 2004; Bosch and Schots, 2010). This is the first proof of concept showing the production of a mammalian glycosyl-transferase can alter the glycosylation pathway of plant cells (Palacpac *et al*. 1999).

Current status of plant derived vaccines in pharmaceutical industry

Some of the plant derived vaccine antigens could soon reach the commercial production level in order to meet the demands of the immunization programmes because of their higher immune efficiencies not only in animal models but also in ferrets or non-human primates too (Yusibov and Rabindran, 2008; Tiwari *et al.* 2009;Gomez *et al*. 2010; Malabadi *et al.* 2011). A review article published by Tiwari and co-workers (2009) in a journal viz. *Biotechnology Advances* has highlighted the outcome and results of the most of the clinical trials of plant derived vaccines (Malabadi *et al*. 2011). The results of clinical trials of plant derived vaccine antigens have been successfully published and several are ready to get clearance for phase II trials (Yusibov and Rabindran, 2008; Tiwari *et al.* 2009; Obembe *et al.* 2011; Malabadi *et al.* 2011). The phase I clinical trials of plant derived vaccine antigens showed positive response in terms of safety, and induce sufficiently high immune response in healthy subjects (Tiwari *et al*. 2009; Obembe, 2010; Malabadi *et al*. 2011). The clinical trials of hepatitis surface B antigen expressed in potato (Arizona State University, USA and Roswell Park Cancer Institute, USA), and lettuce (Thomson Jefferson University) also confirmed the boosting of anti-HBsAg antibodies in serum (Thanavala *et al*. 2005; Tiwari *et al.* 2009; Obembe *et al*. 2011; Malabadi *et al.* 2011). The transgenic lettuce expressing HBsAg also triggered specific serum-IgG response at levels considered to be protective (Kapusta *et al*. 1999; Tiwari *et al*. 2009; Obembe, 2010). Increased serum rabies-specific IgA antibodies were detected in human volunteers against rabies vaccine antigen made in spinach by using plant viral vectors (Thomas Jefferson University, USA) (Tiwari *et al.* 2009; Obembe, 2010; Obembe *et al.* 2011). Clinical trials on pigs using corn derived

edible vaccine antigen for transmissible gastroenteritis virus by ProdiGene (USA) (Lamphear *et al*. 2004; Tiwari *et al*. 2009). They found neutralizing antibodies in piglets. This vaccine antigen was found to be effective in boosting lactogenic immunity (Tiwari *et al.* 2009; Obembe *et al*. 2011). Human intrinsic factor, to be used against vitamin B12 deficiency which was produced in transgenic *A. thaliana* has been taken up for the phase II clinical trials by Meristem Therapeutics, France (Tiwari *et al*. 2009; Obembe *et al*. 2011). The CaroRx antibody expressed in transgenic tobacco against dental caries has been submitted for phase II clinical trials by Planet Biotechnology Company (Tiwari *et al*. 2009; Obembe *et al*. 2011; Obembe, 2010). One of the US based company viz. Dow Agro Sciences LLC received regulatory approval for a plant-made vaccine from the USDA's Centre for Veternary Biologists in 2006 (Tiwari *et al.* 2009; Obembe *et al.* 2011; Malabadi *et al.* 2011). This plant culture derived veterinary vaccine antigen that protects poultry from Newcastle disease (www.thepoultrysite.com) also met the requirements of FDA (Tiwari *et al*. 2009; Obembe *et al*. 2011). The transgenic plant-derived LTB antigen was the first antigenic protein used in the phase I human clinical trials approved by FDA. During the trial, 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; Tiwari *et al.* 2009; Obembe *et al*. 2011). In phase I clinical trial, transgenic potato tubers carrying a gene for Norwalk virus capsid protein (NVCP) fed to human volunteers at Boyce Thompson Institute for Plant Research USA, and confirmed the development of anti-NVCP antibodies in serum (IgG, IgM) and stool (IgA) (Tacket *et al*. 2000; Tiwari *et al*. 2009;Obembe, 2010; Obembe *et al*. 2011; Malabadi *et al.* 2011). In another development, 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 has been submitted for phase II clinical trials (Basaran and Rodriguez-Cerezo, 2008; Tiwari *et al.* 2009; Obembe *et al*. 2011; Malabadi *et al.* 2011). FDA also approved the first plant derived injectable purified antibody fragments as a vaccine in human trials for non-Hodgkin lymphoma therapy as an efficient route of immunization (McCormick and Palmer, 2008; Tiwari *et al*. 2009; Obembe *et al*. 2011). In another development, a Canadian based company SemBioSys has completed Phase II trials of insulin produced in transgenic safflower, and have filed an Investigational New Drug Application with the FDA, and submitted a Clinical Trial Application to European authorities (SemBioSys 2009; Penney *et al*. 2011). Medicago Inc (Canada) are currently undergoing Phase II trials for their avian influenza vaccine produced transiently in tobacco, after receiving clearance from Health Canada (Medicago 2010; Penney *et al.* 2011). *Taliglucerase alfa* produced in stable carrot cell cultures is used to treat Gaucher disease. Protalix Bio-Therapeutics has just completed a Phase III trial and the product was approved by the U.S Food and Drug Administration (FDA), who have since accepted a New Drug Application and granted a Prescription Drug User Fee Act action date in early 2011 (Protalix BioTherapeutics 2010; Penny *et al.* 2011). Therefore, results of clinical trials confirmed the potentiality of transgenic plant biotechnology in diagnostic and therapeutic industry. Plant derived vaccines will likely replace the traditional vaccines in pharma-ceutical industry in the future (Tiwari *et al*. 2009; Penny *et al*. 2011; Obembe, 2010; Obembe *et al.* 2011; Malabadi *et al.* 2011).

Disadvantages of plant derived vaccine antigens

Plant derived vaccine antigens have many challenges. The major limitation of this system is the relatively low protein yields, protein degradation and incorrect post-translational modifications of protein (Daniell *et al*. 2009; Tiwari *et al.* 2009; Desai *et al.* 2010; Malabadi *et al*. 2011). Recently these issues have been addressed by improving the protein expression levels in plants by some of the important steps by using ER retention signal sequences like KDEL and HDEL, by the use of a leader sequence, choice of promoter, choice of vectors, copy number, methods of transformation, integration, stability of a transgene, transcription, role of introns, codon optimization and translation (Desai *et al.* 2010; Malabadi *et al.* 2011). Development of codon optimized gene's for plants during expression studies have improved the protein yields up to 200mg/g Fw of plant tissue. This high expression level was achieved by combination of several factors *viz*. by inserting the plant signal peptide at the N-terminal end, and C-terminal endoplasmic reticulum (ER) retention sequence for plants (Saejung *et al.* 2007). There are other efforts to avoid non-mammalian glycosylation patterns include the prevention of late glycosylation by directing proteins to the endoplasmic reticulum through addition of KDEL sequence-tags, introduction of point mutations to eliminate glycosylation completely, or the humanization of the plant glycosylation machinery (Hellwig *et al.* 2004; Malabadi *et al.* 2011). Expression of protein in ER has drastically reduced the degradation, and resulted in the higher yield of protein accumulation (Desai *et al*. 2010; Tiwari *et al*. 2009; Malabadi *et al.* 2011). Gene silencing is another major problem during the expression of vaccine antigenic proteins in transgenic plants. This is resolved by the use of *Agrobacterium*, which tends to result in fewer copies of transgenes than biolistic transformation (Malabadi and Nataraja, 2007a; Tiwari *et al*. 2009; Desai *et al*. 2010; Malabadi *et al.* 2011). Biosafety, risk assessment, and public acceptance of transgenic plants producing vaccine antigens is another issue, which have to be dealt and widely discussed by other researchers somewhere, which is beyond the scope of this review paper (Malabadi *et al.* 2011). Furthermore, cross contamination of transgenic plants with other food crops was resolved by applying the containment approached-technology, thus limiting the environmental exposure of transgenic products (Tiwari *et al*. 2009; Malabadi *et al.* 2011).

Concluding remarks

Plant derived vaccine antigens could be produced at a larger quantity and found to be properly assembled and functional (Ma *et al.* 2005). Dendritic cell targeting approach using plant derived vaccine antigen may play an important role (Malabadi *et al*. 2011). This would help in the early diagnostic tests in the patients suffering from human infectious diseases. Nevertheless, there is a major difference between plant and animal glycosylation steps in terms of complex glycans, but till today there is no report of loss of any structure or function of expressed proteins in plants (Palacpac *et al*. 1999; Ma *et al*. 2005; Bosch and Schots, 2010; Malabadi *et al.* 2011). As discussed and reviewed by many research workers that, the use of plants as a platform for the production of novel antigenic proteins has a major advantage in terms of scale up, cost effectiveness, and enormous amount of desired protein could be produced (Ma *et al*. 2005;Obregon *et al.* 2006). Further growth requirements of the plants are very simple as compared to bacteria and animal cells, which require costly chemicals, equipment and technically- trained manpower (Ma *et al.* 2005). Therefore, use of

plants is more feasible and considered as one of the potential expression system since plants avoids many of the safety and ethical issues (for example use of animals) associated with other expression systems such as contaminating mammalian viruses or prions (Ma *et al.* 2005; Malabadi *et al*. 2011). In this latter regard, it was mentioned earlier and discussed widely that, scientists in Cuba have developed the facilities for producing a monoclonal antibody at the rate of 15 g/ week (Ma *et al.* 2005). This alone will spare the use of 300,000 mice per year, and found to be very expensive. On the other hand use of plants could save lots of money and fairly invested for vaccine antigenic protein (Malabadi *et al*. 2011).

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