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Review

Transgenic plants for the production of immunogenic proteins

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Abstract: The vaccines are very effective vehicles for the prevention of various diseases. The cost of production and the need of cold-chain limit the vaccination programs especially in low-income countries. Hence, the plants offer a suitable platform to produce the immunogenic proteins with reduced cost without a requirement for cold-chain. The transgenic plants expressing antigens might be either used for the purification of the recombinant proteins or consumed as a food for the oral administration of the plant-based vaccine. A variety of plants have been used as the expression system, such as tobacco, rice, maize, carrot, soybean, potato and barley, each plant possessing pros and cons. *Agrobacterium*-mediated transformation, biolistics or electroporation techniques are used for the transformation of the plants, and the transgenic plants can be utilized for the development of vaccines against bacterial pathogens such as *Mycobacterium tuberculosis*, *Bacillus anthracis*, and viral diseases like Human Papillomavirus, Human Immunodeficiency Virus, as well as the diseases like cancer, synucleopathies or atherosclerosis.

Keywords: plant-based vaccines; recombinant proteins; transgenic plants; vaccine development

1. Introduction

Vaccination has a vital role in the control of many diseases. The vaccines are developed and produced in the industrialized countries, but the need is quite high in low- and middle-income countries facing millions of deaths annually because of the inadequate vaccination. In addition to economical issues, another difficulty for the vaccination is requirement for cold-chain during transfer and storage of vaccines, especially in tropical countries [1,2]. These problems can be solved by the utilization of plants. In some cases, transgenic plants can act as plant-based vaccines expressing immunogenic proteins, and they serve as biofactories since the plant biomass is suitable for

utilization as an oral vaccine [3]. The recombinant protein is assumed to be bioencapsulated by the plant cell walls, but this is not always valid because plant-based vaccines may be formulated as powders of plant biomass in which the cell walls are damaged [4]. Moreover, the stability of antigen can be increased via co-purification with starch fractions like in maize kernels, or incorporation into protein bodies like in rice [5].

The orally administered transgenic plant should be digested, and the antigens are required to appear near the lymphoid follicles, Peyer's patches (PP), in the small intestine. The antigens are sampled by M cells in the PP using three possible pathways: (i) nonspecific transcytosis mediated by M cells, (ii) transcytosis mediated by specific receptors, and (iii) Lyso dendritic cells (DCs) immediately below M cells, having strong phagocytic and antigen sampling capability. The intestinal IgA antibodies can be produced in the first two pathways, while DCs may aid to induce systemic IgG levels [3,6]. In addition to utilization of transgenic plants expressing antigenic proteins as edible vaccines, these plants can also be used for production of immunogenic proteins as an ingredient of a subunit vaccine. The recombinant proteins are purified from the transgenic plants, and used for the development of injectable vaccines [7].

The tobacco plants (*Nicotiana tabacum* and *N. benthamiana*) have been preferred for the expression of immunogenic proteins in several studies [7–9] but other plants such as potato [10], maize [11], barley [12], rice [13], sunnhemp [14], soybean [15], and carrot [16] have also been used for the production of antigenic proteins.

The transgenic plant systems are used for the development of plant-based vaccines against bacterial pathogens such as *Actinobacillus pleuropneumoniae* [17], *Bacillus anthracis* [9], enterotoxigenic *Escherichia coli* [18], *Mycobacterium tuberculosis* [7], and against the viral pathogens such as the Human Immunodeficiency Virus [8], influenza virus [19], Human Papillomavirus [20] as well as against the diseases such as atherosclerosis [21], and synucleinopathies [22].

2. Plant transformation methodologies

In order to obtain the plant-based vaccines, the gene encoding the immunogenic protein is introduced into a plant using a suitable transformation technique. The transferred DNA may be (i) expressed for only a short period of time following the transformation, called transient expression, where just a small fraction of the DNA is introduced into the cell by direct gene transfer methods, and (ii) stably integrated into the chromosome of the cell. The transferred DNA in most cells is lost within time following cell divisions in transient expression. Fortunately, the transient DNA is expressed in some cells forming the basis of practical transient assays used for the analysis of gene expression and rapid monitoring of gene transfer. Different variables associated with gene transfer have been optimized using transient expression [23–25]. The stable transformation occurs when DNA is integrated into the plant nuclear or plastid genomes. The gene is expressed in regenerated plants and inherited in subsequent generations. For stable transformation, the developmental potential of the transformed cells is very important. Ideally, the transformed cells should be capable of regeneration into fertile plants. In general, the smaller the number of cells required for regeneration, the better the culture system is for gene transfer. Therefore, a small cell cluster size gives a larger ratio of transformed to nontransformed cells [26,27].

Since the first reports of tobacco transformation experiments in 1984 [28–30], several fundamental processes such as gene expression, cell metabolism, or plant development have been studied using gene transfer experiments. One of the most efficient methods for gene transfer employs *Agrobacterium tumefaciens* and takes advantage of the naturally evolved crown gall-inducing mechanisms of DNA transfer present in this common soil pathogen. Much has been learned about the mechanisms of this form of DNA movement and subsequent crown gall induction. This information has been applied to develop methods that result in the formation of gall-free, genetically transformed plants [31].

Later, particle bombardment was described as a method for the production of transgenic plants in 1987 [32]. Particle bombardment, or biolistics, is a commonly used method for genetic transformation of plants and other organisms [33,34]. In this technique, thousands of DNA molecules are coated on the metal particles which are shot at target cells or tissues using a biolistic device or gene gun. The DNA molecules are released from the particles that lodge inside the cells, and some of these molecules may be stably incorporated into the plant chromosomes. The basic bombardment procedures described are applicable to the wide range of plants genotypes [27], especially to the ones with available embryogenic cell cultures [35,36].

The difficulty of transforming some of the major crop plant species is a barrier to more widespread use of genetic manipulation techniques. Electroporation based transfection of protoplasts is described as an alternative to the previously mentioned transformation techniques, routinely resulting in transgene expression frequencies up to 90% [37]. The overall efficiency of the procedure depends collectively on numerous key parameters, including protoplast viability, DNA concentration, purity, topology and electrical conditions.

In addition to introduction of the DNA molecules into the nuclear genome, they can be transferred to the genomes of the organelles, chloroplast and mitochondria. For instance, Sidorov et al. [38] described the stable chloroplast transformation for potato. The gene expression in chloroplast has advantages such as reduced gene dispersal due to the maternal inheritance, high copy number of the plastids, simultaneous expression of several genes under single promoter, and position effect and gene silencing are avoided by homologous recombination [39]. High-efficiency transformation techniques for mitochondria depending on biolistics have also been developed [40]. For example, Chuah et al. [41] reported the intracellular delivery of exogenous DNA to the mitochondria using a combination of mitochondria-targeting and cell-penetrating peptide carriers in *Arabidopsis thaliana*.

3. The plants preferred for the expression of immunogenic proteins

The tobacco plants (*N. tabacum* and *N. benthamiana*) are used to produce immunogenic proteins in many studies but the plants other than tobacco were also used for the expression of genes encoding antigenic proteins. Kehm et al. [10] produced Puumala virus nucleocapsid protein (PUU-S) in *N. tabacum* and *Solanum tuberosum* (potato). The expression of the PUU-S was found to be stable over four generations at an amount of 1 ng/3 mg dried leaf tissue. The leaf extracts from transgenic tobacco and potato plants were used to immunize the New Zealand white rabbits, and the serum was shown to recognize the PUU-S protein. Lamphear et al. [11] vaccinated the previously sensitized gilts orally with the seeds (corn) of transgenic *Zea mays* (maize) expressing the S protein of porcine transmissible gastroenteritis virus (TGEV), and induced levels of neutralizing antibody were obtained in the serum, colostrum and milk. Additionally, Joensuu et al. [12] expressed glycosylated

F4 fimbrial adhesin FaeG from ETEC in *Hordeum vulgare* (barley) endosperm yielding 1% of grain total soluble protein (TSP). The gene was targeted to the endoplasmic reticulum and expressed in a glycosylated form. The mice were subcutaneously immunized using TSP from transgenic barley grains, and F4-specific antibody response was obtained. In another study, VP2 protein from the infectious bursal disease virus (IBDV) was produced in *Oryza sativa* (rice) up to 0.678–4.521 μg/mg of the seed TSP. The transgenic rice seeds were used to immunize the chickens orally, and induced specific humoral responses as well as protection against IBDV challenge were obtained [13]. Rao et al. [14] obtained VP1 protein from foot-and-mouth disease virus (FMDV) in transgenic *Crotalaria juncea* (sunnhemp) plants using *Agrobacterium*-mediated transformation. The guinea pigs were immunized intramuscularly with the proteins from transgenic plants or orally with the leaves of the these plants, and the formulations were induced both humoral and cellular immune responses providing 66% protection against FMDV challenge. Moreover, the gene encoding nucleocapsid N protein (ORF7 antigen) of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) was introduced in *Glycine max* (soybean) via *Agrobacterium*-mediated transformation. The recombinant protein accumulated in seeds of the transgenic plants up to 0.65% of the TSP. The intragastric immunization of mice with the transgenic soybean seeds induced humoral and cellular immune responses [15]. Recently, the N protein and a deletion mutant of the Gn glycoprotein from the zoonotic Rift Valley Fever Virus were produced in *A. thaliana* using *Agrobacterium*-mediated transformation. The oral vaccination of mice using transgenic plant conferred elevated IgG antibody responses [42].

4. The pros and cons of plants for antigen production

Vaccines are desired to have the properties of low-cost production and distribution to all over the world without a need of cold-chain. The plants can be used as bioreactors to produce high yields of antigenic proteins with low-cost, and the expression of the protein in seeds may act like encapsulation. Additionally, there are many plants that can be cultivated in various regions in the world. Moreover, the requirement of technical knowledge and labor for the administration, and the need of cold chain are less for plant-derived vaccines. Also, expression of antigens in plants lower the risk of contamination with human or animal pathogens [3,5,43].

There are some concerns for the utilization of transgenic plants to produce antigens. The transgenic plant expressing recombinant protein might be used directly for consumption as a food, but some plant species contain toxic materials like secondary metabolites and there is a risk of oral tolerance or allergy. If the recombinant protein will be used after purification from the plant, problems may occur related with the low rates of stability and/or expression. The protein processing systems are very similar in animals and plants; however, plants-specific oligosaccharides such as α-1,3-fucose and β-1,2-xylose may affect the function of the antigen. The environmental contamination risks and biosafety should also be considered [3,5,43–45].

A variety of plants are used for the expression of immunogenic proteins. Tobacco plants (*N. tabacum* and *N. benthamiana*) are widely preferred for the development of plant-derived vaccines due to the low-cost of production with high number of seeds which can be stored for long period of time, and high number of harvest time per year as well as the risk for contamination of human food chain is less. The disadvantage of tobacco plants is involvement of toxic compounds such as nicotine. *A. thaliana* is often used as a model plant with the advantages of small genome size, easily transformation, self-pollination, and high number of seeds but it has a low biomass. Potato (*S.*

tuberosum) is also used for the expression of antigens as the propagation of this plant easy and can be stored for long time without refrigeration. However, cooking of the potato may cause denaturation of the antigenic proteins. Banana (*Musa* sp.) has the advantages of no need for cooking and growth in developing countries, yet it is disadvantageous in terms of the long time for maturation and the fruit bearing of transformed banana trees, additionally the fruits are spoiled rapidly after ripening and contain low amount of protein. The rice (*O. sativa*) is safe to be used in baby food with low allergenic properties and can be preferred for high levels of expression of immunogenic proteins but special greenhouse conditions might be required as it grows slowly. The widely cultivated plant tomato (*S. lycopersicum*) grows quickly and same doses of immunogenic protein can be obtained from different batches, but the its fruits are spoiled rapidly. Maize (*Z. mays*) is broadly used for molecular farming with large grain size and high yield of biomass. In vitro manipulation and commercialization processes of maize are well-established, but the cross-pollination and contamination risk of human food chain are the disadvantages of this plant (Table 1) [43,46].

Table 1. The advantages and disadvantages of most common plants used for the expression of antigenic proteins [43,46].

Plant name	Advantage	Disadvantage
Tobacco (*Nicotiana* spp.)	• low-cost of production • high number of seeds • storage for long period of time • high number of harvest time per year • less risk for contamination of human food chain	• involvement of toxic compounds such as nicotine
Arabidopsis thaliana	• small genome size • ease of transformation • self-pollination • high number of seeds	• low biomass
Potato (*Solanum tuberosum*)	• ease of propagation • storage for long time without refrigeration	• denaturation of the antigenic proteins while cooking
Tomato (*Solanum lycopersicum*)	• quick growth • obtaining same doses of immunogenic protein from different batches	• rapid spoilage of fruits
Banana (*Musa* sp.)	• no need for cooking • growth in developing countries	• long time for maturation and fruit bearing of transformed banana trees • rapid spoilage of fruits after ripening and containing low amount of protein
The rice (*Oryza sativa*)	• safe to be used in baby food with low allergenic properties • high levels of expression of immunogenic proteins	• requirement of special greenhouse conditions for slow growth
Maize (*Zea mays*)	• large grain size • high yield of biomass • well-established in vitro manipulation and commercialization processes	• cross-pollination • contamination risk of human food chain

5. Production of bacterial antigens

There has been many attempts for development of plant-derived vaccines against a variety of bacterial diseases. The gene encoding an ApxIIA fragment from *A. pleuropneumoniae* was codon-optimized, and introduced into embryogenic callus of rice using particle bombardment technique. An amount of 250 mg/g antigen was obtained in lyophilized samples of transgenic rice callus. The intranasal immunization of mice with this antigen boosted the level of secretory IgA [17]. Additionally, Mamedov et al. [9] obtained a non-glycosylated antigen, a key component of the anthrax toxin, from *B. anthracis* in *N. benthamiana* by co-expression with peptide-N-glycosidase F (PNGase F) of *Flavobacterium meningosepticum* and showed that the protein was functionally active and immunogenic protein induced significant amount of toxin-neutralizing antibody responses in mice. Also, a fusion gene encoding the B subunit of heat-labile toxin (LTB) with the heat-stable toxin (ST) from enterotoxigenic *E. coli* (ETEC) was obtained and transferred into *N. tabacum* via *A. tumefaciens*-mediated transformation. The LTB:ST fusion was expressed in tobacco at an amount of 0.05% TSP, as a pentamer with antigenic determinants from both LTB and ST. The transgenic plant was administered orally to mice eliciting humoral immune responses [18].

A number of studies were conducted on the plant-based vaccines against tuberculosis [7]. Pepponi et al. [47] fused the Ag85B and Acr antigens of *M. tuberculosis* to a heavy chain antibody against Acr protein. The chimera was introduced into tobacco via *Agrobacterium*-mediated stable transformation, and 0.2% of the TSP was obtained with nuclear expression under the CaMV35S promoter. Intranasal immunization of mice induced the IgG and T-cell mediated responses as well as reduced the infection in challenged mice (TSP). Besides, Uvarova et al. [16] expressed ESAT-6 and CFP10 antigens driven by the CaMV35S promoter in carrot. Oral administration of the transgenic plants induced IgG systemic responses and T-cell mediated responses in mice. Moreover, Lakshmi et al. [48] fused ESAT-6 and Mtb72F (Mtb32/Mtb39) proteins to CTB or LipY as carriers, and chloroplast expression was obtained in tobacco and lettuce. The proteins inhibited hemolysis of red blood cells.

6. Production of viral antigens

Many studies have been conducted on the production of antigens from the human immunodeficiency virus (HIV) in plants to be used against Acquired Immunodeficiency Syndrome (AIDS). The *C4V3* synthetic gene encoding V3 loop and C4 domain from the glycoprotein (gp) 120 subunit of the HIV viral envelope was introduced into the tobacco chloroplasts. The plant-derived C4V3 protein was administered to the mice orally, and conferred humoral and cellular immune responses [8]. Moreover, a multi-epitope chimeric protein from gp120 and gp41 of HIV was produced in the moss *Physcomitrella patens* under carbonic anhydrase promoter yielding up to 3.7 µg/g fresh weight in protonema cultures. The mice were immunized subcutaneously using moss biomass, and humoral responses were elicited against the ELDWKA epitopes included in the chimeric protein [49]. Additionally, Kessans et al. [50] obtained enveloped HIV-1 virus like particles (VLPs) composed of Gag and a deconstructed form of gp41 (Dgp41) in *N. benthamiana*. Immunization of mice with these VLPs using prime-boost strategies (systemic and mucosal priming with systemic boosting) induced humoral responses against both the Gag and gp41 antigens as well as Gag-specific CD4 and CD8 T-cell responses.

A number of studies were also performed for the expression of antigens from other viruses in plants. The trimeric hemagglutinin (HA) protein (tHA-BC) from A/California/04/09 (H1N1) strain of influenza virus was produced in *N. benthamiana* plants. The purified tHA-BC conferred high levels of serum hemagglutination inhibition antibodies and protection against a lethal viral challenge in mice [19]. A fusion gene comprised of modified Human Papillomavirus (HPV)-16 L1 protein and glutathione-S-transferase (GST) was introduced in tobacco chloroplasts via biolistic transformation. The transgenic plants were shown to be identical to wild type plants yielding fertile flowers, and L1 protein was accumulated in their leaf extracts [20]. Similarly, the HPV-16 L1 fusions with different epitopes from the minor capsid protein L2 were introduced into *N. benthamiana* via *Agrobacterium*-mediated transformation. The chloroplast expression system produced up to 1.2 g recombinant protein per kg plant tissue. The fusion including amino acids 108–120 of L2 was found to be the most successful candidate vaccine eliciting anti-L1 and anti-L2 immune responses in mice as well as neutralizing antibodies against HPV-16 and HPV-52 pseudovirions [51]. The rotavirus antigens were produced in plants, too. The capsid proteins VP2, VP6 and VP7 of rotavirus were co-expressed in *N. tabacum*. The real-time PCR and Western blot analyses showed that the highest expression level was obtained for *vp6* and the lowest for *vp7*. The extracted proteins from transgenic plants were mixed with cholera toxin as an adjuvant, and administered to mice orally. The formulations induced serum IgG and fecal IgA levels, VP 2/6/7 to be more successful than VP 2/6 [52]. In order to increase the expression level and stability of rotavirus VP6 protein in *N. tabacum* chloroplasts, Inka Borchers et al. [53] altered its 5'-untranslated region (5'-UTR) and the 5' end of the coding region. The VP6 proteins were shown to be in the trimeric form as in the rotavirus capsid but susceptible to proteolysis at its N-terminal region.

7. Production of immunogenic proteins related to other diseases

In an attempt to develop a plant-based atherosclerosis vaccine, a chimeric protein was obtained in transgenic *N. tabacum* consisting of C-terminal of cholera toxin B (CTB) together with ApoB100 and CETP epitopes (CTB:p210:CETPe) to target both the p210 epitope of ApoB100, which is the main apolipoprotein of the low-density lipoprotein (LDL), and amino acids 461–476 at the C-terminal of cholesteryl ester transfer protein (CETPe). The mice were subcutaneously immunized with biomass from the transgenic plants producing CTB:p210:CETPe, and humoral responses against both ApoB100 and CETP epitopes as well as human serum proteins were obtained [21]. In another study, the extracellular (EC) domain of the rat epidermal growth factor-related protein 2 (ErbB2) tyrosine kinase receptor was expressed in *N. benthamiana* with an optimization of human codon usage, and the effect of a 23 amino acid transmembrane (TM) sequence on the protein accumulation was evaluated. All ErbB2 variants were transiently expressed in tobacco, but the TM was damaging for the accumulation of rErbB2 EC. The transgenic plants expressing ErbB2 without TM was used for the immunization of mice, and increased immune responses were obtained together with a potent antitumour activity against $ErbB2^+$ mammary cancer [23]. Arevalo-Villalobos et al. [22] expressed a chimeric protein (LTB-Syn) in transgenic *N. tabacum*, to be used against synucleinopathies, composed of the signal peptide of a vegetative protein from *G. max*, the LTB from *E. coli*, a linker peptide, an endoplasmic reticulum retention signal, and three epitopes from α-synuclein (α-Syn). The fusion protein was produced yielding 0.15 μg/g fresh weight, and oral vaccination of the mice with tobacco-derived LTB-Syn elevated the serum IgG levels. Additionally,

Voepel et al. [54] transiently expressed a fusion protein (CCT) comprised of three *Plasmodium falciparum* antigens in the transgenic *N. benthamiana*. The recombinant CCT protein was accumulated up to 2 mg/g fresh leaf weight, and used to vaccinate the mice intraperitoneally. The CCT-specific antibodies specifically recognized *P. falciparum* sporozoites inducing up to 35% inhibition of sporozoite invasion.

8. Conclusion

Vaccination is a very effective way of protection from infectious diseases. People all over the world deserve to reach this facility. However, vaccination rates are lower in poor countries due to the high costs though the infectious diseases are more common in those places. Production of antigenic proteins in plants to be used as vaccines could be a solution for reachability of the vaccines in low-income countries. The studies on the production of plant-based vaccines should be intensified especially for the diseases common all over the world, such as tuberculosis and AIDS. The stable expression of the antigens in plant seeds might be useful for long-term storage.

Conflict of interest

The authors declare no conflict of interest.

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