



REVIEW ARTICLE

Plants as factories for the Production of Pharmaceutical recombinant proteins

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ABSTRACT

Nowadays, transgenic plants are used as an attractive alternative to produce recombinant pharmaceutical proteins. Recombinant proteins such as vaccines and antibodies can be produced in plants via two systems: stable genetic transformation and transient expression. Stable transformation is included of integrating a gene into the plant nuclear or chloroplast genome. The transient expression can be resulted by using plant viral technology. The use of biopharming technology to produce vaccines in plants overcomes some of the major problems of traditional vaccination. This technology eliminate hazard of contamination with animal pathogens and provide a cheaper and safer alternative method. However, there are some challenges in biopharming such as purification and glycosylation. In this article, expression systems of plant vaccines, antibody production in plants and its application, challenges in biopharming and future perspective are discussed.

Key words: Biopharming, Recombinant protein, Transgenic plant, Vaccination.

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INTRODUCTION

Infectious diseases led to more than 45% of total deaths in developing countries [1]. Vaccination is the most effective facility to prevent infectious diseases. More than 30 million children in the world are not immunized against treatable or preventable diseases [www.care.org/campaigns/children_poverty/facts.asp], because the currently used approaches to vaccine production are technologically complex and costly. Development in molecular biology techniques during the 1980s, helped in the advance 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 used for commercial production of vaccines, these systems are expensive and their scale up is not easy [2,3]. Subunit vaccines have tended to be expensive, requiring substantial investment in facilities for production and purification. Moreover, the purified product tends to be subject to heat destabilization, and refrigeration is required for storage and transport, which may present problems in developing countries, which often lack adequate health care facilities. Consequently, there is considerable interest in new methods of producing cheap and stable subunit vaccines [4]. Production of vaccines in plants eliminates some current obstacles. The simplistic requirement of plants for sunlight, water and minerals makes them an inexpensive means of correctly processing and expressing proteins that can be quite complex. Expression of vaccines in plant tissues omits the risk of contamination with animal pathogens, provide a heat-stable environment, and enables oral delivery, thus eliminating injection-related perils [5]. Edible plant vaccines prepared a promising example of a new strategy that combines the innovations in medical science and plant biology to make affordable pharmaceutical products [6]. In recent years, plant-based novel production systems helped at developing "edible" or "oral" vaccines have also been discussed [3,7,8]. A variety of gene expression and protein localization systems now available for plants allows stable accumulation of the recombinant proteins in target plant tissue [9]. In this review, we focused on the plants as bioreactors for production of recombinant proteins, types of transgenic plant vaccine

systems, Steps in the production of transgenic plant vaccines, antibody production in these plants and challenges in biopharming.

Production of recombinant pharmaceutical proteins in plants (Biopharming)

In the last decade, plants have become an increasingly popular option for the production of recombinant protein [4,10]. Transgenic plants provide a potentially stable and cheap propagation source. However, the development and selection of a suitable transgenic line can take many months, and the production at high yield is often not attainable or stable, often owing to the phenomenon of post-transcriptional or siRNA-dependent gene silencing. This may be triggered by the high concentrations of any particular mRNA, and in any case is reset at meiosis, leading to both immediate and potential long-term production instability [11,12]. Therapeutic proteins have been produced in plants since 1986, when human growth hormone was expressed in engineered tobacco and sunflower callus cultures [13]. Some of helpful fusion partners for recombinant protein expression include *Escherichia coli* heat-labile enterotoxin (LT-B), *Cholera vibrio* toxin B subunit protein (CTB) and *Clostridium thermocellum* lichenase (LickM), the last of which is expressed through a TMV-based system [14, 15, 16]. One of the greatest hardships of in planta protein expression has been achieving enough yields of the protein of interest to warrant further development. Whilst the challenge to improve yields has been addressed for many proteins with recombinant protein yields of up to 80% of total soluble protein (TSP) reported using transient viral based systems [17], 51% of TSP reported for stable chloroplast transformation [18] and up to 37% of TSP in seed derived from stable nuclear transformation [19]. To achieve high levels of protein in plants, several options must be addressed. The appropriate promoters, enhancers, and leader sequences for the expressed protein must be determined. In addition, it may be necessary to optimize the codon usage and to remove mRNA-destabilizing sequences and polyadenylation signals from the foreign gene [20]. There are now some examples demonstrating the successful expression of subunit vaccine candidates in transgenic plants (Table 1).

Table 1- Expression of vaccine antigens in plants.

Plant/tissue	Promoter	Pathogen/causing agent	Disease	Antigenic protein	References
Potato/tuber, callus	Mannopine synthase	V. cholerae and rotavirus	Cholera and Gastroenteritis	CTB- Rotavirus enterotoxin protein (NSP4)	[21]
Tobacco/leaf (chloroplast)	Plastid rRNA operon (Prrn)	V. cholerae	Cholera	CTB	[22]
Tobacco/leaf	CaMV35S	V. cholerae	Cholera	CTB	[23]
Tobacco/leaf	CaMV35S	V. cholerae	Cholera	CTB-InsB3	[24]
Tobacco/leaf	CaMV35S	V. cholerae	Cholera	CTB	[25]
Tomato/fruit	Fruit specific E8 Endosperm	V. cholerae	Cholera	CTB	[26]
Rice/seed	specific GluB-1	V. cholerae	Cholera	CTB	[27]
Carrot/leaf, root	CaMV35S	E. coli	Diarrhea	LTB	[28]
Tobacco/chloroplast	plastid 16S rRNA gene promoter (Prrn)	Enterotoxigenic E.coli (ETEC) strains	Diarrhea	LTB-Heat stable toxin (ST)	[29]
Potato/tuber	CaMV35S	Porcine epidemic diarrhea virus (PEDV)	Diarrhea	Neutralizing epitope of PEDV (COE)	[30]
Tobacco/leaf	CaMV35S	Porcine epidemic diarrhea virus (PEDV)	Diarrhea	Neutralizing epitope of PEDV (CO-26K)	[31]

Transgenic Plant Vaccine Expression Systems

Vaccine antigens can be produced in plants using two different systems: stable genetic transformation and transient expression. Stable transformation produces a genetic line that can be multiplied either by (stem) cuttings or by seeds resulting from sexual reproduction [32,33]. Transient expression uses a

recombinant plant virus that carries the vaccine gene and by systemic infection, causes the plant to express the antigen [34].

The stable genetic transformation can be broadly divided into those using nuclear transgenic technology and chloroplast transplastomic technology and the transient expression can be achieved by using plant viral technology [35]. Each of those technologies are described in the following sections.

Nuclear transgenic system

Nuclear transformation is most often resulted with a plant pathogen, *Agrobacterium tumefaciens*, that can efficiently transport DNA into plant cells, targeting the nucleus to cause chromosomal integration at random sites [36]. The ease of nuclear transgene methodology, started by *Agrobacterium*-mediated delivery of DNA, facilitated this approach, which was the dominant strategy for several years [37]. Strains of *Agrobacterium* have been engineered to delete the virulence genes that cause tumor growth in plants but remain those that mediate efficient transfer of the DNA that lies between specific border sequences. Once the transgene is integrated, it is expressed and inherited in typical Mendelian fashion [38]. Advantages include the ability to scale up production of large rates of vaccine antigens from transgenic seed stocks and the possibility of expression in fruits or other edible plant organs, enabling oral delivery of minimally processed materials [37]. Some agronomically important plant species such as soybean and most cereal grains, are recalcitrant to *Agrobacterium* transformation [38].

Chloroplast transplastomic system

In chloroplast technology, foreign genes are integrated into the chloroplast genome by homologous recombination [39]. Foreign genes have been integrated into the tobacco chloroplast genome, giving up to 10000 copies for each cell and resulting in the accumulation of recombinant proteins at up to 47% of the total soluble protein [40]. Chloroplast expression minimizes the risk of foreign gene transfer through pollen from genetically modified crops to other related crops or weeds owing to maternal inheritance of transgenes [41, 42]. In addition, gene silencing has not been observed with chloroplast transformation, whereas it is a common phenomenon with nuclear transformation [43]. Also, chloroplasts can process eukaryotic proteins, covering enabling correct folding and the formation of disulfide bridges [44].

Plant viral system

Plant virus expression systems are potentially more efficient than the establishment of transgenic plants, because viral infections are quick and systemic, resulting in high yields of virus and viral gene products [34]. Expression of recombinant protein using viral vectors is a rapid and relatively simple method for examining proteins and desired characteristics since the infected plants can produce high amounts of protein within 1-4 weeks of inoculation. This method needs less investment in time compared to the use of transgenic plants [20]. In this regard, NVCP expressed in transgenic tobacco and potato also assembled VLPs and stimulated serum IgG and gut IgA specific for NVCP when fed to mice [45].

Steps in the production of transgenic Plant Vaccine

Successful development of vaccine antigens against human and animal pathogen(s) in plants needs 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 used for introducing the gene in the target plant species [46]. The mechanism of a plant vaccine production, has several stages that are briefly described (Fig. 1). First, the gene of antigens should be identified and isolated. Second, we tried to cloning gene in an expression vector and create stable transformations in plant tissue. The transformed plants were selected and regenerated. With extraction of total protein content, antigen of plant tissue detected and isolated, then immunogenicity and safety tests is carried out by feeding the animals [47].

Antibody production in plants and its application

Plants have proven to be production systems for many forms of antibodies. In the last decade, the expression and assembly of immunoglobulin (Ig) heavy and light chains into functional antibodies was first shown in transgenic tobacco. These immunoglobulins include full-sized IgG and IgA, chimeric IgG and IgA, secretory IgG and IgA, single-chain Fv fragments (scFv), Fab fragments and heavy-chain variable domains [48]. One of the most effective demonstration of the power of a plant-produced protein was in the monoclonal secretory antibody against *Streptococcus mutans* adhesion protein produced in a transgenic plant. It could prevent microbial colonisation in the human oral cavity [49]. The second antibody, a humanized anti-herpes-simplex virus (HSV) antibody made in soybean, was effective in the prevention of vaginal HSV-2 transmission in a mouse model [50]. Although recently potatoes, soybean, alfalfa, rice and have also been used successfully but most antibodies expressed to date have been in tobacco, [50, 51, 52]. There are some applications for plant antibodies. One of them is therapeutic applications. Therapeutic applications of plantibody are the treatment of infectious disease, inflammation, autoimmune disease or cancer. Plant produced the world's first clinically tested Plantibody, CaroRx. CaroRx binds specifically to *Streptococcus mutans*, the bacteria that cause tooth decay, and prevents the

bacteria from adhering to teeth. [53]. Another application for plant antibodies is immunization. Oral vaccines offer convenient immunization strategies for implementing universal vaccination programs throughout the world [32].

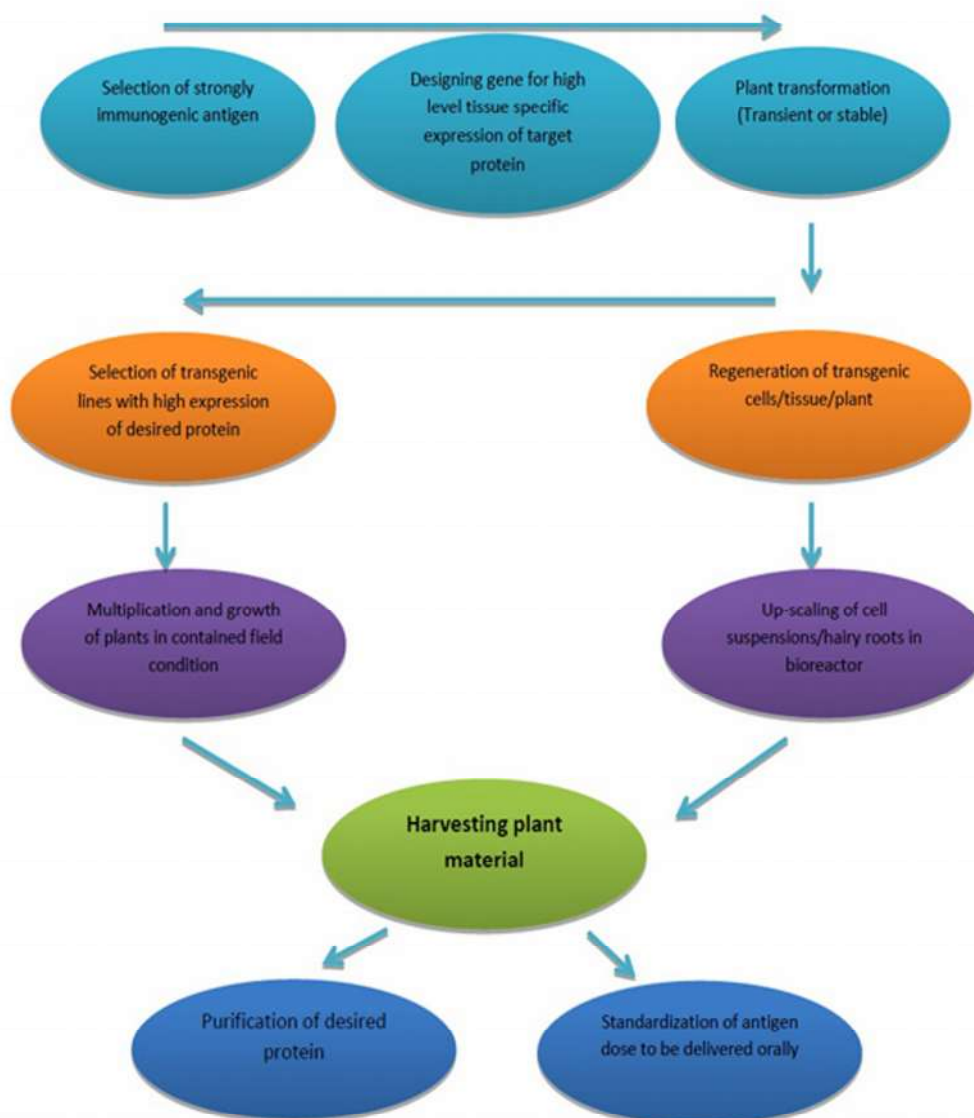


Fig 1- Steps in the production of plant-derived vaccine

Advantages and challenges in biopharming

The production of recombinant proteins in plants has many potential advantages for creating biopharmaceuticals related to clinical medicine. First, plant systems are more economical than industrial equipments using fermentation or bioreactor systems. Second, the technology is already available for harvesting and processing plants and plant products on a large amount. Third, the purification requirement can be omitted when the plant tissue containing the recombinant protein is used as a food (edible vaccines). Fourth, plants can be directed to target proteins into intracellular compartments in which they are more stable, or even to express them directly in certain compartments (such as chloroplasts). Fifth, the rate of recombinant product that can be produced approaches industrial-scale levels. Last, health risks arising from contamination with potential human pathogens or toxins are minimized [48]. However, there are some remaining challenges in biopharming that is described below:

Purification:

Downstream processes of plant system for biopharming such as extraction and purification of proteins as a key step, represents more than half of total production cost. Thus, efficient purification procedures for plant production systems are as important as effective expression level. Currently, for purification of antibody expressed in plants, an affinity purification protocol exploiting protein A-based matrices is mainly used. Large-scale purification has been using protein A streamline chromatography [54]. Alternative methods including the use of oleosin- or polymer-fusions to facilitate purification of recombinant proteins have been discussed recently and may also be effective to antibody molecules [48].

Glycosylation:

Plants have *N*-glycosylation capability similar to those of mammalian cells. However, *N*-glycosylation patterns processed in plant cells differ from those of mammals and humans. In plants, *N*-linked glycans include $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose instead of $\alpha(1,6)$ -fucose in mammals. Furthermore, the plant *N*-glycan hardly carries galactose and lacks sialic acid [55]. A method that has been widely adopted in cases where glycosylation-dependent effector functions are not needed, is the removal of related peptide recognition sequences for *N*-glycosylation. In an elegant approach towards the humanisation of plant glycans, human β -1,4-galactosyltransferase was stably expressed in tobacco plants [55]. Another approach is to retain the antibodies in ER to avoid plant-specific glycan residues such as β -(1,2)-xylose and α -(1,3)-fucose [56]. When the ER-retention signal KDEL/HDEL fused to proteins, the signal retains proteins in the ER [57].

FUTURE PERSPECTIVES

Plant-derived biopharmaceuticals should meet the same standards of safety and performance as other production systems [48]. However, the application of plant expression systems to produce human or animal vaccines have many advantages, such as well-established cultivation, low cost of production, no need for "cold chain" delivery, quick scale-up, simple distribution by seeds, ease of genetic manipulation, oral delivery, and low-health risks from human pathogen and toxin contamination, etc. Plant expression "biopharming" systems represent a cheaper and safer alternative method for antibody production [58]. Also, A number of infectious diseases, including smallpox, anthrax and plague have recently raised concern for their possible use in actions of bio-terrorism. The economic and technical advantages offered by plant vaccines propose these vaccines as ideal substitutes for traditional vaccines. Research on plants that produce antigens against major pathogens feared in case of bio-terrorism is already under way [59].

CONCLUSION

Like any biotechnology detection, the production of human vaccines in plants will be feasible not only on its technical merits but also on its social acceptance. Future research will show how to increase the expression amount of interested genes, and whether these transgenic plant vaccines meet the standards of quality (purity, safety, potency and efficacy) determined for vaccines by the World Health Organization or not.

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