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Plants as Bioreactors- A Review

Mathangi Ganapathy*

Centre for Biotechnology, Anna University, Chennai, Tamil Nadu, India

Abstract

Currently available systems for the commercial production of recombinant subunit vaccines include bacteria, yeasts, insects and mammalian cell cultures. Each of these systems has specific benefits, but overall, their application is limited by insufficient scalability, cost, safety and target integrity. Plant-based production platforms remains attractive as an alternative due to high scalability, cost-effectiveness and greater safety. Vaccines have been developed against viral, bacterial, parasitic and allergenic antigens for human as well as animal use using plant expression systems. Stable integration of transgene into the nuclear or chloroplast genome in many plants (e.g. tobacco, tomato, potato, papaya, carrot) for the production of subunit vaccines have been reported, effective expression has also been achieved by transient expression. Many plant produced recombinant proteins have shown immunogenicity, several have been shown to work effectively in animal models. This review tries to give an update of plant produced recombinant proteins, the future and limitations.

Keywords: Plant produced recombinant proteins; Plant produced pharmaceutics; Gene transformation; Transplastomic technology; Vaccine; Immunological response

Ever since the discovery of genetic manipulation and the establishment of various methods for effective transformation of cells, the use of plants as an expression system for the production of recombinant therapeutic proteins have been advocated. This has been an exciting as well as controversial concept. Plants produce large biomass hence plants can produce large quantities of recombinant proteins at low cost, this would be commercially viable. At the same time, care has to be taken about the contamination of food crops or products because of transgene integration and expression, humans may develop immunotolerance due to oral dosage of vaccine as well as the problem caused by illegal or unethical cultivation. Hence regulatory issues have to be stringent.

Stable nuclear transformation involving the incorporation of exogenous gene into the nuclear genome of the plant can be done by either Agrobacterial infection or biolistic gene delivery. Decreased costs and simplification of production process are the results of stable gene delivery. The exogenous proteins thus produced can be targeted to various organelles for standard eukaryotic post translational modifications. For rapid production of large amount of recombinant proteins, transient expression is the best method. One method of achieving this would be by using viral coding sequences via *Agrobacterium tumefaciens*. The other is by agro infiltration, i.e., infiltration of a suspension of recombinant Agrobacterium into plant tissue [1,2]. This has been specially developed as a rapid and high yield strategy for the production of clinical grade biopharmaceuticals [3,4]. Plastid transformation is another efficient alternative. The major advantage here is that the public anathema against GM plants can be reduced; the transgene cannot be transferred as pollen does not contain chloroplast [5]. High yield of recombinant therapeutic proteins have been obtained (3-6% of TSP) using tobacco chloroplast transformation [6-15].

Using biotechnological approach, transgenic plants have been used to produce therapeutic proteins, edible vaccines, antibodies for immunotherapy and proteins for diagnostics [16-30]. In all these cases, the therapeutics or proteins expressed in the plant tissues are either purified and used or the plant tissue can be processed to a form which can either be applied topically or taken internally. Fermentors and bioreactors can be replaced with green houses with appropriate biological containment or plant growth chambers which will reduce upstream facility. Plant tissues can be processed for oral delivery of edible proteins which will reduce downstream processing too.

In spite of more than twenty years of research and reports about the efficacy of plant produced vaccine related products very few products have gone all the way through the production and regulatory hurdles [31].

Current Status

Literature survey over the years for plant produced antigens or vaccines describes the expression of different vaccine antigens in different plant expression systems (Table 1). For the commercial production of pharmaceutical products the plants chosen should express the proteins with high efficiency in a large scale. Also such systems need to gain safety and regulatory approval.

The types of plants and the types of plant tissue used for the production of therapeutic proteins include leaf and stem tissues of various species and varieties of tobacco (*N. benthamiana, N. tabacum*), *Arabidopsis thaliana* [32-34], alfalfa [35-37] and potatoes [38-41], seeds of rice, beans, maize and tobacco [42-45]; root vegetables like carrots [46,47], fruits like tomatoes and strawberries [48-54]; aquatic weeds like *Lemna* sp [55,56]; hairy root cultures derived from various plants via *Agrobacterium rhizogenes* transformation [57-59]; single cell cultures of the algae Chlorella [60,61] and Chlamydomonas [62,63]; suspension cell cultures of tobacco [64-67]; transformed chloroplasts of a variety of plant species [68-70].

Transgenic single-cell cultures offer the advantages over whole plant systems of a high level of contaminant and possibility of producing proteins in bioreactors under Good Manufacturing Conditions (GMP)

***Corresponding author:** Mathangi Ganapathy, Centre for Biotechnology, Anna University, Chennai, Tamil Nadu, India, Tel: 91-9444336040; E-mail: mathangig@gmail.com

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Epitope source	Plant host	Reference
DPT polypeptide of *C. diphthereae, B. pertussis, C. tetani*	Tomato	Soria-Guerrra et al. [51,188]
E6 and E 7 of HPV	Tomato	Paz De la Rosa et al. [189]
PA of *Bacilllus anthracis*	Tobacco	Lee et al. [190]
VP2 of CPV	Tobacco	Ortigosa et al. [191]
FMDV	*N. benthamiana*	Andrianova et al. [192]
NP of H1N1 Influenza A virus	*Vigna uniguiculata*	Meshcheryakova et al. [193]
Hepatitis B surface antigen	Maize	Hayden et al. [25-27]
Cholera toxin subunit B	Maize	Karaman et al. [194]
Human epidermal growth factor	*N. benthamiana*	Thomas and Walmsley [24]

Table 1: Some examples of vaccines, therapeutic proteins expressed in plants.

conditions, as in currently the case with conventional fermentation or cell culture techniques.

Generally the use of plants for production of therapeutic proteins means a lower cost of production and easier expansion for large volume production than cell culture systems. Plant expression systems can potentially produce hundreds of kilograms per year of a purified protein whereas the cost of a similar production capacity using mammalian cell cultures may be prohibitive [71].

The antigen can be expressed in the cytoplasm and remain there or localized into any plant compartments like vacuole, chloroplast or Endoplasmic Reticulum (ER) by means of specific signal peptides. However the stability of the expressed antigen in the appropriate compartment has to be checked. Also, the level of protein expression for economical extraction, apparently calculated to be 1% of TSP, is very rarely realized [33].

Stable integration, selection and maintenance of transgenic plants take time. Even when it has been achieved, the high level of expression is not maintained in subsequent generations. This might be due to post transcriptional gene silencing or si RNA mediated gene silencing. Also in the case of nuclear transformation expression can be varied because of meiotic segregation. Li et al. [72] reported the stability as well as immunogenicity of human rotavirus VP 7 protein expressed in transgenic potato for 50 generations. However this is the only report where expression study has been done for so many generations. This seems to be an area where much work has not been done.

The steps involved in the production of recombinant proteins from plants include: (i) choice of the host species and optimization of coding sequence of the target gene in relation to the host, (ii) selection of expression cassette and creation of the expression vector, (iii) integration of the gene construct into the plant genome and regeneration of plants expressing the desired protein, (iv) identification and stabilization of the plant line for commercial production, recombinant protein.

Selection of the host plant depends on the type of protein, i.e., the form of the recombinant protein which is to be finally used. The life cycle of the host, biomass yield, containment and scale-up costs are other deciding factors. Success largely depends upon the understanding of species- or tissue-specific factors that affect the recombinant product. Self-pollinating species are advantageous over cross pollinated plants as the spread of transgene through pollen can be reduced. This can also be addressed by using plants which can be grown in containment, e.g. Tomato which can be grown in green houses. Further, the use of plant cell cultures addresses the issue of containment where dedifferentiated cells such as in calli or cell suspensions are used and can be grown on industrial scale using fermenter [73].

Green leaf expression or constitutive expression is easy to achieve and the rate of protein expression would be high. One of the major disadvantages associated with the leafy crops is their limited shelf life, hence immediate processing of the harvested material has to be done. Also purification strategies would have to be optimized to separate the protein of interest from leaf constituents like pigments, alkaloids and other secondary metabolites.

Seeds accumulate a large amount of protein during developmental stage, hence expression via seed specific promoters is preferred in many cases, this also has the advantage that purification would be easier. Also proteins can be stored for a longer time in storage and proteins normally do not degrade under ambient temperature. The only disadvantage is that it takes a long time for seed set depending on the lifecycle of the plant, hence transgene expression can be assessed only then.

Several cereals including rice, wheat, barley and maize have been investigated [74,75].The first plant derived commercialized product was produced in maize [76,77]. Cereal plants have been adopted as a production platform by the plant biotechnology enterprises like Ventria Bioscience (http:// www.ventria.com). A rice based cholera vaccine Muco-Rice CTB was shown to be stable at RT for 8 months, as well as resistant to pepsin digestion [78]. An ETEC subunit vaccine produced in soybean seeds was found to be stable for 4 years [79]. Recent publication from Hayden et al [26] shows that oral delivery of wafers made from HBsAg-expressing maize germ induces long-term immunological systemic and mucosal responses.

Oil crops offer unique production advantages of inexpensive downstream processing to obtain the desired proteins if they are targeted to the oil bodies. Oleosin, a plant protein, is present on the oil body surface. The hydrophobic central core part of the oleosin remains inserted into the oil body while the amphipathic and less conserved N and C-terminals reside on the surface [80]. The protein in question can be targeted to oil bodies as an oleosin fusion which can be later removed by centrifugation-based methods that separates oleosin fused protein from most of the other contaminants [81]. The oil bodies and proteins associated with them can be easily separated from the majority of other seed cell components by floatation centrifugation, taking advantage of their low density. To facilitate the recovery of pure protein, a specific protease cleavage site can be inserted between oleosin and the desired recombinant protein. An example of oil crop being utilized for this purpose is the oleosin fusion platform that has been developed by SemBioSys Genetics Inc. (http://www.sembiosys.com/), where the recombinant proteins are targeted to oil bodies of safflower seeds. Thrombin inhibitor, hirudin, was produced in transgenic seeds of *Brassica napus* using this strategy .The engineered oil bodies with their associated proteins can be used as affinity matrices for the selective, non-covalent binding of desired target molecules. For this, the oil-body proteins may be genetically fused to a ligand having specificity for the desired target molecule [82]. The expression of recombinant protein

as translational fusion with oleosin protein exposes the recombinant protein to cytosol, but at the same time it protects the foreign protein from cytosolic degradation. Because the fusion protein is not exposed to the environment of ER lumen, it avoids the posttranslational modifications.

The use of moss as a bioreactor is one of the major innovations in the manufacturing of biopharmaceuticals, which is cost effective and at the same time, avoids risks associated with environmental release of transgene. Moss allows humanization of glycosylation patterns and also time taken to reach the market is comparable to traditional systems. A transient expression system allows feasibility studies within 10–12 weeks and stable production strain development takes 4–6 month. Cultivation in suspension allows up-scaling of the photo bioreactors up to several 1000 L. Heterologous proteins are secreted into the medium. Downstream processing from this low salt medium involves fewer purification steps. They have high vegetative growth rate which shortens the production cycle. Genome engineering is greatly facilitated by the availability of Physcomitrella genome sequence. Facilities for production under GMP standards as well as long term storage are being processed [83-85].

Other aquatic plants and green algae (Chlamydomonas, Wolffia, Spirodela, Chorella, etc.) can also be used for the production of recombinant proteins. The potential of *C. reinhardtii* as a bioreactor has been demonstrated by the expression of hGAD 65 against type I diabetes [86], D2 fibronectin domain *of Staphylococcus aureus* as a fusion protein with CTB under the control of rbcL UTRs which provided 80% protection against lethal challenge in immunized mice [87]. Further, these can be grown under containment. Aquatic plants and green algae can be an alternative to open field plants [88-90].Though expression levels are highly variable by gene, improvements in codon optimization [91,92] and characterization of gene regulatory elements [93,94] combine to increase the level of transgene expresssion. *C. reinhardtii's* success as a future platform for the production of recombinant proteins has been reviewed [95].

Plant suspension cultures can be used to express the recombinant proteins. Many reports show how plant cells can produce diverse pharamaceutical proteins such as cytokines, growth regulators, nutraceuticals, etc. [96-98]. Some of these products have entered clinical development others are used in diagnostics, research or veterinary applications. Close to market products include gastric lipase for the treatment of cystic fibrosis, (Meristem therapeutics France), glucocerebrosidase for the treatment of Gaucher's disease (Protalix Biotherapeotics, Israel), anti-inflammatory molecules like lactoferrin, lysozyme (Ventria, USA). The first plant derived peptide likely to reach market is insulin produced in Safflower seeds by the Canadian company SemBioSys Genetics (http://www.sembiosys.com).

To achieve high level of transcription, the strength and expression profile of the key regulatory element "promoter" which drives the transcription, play an important role. The promoters that induce the expression of genes irrespective of the environment or developmental factors are called as constitutive promoters. They are generally used for the production of recombinant proteins in all the tissues of a plant. The cauliflower mosaic virus 35S promoter [99] has been used extensively for this purpose and high-level expression of recombinant proteins has been achieved [100]. It is more effective in dicots than monocots probably because of the differences in quality/quantity of regulatory factors. CaMV35S promoter has been used to produce several antigenic proteins in plants including CTB, LTB, HBsAg protective antigen, rabies virus glycoprotein G, SARS virus glycoprotein S [101-107] and other products of therapeutical antibodies, spider silk, SMAP-29 peptide, streptavidin, avidin and adiponectin [108-114].

Ubiquitin promoter is another most commonly used constitutive promoter Stoger et al. transformed rice with the gene encodingscFvT84.66 under the control of maize ubiquitin promoter or enhanced CaMV35S promoter. The expression levels of recombinant antibody were found to be comparable in the leaf tissue using either promoter. Using ubiquitin promoter, expression levels in the leaf tissue and seeds were comparable, but recombinant protein was not detected in seeds of transgenic plants when CaMV35S promoter was used [115]. Several molecules including CTB, LTB, HBsAg, human interferon, avidin or aprotonin have been produced in plants using ubiquitin promoter [116-121]. Generally, transgene expression varies in the transgenic plants generated using the same construct, even in the same environment. It may be because of position effect, transgene copy number or silencing [122-127]. There is a possibility to engineer desirable elements in the expression cassette to obtain the uniform expression levels.

Nuclear matrix attachment regions (MAR), also called global regulatory sequences, that are thought to influence gene expression, can be used to enhance the transcriptional activity of the transgene. They are supposed to place the surrounding loci in the regions which are suitable for recruitment of transcription factors to promoters [25,26]. Further, these AT-rich elements have been shown to reduce position effect by forming chromatin loops and therefore, increase transgene expression [127-131]. Also these have been found to maintain the expression level in subsequent generations [132].

Once the protein is synthesized, it undergoes several modifications before final delivery to its target. These modifications include glycosylation, phosphorylation, methylation, ADP-ribosylation, oxidation, acylation, proteolytic cleavage involving the polypeptide backbone and non-enzymatic modifications like deamination, glycation, racemization and spontaneous changes in protein confirmation [133]. The glycosylation machinery of ER is conserved in most of the species and adds similar glycans that belong to oligomannose category [134,135]. Yeast, insects, mammals and plants attach high-mannose glycans to the same Asn residue in the ER, but they differ in trimming and further modification of the glycans in the Golgi apparatus. Plants have the capacity to add complex N-linked glycans with a core substituted by two N-acetylglucosamine residues which is similar to the glycosylation pattern observed in mammals [136]. However, plants do not add galactose and terminal sialic acids but add plant-specific α-(1,3)- fructose and β(1,6)-xylose residues ,which are not desirable [137,138]. Various strategies have been developed to humanize the glycan patterns generated by transgenic plants.

Plant made vaccine

Dow Agrosciences in 2006 announced that it had received the first regulatory approval for plant made vaccine against Newcastle Disease Virus from USDA. As a part of the approval process, USDA verified that the plant produced protein is equivalent to other Newcastle vaccines. Although this never came forward as a commercially available product the formulation advanced through USDA Center for Veterinary biologics regulatory approval. Hernandez et al [139] had also showed the efficacy of orally delivered papaya produced anticysticercosis vaccine and its potential as a low cost alternative of immunization. Major et al [140] showed that intranasal vaccination with a plant-derived H5 HA vaccine protected mice and ferrets against highly pathogenic avian influenza virus challenge. Protective antigens of multiple strains of

influenza have been transiently expressed in *N. benthamiana* using an Agrobacterium mediated transient expression system. The agro-infiltrated plants produced large amounts of protective antigen from H5N1 (AIV) and H1N1 (human) strains [141,142]. The production of this antigen was performed in less than 3 weeks from the release of viral sequence to purified product. This plant made influenza vaccine has completed Phase II human trails. Mortimer et al [143] has reported the development of a plant-based platform for producing influenza vaccines locally, in South Africa. This was done with an idea to set up platforms in developing countries for vaccine production which would be helpful in times of pandemics.

Recently Hayden et al [26] compared the long term immunological memory against HBsAg the hepatitis B surface antigen in mice injected with a primary dose of Recombivax® and boosted with orally-delivered HBsAg maize germ wafers, control wafers, or parenterally-delivered commercial vaccine (Recombivax®). They have reported that mice boosted with HBsAg orally administered wafers displayed increase in mucosal IgA titres in fecal material and steep increase in in serum IgA whereas mice boosted with Recombivax showed no detectable levels of IgA. They have concluded that oral delivery can provide long term immunoresponses mucosally and systemically. This was a follow up paper from the same group which had reported earlier that oral feeding of supercritical fluid extraction treated maize material to mice elicited robust IgG and IgA responses which was comparable to injected commercial vaccine, Recombivax® [25]. Latest publication from the same group have reported the biochemical and biophysical characterization of maize-derived HBsAg and established that SFE-treated maize material is a viable oral vaccine candidate and this major advancement in the development of the first oral subunit vaccine [27].

Plant made antibodies

There are reports of many plant produced antibodies in literature with applications ranging from diagnostics [30,144-147]; to cancer treatment [148-153]; prevention of tooth decay [154-156]; prevention of plant disease [157,158]; and preventing sexually transmitted diseases [75,114,159]. de Muynck [160] has given a comprehensive review about this. Different subclasses of antibodies (IgG, IgM) have been expressed in different plant species but *Nicotiana* species predominate [161,162].

He et al. [163] demonstrated that WNV DIII antigen (West Nile Virus) and E 16 monoclonal antibody were produced at high levels, could be purified easily and were effective in identifying WNV and also in detecting human IgM response to WNV detection. Ganapathy et al [30] reported the efficacy of plant produced *Wb* SXP1 as comparable to E.*coli* produced *Wb*SXP1 in the diagnosis of Lymphatic filariasis, a neglected tropical infectious disease. Immunological screening using clinical sera from patients indicates that the plant-produced protein is comparable to *E. coli*-produced diagnostic antigen. These reports further substantiated that plants could serve as cost effective platform for diagnostic protein production, especially towards infectious and parasitic diseases which are prevalent in tropical countries.

Advanced plant and mammalian glycosylation differ in regard to types of sugar moieties added and the type of linkages [164].This difference in glycosylation might result in the identification of antibodies of non-human origin being seen as antigen by patients [165,166]. Plant specific glycosylation can also induce immune response. Plants now have been genetically modified to mimic typical animal glycosylation pattern by either inactivating the native enzymes or by expressing heterologous enzymes responsible for mammalian like glycosylation [160,167-169].

Two successful plant made antibodies have made to human clinical trials. Planet Biotechnology Inc. produced the world's first clinically tested antibody CaroRx™ in tobacco which specifically binds to bacteria that cause tooth decay and prevent adhesion of the organism to tooth. This is undergoing Phase II clinical trials.

In July 2008, Large scale Biology Corp reported the success of first human clinical trials testing of a plant made vaccine against cancer. A transient expression system produced patient specific recombinant idiotype vaccine against follicular B cell Lymphoma in tobacco. 16 patients immunized with their own individual therapeutic antigen showed no serious adverse effects, 70% of the patients developed cellular or humoral responses, 47% developed antigen specific response. In 2009, Bayer started the clinical development of this plant made antibody vaccine submitting Phase I study protocol to US FDA.

Additional therapeutic proteins

There have been many reports of therapeutic s expression in plants including anticoagulants [170]; thrombin inhibitors [170]; HIV [171]; Diabetes [172]; Liver cirhosis and burns [173]; Hepatitis [170,174]; anemia [173]; hemophilia [175]; organophophate poisoning [176]; Hypertension [177] etc. Shenoy et al [28] reported that the oral Delivery of Angiotensin-Converting Enzyme 2 and Angiotensin-(1-7) Bioencapsulated in Plant Cells Attenuates Pulmonary Hypertension. Further this also provided proof-of-concept for a novel low-cost oral ACE2 or Ang-(1-7) delivery system using transplastomic technology for pulmonary disease therapeutics.

Taking advantage of the high number of chloroplast genomes per cell, Daniell's group optimized technology for chloroplast transformation and gene expression. Oral administration of factor VIII or FIX antigens expressed in transplastomic tobacco plants suppressed inhibitor formation and anaphylaxis in hemophilic mice. A combination of protection from digestion offered by bio encapsulation in plant cells and fusion to the transmucosal carrier cholera toxin B (CTB subunit, thereby targeting gut epithelial cells) resulted in efficient tolerogenic delivery.

The first plant made therapeutic to reach phase II human trials was made by Biolex therapeutics, regarding Locterin a plant made controlled release interferon alpha treatment for chronic hepatitis [174]. First plant made therapeutic to reach phase III trials was carrot suspension cells derived Gauchers disease therapeutic developed by Prolix BioTherapeutics [178]. Human cereberoside expressed by carrot cells (human pr GCD) had high batch to batch enzymatic activity. In December 2009, Pfizer and Protalix entered an agreement to develop and commercialize pr GCD. However in early 2011, FDA declined the approval of the drug asking for additional data from existing studies, but not asking for additional trials.

U.S. Food and Drug Administration granted approval for ELELYSO, a product of Protalix Biotherpeutics and Pfizer for injection in May 2012 as a hydrolytic lysosomal glucocerebroside-specific enzyme ELELYSO, which is branded as UPLYSOTM (alphataliglicerase) in Latin America, which is a plant cell-expressed form of the glucocerebrosidase (GCD) enzyme. This enzyme is indicated for long-term enzyme replacement therapy (ERT) for adults with a confirmed diagnosis of Type 1 Gaucher disease. Approvals have also been granted by the applicable regulatory authorities in Uruguay, Mexico, Australia, Canada, Chile and other countries. (www.protalix.com) SemBioSys has also completed Phase I and II trial of safflower produced insulin grown in seed bioreactor Using Seed crops, ORF Genetics also produces various growth factors and cytokines in transgenic barley for use in cosmetics.

Limitations of Plant Vaccines

There may be development of immunotolerance to vaccine peptide or protein. Consistency of dosage form differs to fruit, plant, and generation of the plant. There have been reports of fruit specific expression of vaccines in tomato [179-182] and banana [183] and also in potato tuber [184,185]. Stability of vaccine in fruit is not known. Rigano and Walmsley [186] reported the expression of a fusion protein containing LTB and a species specific immune contraceptive epitope in fresh tomato fruits. As the shelf life of the fruits are less, the fruits were pooled and freeze-dried. Freeze-drying tomato fruit concentrated antigen 16-fold and extended shelf life to 5 months (before materials were used). These materials also proved to be immunogenic in animal trials [180]. Certain plants like potato cannot be eaten raw and cooking may change the properties of vaccine. The efficacy of freeze dried and stored potato based vaccine has also been reported [187]. Evaluating dosage requirement is tedious in case of plant vaccine. Selection of best plant is difficult. There has not been much reports regarding the stability and or characterization of vaccines in fruits.

The Future

Though the benefit of plant made pharmaceuticals have been pointed out reportedly it is being implemented only now due to investment by big pharmaceutical companies. Plant based systems have been able to reproduce a wide variety of human proteins, including those that have multiple subunits expressed and assembled in plants as well as proteins and vaccines requiring Co expression of additional modifying enzymes. While raw edible vaccines are unfeasible for human therapy, it may not be necessary to fully isolate the target protein from plant material. A middle ground of dried and ground plant material may be more suitable for oral delivery of some vaccines and therapeutics. This would be an excellent option for the production of veterinary medicines where recombinant feed could contain vaccine antigens and would be useful and cheap for developing nations [188]. If yields can be standardized, there is potential for delivery of therapeutics in unprocessed plant material, especially in veterinary field where the dosage has a wide active range. It is probable that partially purified vaccines would be first introduced for veterinary purposes and then progress to humans.

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