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REVIEW PAPER

Rapid Production of Therapeutic Proteins using Plant System

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ABSTRACT

Plant molecular farming is simply defined as the production of proteins therapeutics (PT) in plants, which involves transient gene expression in plants and purification of expressed protein to a great scale for diagnosis, treatment and other applications. This is the rapid, economical, safe and reproducible approach for the production of PT as compared to bacterial and mammalian systems. Protein yield and post-translational modifications are the major roadblocks that can be overcome by high expression strategies includes over expression constructs, suitable plant host systems and glycoengineering of proteins. The inherent ability of ideally producing safe, functional protein is the most striking phenomenon recognised by the pharmaceutical industries and developed many therapeutic products within few weeks to meet escalating demands during pandemic/epidemic outbreaks recently.

Keywords: Plant biopharming, Plant molecular farming, Agroinfiltration

1. INTRODUCTION

The era of recombinant therapeutics production initially started with *E. coli* still being successful only with the simple proteins like insulin, growth hormones that are not glycosylated and or that, while glycosylated in their native form, do not require glycosylation to exert pharmacological activity (such as IFN-α, IFN-β, IFN-γ, interleukin-2)[1]. Rather than *E.coli* yeast system (Pichiapastoris and Saccharomyces) specifically employed for producing glyco protein but some of those were hyper antigenic may not be suitable for the therapeutic purpose[2,3]. Most of the human protein are glyco protein and needs glycosylation for their proper function. Mammalian cell culture system is commonly used for the production of the glycoprotein. For the production of more complex proteins requiring post-translational modifications (like glycosylation–addition of carbohydrate moiety to the protein) the Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, mouse myeloma cell lines were mainly used and commercialised. The main advantage of this is the glyco protein produced is not immunogenic in human or animal[4-8]. However, this is a very expensive system for production of recombinant protein. Both the microbial and mammalian cell culture system is expensive to process that requires huge capital investment, trained main power to operate the system and operation cost is high thereby increasing the cost of the recombinant protein produced that is being commercialised[9-13].

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This is an entry point where the plant's systems were recognised for their simple growth requirements, the advantage of post-translational modification, scalability, targeted approach for organ/sub cellular compartments. The plant is a cheap alternative for the production of the recombinant protein. It requires less capital investment and scale up can be easily done in the case of requirement. It is estimated that the plant-derived recombinant protein can be 10-20 fold less expensive than the fermenter based recombinant protein production. Also, the initial investment for the establishment is less and scale up is possible since it is following simple plant grower's strategies for mass propagation. Hence, the focus protein therapeutics production in the bacterial and mammalian systems is slightly moved to plant genetic engineering based technique called 'Plant Molecular Pharming'. The inherent potential of plant system for protein production is the striking phenomenon led researcher's attentions to overcome the bottlenecks in the existing system are in routine practice. The most appropriate substitute is planted molecular farming since it is free from animal pathogen contamination risks, more yield percentage and economic. Since plants have long been used for the production of many protein therapeutics, there are many shortcomings affect the plant produced protein therapeutics to meet commercialisation standards. Recent developments in the plant molecular farming especially the development of simplest protein purification strategies, deconstructed viral vectors, glyco engineering and magnifection technology[12,14-23] were the most astonishing developments added more value to the plant-based therapeutics that are already commercialised and in various phases of clinical trials.

2. PLANTS AS A PROTEIN PRODUCTION HOST

Plant cells are versatile by means of combining the advantages of post-translational modification, simplicity and economic requirements for scale-up and obligatorily free from potential animal pathogen contamination. Heterologous protein expression and its stable functionality may give success in the bacterial system, but in most of the cases, plasmid instability and proteolytic degradation in the system makes it is unfeasible to commercialise the recovered product[24]. Plant biopharming is the boon for such conditions, to express the grams of functional proteins per kilogram of biomass. Several studies have proven that product recovery is simple, rapid and devoid of pyrogens.

2.1 Conventional Approaches

Plant cells can be cultivated under in-vitro conditions by hairy root, shootyteratomas, immobilised cells and suspension cell cultures. So, when compared to all these techniques cell suspension culture is of a greater advantage because they are amenable to GMP procedures and can be cultivated in large-scale using bioreactors[25]. Several plant species are used for suspension cultures; the most commonly used ones are the domestic crops including tobacco, rice, alfalfa, tomato and soybean. Among this tobacco cultivar bright yellow (BY-2) and *Nicotianatobacum* 1 (NT-1) are used for recombinant protein production[26,27]. The increased production of recombinant proteins in the cell suspension culture can be achieved by the use of a suitable promoter, signal sequence and a terminator. Hence, the recombinant proteins that are produced in large-scale can be obtained by downstream processing.

The production of pharmaceutical proteins using transgenic plants has also gained some attention over the last decade. Here the stable transgenic plants are produced by means of *Agrobacterium* –based vector-mediated gene transfer. Using this approach a whole range of therapeutic proteins has been produced in the plant tissue. Depending upon the use of a specific promoter, expression can be achieved uniformly throughout the plant or it can be limited up to the expression in plant seed, organs and sub-cellular organelles[28–33]. Apart from this, the focus of future research is in the production of oral vaccines in the edible plants/ fruits, such as tomatoes and bananas. This particular approach is elegant by which the consumption of plant materials provides an inexpensive, efficient and technically straightforward mode of large –scale vaccine delivery, particularly in poorer world regions. Since the generation of the stable transgenic plant for the production of recombinant protein is a time-consuming process transient mode expression has been adapted to produce proteins in large scale.

2.2 Plant Biopharming Concept

Plant biopharming follows plant grower strategies to propagate plants. Plant molecular farming method involves the use of disarmed strains of *Agrobacterium tumifaciens* employed for introducing recombinant plasmids into the plant intracellular spaces through syringe infiltration or vacuum infiltration techniques. This results in ectopic integration into the plant genome. Gene expression occurs 3-5 days of post infiltration immediately. This comeback is rooted from the perception that the usage of plant-based products is the safer and holistic approach.

Transient expression is feasible to produce a lot of proteins by using high expression vectors. Agroinfiltration is one of the most widely used techniques for transient gene expression initially known for host-virus interaction studies. Agroinfiltration followed by expression studied within a short period of time and can be assayed directly without any complex steps or time consuming procedures[34]. The basic protocol for the production of protein therapeutics in plant system comprising the three major steps,

(a) Construction of plant expression vector
(b) Host selection, and
(c) Agroinfiltration of recombinant constructs into the production host plant.

2.2.1 Construction of Plant Expression Vector

The expression constructs, design, determines the level of transcription and translation of the heterologous genes in the different species. Optimised factors for the transcription, translation, post-translational events and gene regulatory elements incorporated into the vectors achieve redress expression of the desired protein. Genes, those originally designed for the bacterial system is simply got modified to shuttle in the plant system by the addition of legumin signal peptide[35]. To improve the intracellular stability and yield percentage KDEL motif is incorporated into the sequence, which translocates the sequence into ER[24,36,37]. Irrespective of tissues constitutive promoters transcribe the genes effectively; this may be either good enough for monocotyledon and dicotyledonous plants. Largely CaMV35S has been used as a strong constitutive promoter for the PT production in dicots, for monocots ubiquitin-1 promoter[38]. A study compared CaMV 35S, subterranean clover stunt virus (SCSV), segment 4 (S4) promoter element and double promoter (S4S4) promoter efficiency in three different plant expression system, among these three CaMV35 leads the expression actually than S4, and even S4:S4, further it proves the theoretical potential of producing 153 μg/g[24]. The highest level of 2% TSP was achieved by expressing the α-trichosanthin gene under the transcriptional control of tobamovirussubgenomic promoter in the plant viral RNA vector[39]. Hence, plant viral vectors serve as an excellent carrier for the expression of multi-unit proteins like antibodies and it is more rapid as compared to the bacterial vectors, where regeneration after transformation, hardening and sexual crossing of plants to achieve multiple subunit assemblies are a highly time-consuming process[40]. Further improvement has made in the viral vector construction by following deconstructive approach and designed the novel, high expression vector system combines the advantages of viral vectors and non-viral vector systems called 'Magnifection technology'[18,29,41]. This scenario is most beneficial in many aspects by using *Agrobacterium* as an infective agent, high expression levels of viral vectors by incorporating viral regulation accessories, post-translational modification and simple growth requirements of plants.

2.2.2 Host Selection

Host selection is the important parameter which reinforces the absolute yield of the recombinant protein in the transgenic plants. Tobacco was the first plant to express a recombinant antibody in 1988. Several reports show the potentiality of the use of various plants such as *Nicotianatabaccum* and *N. benthamiana*, cereals (rice, wheat, maize), legumes (pea, soybean, alfalfa), leafy vegetables (Lettuce and Amaranthus, Spinach, Alfalfa) and fruit and root crops (tomato, potato).

Before making a choice, the requirement has to be customised as to reduce the production and storage cost. For instance, targeted expression of antibodies in potato tubers and barely seeds enhances the intracellular stability further it can be stored even at room temperature and reduces the post-harvest storage[24] cost, if the antibody is expressed in the leaves has to be stored and transported in frozen condition to maintain the stability of the protein. Selection of less maintenance, easy growing crop, minimises the production cost. Apart from this concern, food crops should be avoided to ensure the biosafety and ethical issues.

2.2.3 Agroinfiltration

Agroinfiltration is the technique used to study the plant-pathogen interaction, but now the technique has been adapted to study transient gene expression. Simply, the recombinant plasmid carrying *Agrobacterium* culture is introduced into the intracellular spaces of the leaves by making a small nick in the abaxile side of the leaf tissue or through the stomatal openings. The agro solution is injected into the leaf by placing the tip of the needless syringe in the leaf and simultaneously applying gentle counter pressure to the other side of the leaf (Fig. 1). The *Agrobacterium* solution will spread into the air spaces inside the leaf. Another way of introducing *Agrobacterium* cultures into leaf intracellular spaces by applying vacuum pressure. Vacuum infiltration is often used in labs to scale-up agroinfiltration for the production of test batches of protein. In vacuum infiltration, the plant tissue/leaf disk/the whole plantlet is submerged into the *A. tumefaciens* culture and subjected to decreased pressure followed by rapid re-pressurisation (Fig. 2). By vacuum infiltration, almost all the parts of the leaf are infected by *Agrobacterium* and also this is the preferable method for the large-scale production of proteins in various plant species, such as lettuce and Arabidopsis that are not amenable for syringe infiltration. Agroinfiltrated plants were maintained in optimal condition for 4-6 days and followed by downstream processing of infiltrated tissues, which includes homogenisation harvested leaf tissues (4-6 days of post infiltration (dpi)), column purification either in native/denaturing conditions, dialysis of purified fractions to remove excess salts, concentration and the plant produced protein function is confirmed further by western blotting and other functional assays.

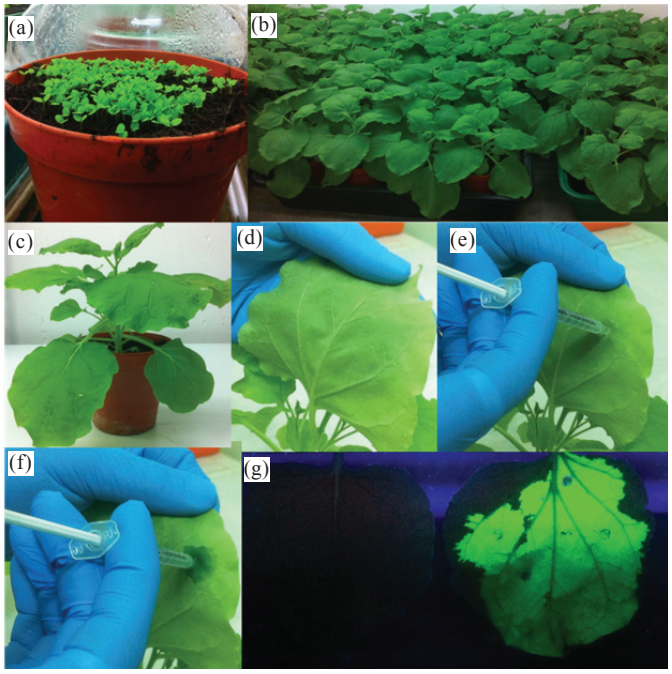

Figure 1. Syringe infiltration technique and GFP expression in leaves observed under UV light after 4 days of post infiltration; (a) *N. benthamiana* seedlings, (b) Propagation of *N. benthamiana* plantlets in plant containment room, (c) Well grown plantlet, (d)(e) and (f) Syringe infiltration of pEAQ-GFP construct into the abaxial side of the leafs, and (g) GFP expression analysis under UV transilluminator.

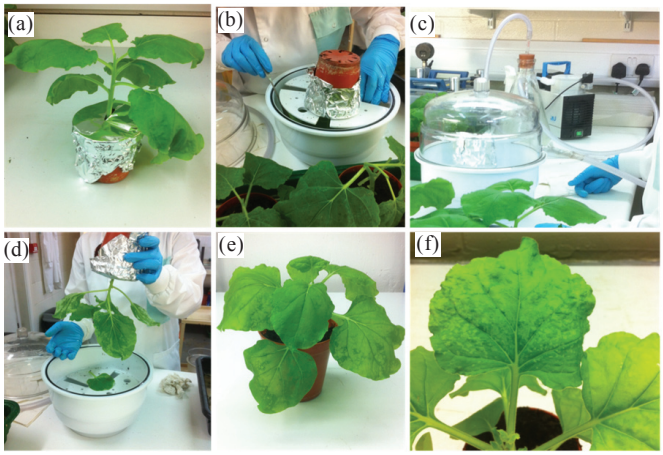

Figure 2. Vacuum infiltration technique: (a) Plant preparation for *agroinfiltration*, (b) The plant is immersed in the *Agrobacterium* solution in the vacuum chamber, (c) Vacuum infiltration setup, (d) After vacuum re-pressurisation the plant is infiltrated and carefully removed from the chamber, (e) completely vacuum infiltrated plant, and (f) Infiltrated leaf after 4 dpi.

3. RAPID PRODUCTION OF THERAPEUTICS DURING EPIDEMIC/PANDEMIC EMERGENCY

Pandemic (an epidemic disease spread over an ample region)/epidemic are sudden outbreak of a disease causing widespread and mass destruction within a short period of time. The epidemic/pandemic diseases are considered as a global threat that accounts for about >60 per cent human illness[42], especially the countries with more diversity and huge population are at high risk. Therefore pandemic/epidemic planning and preparedness is necessary to reduce transmission of the pathogenic strain, to control hospitalisations, deaths

Table 1. Current status of plant derived therapeutics developed against epidemic/pandemic diseases

Product	Disease	Plant	Status
Vaccine			
VLPs[50]	Blue tongue virus (BTV)	*Nicotiana Benthamiana*	-
VP6 gene[42]	Rotavirus	Chenopodium leaves	On market
DPP4-Fc[48]	Middle east respiratory syndrome corona virus (MERS-CoV)	Tobacco	-
hE16[51,52]	West Nile	*Nicotianabenthamiana*	On market
PA83 and DIV[53]	Anthrax	Brown Mustard, Tobacco and *Nicotianabenthamiana*	On market
Vibrio cholera[54]	Cholera	Rice and Potato	Phase I
HA[55]	Influenza virus (H5N1)	*Nicotiana Benthamiana*	Phase I
HA[56]	Influenza virus (H1N1; 2009 pandemic)	*Nicotiana Benthamiana*	Phase I
Hepatitis B antigen (HBsAg)[54]	Hepatitis B	Lettuce and potato	Phase I and Phase II
Antiviral (griffithsin)[57]	Severe acute respiratory syndrome (SARS)	*Nicotianabenthamiana*	On market
F1–LcrV fusion[58]	Plague	Tomato, Tobacco and *Nicotianabenthamiana*	On market
B5[59]	Small pox	Tobacco and*Nicotianabenthamiana*	On market
Capsid protein[60]	Norovirus	Potato	Phase I
LTB[60]	Enterotoxigenic *E. coli*	Potato	Phase I
HA (H7; VLP)	Influenza virus (H7N9)	*Nicotiana Benthamiana*	Phase I
HA (VLP) (seasonal; quadrivalent)	Influenza virus	*Nicotiana Benthamiana*	Phase I
Antibodies			
Antibody against hepatitis B[54]	Vaccine purification	Tobacco	On market
CaroRX[54]	Dental caries	Tobacco	EU approved as medical advice
DoxoRX[54]	Side-effects of cancer therapy	Tobacco	Phase I completed
Fv antibodies[54]	Non-Hodgkin's Lymphoma	Tobacco	Phase I
IgG (ICAM1)[54]	Common cold	Tobacco	Phase I
RhinoRX[54]	Common cold	Tobacco	Phase I completed

and to maintain essential services to reduce the economic and social impact of the pandemic outbreaks. Even though, we experienced many unforeseeable outbreaks like Chikungunya, Ebola and zika over proper pandemic planning. Chikungunya and Swine flu is a good example could describe the real difficulties we faced during recent outbreaks. This indicates still we need to explore, adapt and develop technologies to produce diagnostics and therapeutics in rapid manner to meet the demand in pandemic emergencies. Plant molecular farming one such convenient technology to adapt easily without huge investment to produce tons of doses rapidly. Plant molecular farming approach is most extensively used and it allows for the production of proteins in large quantities within a short time frame, this is particularly an attractive feature in case of pathogens that may be used for bioweapons and for epidemics[43]. Hence, plant-based vaccines that are transiently produced are with the native immunogenic properties stimulating both humoral and mucosal immune response[44].Many industries like Medicago, Fraunhofer Center for Molecular Biotechnology/iBio, Fraunhofer IME, Icon Genetics, VAXX and Mapp Biopharmaceutical/LeafBio were the pioneer in plant based therapeutics productions. Medicago and Fraunhofer Center for Molecular Biotechnology/iBiohas developed virus like particles for vaccination against H1N1 and H5N1 within three weeks of receiving sequence information[45-47].MappBiophamaceutical Inc., a USA based company has produced a drug in tobacco leaves called ZMapp, which has been used to combat Ebola virus outbreak in Africa. Middle East respiratory syndrome corona virus (MERS-CoV) is an emerging pandemic disease. Due to its high mortality rate

it caused panic in South Korea during 2015 and currently, no effective drugs are available to treat this disease. Plant Biotechnology Inc a USA based company produced an immunoadhesion (DPP4-Fc) in transgenic tobacco plants. DPP4-Fc strongly binds with MERS-CoV and it prevents the virus from infecting the lung cells[48]. Keeping in view the practical need of new technology for the production and delivery of inexpensive vaccines, especially in the developing country like India plant derived vaccines is the best option in hand to combat pandemic diseases.

4. CHALLENGES TO OVERCOME

Post-translational modification (PTM) is the major road block in the commercialisation of the plant based protein therapeutics, even though it has many ethical issues regarding environmental concerns about the transgene containment, antibiotic resistance, food safety and so on. Such regulatory hurdles can be easily overcome by good manufacturing practices (GMP) devised by the pharmaceutical industries. Still, PTM remains the daunting challenge to be tackled[49].

The major technological challenge to be addressed by researchers is to ensure that the structure of the engineered protein results in functionality that is equivalent to that of the native form. Most human and other mammalian proteins are biochemically modified at their carboxy- and/or amino-termini, and/or at the side chains of amino acids, during translation and post- translation events (covalent attachment of sugar chains i.e. glycosylation). Another challenge to researchers working on a suitable plant expression system for human therapeutics is inadequate information about a plant's capacity to post-translationally modify human proteins, with glycosylation being a particular concern. Diverse glycoforms are rich in plant expression system as compared to the other mammalian expression system. Still,this is sufficient for the production functional antibodies in plant system where glycation is not affecting the confirmation and its function. More than half of the oligosaccharides N-linked to the plantibody have β (1,2)-xylose and α (1,3)-fucose residues linked to the core Man3GlcNAc2. Complex N-glycan structures were reported on the plantibody Guy's 13; these structures were common in plant extracellular proteins. This is the evidence, which supports the antibody production in plant system is secreted and stored at the apoplast/vacuoles. Meanwhile, glycosylation is not anignorable modification in immunoproteins that happens during and after translation in amammalian system in order to provide resistance against protease cleavage, solubility and confers other physiochemical properties. Also, it performs some biological functions including antigenicity and immunogenicity.Plant system has altered glycans having β(1,2)-xylose residue and α(1,3)-fucose residues linked to the proximal N-acetylglucosamine, it is absent in animal system, this is how plant glycosylation collapse the function and lifetime of the protein therapeuticsin vivo[38].Since the glycosylation pattern of plant and animal system has many differences, it has no effect on the antigen binding or specificity, and still, the immunogenicity and other concerns need to be addressed through developing inventions in glyco-engineering.

5. CONCLUSION

Emergence plant molecular farming fanfares the potential of protein therapeutics production in plant system in past two decades. Even though more than 90 per cent of plant-based protein production is from stable transgenics, transient gene expression by agroinfiltration is more prominent by means of its rapidness, safe, economical and reproducibility in nature. High expression construct design with viral deconstructive approach 'magnifection technology' and use of viral regulatory pro vector systems are noticeable milestones in the plant molecular farming field. On the other hand selection of suitable plant hosts system also the important parameter which reinforces the absolute yield of the PT, while food crops should be avoided to address bio-safety related concerns. Even though the system has many advantages, still it has some latent issues like post-translational modification (PTM) and other biosafety and ethical issues will hinder the commercialisation potential. Biosafety like hurdles may be overcome by good manufacturing practices, and PTM is the major concern need to be addressed to produce humanised therapeutics by glycoengineering, which will mimic the mammalian proteins.

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