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Review Article

Plant Expression Platform for the Production of Recombinant Pharmaceutical Proteins

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Abstract

Plant Molecular Farming is a recent emerging field which relies on plant biotechnology and recombinant DNA techniques to produce recombinant proteins in plants. In this review, we describe the importance of plants for recombinant protein production for pharmaceutical and therapeutic purposes. This review also describes the importance of plants as an alternative expression system for the production of recombinant antigens and monoclonal antibodies with the purpose to develop cheap and safe vaccines against infectious diseases. Apart from this, economics, scalability and challenges of plant made therapeutics are discussed.

Keywords: Tobacco, Recombinant Protein, Vaccines, Molecular Farming

Introduction

Nowadays vaccines became an essential need to maintain public health; hence the demand for vaccine production significantly increased, as new diseases are emerging in our day-to-day life. An effective vaccine should be safe, easy to administer, stable, inexpensive and should provide protective immunity that lasts for long period and has less side effects. Still there is no vaccine available for several human diseases due to limitations like high cost and low manufacturing technologies, which make the vaccine inaccessible in developing world. The collaborative efforts of researchers, e.g. protein chemists and immunologists and chemical engineers are vital to the successful development of vaccines against a wide range of diseases that can reach the global population.

Regular immunization programs have gradually reduced the most of infectious diseases worldwide. However, still there are major hurdles in the vaccine production pipeline *i.e.* manufacturing, transportation and storage, which make them unaffordable to most of the developing/underdeveloped countries, where the vaccines are essentially needed. Recombinant DNA technology opens the avenue for the development of new vaccines/drugs that revolutionized the field of vaccinology. In earlier days, the production of recombinant therapeutic proteins are mainly restricted to mammalian cells as mammalian cells carry out post-translational modifications that significantly enhance the protein bioactivity [1]. To date, most of the recombinant proteins approved by FDA are produced in mammalian cells [2]. Even though there are other transgenic systems like yeast, bacteria and insect cell line are available for therapeutic recombinant protein production, every system has its own shortcomings excluding their acceptance by medical/research community. Now plants are emerging as a potential competitor to conventional expression systems, due to its practical, economic and safety advantages.

Plants as an expression system for therapeutic protein production

Plant Molecular Farming is an emerging technology that relies on recombinant techniques in order to produce therapeutic proteins in plants for vaccine development. Humans have always depended on plants for their food source; however recent advances in molecular biology have changed the paradigm of plant as a food source to so-called plant as green bio-factories to produce valuable recombinant/therapeutic proteins in demand. The advantages of using higher plants for the rationale of therapeutic protein production and vaccine design includes: (1) Lower production costs of large scale production and convenient system when compared to transgenic animals, fermentation or bioreactors; (2) Facilities already exists for the planting, harvesting and processing of plant material; (3) No contamination of therapeutic protein with any human pathogens when produced in the plants. (4) Protein produced with correct folding, more or less similar glycosylation pattern like eukaryotes [3,4]. Apart from these advantages, recent advances achieved in the expression levels both in stable and transient based plant transformation methods makes this plant host system as a promising system for the production of various biopharmaceutical proteins.

Plant based production systems

The therapeutic protein production in plants can be achieved by developing stable transgenic lines or transient expression systems or using plant suspension cultures [5]. Development of stable transgenic plants for the recombinant protein production provides the transgenic seed bank but with drawbacks as, it needs sterile environment and also its time consuming. Nowadays transient expression system (Agroinfiltration and Viral based vectors) is used as a production platform, which has several advantages over stable expression *i.e.* less time with more protein expression, consistency in protein accumulation, advantage of scalability and without any biosafety concerns, which makes this method more attractive [6]. Agroinfiltration is the method of choice however viral vector based transient gene expression has gained momentum due to its rapid gene-to-protein cycle [7,8]. Plant suspension cultures can also be used, where the recombinant proteins expressed in plant cell suspensions can be secreted into the culture medium [3].

Transient gene expression

The genetic transformation of plants with the aim to introduce

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specific traits in plants has revolutionized the plant biology and biotechnology. This has led to the development of crops with desired traits [9]. Plant genetic transformation is commonly performed *via,* two different approaches; (i) Stable transformation or *Agrobacterium*-mediated gene transformation and (ii) Direct gene transfer or transient transformation or physical method of gene transfer [10].

Tobacco, *Nicotiana* sp. is a member of the nightshade (Solanaceae) family. Tobacco is being used as a model plant for several *in vitro* studies. It has been proved to be an extremely versatile system for all the aspects of cell and tissue culture research. Although tobacco and *Arabidopsis* are the two facile plants that are easily to transform but it takes several months to produce to a stable transgenic line [11]. This problem can be overcome by the transient expression of proteins and this approach is the method of choice for high expression of foreign proteins in plants. Transient expression of proteins is quick and inexpensive that produces high yields of protein within few months that makes plants as an attractive system for therapeutic vaccine production [5]. Transient expression is achieved either by epichromosomal expression of *A.tumefaciens* on the infiltrated leaves (Agroinfiltration) or by viral expression vectors [12]. In viral expression vectors, the desired gene is cloned between the genetic materials of the plant virus, most preferably RNA virus and allowed to infect the plants to produce the target protein. Since the viral vectors do not get incorporated into the plant genome, the expression is transient that will yield more protein in less time [13].

Agroinfiltration is the commonly used method for transient expression of proteins, where the foreign gene in T-DNA is moved into the host cell nucleus by bacterial proteins [7]. Using agroinfiltration, it is easy to produce multi-component protein complex by means of infiltrating the plants with different combinations of recombinant *Agrobacterium viz.* Full length antibody can be assembled *in vivo* by expressing both light chain and heavy chain simultaneously in the host system by agroinfiltration, which is quite difficult in the transgenic system, where this can be achieved easily by crossing of two different transgenic lines [5].

Agroinfiltration in plants can be performed by vacuum infiltration or syringe infiltration Vacuum infiltration is more convenient than syringe infiltration in which plant tissue is submerged into the *A. tumefaciens* culture and subjected to decreased pressure followed by rapid repressurization [14-16]. By vacuum infiltration, almost all the parts of the leaf are infected by *Agrobacterium* and also this is the preferable method for the plant species such as lettuce and *Arabidopsis* that are not amenable for syringe infiltration [17]. Furthermore there have been several previous reports of the successful production of vaccine antigens/antibodies in plants by agroinfiltration [17-19]. Hence the transient expression system is better placed than the stable transgenics.

Post-translational modification

Post-Translational Modification (PTM) is the pre-requisite for the stability and biological action of recombinant therapeutic proteins produced in any host system that makes the plant expression system more unique when compared to prokaryotic expression systems. PTMs include disulfide bond formation, phosphorylation, proteolytic cleavage and glycosylation, which have a great impact on the biological function of proteins. Most of the human proteins are glycosylated at the asparagine residue located in a specific amino acid sequence termed by *N*-linked oligosaccharides. There are three types of glycosylation (1) *N*- linked glycosylation, where glycans were linked to an Asn residue of Asn-Xaa-Ser/Thr consensus sequence, where Xaa is any amino acid), (2) *O*- linked glycosylation, where glycans were linked to a Ser or Thr, and (3) *C*-linked glycosylation where glycans were linked to tryptophan [20].

Protein glycosylation takes place in two compartments; initial glycan synthesis occurs in the endoplasmic reticulum (ER) is conserved among the eukaryotes, whereas the final glycan biosynthesis and processing occur in the golgi apparatus, which finally yields a mixture of glycoforms [21]. The protein glycosylation in plants is slightly different from the mammalian cells; the lack of sialic acid, addition of xylose and change in the core fucose linkage (β1, 6 -> β1, 3) are typical in plant glycoproteins [22]. However, these differences had less impact on the recombinant protein function and now the progress is made towards glycan engineering in plant expression systems. The inactivation/*de novo* expression of plant glycosyltransferases helps to redesign the glycan structures in the plant glycoprotein for the production of protein glyco-variants similar to protein expressed in mammalian cells [21].

Plant expression system for vaccine antigen and antibody production

In earlier days, where the transgenic plant technology was young and growing, there is a strong belief among pharmaceutical companies that the plants take minimum of one year to produce milligrams of recombinant protein. Recent developments in the transient protein expression in plants, which is based either on agroinfiltration or viral-based expression vectors made this as a false belief, as this technology allow more protein accumulation within a very short period [23]. As the protein accumulation in the expression system increases, it will eventually reduce the downstream processing costs. However, it largely depends on the expression level and the tissue in which the protein is expressed. The cost of production of antibodies in plants may vary from $0.10 to $1 per gram that depicts the 1000 fold reduction in the manufacturing costs when compared to cell lines [24]. The approximate cost for production of therapeutic antibodies from different expression systems are summarized in the table 1 [22].

Several new vaccines are in the pipeline against many infectious diseases including life threatening Human Immunodeficiency Virus (HIV), tuberculosis etc. Since the approval of first monoclonal antibody by Orthoclone in 1986, the antibody molecules continue to fascinate researchers. More than ever almost >150 mAbs are in clinical development right now for different therapeutic applications [25]. The realization of high cost associated with mammalian cell culture technology has led to the search for low cost alternatives for

Table 1: Cost of Production of Therapeutic Antibodies [22].

Production System	$/g
Chinese Hamster Ovary Cells (CHO)	300
Transgenic Chikens/Eggs	1-2
Transgenic goats/milk	1-2
Microbial fermentation	1.00
Plants	0.10

Table 2: Comparison of different expression hosts for therapeutic protein production [28].

System	overall Cost	Production time scale	Scale-up Capacity	Product quality	Glycosylation	Contamination risks	Storage cost
Bacteria	Low	Short	high	Low	None	Endo toxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low Risk	Moderate
Mammalian cell culture	High	Long	very low	Very High	correct	Virues,Prions and oncogenic DNA	Expensive
Transgenic animals	high	Very long	low	Very High	correct	Virues,Prions and oncogenic DNA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor Differences	Low Risk	Moderate
Transgenic plants	Very low	Long	very high	High	Minor Differences	Low Risk	Inexpensive

therapeutic protein production. Recently, the antibody expression in plants has rapidly increased, depicting its importance as preferable alternate host system. Several studies have explored the possibilities of using plants as a cheap and effective alternative system for therapeutic protein expression [19,26-30]. The ability of plants to assemble and process the multimeric complexes has advanced from dimeric IgG to complex secretory antibodies [31]. Currently plants are the most productive and economical expression system to produce secretory IgA, which requires simultaneous expression of four genes to produce functional antibody [32].

Similarly, Several antigenic vaccines like, *Streptococcus mutans* surface protein antigen A [33], hepatitis B surface antigen [34], the *E. coli* heat–labile enterotoxin responsible for diarrhea [35], the Norwalk virus capsid protein [36] and the rabies virus glycoprotein [37] were expressed in plants that proved to elicit potential immune responses in animal experiments. The list of protective antigens that were expressed in plants is increasing and nowadays apart from human pathogens, the antigen from animal pathogen was also expressed in plants. Khandelwal et al. [38] reported that the hemagglutinin protein of Rinderpest virus expressed in tobacco elicited high antibody titres in the mice. Even though tobacco is the most preferred system, there is many other plant species such as tomato, potato, banana, corn and lettuce are also used for vaccine production.

Economics and scalability

Recombinant protein can be produced in plants at the cost of 2-10% and 0.1% of the cost of microbial fermentation and cell culture systems respectively [39]. Not all the proteins are expressed at high levels in plants, but yields of 0.1-1% Total Soluble Proteins (TSP) are sufficient to make plants economically viable [3]. Above 85% of the total cost for the recombinant protein production is spent for downstream purification rather than production, regardless of the expression system [40]. However, the downstream production costs can be reduced if the recombinant proteins are expressed in fruits and vegetables like in the case of edible vaccines. Scalability is one of the factors to be considered that determine importance of expression systems. The concentration of desired protein in the starting material and downstream processing cost was inversely proportional in which high accumulation of the product significantly reduces the processing/ production cost. By using viral vector based transient expression, targeting the gene expression to seeds or edible parts may further increase the expression level, which ultimately reduce the production cost [41]. The overall production cost, advantages and disadvantages of different expression systems are given in the table 2. As plants offer many advantages over other expression systems, recently plants are used as an attractive alternate entrant for the expression of vaccine candidates and therapeutic proteins. Furthermore, several therapeutic proteins produced in plants are in clinical trials and few have reached the market.

Conclusion

Even though the possibility of expressing foreign proteins in plants has been well established for past few years, plant molecular farming is still at an emerging stage of development. Currently, countries including India, Japan, Korea and European community are involved in developing plant vaccines against several human diseases. There are still few practical and regulatory issues, which need to be addressed to make this plant production system realistic. Major challenges concerning the production of recombinant protein on commercial basis are biosafety and risk assessment. However, recent advances in regulatory approval made the plant expression system much more pragmatic. Still greater research emphasis is needed to establish safety, efficacy and functional equivalence of the therapeutic proteins that would help molecular pharming to move forward. Although plant molecular pharming industry faces hurdles now and then, undoubtedly it will definitely find its way into future programs and that day is not too far.

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