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Advances in plant molecular farming: More than a medicine for achieving sustainable biomanufacturing

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Abstract
Plant molecular farming is a new and promising industry involving plant biotechnology and it is considered as a cost-effective technology that has grown and advanced tremendously over the past two decades. The development and improvement of the transient expression system has significantly reduced the protein production timeline and greatly improved the protein yield in plants. Plant based manufacturing can make the greatest impact, focusing on commercialized products or biopharmaceuticals including recombinant vaccine antigens, monoclonal antibodies, enzymes, and growth factors that are used as research-grade or diagnostic reagents, cosmetic ingredients, and biosensors or biocatalysts. Plants have emerged as a promising alternative expression system for production of pharmaceutical proteins because they offer several potential advantages, including low production costs, ease of scale-up to commercial quantities of production and reduced risk of product contamination by mammalian viruses or toxins. In this chapter, we discuss the technological basis of molecular farming in plants, with a focus on host systems and approaches/strategies developed to maximize protein yields and to ensure efficient recovery and purification of plant-made recombinant products.

Keywords: molecular farming, protein stability, biomedicines

Introduction

Recombinant proteins are complex exogenous ("foreign") proteins that are produced in expression hosts, and mainly used as medical diagnostic reagents and in human healthcare as vaccines, drugs, or monoclonal antibodies. The industry is focusing mainly on already established production platforms using prokaryotic and eukaryotic expression host systems such as Escherichia coli, a selection of yeast, insect and mammalian cell cultures, due to their well-defined processes in-line with current good manufacturing practice. Moreover, industrially established mammalian and other cell cultures have stringent regulatory approval in place, which hinders the industrial acceptance of the new technology or production system. Bacterial expression systems offer rapid production with high product yield, whereas *Saccharomyces cerevisiae* and *Pichia pastoris* (yeast) offer post-translational modifications (PTMs) which are essential for functional activity of the recombinant proteins. The majority of the approved recombinant biopharmaceuticals are produced in mammalian cell lines. However, all the production systems have their own merits and setbacks such as production time, high operating costs, protein yield, and chances of contamination with pathogenic microorganisms, limited post-translational modifications, and regulatory approval. In order to compete with the established platform, the new expression system must have unique advantages that can overcome the limitations of the existing ones. Plants offer several potential benefits over conventional expression platforms and prove the reliability of the system for the production of highly valuable proteins. Advancements in plant molecular farming approaches in the recent decade have made plants an attractive manufacturing system that can even achieve commercially relevant production levels in a short period. As the progress is continuously being made in this ever-growing field, here in this review, we summarize the importance and prospects of plant expression systems for the cost-effective production of recombinant proteins. Potential vaccine candidates, monoclonal antibodies, and industrial enzymes expressed in plants are also described. The practice of using plants for high-value recombinant protein production ranging from pharmaceutical therapeutics to non-pharmaceutical products such as antibodies, vaccine antigens, enzymes, growth factors, research or diagnostic reagents, and cosmetic ingredients has improved over time and advanced significantly in recent decades, which in turn has led to a major paradigm shift in the pharmaceutical sector.
The aim of the study is to review the technologies of molecular farming, advantages and limitations, challenges, biosecurity and public acceptance of PMF.

Plant transformation strategies

Basically, PMF works on the principles of recombinant DNA technology, where the gene of interest is transferred and expressed in suitable host. For this purpose, two general methods ie., stable/ permanent expression systems and temporary/ transient expression systems.

Stable/permanent expression systems

1. Stable nuclear transformation

It is done by transferring the gene or genes of interest into the nuclear gene of the host plant and there by altering the genetic makeup the host plant in such a way that the host plant can produce the exogenous product. Thus far, this is the most used

method to produce proteins of pharmaceutical interest. Major advantage of this system is its capacity to express stable the introduced gene and ability to scale up the production simply by increasing the area under cultivation. Another advantage is the long-term production capacity of this system since the entire plant will be stable transformed into a bioreactor and it can be stable transferred generation after generation since the nuclear genome is involved (Tremblay *et al*. 2010) [40]. In addition to the above, it also allows the expression of the genes in edible plant parts such as leaves, seeds or fruits making it suitable for oral consumption. This will eliminate the tedious and expensive downstream process which in turn cut the cost of production drastically. There are some disadvantages of this system such as long production cycle of some crops (6 – 9 months) along with some biosafety issues. Using whole plant as a bioreactor many accidently escape into wild population through seed mixing or by cross pollination through pollen grains of transgenic crop causing ecological problems (Pilson and Prendeville, 2004; Commandeur *et al*., 2003) [28, 11].

2. Stable plastid transformation (chloroplast transformed plants)

Plastid transformation offers a useful alternative method to the former one in the production of therapeutic proteins in plants. The key advantage with this system is the transgenic containment. Here the foreign genes are integrated into the chloroplast DNA where it shows maternal inheritance and normally the pollen grains are devoid of chloroplast. It will clear the ecological issue related to the genetic contamination by transgenic crops (Cardi *et al*., 2010; Meyers *et al*., 2010) [9, 27]. Another advantage is the ability to accumulate high level proteins in the cell. The plastid genome is highly polyploid. A typical tobacco leaf cell contains as many as 100 chloroplasts holding up to 100 genome copies per chloroplast. This itself the ability to amplify the introduced genes in plastids (Chebolu and Daniell, 2009) [10]. In addition to these, chloroplast bioreactors has precise integration of transgenes by homologous recombination, whereas in case of nuclear transformation in plants that occurs by the random integration of transgenes into unpredictable locations leads to varying levels of expression and, in some cases, gene silencing, the ability to express multiple genes in a single transformation event (transgene stacking), as well as the ability to perform the complex post-translational modifications such as disulfide bond formation, protein lipidation, folding and assembly may takes place (Verma and Daniell 2007 ; Chebolu and Daniell 2009 ; Tremblay *et al*., 2010) [42, 10, 40]. All these advantages bring more attention towards chloroplast transformation for optaining high levels of stable expression of pharmaceutical proteins in plants. Ruhlman *et al*. in 2007 reported a 160-fold increase in the accumulation of CTB-insulin fusion protein through chloroplasts transformation as compared to its nuclear-transformed tobacco plants. Other examples demonstrating the potential of chloroplast transformation to achieve high yield of foreign proteins in plants include. The expression of human immune deficiency virus (HIV-1) Gag (Pr55gag) polyprotein, a primary HIV vaccine candidate, in tobacco at expression levels up to 7–8% of TSP (Scotti *et al*., 2009) [35], expression of the p24 and Nef antigens, also HIV vaccine candidates, as a fusion protein in both tobacco and tomato chloroplasts (up to 40% TSP) (Zhou *et al*., 2008) [45], and the expression of human papillomavirus type 16 (HPV16) L1 structural protein, a vaccine candidate for cervical cancer, in tobacco with expression levels up to 24% TSP (Fernández-San Millán *et al*. 2008) [14].

Despite this, the great potential of plastid transformation has some functional limitations. It is unable to perform glycosylation like bacteria, a necessity for many pharmaceutical glycoproteins including monoclonal antibodies (Tremblay *et al*., 2010) [40]. Moreover, particle bombardment is the common method used for chloroplast transformation, and thus far, has been achieved routinely only in a few plant species (Bock, 2007; Singh and Verma, 2010) [7, 38], even though plastid transformation in tobacco was reported over 20 years ago (Svab *et al*., 1990) [39]. Unfortunately, tobacco is inedible and highly regulated, being rich in toxic alkaloids. It is also envisaged that protein stability will change over time even with refrigerated condition (Horn *et al.,* 2004) [22].

3. Plant cell suspension culture

It can be considered as a hybrid system which combines merits of plant-based production system with those of microbial and animal cell cultures (Xu *et al.,* 2011) [44]. Which means, as a production system, it maintains the merits of whole-plant systems, i.e., product safety, easy scale-up, post-translational modifications and ability to synthesize correctly folded and assembled multimeric proteins in it. On the other hand, suspension culture, like bacteria, have relatively rapid doubling times and can be grown in simple synthetic media using conventional bioreactors. This system guarantees sterile in vitro conditions, coupled with high-level containment (Franconi *et al*., 2010) [16]. The purification system and its downstream process are cheaper and easier since plant cells have the potential to secrete the expressed proteins into the cultural medium (Kim *et al.,* 2008). Recombinant glycoproteins derived from suspension culture have reduced N-glycosylation heterogeneity, owing to the uniformity of cultured plant cell populations (Liénard *et al*., 2007; De Muynck *et al.,* 2009) [13] makes stable product. It is also considered as a rapid method for biopharmaceutical production which is highly demanded during disease outbreaks and rapid spread situation. It is because there is no need to produce transgenic plants from the transformed cells, instead we can directly produce the proteins from the transformed cell lines. Which will reduce the duration by at least two times (Shaaltiel *et al.,* 2007; Aviezer *et al.,* 2009) [36, 5]. Tobacco cell suspension culture-derived Newcastle disease virus (NDV) vaccine, produced by Dow AgroSciences (Indianapolis, IN, USA), has obtained regulatory approval from the US Department of Agriculture (USDA), representing the world's first plant-derived vaccine approved for veterinary application. Cell suspension cultures derived from rice, soybean and tomato have also been used for recombinant proteins production (Hellwig *et al.,* 2004) [19]. Protalix Biotherapeutics (Carmiel, Israel) has developed the use of suspension cultures of carrot cells to produce recombinant human glucocerebrosidase for the treatment of Gaucher's disease (Shaaltiel *et al.* 2007) [36], and preclinical and phase I human trials showed that the plant cell-derived protein is safe to use (Aviezer *et al.*, 2009) [5]. However, suspension culture is still not the best production platform the plant system does offer, as the overall yield and usability is somewhat limited by the reduced level of recombinant protein production during the late stationary phase due to increased proteolytic activity (Corrado and Karali, 2009). Besides, the system is still limited to small number of well-

characterized plant cell lines such as tobacco, rice, carrot, or Arabidopsis and each species require special media for culturing (Breyer *et al.,* 2009, Hellwig *et al.,* 2004) [19].

Temporary/ transient expression systems

Transient expression system provides another plant-based platform for recombinant pharmaceutical production. The system offers several advantages over stable gene expression systems. One of the most important advantages is its speed of protein production (Rybicki, 2010) [34]. Useful amounts of target proteins can be generated within a short period matter, which is not achievable via stable gene expression. This may be of critical importance in ensuring a rapid and effective response to a sudden outbreak of an infectious disease, such as corona disease. There are instances where this system supported effectively in controlling human disease outbreaks. During 2009 influenza A/H1N1 pandemic that would require a recombinant vaccine product to be made in enough quantities within a very short time period in order to meet the needs of a pandemic (Tremblay *et al.,* 2010, Vézina *et al.,* 2009) [40, 43]. The simplicity and ease of performance will add more to its attractiveness. Moreover, there is no chromosomal integration of foreign genes, so its expression is not affected by position effects as often observed in nuclear transformation (Sheludko, 2008) [37].

There are mainly three methods for achieving transient expression in plants viz., agroinfiltration method, virus infection method and magnifection technology.

1. Agroinfiltration method: This method was developed by Kapila *et al.* (1997) [23], involves infiltration of a suspension of recombinant Agrobacterium tumefaciens into tobacco leaf tissue, which facilitates the transfer of T-DNA to a very high percentage of the cells, where it expresses the transgene at very high levels without stable transformation. This method has now been developed into a very rapid, high-yielding transient expression strategy for producing clinical grade biopharmaceuticals (Vézina *et al.,* 2009; Pogue *et al.,* 2010; Regnard *et al.,* 2010) [43, 29, 31].
2. Virus infection method: This method exploits the ability of plant viruses such as tobacco mosaic virus (TMV) and potato virus X (PVX) to be used as vectors to deliver foreign genes into plants, without integration (Porta and Lomonossoff, 2002). Both expression platforms infect tobacco plants and then transiently express a target protein in the plant tissue. A study conducted by Varsani and co-workers in 2006, were able to successfully obtain protein yield as high as 17% of the total protein, using virus infection method. The major advantage of this system is rapid and high level of production of recombinant protein as plant viral vectors can systemically infect all cells of a plant after inoculation, generating many transcripts of the transgene (Fischer and Emans, 2000). However, as with other fresh plant-based production systems, the recombinant protein must be processed immediately to prevent tissue degradation and protein instability. There is a concern about the spread of this protein to the environment and there are limitations in the size of gene sequence to be inserted into virus genome for successful transfer. Apart from this, Large Scale Biology Corporation, a pharmaceutical company, has adopted this system to produce idiotype vaccines for the treatment of B-cell non-Hodgkin's lymphoma, which have successfully passed the phase I clinical testing (McCormick *et al.,* 2008).
3. The magnifection technology: Both these methods are limited in their inability to achieve high-level co-expression of two or several polypeptides necessary for the assembly of heterooligomeric proteins (Giritch *et al.,* 2006). To overcome this pitfalls, Icon Genetics (Halle, Germany) has developed a TMV-based "deconstructed" viral vector system, known as magnICON, for the over-expression of foreign genes. This system combines the advantages of Agrobacterium- mediated delivery with the speed and expression level/yield of a virus. The coat protein responsible for systemic movement of the noncompeting virus strains was removed and the systemic delivery of the resulting viral vectors to the entire plant using Agrobacterium as the vehicle of delivery and primary infection (Gleba *et al.,* 2005). This method improved infectivity along with enhanced amplification and ultimately lead to high-level co-expression of several polypeptides, which were able to assemble functional heterooligomeric proteins at much increased levels up to 100-fold, including a full sized IgG (Giritch *et al.,* 2006) and Yersinia pestis antigens fusion protein F1–V (Rosales-Mendoza *et al.,* 2010).

Why should we use transgenic plants as bioreactors?

When we compare different production systems, there exist a number of reasons to shift into transgenic plants as bioreactors. The following reasons justifies PMF as a promising platform for biopharmaceuticals.

- Plants are free of human pathogens and bacterial toxins, hence the products derived from plants are safe to use.
- Being eukaryotic, plants are capable of doing post-translational processing, reduces processing cost.
- There is scope for utilizing plant breeding methods and chances of obtain active recombinant multi-chain proteins through sexual crosses (possibility of producing antibodies without application of a double transformation).
- Plants are autotrophs, they utilize natural resources for growth and development, reducing the costs of production (plants can produce biological materials by the use of carbon dioxide, solar energy, and inorganic materials).
- The scale of production can be manipulated regarding scalability by increasing the area under cultivation.
- Using cereals for PMF production reduce the costs of storage and transportation of recombinant proteins since, dry seeds can be stored for long without affecting quality.
- Since plant parts are edible, we can avoid purification step and cost of production furthermore.

Host plant selection

Choice of suitable host plant can be considered as a central most important factor. Since it is a huge task to produce plant based recombinant protein production which demands technological support as well as economical support hand in hand a wise selection should be done. The host plant selected should be amenable for the available method of transformation, protein stability, high biomass production, storage characteristics, ease of transport, value of recombinant proteins, maintenance costs, its

availability for workers, required area, duration of production cycle, cost of subsequent products, and edibility (Fischer *et al.*, 2004; Schillberg *et al.,* 2005). So far the most commonly used host plant is tobacco which is highly amenable for transformation and regeneration along with almost all economic benefits hand in hand (Biemelt and Sonnewald, 2005; Fischer *et al.,* 2004; Stoger *et al.,* 2005a). However, tobacco plant contains high amount of toxic compounds and other alkaloids which demands specialized downstream processing (Menassa *et al.,* 2001). But it should be considered for the production of PMF proteins which are not meant for human consumption and this will be an alternative source for tobacco farmers. By considering the toxic production in tobacco now alternative leafy vegetables are being tested for PMF such as lettuce and alfalfa (Rosales- Mendoza *et al.,* 2010). Even though there is a high amount of biomass production and through the application of nutrients to an extent we can increase the production, it demands immediate processing or special storage facilities to maintain the protein stability. Sometimes the expression of foreign protein molecules will interfere with plant growth. In this respect seed based production system will be more ideal. It not only provides easy handling but also long-term storage with minimum energy and maximum protein stability (Stoger *et al.,* 2002; Nochi *et al.,* 2007). It will be a path to edible vaccines – a needle free remedy (Lamphear *et al.,* 2002; Takagi *et al.,* 2005). In this regards using cereals such as rice, wheat, maize etc. are gaining preference. They can be easily transformed, amenable for scaling up, easy storage and transportation. In addition self-pollinated crop will be act as a genetic containment

Challenges ahead

Even though plants offer a handful of benefits over the conventional methods there still exist several obstacles for widespread acceptance of plants for biopharmaceutical production. The relative low yield poses one of the major challenges which is very relevant to be handled. For the past several years scientists were practicing different methods to address the low production challenge and many of them are promising in this.

In plants the gene activities are regulated at different levels viz., transcriptional regulation, translational regulation etc.

The problem of product shortage or the same recombinant proteins

1. Optimization of expression of transcripts: Building promoters are widely used to optimize the expression of transcripts, such as cauliflower mosaic virus 35S RNA promoter and maize 1-ubiquitin promoter, respectively, which are suitable for both monocot and dicot plants. Tissue specific and organ-specific promoters will stimulating the expression of transgenes such as antigen vaccine HBsAgM, and Human interferon-α in specific tissues or organs, such as tubers, seeds, and fruits (He *et al.,* 2008; De Jaeger *et al.,* 2002; Masumura *et al.,* 2006). It will enable the development of edible vaccines where the particular gene is expressed in edible portion of the plant such as fruits, seeds etc. it also avoid accumulation of foreign genes in vegetative plant parts which may have a negative impact on plant growth. Use of transcription factors will help in boosting the gene expression (Yang *et al.,* 2001). Besides the transcriptional stability can be ensured by co-expression of the specified gene and suppressor of RNA silencing (Voinnet *et al.,* 2003). Autonomous artificial minichromosomes, a new technology has been described to have enormous possibilities at transgenic level by providing gene stability owing to the absence of gene silencing and position effect in the construct (Ananiev *et al.,* 2009).
2. Optimization of protein stability: Even though each step in transgenic breeding is a challenging once, protein stability in a foreign genetic background is most crucial and challenging one so far (Schillberg *et al.,* 2005). To enhance protein stability, some intracellular targeting might be needed. It will also help in determining the type of processing and use of some techniques such as fusion and affinity tagging will enhance the downstream process and protein purification (Fischer *et al.,* 2004). Intracellular targeting for example with storage vacuoles have been discovered for accumulation of recombinant proteins (Yung *et al.,* 2001). Even fusion of full size large protein molecule with stabilizing partner brings high level of accumulation and recovery in secretary pathway (Benchabane *et al.,* 2008; Floss *et al.,* 2009). Fusion of proteins with stabilizing agents like Cathepsin D inhibitors in tomato will act as stabilizing agents and protect the recombinant proteins from cytosol degradation (Schillberg *et al.,* 2005; Goulet *et al.,* 2010). Targeting proteins to chloroplast will be use full for those proteins which do not require any post translational modification, such as glycosylation proteins (Moloney and Siloto, 2004). Using all these techniques we can increase the protein stability, still it will be better to have extracellular peptidase free host plants (De Muynck *et al.,* 2010).
3. Post translational challenges: The newly formed protein molecules should undergo further processing such as folding or binding with other biomolecules to increase biological activities, solubility and other such biological functions. Glycosylation is a post translational activity in which a carbohydrate/ sugars are covalently binds to protein to enhance its biological activities. But the pattern of glycosylation differs in plants and animals. Plants add residues of α (1, 3) fucose and β-(1,2) xylose to N-glycans of their protein, but animals add residues of (1 and 6) fucose, glucose, and sialic acid to N-glycans. Hence the plant derived animal proteins should undergo in vitro modifications so that it can perform perfectly in humans. But this will add to the cost of proteins due to this extra downstream process.

Downstream processing of recombinant proteins

Protein recovery and purification is another critical step in PMF. It represents a significant production cost of plant derived recombinant proteins. It is considered as an important factor in evaluating economic feasibility of plant based protein production systems. Compared to the conventional methods such as yeast and bacterial based system, PMF demands much more attention owing to the complexity of plant system. The processing and recover may vary according to plant parts and type of proteins to be extracted. Perishable plant parts need special attention such as in the case of leaves, which require either immediate processing or frozen storage. The purification systems can be broadly classified in to affinity fusion based system and non- affinity

fusion based system. There are new methods to make the tedious downstream process a little easier such as tagging the proteins to excretory systems, attaching to some oils etc. are being focused (Boothe *et al.,* 2010; Nykiforuk *et al.,* 2006; Nykiforuk *et al.,* 2010).

Biosafety concerns

Like GM crops, PMF also face biosafety problems from the general public. The problems may due to the cultivation of transgenic plant and the related issues regarding environmental safety and the other one regarding the safety of the protein produce from PMF. As we proceeds with PMF we should be able to handle the following concerns.

- Risk of co-mingling with food/feed crops: It can be avoided to an extent by growing non-food and feed crops. But when it comes to edible vaccines, farmers have to special concerns such as providing physical containment, growing less common variety, different sowing time, special equipment for cultivation and harvest etc.
- Risk of unintended gene spread: There is a chance that the recombinant DNA can escape in to related species or varieties and create biological contamination. Using greenhouses, closed containers, cell suspension cultures, self-pollinated crops, male sterile lines as well as using chloroplast transformation will provide protection against biological contamination of trans genes to an extent.
- Horizontal gene flow: It deals with the chance of gene to microorganisms, especially when antibiotic resistant genes are under consideration. But there is not yet any report on this aspect, still using cell suspension culture will act as containment in such cases.
- Plant bioreactors are meant for supporting human health and well-being. Hence it is prime important to handle public concern regarding PMF and derived proteins. Government with expert's advice can formulate policies and other regulatory activities so that there will be social acceptance of the same.

Conclusion

By considering 2019 corona out break and other disease out breaks happened in different parts of the world which took life and created economic disasters, seeks good, stable therapeutical proteins and other chemicals to support human life. All these efforts should support every human being irrespective of economic status and age. Also it should ensure rapid multiplication so that it will be useful during disease out breaks. So no doubt, PMF is the key to attain all these goals along with which it will help the farming communities indirectly. We can hope for a ' Pharmed drug' in future.

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