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Chapter 2

Molecular Farming in Plants

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Abstract

Plant molecular farming describes the production of recombinant proteins and other secondary metabolites in plants. This technology depends on a genetic transformation of plants that can be accomplished by the methods of stable gene transfer, such as gene transfer to nuclei and chloroplasts, and unstable transfer methods like viral vectors. An increasing quest for biomedicines has coincided with the high costs and inefficient production systems (bacterial, microbial eukaryotes, mammalian cells, insect cells, and transgenic animals). Therefore, transgenic plants as the bioreactors of a new generation have been the subject of considerable attention with respect to their advantages, such as the safety of recombinant proteins (antibodies, enzymes, vaccines, growth factors, etc.), and their potential for the large-scale and low-cost production. However, the application of transgenic plants can entail some worrying concerns, namely the amplification and diffusion of transgene, accumulation of recombinant protein toxicity in the environment, contamination of food chain, and costs of subsequent processing. The given threats need to be the subject of further caution and investigation to generate valuable products, such as enzymes, pharmaceutical proteins, and biomedicines by the safest, cheapest, and most efficient methods.

Keywords: Molecular farming, transgenic plants, biomedicines, protein stability

1. Introduction

Molecular farming is a biotechnological program that includes the genetic modification of agricultural products to produce proteins and chemicals for commercial and pharmaceutical

purposes. A vast majority of developing countries cannot afford the high costs of medical treatments resulted from the existing methods. Hence, we need to produce not only the new drugs but also the cheaper versions of the present samples in the market. Molecular farming can offer efficient solutions for the current growing need for the biomedicines [1]. Plants provide an inexpensive and simple system for the production of valuable recombinant proteins on large scale, and compared to the other production systems, they have numerous advantages in terms of economy, safety, and applicability. Though using transgenic plants has entailed some sorts of limitations and concerns, the optimization has been operated for solving the existing problems. Normally, the production of pharmaceutical proteins has been largely concentrated by the technology of molecular farming in plants, also plants can be used for the production of food supplements, biopolymers, industrial enzymes, and proteins in the investigations (avidin, β-glucuronidase, etc.). Prior production systems, including bacteria, microbial eukaryotes (yeasts, double-stranded fungi), animal cells, and transgenic animals, as a result of their limitations, were replaced by transgenic plants. The primary recombinant pharmaceutical proteins, extracted from the plants (hormones of human growth), and the first recombinant antibodies were generated from transgenic plants, respectively, in 1986 and 1989 [2, 3]. In 1997, the first recombinant protein, avidin (egg protein) was produced in a transgenic maize for industrial uses [4]. These applications proved that plants can be converted into bioreactors to produce a wide range of recombinant proteins. Many years had already passed when it was proved that plants were even able to produce several complexes of functional mammal proteins with the pharmacological functions, such as human serum proteins, growth regulators, antibodies, vaccines, hormones, cytokines, and enzymes [5]. An increasing request for the biomedicines was aligned with the high costs and inefficiency of existing production systems [6] including yeasts, bacteria, animal cells [7], and transgenic animals [8].

The aim of this study is to review the technologies of molecular farming, limitations and advantages of plant systems, challenges, bio-security, public acceptance of molecular farming.

2. The strategies of plant transformation

Plant molecular farming depending on the production of transgenic plants has been operated by two general methods as the following:

2.1. Stable or permanent expression systems

a. Stable nuclear transformation: Stable nuclear transformation refers to the integration of genes or nominated foreign genes into the nuclear genome of plants, which results in the change of genetic structures and consequent expression of transgenes after integration with the host genomes. The largest amount of recombinant proteins has been produced by one of the most common methods of stable nuclear transformation. A method exploited for aggregating proteins in dried beans of maize culminates in a long-term storage in the beans at the room temperature without decomposition of proteins [9]. In addition, it has a considerable

potential for producing crops like cereals that actually grow everywhere. However, a long production cycle for some crops and their potential collisions with natural species or food products have restricted the wide acceptance of this method [10].

b. Stable plastid transformation: Plastid transformation offers a remarkable solution in comparison to that of nuclear transformation since it has numerous advantages including preventing transgene escape through amphimixis (because plastids are inherited through the maternal tissue in the majority of species.) and absence of chloroplasts in pollen and consequent improbability of their transfer, which reduces environmental concerns [11, 12]. The transformed transgenic plants with homoplasmic chloroplasts (all chloroplasts carry transgenes) were selected after several generations of plant regeneration from bombarded leaf explants. Selection was conducted on a medium containing spectinomycin or combined with streptomycin. The researchers [13, 14] have already extracted a human pharmaceutical protein, more than 3% to 6%, from the total soluble proteins in the chloroplasts of tobacco. Recently, Oey et al. [75] reported a very high level (70% of an entire soluble protein) for a protein antibiotic with the chloroplast system, which, till today, has been the highest concentration of recombinant proteins. Despite this, the great potential of plastid transformation has some functional limitations. Although this technology has been developed in other species such as tomatoes, lettuce, soy, and eggs [15, 16], in the current situation, chloroplast transformation only in tobacco is practically possible, but unfortunately this plant is inedible and full of poisonous alkaloids; in addition, long lasting storage in refrigerators will bring about changes in protein stability [9].

c. Plant cell suspension culture: This method involves the removal of cell walls and gene transfer to the obtained protoplasts and suspension culture. The purification system and its downstream processing are cheaper and easier [17]. In addition, the use of suspension culture can decrease heterogeneity in proteins and sugar (N-glycans) regarding the uniformity of the type and size of cells [5]. Furthermore, as a fast system there is no need for the production of transgenic plants; however, the cell lines can be produced after a few months [18, 19]. Some samples of plant suspension cultures can be used for producing biomedicines, including vaccines of Newcastle disease virus of chicks approved by the Center for Veterinary Biology and recombinant human glucocerebrosidase for treating diabetes (www.protalix.com) [19]. Though this method is cheaper, safer, and easier in comparison to the other methods of genetic manipulation, cell suspension has not yet been suggested as an optimal production choice of production in plant systems. This is due to a belief that the ultimate products and their usability are constrained by reducing the level of recombinant proteins during the stationary phase because of the enhanced proteolytic activity [20].

2.2. Temporary or transient expression systems

A transient production may be the fastest system for plant molecular farming [21]. Nowadays, these are the systems routinely applied for verifying expression constructs during a few weeks for a significant amount of proteins [22]. The given systems include the following methods:

a. Agrobacterium transformation method: Infiltration of recombinant agrobacterium suspension into tobacco leaf tissue is achieved without stable gene transfer, which facilitates the transfer of T-DNA to a very high percentage of cells, where the transgenes are expressed at a high level without a stable transfer of genes. Presently, this method has been very efficient for the production of clinical biomedicines with a fast expansion [22-24].

b. Viral infection methods: The viral infection method depends on the capability of plant viruses, such as tobacco mosaic virus and X potato virus, which functions as a vector to convey foreign genes into plant genomes without fusing with the genome of that plant [25].

c. Magnifection system: Expression systems based on viral vectors and agrobacterium methods suffer from some constraints for the co-expression of two or more polypeptides required for the production of *hetero*-oligomeric proteins [26]. Thus, a new transient expression system known as MagnICON technology has been developed by Icon Genetics Company. This method includes removing coat proteins (responsible for systemic movement) of non-competitive virus stains and systemic delivery of the derived viral vectors to all of the plants using agrobacterium as the medium of primary infection. This method not only optimizes the infection but also significantly increases proliferation, and finally results in the co-expression of several polypeptides and the rise of functional protein production more than 100 times.

3. The advantages of utilizing transgenic plants as bioreactors

Comparison of different expression systems (see Table 1) reveals the advantages of plants in comparison with other expression systems as follows:

- The healthiness of derived products (plants cannot be the host of human pathogens and bacterial toxins).
- Capability of post-translational processing (respecting the features of eukaryotic cells).
- The possibility of using breeding methods and sexual crosses to obtain active recombinant multi-chain proteins (therefore, there is the possibility of producing antibodies without application of a double transformation).
- Reducing the costs of production (plants can produce biological materials by the use of carbon dioxide, solar energy, and inorganic materials. Moreover, the scale of production can be manipulated regarding scalability).
- Reducing the costs of storage and transportation of recombinant proteins (when they are produced in dry textures like grains).
- Removing the purification step (when the plant tissues containing recombinant protein are edible) [1].

Characteristics	Bacteria	Mammalian cell culture	Transgenic plants	Plant cell culture
Production cost	Average	High	Low	Low
Post-translational modifications	No	Yes	Yes	Yes
Function	High	Average	High	High
Protein stability	Yes	Yes	Yes in seeds	Yes

Table 1. Comparison of Various Expression Systems for Producing Recombinant Proteins

4. The limitations and optimization of plant production systems

4.1. The problem of product shortage or the same recombinant proteins

4.1.1. 3.1.1. Optimization of expression of transcripts

To optimize the expression of transcripts, a widely used strategy is the use of building promoters, such as cauliflower mosaic virus 35S RNA promoter and maize 1-ubiquitin promoter, respectively, suitable for spilt-cotyledons and single-cotyledons [27]. Tissue-specific and organ-specific promoters are used for stimulating the expression of transgenes (antigen vaccine HBsAgM, single-chain variable fragment Maureen G4, and Human interferon-α) in some tissues or organs, such as tubers, seeds, and fruits [28, 29]. The given specific expression of tissues prevents the accumulation of recombinant proteins in vegetative organs, which can have a negative impact on plant growth; for example, *palatine* is a gland-specific promoter; i.e., the protein is expressed in the gland but not in leaves; and also ubiquitin promoter is specified for the embryonic tissues of plants. Transcription factors (e.g., AlcR) can act as the invigorator of promoters to increase the level of transgene expression [30]. The stability of transcripts of genes can be achieved by co-expression of the specified gene and an RNA silencing inhibitor [31].

4.1.2. Optimization of translation

Expression constructs can be designed for guaranteeing the efficiency of translation and the sustainability of transcripts. As an instance, the removal of 5' untranslated region and natural ′3 for foreign genes and introducing the leader sequence of tobacco mosaic virus RNA, RUB13 rice polyubiquitin gene, alfalfa mosaic virus, or tobacco viruses in the expressions, all, individually, have shown a significant increase in the level of transgene expressions [32, 33].

In addition to the leader sequences, expression cassettes can be designed with the AU-rich sequences in 3' untranslated regions, which may change or be removed as the editing sites for ensuring the stability of transcript. It is also proved that every organism shows codon usage deviations that may be the subject of importance for adapting the coding sequence of heterologous genes for the host gene to optimize the efficiency of translation. In this regard, the site of initial translation from heterologous protein to pair with Kozak consensus sequence, with

the application of GCTTCCTCC sequence, started after codon or ACC, or ACA had been changed before that. It is better to unscientifically estimate codon changes rather than their real amount considering the changes in the expression level of transgenes in similar systems and the use of similar structures. To this end, an increase of codon combinations of (A/G)(a/c)(a/g)AUG and (A/g)(u/C)(g/C)AUG for the optimal operation of translation was, respectively, reported in Arabidopsis and rice. The given change in transgene expression could be due to the position effect, number of transgene copies, or gene silencing.

Regarding the effect of position, expression cassettes can be designed to have nuclear matrix attachment regions for ensuring the transgene insertion in proper sites for stimulating transcription factors for promoters. Furthermore, the problem of position effect can be prohibited by targeting the transgene to plastids. To optimize the production of single-cotyledon transgene, the strategies that include the use of specific genetic elements containing cAMP response elements for a simultaneous transfer with transgene in T-DNA are used. In addition, one new technology, including the structure of an artificial autonomous mini-chromosome, can genetically materialize excellent possibilities with several advantages, namely genetic stability due to the absence of gene silencing and position effect.

4.1.3. Optimization of protein stability

To optimize the stability of recombinant proteins, known as the most important limiting factor for the function of molecular farming [34], the targeting of proteins into certain intracellular parts is demanded. The intracellular targeting not only increases protein stability but also determines the processing type of dependent protein. This can be applied for the optimization of isolation and procedures of purification by the fusion proteins and targets with high affinity [27]. Targeting of proteins can be done by the following pathways and organelles,

- The intracellular parts, like *protein storage vacuoles,* have been discovered for the accumulation of recombinant proteins [30].
- Cathepsin D *inhibitor* can act as the agent of stability *of protein structures* to protect the targeted recombinant proteins in the cytosol of plants [35]. Recombinant protein production through this signal has been proved to be very effective and economical [36-38].
- To protect proteins from cytosolic degradation, these proteins can be targeted by fusion to a C-terminal tail without a forced passing through the lumen of the endoplasmic reticulum to the membrane surface [39]. To enhance the ease of purification, proteins can be fused to *oleosin* proteins as oil bodies in order to target protein expression with the oil bodies of seeds.
- The proteins, like in glycosylation, that do not need post-translation modifications for their activity, can be targeted to chloroplast since post-translation modifications are not conducted in these organelles [40].
- Targeting for accumulation in endoplasmic reticulum is accomplished by two methods: one is adding SEKDEL endoplasmic reticulum signals to the end of C-protein, and the other is using fused N or C signals with y-zein. Endoplasmic reticulum is an oxidizing environment with high amounts of *chaperone proteins and low levels of proteases.* This pathway is suitable

for the proteins that need post-translational modifications (e.g., glycosylation) [76]. The breakdown of proteins by proteases (proteolytic degradation) outside the cell is another noticeable factor for investigating the plant-based production of biomedicines.

4.2. Challenge of glycosylation (protein quality)

Glycosylation refers to the covalent binding of sugars to proteins in order to increase close-packing, biological activity, solubility, and biological functionality [5]. Glycosylation takes place in plants in the secretory pathway of endoplasmic reticulum and golgi apparatus. The glycosylation patterns of plants and animals differ in the composition of N-glycans; 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. These differences can be problematic for humans when medical animal proteins extracted from plants are used (Krupp et al., 2003); consequently, a correct human N-glycosylation demands a plant engineering. A number of strategies for changing the pattern of N-glycosylation in plants have been elaborated as following [71]:

- The use of purified enzymes of β-(1,4) galactosyltransferase and Sialyltransferase for making glass transitions in the recombinant proteins derived from plants.
- Co-expression of β-(1,4) galactosyltransferase human enzyme with the target transgene in transgenic plants.
- Prohibiting the activity of fucosyltransferase and xylyltransferase enzymes.
- Targeting pharmaceutical proteins to the endoplasmic reticulum in order to avoid the addition of protective N-glycans.

4.3. Selecting appropriate host plants

Major economical factors in appointing an appropriate host include the total biomass yield, 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 [27, 34]. In addition to the economical analysis, a sufficient host should be appropriate in terms of transformation and regeneration [34, 72]. In addition to the high potential of tobacco for transformation and regeneration, it has the majority of the aforementioned economic benefits [27, 41, 42]. However, tobacco (except the *cultivar 81 V9*) [43] contains high amounts of toxic combinations, nicotine and other alkaloids, that cannot be removed during the purification process. In spite of this, alternative forage crops like alfalfa and lettuce are being investigated and discovered as a suitable host for molecular farming [44]. However, forage plants generally suffer from the problem of instability of expressed proteins, by which drying and freezing of the leaves and immediate processing following the harvest have been inevitable [27]. The seed-based expression of proteins is considered to be more ideal regarding the fact that it neither affects the growth of plants nor needs the freezing of leaves or immediate processing after harvest, and it allows the long-term storage of proteins at a limited temperature without decreasing the level of activity [45, 46]. In this regard, grains, especially rice and

corn, have been cited as the superior ones. Maize has abundant advantages, such as having the highest rate of biomass yield among food crops and ease of transformation and production increase [47]. The high amount of protein (20%-40%) in the grains of legumes with remarkable levels of self-pollination in soy and peas is the main reason for transgenes of these plants for protein accumulation [48-50].

5. Predicting the intracellular localization of the recombinant protein

The importance of intracellular localization of proteins is due to the functional consequences of proteins. Therefore, the problem of intracellular localization of amino acid sequences has been the subject of great attention in the community of bioinformatics. Thus, various methods, like searching for targeted signals, have been presented with respect to a prediction that various proteins are produced in different intercellular segments [51].

6. Proteins and biomedicines produced in plants

Plants are able to produce those bacterial and viral recombinant antigens that preserve the capability of making the structures Type IV similar to those witnessed in mammalian systems, and the post-translational modifications are operated to maintain the biological activity of proteins. The most important issue is vaccine production in the edible tissues of transgenic plants, which is a very safe and effective method in vaccination.

The biomedicines produced in plants are as follows:

- Antigens for the production of edible vaccines: Antigens, used for generating an immune response resulting in immunity against diseases in human proteins, are expressed from different pathogens in plants. Those vaccines derived from plants have been so far induced immunity against rabies virus, hepatitis B, rotavirus, HIV, and other pathogens.
- Monoclonal antibodies: Widespread application of antibodies has lead to the study of new methods in order to strengthen efficiency and reduce the cost of producing antibodies. Among the studied methods, using transgenic plants as bioreactors are known as the most efficient one. While designing therapeutic antibodies in the production of recombinant expression systems, the apprehension of the functioning mechanisms of antibodies is essential. Although the primary function of antibodies is actualized by binding to antigens, it does not act as a protective performance. Some antibodies have a direct neutralizing impact, for instance blocking the bacteria or the active sites of the pathogenic factors such as enzymes. The antibodies produced in plants incorporate Immunoglobulin G (IgG) and Immunoglobulin A (IgA), IgA and IgG shimmer molecules, IgG and IgA secreted molecules, Single-Chain variable fragment, fragment antigen-binding, and second variable of heavy and light chains [52-54].

- Pharmaceutical proteins: Some samples of biomedicines recently expressed in plants include *erythropoietin, interferon*, hirudin, aprotinin, Leu-enkephalin, somatotropin of human growth hormone [55, 56].
- Non-pharmaceutical proteins derived from plants or industrial proteins belong mainly to the enzymes that include avidin, trypsin, aprotinin, β-*glucocerebrosidase, peroxidase* and *cellulose*, etc., listed by Basaran and Rodriguez-Cerezo [73] and now available in the market. Molecular farming of destructive enzymes of the cell walls such as cellulose, *hemicellulase*, xylanase, and particularly *ligninase* provide a great status for the biofuel industry respecting cellulosic ethanol [57, 58].

7. Molecular farming and metabolic engineering, an opportunity for producing plants with a high technology

Molecular farming and metabolic engineering make the production of new high-tech products possible. There is a driving force backing molecular farming that makes its costs much less than traditional farming. *Chlamydomonas reinhardtii*, as a unicellular alga, is one of the most recent production projects examined by Franklin and Mayfield. *C. Reinhardtii* is the only plant whose transformation was operated in its all segments containing DNA (nucleus, plastid, and mitochondria). Unique features of the moss system bring about the possibility of removing target genes and purification of the proteins secreted from the culture medium. The target gene was omitted to get rid of the nuclear genes for glycosylation. The first step towards the long-term goals of reengineering mechanism in modifications of plant proteins is setting a new standard in all systems of plant expressions in order to humanize the biomedicines produced in plants [59].

8. Purification and downstream processing of the recombinant proteins

Recovery usually includes the process and breakdown of plant tissues, protein extraction, solid-liquid separation, and protein concentration while purification encompasses safety protection, liquid-liquid extraction, membrane filtration, chromatography, etc. The processing of leaves requires a particular attention; leaves should be processed immediately after the harvest or frozen to prevent protein degradation by proteases, whereas seeds can be stored for a long period of time due to the less probability of destruction of recombinant proteins expressed in seeds. Using the secretory systems of cells can also be beneficial since disintegrating plant cells throughout recovery is not required; thus, the release of phenolic compounds can be avoided while the recombinant proteins can be unstable in culture mediums. Another way of facilitating the recovery of proteins is utilizing continuous labels. Protein labels must be removed after purification so that the structure of purified protein can change into its natural position. The technology of oleosin fusion, through which the gene sequence of recombinant proteins is fused to the sequence of a special internal oil protein called oleosin in

safflower and canola, is separated after the digestion of internal protease following protein purification [1].

9. Costs of subsequent processing

The costs of subsequent processing of the recombinant proteins derived from plants have been estimated about 80% of the total production costs [60, 61]. This is why so much attention has been paid at sufficient strategies for reducing the costs to the least amount. The application of watery textures like tomatoes as a production system has been expanded because of their potential for reducing the costs via the ease of extracting from their textures in comparison with those of dry tissues like grains [34, 62]. In addition, tomatoes are highly regarded as a reputed host crop in terms of its bio-safety because these plants grow in greenhouses without worrying about the preservation of transgenic plants.

Nowadays, oil bodies of oilseed agricultural products, like the seeds of safflower and mustard, are being exploited by the application of oleosin fusion technology developed by SemBioSys Genetics in order to facilitate the purification of recombinant proteins and reduction of subsequent costs (http://www.sembiosys.com/). The strategies including targeting of recombinant proteins for the seeds of oilseed agricultural products as an oleosin fusion facilitate the extraction of fused proteins from oil bodies and the release of the recombinant proteins from their fusion partner; one example can be the accumulation and purification of biologically active human insulin, apolipoprotein *A-I* (Milano) and human growth hormone in safflower [63-65].

There are several recombinant proteins derived from plants that were the basic idea of edible vaccines, directly eaten as fruits (tomatoes and bananas) and vegetables (lettuce and carrots); accordingly, no processing costs will be demanded by the elimination of processing, [66]. Bananas, as a fruit host in agricultural products, have particularly attracted lots of customers for the production of edible vaccines, especially for developing countries. This has been widely developed in such countries because of long distance transports and cooling requirements [42]. Apart from the mentioned advantages, high digestibility and palatability of bananas have won a wide public acceptance for the vaccination of children [67, 68]. The sufficiency of potatoes, eaten in raw or low processed forms, for edible vaccines has resulted in their wide production. Potatoes, like seeds, have the advantage of production stability due to a special molecular environment allocated in glands [69].

10. Bio-safety and the challenges in the domain of protein production and biomedicines in molecular farming

The risks of transgenic plants are divided into two categories: one category directly affects humans and the other endangers environment and other organisms. The attack of immune

system can disable these medicines and lead to the stimuli for the allergic reactions, some of which have been elaborated as follows:

- There are some concerns in terms of environmental pollution about the entrance of transgenes into the food chain, which requires a sound management and supervision.
- The other concern refers to the grain transformations using agrobacterium since grains are important crops in the production of pharmaceutical protein.
- The reactions of immune system can disable the medicines produced in plants and be the stimuli for allergic reactions [70].

11. Perspectives upon the commercial production of medicines and pharmaceutical proteins in molecular farming

The development stages and subsequent commercialization of the products is the subject of consideration in the second phase of clinical trials. A number of small biotechnology companies have aimed to commercialize the antibodies produced in plants. It has been estimated that the increasing annual need for secretory IgA will be 13%, and a rate of $25 billion was predicted as the annual income of producing IgA in crops. While there have been great advances in the field of biomedicine production in plants on large scales, fundamental studies are demanded to pave the way for the commercialization of these products. The present problems include the difficulty of low yield of protein, the possibility of harmful effects on the function/performance of proteins due to the differences in glycosylation patterns, and the severe potential impact of expressing plants of biomedicine plants on the environment (e.g., concerns upon genetic limitations) [74].

12. Conclusion

The aim of molecular farming is to produce large quantities of active and secure pharmaceutical proteins with lower prices. With the scientific advances in the field of bio-technology, nowadays, gene transfer methods in plants have considerably developed. These transgenic plants in comparison with other microbial and animal expression systems have various advantages in terms of easy production, cost, safety, etc. for producing pharmaceutical biomolecules. So far, lots of valuable pharmaceutical proteins and antibodies have been produced by the help of this method, which remarkably has helped the treatment of patients especially in developing countries where the production and preservation costs of such medicines cannot be afforded. However, there are some disputes, such as public acceptance, transgene escape and biosecurity, clinical and commercialization investigations of products, etc., which has made it a challenging area, but it is hoped that in near future molecular farming will witness great achievements with the researchers and scholars' efforts.

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