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Mini Review

Molecular Farming: Implication for Future Pharmaceutical Products

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Abstract

The goal of molecular farming is to produce large amounts of active and secure pharmaceutical proteins at a low cost. Nowadays, gene transfer methods in plants have advanced significantly because of scientific advances in the field of biotechnology. Compared to other microbial and animal expression systems, these transgenic plants have several advantages in terms of ease of production, low cost, safety, and so on for producing pharmaceutical biomolecules. So far, many valuable pharmaceutical proteins and antibodies have been produced using this method, which has significantly aided patient treatment, particularly in developing countries where the production and preservation costs of such medicines are prohibitively expensive. However, there are some disadvantages, such as acceptance by the public, transgene escape and biosecurity, and so on, however, it is hoped that with the efforts of researchers, molecular farming will achieve great success in the near future. This mini-review highlights the history of molecular farming, plant transformation strategies, classes of protein within molecular farming, products in the market, products nearing commercialization, advantages of using transgenic plants as a bioreactor, major barriers to broader market penetration and strategies to overcome them, biosafety and challenges in the production of proteins and future prospects.

Keywords: Pharmaceuticals; Recombinant protein; Edible vaccine; Monoclonal antibody; Transformation

Introduction

Plant molecular farming (PMF) is a simple and cost-effective method (1) that involves genetically modifying agricultural products to produce high value recombinant proteins and chemicals for commercial and pharmaceutical purposes. The vast majority of developing countries are unable to afford the high costs of medical treatments resulting from the existing methods. Therefore, not only new drugs but also less expensive versions of existing drugs must be developed using low cost methods (2). Molecular Pharming represents an unprecedented opportunity to produce low-cost modern medicines and make them available on a global scale. The most promising area is infectious disease prevention, particularly in developing countries where access to medicines and vaccines has historically been limited (3).

Typically, molecular farming technology in plants has greatly concentrated on the production of pharmaceutical proteins; however, plants can also be used to produce food supplements, biopolymers, industrial enzymes, and proteins in research (avidin, glucuronidase, etc.) (4). To date, five common platforms, including mammalian cells, bacteria, yeasts, insect cells and plant cells, have been widely used

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to produce recombinant proteins (5). The ability of plants to express various proteins has now been demonstrated. However, only a few products have made it to the market due to some concerns, such as transgene amplification and diffusion, recombinant protein toxicity in the environment, food chain contamination, and subsequent processing costs (6, 7).

Academic laboratories have been critical in elucidating much of the science underlying potential products and will continue to be so they will also need to emphasize downstream process development research as the industry grows. This will supplement their fundamental work on protein expression and provide the foundational knowledge needed to fuel the sector (7). Plant molecular pharming holds significant promise for increasing global access to medicines. The time has come to accelerate the development of traditional products and new concepts (8). Original research articles were searched using relevant keywords in PubMed and Google Scholar. Then the articles were screened and 25 articles were selected to include in the review. The selected articles were reviewed, and relevant findings were incorporated into the mini review.

History of molecular farming

Plant viruses were discovered in the early 1980s. Fritz Kreuzaler and his colleagues confirmed the presence of an assembled full-size antibody after injecting cDNAs into the nuclei of *Acetabularia mediterranea,* an algal species with large cells used as a model organism in cell biology. To confirm the preselection, an anti-idiotype antibody that binds specifically to the assembled antibody was used (4).

The first recombinant pharmaceutical proteins were extracted from plants (human growth hormones), and the first recombinant antibodies were generated from transgenic plants in 1986 and 1989, respectively (6). The first molecular farming products, which were commercialized more than 20 years ago, were avidin and β-glucuronidase produced in transgenic maize plants for industrial use in 1997 (4). Since then, many diagnostic and technical proteins have been produced in plants, and some companies (e.g., Leaf Expression Systems, Agrenvec, and Diamante) have added this protein category to their portfolios (4).

Plant transformation strategies

Currently, there are two general methods for producing protein from plants: 1) Stable or permanent expression systems, and 2) Temporary or transient expression systems.

1. Stable or permanent expression system

Stable transformation entails the incorporation of a foreign gene or genes into the plant's genome. This can be accomplished in dicotyledonous plants through agrobacterium-mediated transformation or in monocots through biolistic delivery (gene-gun) methods. These transformations result in the heritable expression of a stable recombinant protein from generation to generation, making it suitable for long-term recombinant protein production (2). Stable/permanent expression systems can be further grouped as follows:

a) Stable nuclear transformation

To date, the nuclear transformation of a crop species is the most common method of producing protein from plants and has produced all the products available in the market. This system necessitates a method for transferring foreign genes into plant cells, typically via *Agrobacterium tumefaciens* or particle bombardment, in which the genes are taken up and stably incorporated into the host nuclear genome (5, 7). There are several advantages with this method. When carried out on a crop species like grains, the protein product is generally accumulated into the seeds. Then, they are harvested in dry form and stored until processing is completed. Also, this approach can be used for large areas of land at the lowest possible cost. Because crops like rice and corn are grown worldwide, the products have the potential to be produced close to the target markets (6). However, it takes time to develop stably transformed plants,

which can take months or years depending on the plant type used for recombinant protein expression (2). The other disadvantage of this method is that some grains, such as corn have the potential to cross with native species or food crops. Still, some technologies can be used to prevent outcrossing, such as mechanical detasseling or genetically based male sterility. Because of higher manual labor requirements, lower yields, and less effective genetics, such technology generally reduces the system's cost advantage (7).

(b) Stable plastid transformation

A plastid transformation system was first described using the tobacco plant (9). This system is based on the insertion of exogenous DNA into specific chloroplast genome sites via homologous recombination (10). Plastid transformation is a superior solution to nuclear transformation because it has numerous advantages, such as preventing transgene escape through amphimixis. Plastids are inherited through the maternal tissue in most species and the absence of chloroplasts in most species of pollen reduces environmental concerns (7). This system's disadvantage is that protein stability will change over time in any fresh tissue molecular farming system, even when refrigerated. Following harvest, extraction and purification must take place at precise times. Tobacco is a highly regulated crop that is not edible. This system does not appear to be capable of producing large quantities of products or edible vaccines (7). The researchers have already extracted a human pharmaceutical protein, amounting to more than 3% to 6% of the total soluble proteins in tobacco chloroplasts. Recently, a very high level (70 % of an entire soluble protein) was reported for a protein antibiotic with the chloroplast system, which was the highest concentration of recombinant proteins until today (11).

(c) Plant cell suspension culture

In-plant cell suspension culture, cell walls are removed, and genes are transferred to the obtained protoplasts and grown in suspension culture (6). In this system, transgenic plants containing a gene coding for the target protein are grown hydroponically in such a way that the desired product is released into the hydroponic medium as part of the root exudate (7). This method has been utilized as an alternative source to produce high-value bioactive compounds in some plant species (12). Plant cell suspension cultures have several advantages that make them suitable for recombinant protein production. They can be grown under aseptic conditions using traditional fermentation technology and are easy to scale up for manufacturing. The regulatory requirements are similar to those established for well-characterized production systems based on microbial and mammalian cells (13). Purification of the desired product is much easier because no tissue disruption is required, and the amount of contaminating proteins is low (7). Therefore, the purification system and its downstream processing are less expensive and easier to implement.

Furthermore, the use of suspension culture can reduce heterogeneity in proteins and sugar (N-glycans) in terms of cell type and size uniformity. Additionally, as a fast system, no transgenic plants are required. However, cell lines can be produced after a few months (6). The main disadvantage of this system is the lack of ability to produce large (kg) quantities of any protein. Further, this method is relatively costly to operate due to the use of greenhouse/hydroponic facilities (7). Plant cell suspension cultures will almost certainly become the preferred choice among plant-based systems to produce high value recombinant proteins soon because they combine the benefits of all other systems (14).

2. Temporary or transient expression systems

Transgenic plants are commonly used to obtain recombinant proteins or to identify protein localization. However, it takes a long time to create transgenic plants, and the yield of the expressed protein is relatively low. Transient expression systems, on the other hand, allow of rapid and high-level expression of recombinant proteins (15). This is because it can be accomplished through agroinfiltration using agrobacteria, viral vectors, or biolistics. During transient expression, the foreign genetic material does not integrate into the plant's genome (2). Before proceeding to the time consuming and costly stable

transformation, transient expression can be used in pilot experiments. Protein expression problems can be identified and corrected, increasing the likelihood of producing the desired protein via stable transformation. Agroinfiltration allows several genes to be expressed simultaneously, which can aid in research (2). This method is not suitable for any protein that needs to be consumed in large quantities. The product must be processed immediately because storage causes plant tissue degradation (7).

Classes of proteins within molecular farming

Proteins currently produced in plants for molecular farming purposes fall into four categories: 1) parental therapeutics and pharmaceutical intermediaries, 2) industrial proteins (e.g., enzymes), 3) monoclonal antibodies (MAbs), and 4) antigens for edible vaccines.

1. Parental therapeutics and pharmaceutical intermediates

This category includes all proteins that are directly used as pharmaceuticals and used in the production of pharmaceuticals. The list of such proteins is long and growing, and it includes products such as thrombin and collagen (both therapeutics) and trypsin and aprotinin (intermediates). In practice, only high-value proteins will be considered candidates for molecular farming.

2. Industrial protein- enzymes

This category includes hydrolases, which include glycosidases and proteases. Laccase, a fungal enzyme used in fiber bleaching and bio-glue a wood product, belongs to a distinct class of industrial enzymes. Enzymes involved in biomass conversion to produce ethanol are potential candidates for molecular farming. All these products are usually distinguished because they are used in large quantities and thus must be produced at a low cost (16).

3. Monoclonal antibody

This category includes all antibody forms (IgA, IgG, IgM, secretory IgA, and so on) and antibody fragments (Fv). Plants can produce both glycosylated and non-glycosylated forms of them. These plant-derived MAbs (plantibodies) have the potential to alleviate the severe production bottleneck that currently exists as dozens of new MAb products compete for market share.

4. Antigen for edible vaccine

Plants can produce specific protein antigens that, when consumed by an animal or a human, cause a humoral immune response. These edible vaccines were used in protection studies, they demonstrated good efficacy. In some cases, the edible vaccine provided better protection than the commercially available vaccine (17).

Products in the market

Avidin

Avidin is a glycoprotein found in the egg whites of birds, reptiles, and amphibians. It is primarily used as a diagnostic reagent (4). The protein comprises four identical subunits, each of which is 128 amino acids long. Avidin is typically obtained in commercial quantities from chicken eggs white. However, the finished product is relatively expensive due to the high cost of keeping live animals. The production of chicken egg white avidin is done using transgenic corn, which uses an avidin gene with an optimized sequence for expression in corn. The resulting avidin had properties nearly identical to those of avidin derived from chicken egg white (7).

Beta-glucuronidase

Beta-glucuronidase (GUS) is a homotetrameric hydrolase that cleaves linked terminal glucuronic acids in monosaccharides and oligosaccharides as well as in phenols. GUS is a popular visual marker in

transgenic plant research. It was first reported to be commercially produced in transgenic corn, where its properties were compared to GUS extracted from *Escherichia coli* (7).

Trypsin

A more recent introduction, maize-derived trypsin, has significant market potential. Trypsin is a protease used in various commercial applications, including the purification of some biopharmaceuticals. The availability of bovine trypsin derived from maize contributes to the growing market for animal-free reagents. Pharmaceutical companies involve in this market desire to eliminate animal-sourced materials and reduce concerns about product contamination by mammalian viruses and prions (7).

Products nearing commercialization

Many companies have started to produce molecular farming products over the last several years. Several of these proteins are normally derived from animal organs. Due to the possibility of animal pathogens being carried along with these proteins, there is a need for low-cost alternatives (7).

Several Pharmaceutical Derived Protein (PDP) products for the treatment of human diseases, including recombinant gastric lipase for the treatment of cystic fibrosis and antibodies for the prevention of dental caries and the treatment of non-Hodgkin's lymphoma, and Hodgkin's lymphoma are nearing commercialization (18), which are shown in Table 1.

Table 1. Pharmaceutical Derived Proteins that are closest to commercialization for the treatment of human diseases (18).

Product	**Class**	**Indication**	**Company/ Organization**	**Crop**	**Status**
Various single chain Fv antibody fragment	Antibody	Non-Hodgkin's lymphoma	Large Scale Biology Corp	Viral vector tobacco	Phase I
CaroRx	Antibody	Dental caries	Planet Biotechnology Inc	Transgenic tobacco	Phase II
E.coli heat labile toxin	Vaccine	Diarrhoea	Prodigene Inc	Transgenic maise Transgenic potato	Phase I Phase I
Gastric lipase	Therapeutic enzyme	Cystic fibrosis, Pancreatitis,	Meristem Therapeutics	Transgenic maise	Phase II
Hepatitis B virus surface antigen	Vaccine	Hepatitis B	Arntzen Group Thomas Jefferson University/Polish Academy of Science	Transgenic potato Transgenic lettuce	Phase I Phase I
Human intrinsic factor	Dietary	Vitamin B 12 deficiency	Cobento Biotech AS	Transgenic *Arabidopsis*	Phase II
Lactoferrin	Dietary	Gastrointestinal infection	Meristem Therapeutics	Transgenic maise	Phase I
Norwalk virus capsid protein	Vaccine	Norwalk virus infection	Arntzen Group	Transgenic potato	Phase I
Rabies glycoprotein	Vaccine	Rabies		Viral vectors in spinach	Phase I

The advantages of using transgenic plants as bioreactors

There are many advantages of plant-based systems compared to other expression systems. Plant bioreactors are inexpensive. This is mainly due to plants producing biological materials using carbon dioxide, solar energy, and inorganic materials. Plant bioreactors are simple to scale up for agricultural use. Additionally, storage and transportation costs are reduced when recombinant proteins are produced in dry textures such as grains. The purification step is skipped when plant tissues containing recombinant protein are edible (6). As a result, certain costly biopharmaceuticals, such as human lysosomal enzymes, can be produced in plant bioreactors, which is especially useful in developing countries. Plant bioreactors cannot be contaminated by animal pathogens due to post-translational modifications (19).

The most significant advantage of transient expression systems is their production speed, which allows them to produce recombinant proteins in a matter of days. This platform is particularly well-suited for developing emergency vaccines and diagnostics, such as those used to combat novel influenza virus strains and SARS-CoV-2, which are required within a few weeks or months of confirming the virus gene sequence. Protein production capacities become scarce very quickly in such emergency scenarios because the production of other drugs and diagnostics cannot be stopped or delayed in the face of a new disease. Transient expression in plants provides a strategy for quickly closing production gaps (4, 20). Plants also have the advantage of administering various types of proteins, such as recombinant subunit vaccines, in the form of raw or partially processed fruits and vegetables (2).

Major barriers to broader market penetration and strategies to overcome them

There are fewer molecular farming products in the market compared to a large number of research studies due to some limitations of molecular farming, such as low plant productivity, high downstream processing costs, and slow translatability (4).

Low producibility: Many plant-based systems have low protein yields compared to industrial microbial and mammalian production platforms. Despite extensive research to optimize protein expression and stability, recombinant protein levels rarely exceed 100 g/kg fresh weight of plant tissue or per liter in suspension cell cultures (21). In general, protein yields from cell suspensions have been low, averaging 1–5 mg/L cell suspension culture. Furthermore, cell suspension cultures are frequently genetically unstable, whereas hairy root culture necessitates the use of expensive bioreactors and does not exploit the autotrophic capacities of the entire plant. An alternative to these methods is to use the plant's natural rhizosecretion mechanism, which in nature plays a role in nodulation, mycorrhizal colonization, growth inhibition of neighboring plants, nutrient acquisition from the soil, and defence against toxic metals (3). The yields of recombinant proteins produced by rhizosecretion are insufficient for commercialization. Several research groups have used various strategies to increase the yield of rhizosecretion, with varying degrees of success. These include the use of a root promoter or a plant signal sequence, the use of A. *rhizogenes* to induce hairy roots on transgenic tobacco plants, and the co-expression of the Bowman-Birk Ser protease inhibitor.

High costs of downstream processing: Downstream processing refers to the recovery and purification of the recombinant protein from plants (2). Because most host proteins are retained within the cell, the secretion of those proteins to the medium facilitates the production of recombinant proteins by microbes and mammalian cells. In theory, this applies to plant cell suspension cultures, hairy roots, and rhizosecretion systems based on whole plant hydroponic cultivation. Plant cells, on the other hand, secrete a number of host proteins into the medium, including proteases that can degrade the target recombinant protein, making the purification process more difficult (22, 23). To reduce the possibility of protease release, one approach is to develop secretion-based systems for recombinant proteins, which will make harvesting easier. For example, single chain Fv and monoclonal antibody heavy chain were

recovered from the surrounding growth medium of genetically modified tobacco cell suspensions, and *Agrobacterium rhizogenes*-derived hairy roots of tobacco were used to secrete assimilate (3).

Using seed-based expression is more beneficial than using a leaf-based expression which requires special attention. Seeds can be stored for longer periods as there are fewer chances of degradation of recombinant proteins expressed in the seed (6). The use of cell secretion systems may also be advantageous because there is no need to disrupt plant cells during replication, thereby avoiding the release of phenolic compounds. The use of affinity tags is another method of facilitating protein recovery. Protein tags should be removed after purification to restore the purified protein's structure to its native state (2). Another system is oleosin fusion technology, in which the recombinant protein gene sequence is fused to the sequence of an oil body-specific endogenous protein oleosin in rapeseed and safflower, and the protein is separated by an endoprotease digestion after purification (6).

Slow translation to applications: The third barrier is uncertain intellectual property and regulatory landscape compared to industrial microbial and mammalian cell expression systems, which have a long track record, particularly in biopharmaceutical manufacturing. As a result, the industry continues to view molecular farming as a risk and, in most cases, prefers to rely on tried and tested platforms. Molecular farming companies typically have Intellectual Property portfolios for their own expression systems, which should give industrial partners confidence. However, limiting industry partners to individual proprietary technologies effectively locks them in by the limitations of individual platforms, limiting their freedom to operate (4).

The Pharma-Planta Project is a group of scientists who study the intellectual property landscape in relation to plant-based pharmaceuticals (PMP). The goal is to make PMP end products and processes available and affordable to low- and middle-income countries. Because there is a significant patenting activity in the PMP arena, such analyses are critical to removing any potential barriers to ensuring freedom to operate (3).

Biosafety and the challenges of protein production and biomedicines in molecular farming

One of the most challenging issues is public concern about introducing genetically modified crops (2). Even though some of these products may pose a significant risk to the public, many would not pose a risk if they were introduced inadvertently. Many of the products in the pipeline are already in the food supply or are endogenous to humans (7). Lack of communication among the authorities dealing with research, biosafety and trade is an important issue that has hindered developments in molecular farming (2).

There are some environmental concerns about introducing transgenes into the food chain, which necessitates careful management and supervision (6). The long-term impact of molecular farming products on the environment is challenging to assess. An important concern is the food chain contamination with plant-made pharmaceuticals. This could happen as a result of the transfer of genetic material from transgenic plants to food crops. To avoid this, strict rules need to be put in place, such as geographically isolating the transgenic crop and growing in greenhouses instead of open fields and harvesting and processing transgenic plants with separate equipment or properly decontaminating the equipment if the same equipment is also used for food crops. Labeling genetically modified products are critical so that the consumer can choose based on their preferences (2, 24). Another source of concern is the use of agrobacterium in grain transformations, as grains are important crops in pharmaceutical protein production. These products can also lead to immune system reactions, which cause severe allergic reactions (6).

Future prospective of molecular farming

Current mammalian cell-based recombinant pharmaceutical manufacturing systems are incapable of meeting the demands. These systems' manufacturing capacity, safety, and reliability have been deficient on multiple occasions (14). The key to success in the future will undoubtedly be the level of expression of the recombinant protein in plants. The expression level affects the cost of growing, processing, extraction, purification, and waste disposal. Keeping the protein out of pollen can reduce inadvertent exposure to the environment, but this does not remove the possibility that the pollen will outcross with other plants and intermix with food crops. The regulatory agencies impose physical isolation requirements to prevent this from occurring (7).

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 for producing IgA in crops. The challenges 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 (6).

The use of plants in pharmaceutical manufacturing, in general, appears to be improving, especially with the recent approval and licensure of Protalix's carrot cell that produced Gaucher disease therapeutic enzyme. The prospects for animal vaccines in general, as well as possibly human therapeutic vaccines, appear promising. The length and rigor of the human prophylactic vaccine developmental and clinical testing path compared to animal and human vaccines are a massive obstacle to the commercial production of novel bio-farmed vaccines (14, 25).

Transient expression in plants provides a strategy to close production gaps quickly in an emergency. Transient expression allows plants to be grown while the pathogen's genome sequence is being investigated. The plants can then be ready for protein production as soon as antigen sequences are available. Therefore, many academic and industrial groups use this technique to produce diagnostics and therapeutics against SARS-CoV-2 (20).

Conclusion

The goal of molecular farming is to produce large quantities of active and safe pharmaceutical proteins at a low cost. Nowadays, gene transfer methods in plants have advanced significantly as a result of scientific advances in the field of biotechnology. Compared to other microbial and animal expression systems, these transgenic plants have several advantages in terms of ease of production, cost, safety, and producing pharmaceutical biomolecules. So far, many valuable pharmaceutical proteins and antibodies have been produced using this method, which has significantly aided patient treatment, particularly in developing countries where the production and preservation costs of such medicines are prohibitively expensive. However, there are some disagreements, such as public acceptance, transgene escape and biosecurity, clinical and commercialization investigations of products, and so on, which have made it a complex area, but it is hoped that with the efforts of researchers and scholars, molecular farming will achieve great success in the near future.

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