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Review Article

Health Positive Uses of Genetically Modified Food Crops as a Source of Biopharmaceuticals

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ABSTRACT

Molecular farming, or bio-pharming, has recently received much of attention for production of valuable recombinant proteins, with a few already being marketed. The use of whole plants for synthesis of pharmaceutical proteins offers various advantages in economy, scalability and safety over conventional production systems. GM plants are suitable for the inexpensive production of large amounts of functional, recombinant macromolecules, such as blood substitutes, vaccines, anti-cancer antibodies, plasma proteins, enzymes, cytokines and growth factors, and the expressed proteins, ranging from the smallest antigen-binding domains, to full-length, and even multi-meric proteins, are almost comparable to their mammalian counterparts. Delivery of a biopharmaceutical product by direct ingestion of the modified food crops removes the need for purification. Such biopharmaceuticals and edible vaccines can be stored and distributed as seeds, tubers, or fruits, making immunization programs in developing countries potentially cheaper and easier. It is anticipated that this technology has the potential to greatly benefit human health by making safe recombinant pharmaceuticals widely available. Here, we discuss facts, recent developments and perspectives of this field in detail.

1.INTRODUCTION

Formerly, the cost of biopharmaceuticals (biological substances with health-promoting properties) limited their availability. Being in charge for a new wave of high-value drugs based on mammalian proteins, the biopharmaceutical industry currently relies on microbial production systems (mostly *E. coli* and yeast) and cultured mammalian cells, but both are fermenter-based and hence expensive and limited in capacity (Daniell *et al.*, 2001; Streatfield, 2005; Naqvi *et al.*, 2011). As the demand for biopharmaceuticals is expected to increase, it would be wise to supply them in significantly larger amounts, on a cost-effective basis. As major long-term traditional breeding programs have been unsuccessful for this aim, genetic engineering is the most commonly used tool to generate crop lines synthesizing biopharmaceuticals (Acosta & Chaparro, 2008; Flinn & Zavon, 2004). Plant Molecular Farming (PMF) consists of using transgenic plants as production platforms for the synthesis of pharmaceutical or industrial compounds (Breyer *et al.*, 2009). While plants have long been exploited as a source of medicinal compounds, molecular farming has the capacity to provide a novel source of molecular medicines – medications whose medical benefits are understood at a molecular level (Sourrouille *et al.*, 2009; Schillberg *et al.*, 2003). Plants are capable of synthesizing and assembling nearly every kind of complex molecules and have superiorities over traditional microbial and mammalian production systems. This demonstrates the promise of using transgenic plants for the molecular farming of recombinant therapeutics, including vaccines,

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diagnostics, and antibodies (Fischer & Emans, 2000; Hellwig *et al*., 2004; Ko *et al*., 2009).

Since their development in 1990s, plant-produced pharmaceutics have emerged as a brand-new alternative that will benefit especially those who cannot afford the high cost of current treatments (Desai *et al*., 2010). For example, vaccines against hepatitis B and rabies viruses are manufactured by yeast cultures insufficiently for the needs of many developing countries. The overall improvements in expression of various recombinant immunoglobulins and other heterologous proteins in plants, may not only supply an adequate quantity, but also allow new applications, such as topical (mucosal) application or oral delivery (Fischer *et al*., 2000; Alderborn *et al*., 2010). In this short review we debate merits and demerits of plant bio-pharming and how we are moving toward the molecular farming of therapeutic antibodies becoming an economic and clinical reality.

2. Plant-based systems vs. microbial and mammalian cell lines for bio-pharming

2.1. Preferences

Pharmaceutical and industrial applications of Genetically Modified (GM) plants deserve a growing interest. Plants have all the advantages of mammalian cells (i.e. they can express complex molecules and can fold and assemble them properly), yet they are indeed as economical as microbial systems but unlimited in capacity, and have additional advantages, including:

- Rather straightforward and cost-effective culture and processing technology.
- Ability to perform most post-translational modifications needed for giving functional proteins.
- Increased safety to human health of products synthesized in plant systems since the risks arising from the contamination with human pathogens or toxins are minimized.
- A diversity of platforms offering niche concepts, so as long-term storage in some organs (seed, tubers) is achievable and purification processes can be avoided (regarding direct administration of oral vaccines through plant tissues containing the recombinant protein) or greatly facilitated (when recombinant product can be targeted directly into certain intracellular compartments) (Breyer *et al*., 2009; Naqvi *et al*., 2011).

Producing antibodies for 'passive' immunization is one important target of molecular pharming, while 'active' immunization with antigens produced in plants is another emerging field. Beside all the advantages of safety and scalability listed earlier, studies have shown that the antigens produced by plants can induce immune responses at both the mucous and serum levels, and can be administered by injection as well as orally, the latter providing significant cost benefits by removing the necessity for extensive downstream processing (Naqvi *et al*., 2011). Several potential characteristics of plant-derived vaccines could make them particularly attractive for controlling infectious diseases in developing countries:

- The vaccines would be orally active, thus eliminating the need for injection and the associated costs and hazards.
- Oral activity is associated with the ability of plant-derived vaccines to evoke mucosal immunity, which is valuable for a number of infections that are transmitted through the mucosa.
- Plant-derived oral vaccines should be heat stable, thus largely eliminating the need for a cold storage.
- It might be possible to make multi-antigen vaccines either by multiple gene splicing or by mixing various plant-derived vaccines.

So, a very important potential aspect of plant-derived vaccines is that developing countries could launch and carry forward their development and production on a very large scale and at very low cost (Krattiger & Mahoney, 2007).

2-2- Drawbacks

The use of plants as production systems for pharmaceuticals or industrial compounds is a controversial issue. There are several arguments in this context, including risk of GM plants entering the food chain, risk of gene transfer to related species, and co-existence aspects. Adoption of non-food plants for plant molecular farming, although having advantages in term of biosafety (reducing the possibility of unintentional contact and contamination of the food or feed chains), might pose new challenges due to the lack of knowledge about the genetics and biology, the lack of domestication, limited experience with the cultivation and production of toxins or allergens that could interfere with the processing of the desired compound (Breyer *et al*., 2009).

Furthermore, certain forms of posttranslational modification are not carried out in plants or occurred differently. For example, most therapeutic proteins are glycoproteins and N-glycosylation is often essential for their stability, folding, interactions, and biological activity. Recombinant glycoproteins of mammalian origin expressed in transgenic plants largely retain their biological activity. However, they are not ideally compatible with therapeutic applications in humans

because of subtle differences in N-glycan structures (Ko *et al*., 2008; Gomord *et al*., 2010). This differences in glycosylation patterns apparently have no effect on antigen-binding or specificity, but there is some concern about potential immunogenicity in humans (Larrick *et al*., 2001), which is a major barrier to the parenteral administration of plant-made biopharmaceuticals (Ahmad *et al*., 2010). Also hydroxylation of proline residues is not spotted in plants. Additionally, the homogeneity of plant-derived glycoproteins is also important for batch-to-batch consistency. It depends to a certain extent on species. For example, while tobacco produces very heterogeneous glycans, glycoproteins produced in alfalfa have homogenous glycan chains (Lerouge *et al*., 2000; Schillberg *et al*., 2005). Thereupon, research is ongoing to equip plant cells with human-equivalent carbohydrate modification machinery. In addition to in vitro enzymatic modeling glycoproteins, there are two gene manipulation strategies to humanize plant N-glycans. One is retaining the recombinant glycoproteins in endoplasmic reticulum (ER), the site where few specific modifications of N-glycans occurs. The other is inhibiting the plant endogenous Golgi glycosyltransferase and/or adding heterologous mammalian glycosyltransferase (Chen *et al*., 2005; Alderborn *et al*., 2010).

3. Plant-made recombinant pharmaceutical industry: challenges and achievements

3.1. Stability of transformation

Stable plant transformation is defined as the genomic integration of a transgene. Both the nuclear and plastid genomes can be transformed via various transgenesis methods. Stable transgenic plants can be used to produce organs rich in the recombinant protein for long-term storage or direct processing (Fischer et al., 1999; Schillberg et al., 2005). Transient expression through agro-infiltration or viral vectors can be used to test the function of an expression construct before large-scale production in stable transgenic lines. It is also possible to rely completely on transient expression for PMF. In a recently developed second generation of plant viral vectors only the viral elements required for efficient expression of the transgene are maintained. These vectors clearly limit the risk of lateral or systemic dispersal, but require infection of *Agrobacterium* for delivery of transgenes into the cell (Schillberg *et al*., 2005; Gleba *et al*., 2007).

3.2. Expression levels

Pharmaceutical proteins can either be purified from the plant or expressed in edible plants and administered orally. In both cases, high expression levels are of pivotal importance because it makes downstream processing (extraction, purification and characterization) more efficient and cost-effective and means that fewer plant biomass or area are required to achieve a target yield. For edible vaccines, high protein accumulation is absolutely essential because stimulation of the mucosal immune system generally requires much higher doses of the antigen than conventional vaccination by injection into the bloodstream (Naqvi *et al*., 2011; Maliga & Bock, 2011).

Expression levels depend on the site of integration, regulatory elements used to enhance transcription/translation and the stability of the foreign protein. Protein stability is probably the most important factor limiting yields in molecular farming. This can be addressed, at least in part, by appropriate sub-cellular targeting which influences the folding, assembly and post-translational modification of proteins, thus affecting their stability and final yield. For example, recombinant antibodies targeted to the secretory system generally accumulate to much higher levels (>1000-fold) than those synthesized in the cytosol, and further yield increases occur when they are retained in the endoplasmic reticulum rather than secreted to the apoplast (Schillberg *et al*., 2002; Schillberg *et al*., 2005; Daniell *et al*., 2005). Protein yield is also influenced by the choice of genotype/explant for production and expression levels. Certain genotypes express proteins at higher levels than others. The choice of variety also influences the ease of transformation and the ease of purification. Other factors that may be involved include zygosity, which has been shown to boost transgene expression, and the inclusion of purification tags to facilitate extraction and purification (Naqvi *et al*., 2011). It is also important to remember that the ability of the host plant to produce a recombinant protein depends on its spare metabolic capacity. For example, a recombinant protein that is particularly demanding for a rare amino acid is unlikely to accumulate to high levels (Schillberg *et al*., 2005).

3.3. Economics of producing edible vaccines

Extraction plant-made pharmaceuticals usually make up the largest fraction of (80% or more) of total production costs. Because downstream processing represents such a high proportion of production costs in plants, it is important to ensure that optimal purification and processing methods are used in order to maximize the output of high amounts of pure, functional proteins. Tobacco, while advantageous in terms of biomass yield, can contain toxic metabolites that have to be removed. Using edible plant organs as the production vehicle may be advantageous for certain recombinant proteins, such as vaccines and antibodies, as this allows direct oral administration with no processing costs at all (Maliga & Bock, 2011).

It is easier to extract proteins from watery tissues (such as tomato fruits) than from dry material (e.g. cereal grains). However, recombinant proteins can be modified

by proteases and phenolic compounds released from watery plant tissue (Schillberg *et al*., 2005). Production in seeds is advantageous as high levels of the protein can accumulate in a small volume, which minimizes the costs associated with processing. Cereals are advantageous in this context because they are food crops with FDA GRAS status, having been consumed for millennia by billions of people without ill effect (Naqvi *et al*., 2011). For production of edible vaccines, there is a need for robust, high-level regulated gene expression so that vegetative growth and the production phases can be separated and the production of proteins induced prior to harvest. Prototypes of such regulatory elements are metabolite activated ribo-switches and other inducible expression systems, which, however currently still lack the robustness required for practical applications (Maliga & Bock, 2011).

3.4. Risk assessment

As with current GM plants, all transgenic plants intended for molecular farming must go through a thorough health and environmental risk assessment before they can be used. To avoid toxicity or off-target effects, it may be necessary to verify that the favorite metabolite accumulates in a certain tissue (e.g. tubers and seeds) or in specific subcellular compartments (e.g. the vacuole). Production of these products in seeds has an additional advantage in that proteins do not normally interfere with vegetative plant growth, and this strategy also limits adventitious contact with non-target organisms (Fischer *et al*., 2012; Naqvi *et al*., 2011; Breyer *et al*., 2009).

Where containment of transgene is an issue, plant cell cultures grown in industrial scale fermenters are optimal since these are closed vessels. Plants which can be grown in greenhouses are also attractive. In any crop species, chloroplast-expression of the transgene will result in natural containment since functional chloroplast DNA is not transmitted through the pollen (Schillberg *et al*., 2005). This will be discussed later. Various therapeutic proteins have been tested for clinical evaluation including human serum proteins (epidermal growth factor), monoclonal antibodies, such as oral vaccine against Hepatitis B, antigenic peptides for rabies virus, human intrinsic factor, tuberculosis and HIV, antibodies to treat cancer, cardiovascular diseases, gastric lipase in the fight against cystic fibrosis, human lactoferrin and human lysozyme. In some cases, the function of expressed recombinant proteins can be rapidly analysed by expression in microbes or by transient expression in intact or virally infected plants (Fischer *et al*., 1999; Krattiger & Mahoney, 2007; Spok *et al*., 2008). Tables 1 and 2 respectively represent some cases of plant-produced healing bio-molecules studied worldwide and in Iran.

Table 1.

Pharmaceutical proteins produced in maize, intended for administration to humans (adapted from Naqvi *et al*. 2011; Daniell *et al*. 2002).

Produced bio-molecule	Indication	Status
Vaccines		
E. coli heat labile toxin (ETEC LtB)	Diarrhea	Clinical trials
PTGEV, porcine transmissible gastroenteritis virus (oral veterinary vaccine)	Piglet gastroenteritis	Clinical trials
Newcastle disease virus (NCDV) (oral veterinary vaccine)	Protection against viral changes	Tested in chickens. Determination of the appropriate dose
Antibodies		
Monoclonal antibody E559	Rabies	Experimental studies
Avicidin	Colorectal cancer	Clinical trials
Humanized IgG	Therapy use (inhalation): respiratory syncytial virus	Tested in animal models
Humanized IgG	Therapy use (oral): *Clostridium difficile*	Tested in animal models
Humanized IgG	Sperm (topical): contraceptive	Tested in animal models
2G12	HIV	Neutralization of the virus
Monomeric hIgA1	Herpes simplex virus (HSV) (anti-herpes)	Experimental studies
Monomeric hIgA1	SAGA-1 antigen	Experimental studies
Interleukin 13 (IL-13)	Asthma, type I diabetes	Clinical trials
Human Alfa interferon (IFN α-2b & α-2a)	Malignant carcinoid tumors, hepatitis C	Clinical trials
scFv antibody	Against carcinoembryonic antigen	Clinical trials
Other products		
hGC enzyme	Replacement therapy in patients with Gaucher's disease	Clinical trials
Human somatotropin (hST)	Hypopituitary dwarfism, Turner syndrome, chronic renal failure, HIV wasting syndrome	Clinical trials
Human serum albumin (HSA)	Regulation of osmotic pressure of the blood	Clinical trials
Human insulin like growth factor (IGF-	Growth of muscle and other tissues,	Available in Sigma

1)	diabetes	catalogue
Gastric lipase	Cystic fibrosis, pancreatitis	Clinical trials
Lactoferrin	Gastrointestinal infections	Clinical trials
Avidin	Diagnostic use	Available in Sigma catalogue
Trypsin	Wound care/insulin manufacture	Available in Sigma catalogue
Aprotinin	Protease inhibitor in tissue cultures and cardiac surgery	Marketed by Prodigene
Recombinant human proinsulin (rhProinsulin)	Diabetes	Experimental studies

3.5. Chloroplast as bioreactors for bio-pharming

Plastids of higher plants are semi-autonomous organelles with a small circular double stranded DNA with high copy numbers and have their own transcription-translation machinery. The solar powered chloroplast is one of the organelles known as plastids in plant cells and eukaryotic algae. In recent years, the development of optimized expression strategies has given a huge boost to the exploitation of chloroplasts in molecular farming. Besides providing biotechnologists with an ideal site for the overproduction of foreign genes, transgenic plastids offer advantages with reference to biosafety. Since chloroplasts are almost always maternally transferred to the next progeny, they pose lower environmental risks concerning transgene flow from transplastomic plants to the weedy or wild relatives. In this fashion, genes introduced into the chloroplast genomes can move only through seed, whereas the genes introduced into the nuclear genome can move through seed as well as pollen. It also reduces the potential toxicity of transgenic pollen to non-target insects (Daniell *et al*., 2002; Bock & Warzecha, 2010; Bansal & Sharma, 2003).

Arguably, the most alluring feature of transplastomic plants is their enormous capacity to accumulate foreign proteins (up to 46% of the total soluble protein). This is, at least in part, due to the high number of chloroplasts per cell, the large volume of the cell occupied by the chloroplast compartment, and their highly polyploid genomes, with hundreds or even thousands of copies being present in a single cell. The carbon dioxide-fixing enzyme Rubisco provides a case in point for the high protein accumulation capacity of the chloroplast (Maliga & Bock, 2011). With the concept of oral vaccination in mind, expression of a large number of antigens has been attempted in chloroplasts. Moreover, the ability of chloroplasts to form disulfide bonds and to fold human proteins has opened the door to high-level production of biopharmaceuticals in plants. Such folding and assembly can minimize the need for expensive in vitro processing of pharmaceutical proteins after their extraction. Furthermore, foreign proteins observed to be toxic in the cytosol are non-toxic when accumulated within transgenic chloroplasts. Bioencapsulation of pharmaceutical proteins within plant cells offers protection against digestion in the stomach but allows successful delivery to the target tissues. The other advantages include lack of gene silencing (both at transcriptional and translational levels) and position effects (Bansal & Sharma, 2003; Clarke & Daniell, 2011; Maliga & Bock, 2011).

Several recent advances should make chloroplasts even more attractive as biopharmaceutical reactors. These include possibility of engineering multiple foreign genes as operons; transformation without use of antibiotic markers; elimination of resistance genes subsequent to transformation; and the transformation of edible crops such as potato and tomato (Daniell *et al*., 2002). Extraordinarily unprecedented high expression levels have recently been obtained with protein antibiotics derived from phage lytic proteins (so-called endolysins). These proteins efficiently digest the cell wall of pathogenic bacteria and, accordingly, have huge potential as next-generation antibiotics. The chloroplast may be an ideal site for the production of these protein antibiotics because: (1) chloroplasts do not have a cell wall and, therefore, should be able to accommodate large amounts of them and (2) chloroplasts possess a very similar set of proteases as bacteria to which phage endolysin proteins are highly resistant. Importantly, the plastid-produced endolysins from tobacco were highly active and stable and efficiently killed the target pathogens (pathogenic streptococci) (Maliga & Bock, 2011). However, there are limitations of chloroplast transformation:

- Proteins expressed in this way, contrary to those expressed in the nucleus, are devoid of mammalian-type post-translational modification, which limits the range of proteins of commercial interest that can be produced.

- Implementation in new crops will depend on the availability of a genetic line that is suitable for repeated cycles of plant regeneration (to attain homoplasmy) and a suitable selectable marker gene. Prolonged selection procedures under high selection pressure are required for the recovery of transformants

- Transformation frequencies are much lower than those for nuclear genes.

- The methods of transgene transfer into chloroplasts are limited, and they are either expensive or require regeneration from protoplasts.

- These transformation systems are far more successful with tobacco than with other plant species, and in green chloroplasts rather than other plastids (Daniell, 1999; Bock, 2001; Daniell *et al*., 2002; Daniell *et al*., 2005; Gao *et al*., 2012).

To exploit the chloroplast compartment for the production of orally delivered pharmaceuticals, it is important to extend chloroplast transformation to edible crops. Studies predict the feasibility of expressing foreign proteins in the other plastids of edible plants (e.g. in tomato fruit and potato tuber). So far, very few of the chloroplast-produced antigens have successfully been tested in oral vaccination experiments. Moreover, all studies conducted so far have been confined to small experimental animals, and the concept of oral vaccination with transplastomic plants still awaits its validation in large mammals and ultimately in humans (Daniell, 1999; Daniell *et al*., 2002; Tregoning *et al*., 2003; Davoodi-Semiromi *et al*., 2010; Maliga & Bock, 2011).

Table 2- Examples of achievements in Molecular Farming in Iran.

Produced bio-molecule	Indication	Technology	Transgenic plant	Reference
Single-domain monoclonal V_{HH} antibody	Antibody against MUC1 mucin antigen	Nuclear transformation	tobacco, canola	Ismaili *et al*., 2006 Rajabi-Memari *et al*., 2006 Dymyad, 2007
Human Gamma interferon (INF-γ)	Immunodeficiency syndrome tuberculosis, AIDs, Leishmaniasis, Salmonellosis, melanoma & neuroblastoma cells	Plastid transformation/ Nuclear transformation	tobacco, canola, lettuce	Bagheri, 2009 Taheri-javan, 2008 Rahimifar, 2009
Human tissue *type plasminogen activator* (*tPA*)	Fibrinolysis and thrombolysis of blood clot	Nuclear transformation	tobacco, canola	Masoumi-Asl *et al*., 2010 Seifi-Nabi-Abad, 2009
Protective antigen (PA) gene from *Bacillus antracis*	Anthrax	Nuclear transformation	lettuce	Honari, 2008
Human proinsulin	Diabetes	Plastid transformation/ Nuclear transformation	lettuce, potato, tobacco, strawberry, cucumber	Mohebodini *et al*., 2009 Kashani *et al*., 2012
Hepatitis B vaccine	Hepatitis B virus	Nuclear transformation	tomato	Baesi *et al*., 2011

4. CONCLUSION

Bio-pharming (where the expressed protein itself is the desired molecule) along with bio-fortification or metabolic engineering (where the transgene alters the metabolic phenotype and leads to the production of bio-available concentrations of a low-molecular-weight compound or nutrient) can be a promising solution for

health promotion in poor countries (Krattiger & Mahoney, 2007; Acosta & Chaparro, 2008). Plant-derived biopharmaceuticals are cheap to produce and store, easy to scale up for mass production, and safer than those derived from animals. Other advantages of plant-derived therapeutic proteins include elimination of hospitals and health professionals for their delivery and the use of renewable resources for their production (Daniell, 1999; Chebolu & Daniell, 2009; Maliga & Bock, 2011). The regulatory framework for plant-made pharmaceutical and industrial products is evolving, but the emerging regulations should be based on solid scientific facts principles and not risks that are poorly understood and largely unquantified or media-fueled speculations. We must avoid fearing the 'bogeyman under the bed', and must instead study the impact of plant factories rigorously and carefully. It has been suggested that a more practical and productive approach might be to base risk assessments on ecotoxicological principles, in which research problems refer to relevant assessment endpoints and risk hypotheses predict no adverse effects

of transgenic plants on the assessment endpoints (Daniell *et al*., 2002; Naqvi *et al.*, 2011).

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