

# A Critical Review of the Concept of Transgenic Plants: Insights into Pharmaceutical Biotechnology and Molecular Farming

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## Abstract

Using transgenic plants for the production of high-value recombinant proteins for industrial and clinical applications has become a promising alternative to using conventional bioproduction systems, such as bacteria, yeast, and cultured insect and animal cells. This novel system offers several advantages over conventional systems in terms of safety, scale, cost-effectiveness, and the ease of distribution and storage. Currently, plant systems are being utilised as recombinant bio-factories for the expression of various proteins, including potential vaccines and pharmaceuticals, through employing several adaptations of recombinant processes and utilizing the most suitable tools and strategies. The level of protein expression is a critical factor in plant molecular farming, and this level fluctuates according to the plant species and the organs involved. The production of recombinant native and engineered proteins is a complicated procedure that requires an inter- and multi-disciplinary effort involving a wide variety of scientific and technological disciplines, ranging from basic biotechnology, biochemistry, and cell biology to advanced production systems. This review considers important plant resources, affecting factors, and the recombinant-protein expression techniques relevant to the plant molecular farming process.

## Introduction

Currently, critical challenges in soil and water resources, as well as climate change, have resulted in the human population outpacing the available agricultural products. Thus, scientists are attempting to find various ways to increase the quality and quantity of the food, pharmaceutical and the industrial products of plants within limited arable spaces (De La Fuente et al., 2013). Phenotypic selection was the first action taken by humans to establish plant breeding as a reliable classical approach to this problem. Indeed, the history of plant breeding can be traced to the dawn of civilization, 10,000 years ago, when early societies began to shift from being hunter-gatherers to being members of agrarian communities (Tilman et al., 2002).

Gregor Mendel reached the first milestone in modern plant breeding. The results of his plant-breeding investigations led to the discovery of genetic functions through focusing on DNA as the basis of trait heredity (Acquaah, 2009). Although, conventional plant breeding has been used to overcome the lack of sufficient food or feed (Lopes et al., 2012), the success of this method is largely dependent on the breeder's experience and on phenotypic selection; consequently, inaccurate predictions can be made and low-efficiency breeding can occur (Mewett et al., 2007). Moreover, other limiting factors, such as the method being time consuming, the difficulty in finding the most related parents of a new generation of plants, selecting the best crossing method according to the plant traits desired, the high number of back-crosses required and the difficulties in increasing the expression of the favoured traits are the main concerns in plant breeding (De La Fuente et al., 2013).

Genetic modification (GM) is a new method that researchers have been using to increase the yield of plant products by improving certain traits, including the responses of herbs to abiotic and biotic stresses (Tait, 1999; Ashraf et al., 2008). Background genetic transformation is the most significant application of GM; in this process various methods are utilized to introduce desirable traits into the host genome while concentrating on preserving the individual characteristics of the plant (Ziemienowicz, 2013). The most important plants that have been successfully subjected to gene transformation programs are industrial plants, cereal crops, legumes, root plants, vegetables, turf grasses, tropic plants, woody species, medicinal and ornamental plants, as well as fruit plants (Wang, 2006a; Wang, 2006b). These plants and their related species have been presented in Table 1. In gene transformation processes, the gene(s) of interest of donor plants, bacteria or viruses are transferred to host

**Table 1.** Examples of genetically transformed plant species using various transformation methods.

Model Plants	Plants	References	Model Plants	Plants	References
Root plants	Carrot ( <i>Daucus carota</i> )	(Zhang and Huang, 2010)	Ornamental plants	Carnation ( <i>Dianthus caryophyllus</i> )	(Burana et al., 2014)
	Cassava ( <i>Manihot esculenta</i> )	(Xu et al., 2012)		Orchids ( <i>Cymbidium</i> spp., <i>Oncidium</i> , <i>Phalaenopsis</i> )	(Shu-Hong et al., 2015)
	Potato ( <i>Solanum tuberosum</i> )	(Park and Cheong., 2002)		Rose ( <i>Rosa hybrida</i> )	(Condliffe et al., 2003)
	Sweet potato ( <i>Ipomoea batatas</i> )	(Goo et al., 2015)		Petunia ( <i>Petunia hybrida</i> )	(Kamenarova et al., 2005)
Turf grasses	Perennial ryegrass ( <i>Lolium perenne</i> )	(Fischer et al., 2012)	Industrial plants	Sunflower ( <i>Helianthus annuus</i> )	(Sankararao and Rohini., 1999)
	Bermudagrass ( <i>Cynodon</i> spp.)	(Fischer et al., 2012)		Indian Mustard ( <i>Brassica juncea</i> )	(Fischer et al., 2012)
	Switchgrass ( <i>Panicum virgatum</i> )	(Wang, 2006a)		Canola ( <i>Brassica napus</i> )	(Wang, 2006a)
Tropic plants	Banana ( <i>Musa</i> spp.)	(Wang, 2006a)		Cotton ( <i>Gossypium hirsutum</i> )	(Ashraf et al., 2008)
	Pineapple ( <i>Ananas comosus</i> )	(Wang, 2006a)	Legume plants	Alfalfa ( <i>Medicago sativa</i> )	(Wang, 2006a)
	Sugarcane ( <i>Saccharum</i> spp.)	(Ziemienowicz, 2013)		Beans ( <i>Phaseolus</i> spp.)	(Wang, 2006a)
	Citrus spp., coffee ( <i>Coffea</i> spp.)	(Ziemienowicz, 2013)		Soybean ( <i>Glycine max</i> )	(Ziemienowicz, 2013)
	Papaya ( <i>Carica papaya</i> )	(Ziemienowicz, 2013)		Pigeonpea ( <i>Cajanus cajan</i> )	(Ziemienowicz, 2013)
Woody species	Eucalyptus, Pine ( <i>Pinus radiata</i> )	(Ziemienowicz, 2013)		Peanut ( <i>Arachis hypogaea</i> )	(Wang, 2006a)
	Cork oak ( <i>Quercus suber</i> )	(Dunwell, 2009)		Peas ( <i>Pisum sativum</i> )	(Ziemienowicz, 2013)
	Poplar ( <i>Populus</i> spp.)	(Wang, 2006b)			
	Rubber tree ( <i>Hevea brasiliensis</i> )	(Wang, 2006b)		Chickpea ( <i>Cicer arietinum</i> )	(Ziemienowicz, 2013)
Medicinal plants	Ginseng ( <i>Panax ginseng</i> )	(Wang et al., 2005)	Cereal crop	Rice ( <i>Oryza sativa</i> )	(Dunwell, 2009)
	Hemp ( <i>Cannabis sativa</i> )	(Wang et al., 2005)		Maize ( <i>Zea mays</i> )	(Ziemienowicz, 2013)
Nuts and fruits	Blueberry ( <i>Vaccinium corymbosum</i> )	(Dunwell, 2009)		Rye ( <i>Secale cereal</i> )	(Dunwell, 2009)
	Walnut ( <i>Juglans</i> spp.)	(Fischer et al., 2012)		Sorghum ( <i>Sorghum bicolor</i> )	(Dunwell, 2009)
	Strawberry ( <i>Fragaria x ananassa</i> )	(Dunwell, 2009)		Wheat ( <i>Triticum aestivum</i> )	(Dunwell, 2009)
	Apple ( <i>Malus x domestica</i> )	(Ziemienowicz, 2013)		Barley ( <i>Hordeum vulgare</i> )	(Ziemienowicz, 2013)
Vegetable plants	Tomato ( <i>Lycopersicon esculentum</i> )	(Wang, 2006a)	Model plants	Arabidopsis ( <i>Arabidopsis thaliana</i> )	(Wang et al., 2005)
	Lettuce ( <i>Lactuca sativa</i> )	(Ziemienowicz, 2013)		Tobacco ( <i>Nicotiana tabacum</i> , <i>N. benthamiana</i> )	(Fischer et al., 2012)

plants using various methods, such as *Agrobacterium* mediation, particle bombardment (biolistics), the microprojectile gun method, electroporation, a silicon-carbide fibre-based technique, polyethylene glycol (PEG)-mediated protoplast fusion, and liposome-mediated gene transfer, allowing the investigation of their effects (Rivera et al., 2012).

To date, transgenic plants have been engineered for the following purposes: to increase their tolerance of abiotic and biotic stresses (Lau et al., 2014; Wang et al., 2005), to

improve their nutrient uptake (Sahebi et al., 2014), to reduce the effects of harmful agrochemicals and increase crop yields (Ziemienowicz, 2013), to allow phosphorus absorption and reduce the use of fertilizer (Hirsch and Sussman, 1999), to enhance grain production and the growth rate under the condition of low-iron availability (Takahashi et al., 2001) and to increase the aluminium tolerance of papaya and tobacco (De la Fuente et al., 1997). Some of the most significant genes transformed in the four plants of tobacco, rice, potato and Arabidopsis



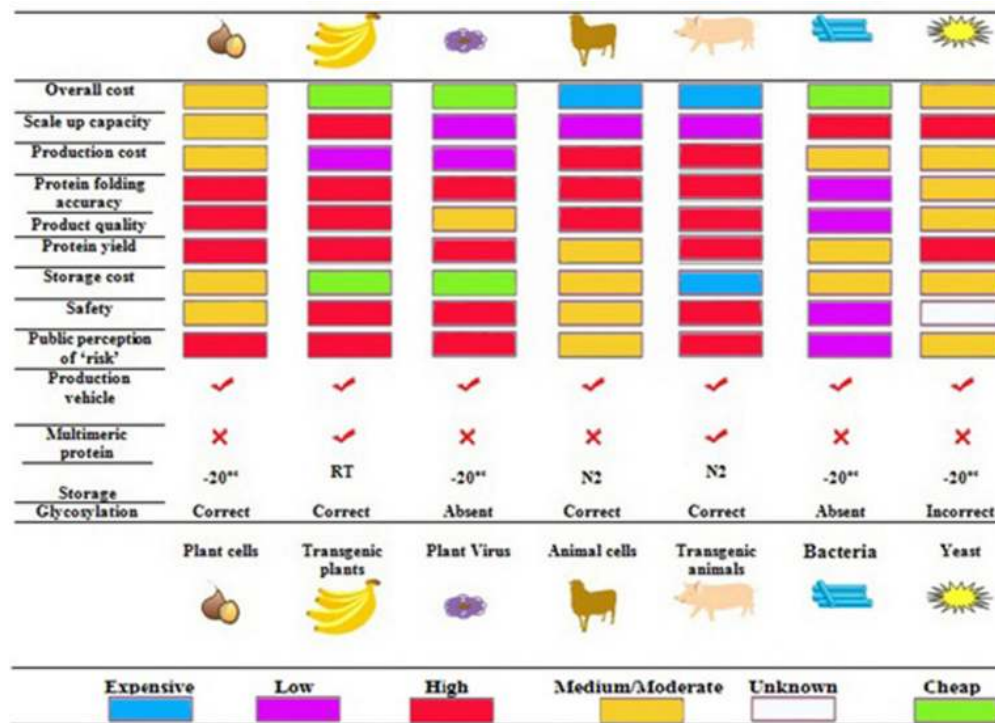
and microbial cells). Accordingly, plant molecular farming is one of the outcomes of this process, and the major source in this field is the transgenic plant cell (da Cunha et al., 2014).

Compared with other transgenic products obtained from transgenic bacteria, fungi, and animals, which are the most common models for recombinant-protein production, plant-based therapeutics are produced with the lowest cost, leading to an economic justification for their use (Häkkinen et al., 2014). The availability of personnel with experience and expertise in planting, harvesting, and processing plant material are some of the other benefits of recombinant-protein production using molecular farming of higher plants. In addition, the dissimilarity of the pathogenic factors of plants and humans decreases the risk of microbial interactions that could negatively affect the quality of the final products. Technically, the stability of recombinant proteins within plants stressed by environmental factors is greater than that of recombinant proteins produced in other hosts. Furthermore, higher plants typically produce recombinant proteins with the correct folding, activity and glycosylation (Schillberg et al., 2005; Yano et al., 2010). Another beneficial characteristic of these systems is that recombinant plants can be stored at room temperature. In contrast, the storage temperature for plant viruses, bacteria and yeasts is -20 °C. The storage condition for cultured mammalian cells is even more stringent because they must be maintained in liquid nitrogen (Faye et al., 2005). Among plant species, plants with watery tissues, such as tomato plants, are more

suitable for molecular farming than are dry-tissue plants, such as cereals. This phenomenon could be related to the ease of extraction of squashy tissues (Horn et al., 2004). Despite all of the advantages of using higher plants, there are limits to the products that can be obtained using plant molecular farming, such as the unknown mechanisms that cause certain post-translational disorders in plant cells. The challenging issue of how to fine-tune the systems that are essential for the preservation of the structural integrity of the nascent recombinant proteins and their activities in their new cellular environments are still debated within the field of plant molecular farming (Walsh and Jefferis, 2006). The advantages and disadvantages of plant molecular farming compared with using other molecular farming systems are presented in Figure 2.

**Factors affecting the expression of recombinant proteins in different hosts**

According to the classification of final plant molecular farming products by Horn et al. (2004), proteins are clustered as parental pharmaceuticals, therapeutical intermediates, monoclonal antibodies (MAbs), industrial proteins, and antigens for use as edible vaccines (Figure 3). As green bioreactors, plants have various advantages, such as the capacity to accomplish eukaryotic post-translational modifications that are necessary for biological activities, such as disulphide bridging and glycosylation, in many different mammalian proteins (Ma et al., 2003). The genetic transformation procedures employed are governed by various vital factors, such as the plant genotype, the



**Figure 2.** Comparison between various hosts in molecular farming.

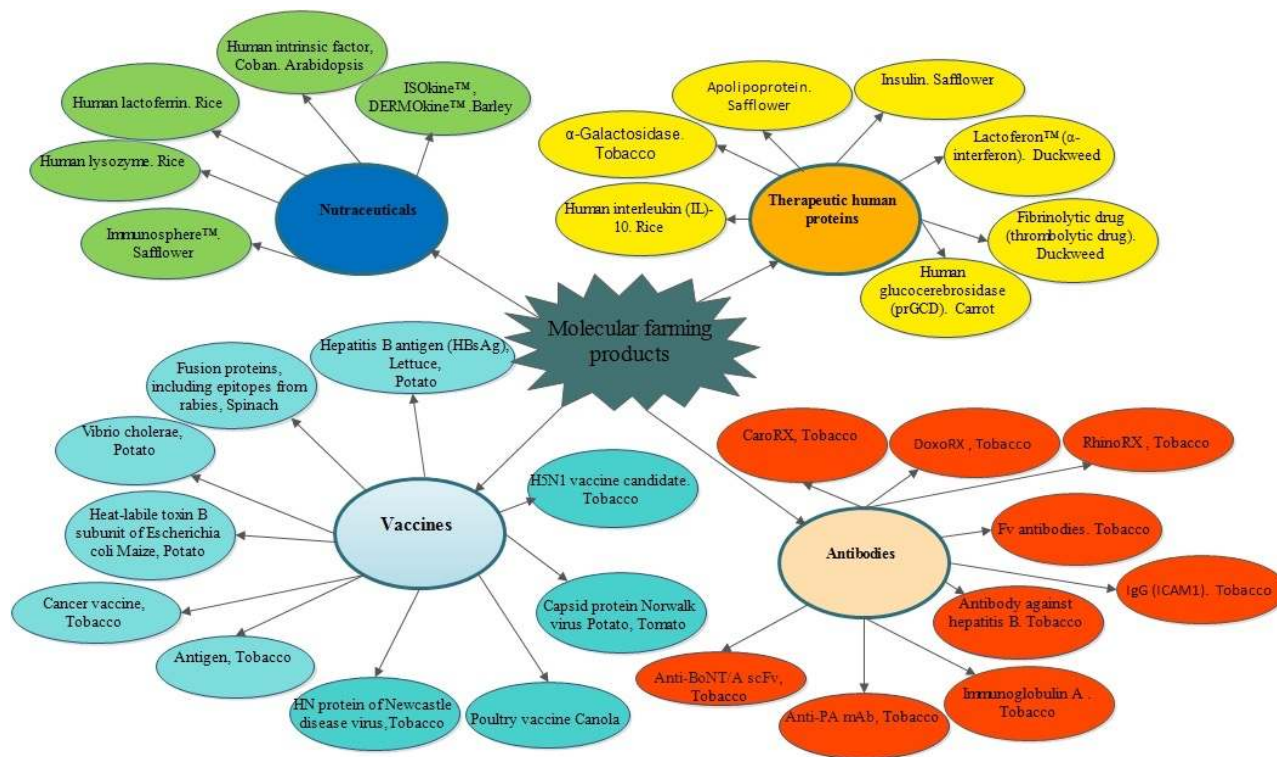
type of explant, the plasmid vector, the agrobacterial strain, and the composition of the culture medium (Kavitha et al., 2010; Sood et al., 2011). These factors play important roles in the transformation process that depends upon the plant species involved. Therefore, efficient plant molecular farming researchers tend to develop new transformation methods and novel construct designs, to incorporate well-defined transgenes and to search for more effective methods of introducing multiple genes into plants (Bregitzer and Brown, 2013; Karimi et al., 2013).

**Types of explants**

As mentioned above, the type of explant involved is one of the most vital factors for genetic transformation. For instance, the use of different sources of plant cells or tissues (e.g., seed, root, shoot, leaf, and shoot and root apical meristems) leads to variable results for callus induction, plant regeneration, and the efficacy of genetic transformation. In this regard, to produce a mass of calli, numerous protocols have been established for different plant-tissue culturing systems. Although these protocols are well-established, difficulties in callus induction in some plants, such as *Indica* rice varieties, are still experienced (Ikeuchi et al., 2013).

**Types of PGRs and plant regeneration**

The complicated procedure of plant tissue culture prompted the development of somatic embryogenesis (SE) methods, in which PGRs play an essential role. Two mainstream PGRs that have been used for plant tissue culturing are auxins and cytokinins. Members of the sub-groups of each family are involved in different metabolic pathways that affect plant physiology. For examples, auxins cause the production of undifferentiated cells called callus cells. Apparently, PGRs induced methylation of DNA, whereas cytokinins affect root formation in tissue cultures. The auxins generally used for the induction of different callus tissues are 2,4-D (2,4-Dichlorophenoxyacetic acid), IAA (Indole-3-acetic acid), NAA (1-Naphthaleneacetic acid), Dicamba, Picloram and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) (Yokoya et al., 2014). In contrast, cytokinins are the best candidates for inducing the production of roots and of plant regeneration via calli. The main function of cytokinins appears to involve the synthesis of proteins necessary for the formation and functionality of the mitotic spindle apparatus (George et al., 2008). BAP (6-Benzylaminopurine), Kinetin, Zeatin and TDZ (Thidiazuron) are the most important cytokinins that display regenerative activity in cultured plant tissues (Alam et al., 2010). Despite the undeniable effects of PGRs on cultured plant tissues, other essential factors, such as sugars, vitamins, salt concentrations, the type of medium and certain chemicals,



**Figure 3.** Examples of plant molecular farming products in different types of plants.



have been found to be a key factors in somatic embryogenesis (Enríquez-Obregón et al., 1999; Kavitha et al., 2010; Murashige and Skoog, 1962).

Achieving the effective gene transformation systems in molecular farming largely depends on the efficiency of tissue culture methods. These methods are mainly affected by the plant source, such as calli, root and shoot. In several plants, the lack of effective regeneration system is the most important limiting factor preventing the successful gene transfer technologies.

### Plant vectors

Scientists have many devices that allow them to achieve their goals of producing transgenic plants. In addition to various methods, such as the gene gun and biolistic techniques, natural bacterial vectors of plants, such as *Agrobacterium tumefaciens*, have been introduced as efficient tools, particularly in the case of cereals. *Agrobacterium tumefaciens* has the ability to transfer a portion of its DNA or so-called transferred DNA (T-DNA) to the genome of a host plant (Sheng and Citovsky, 1996). *Agrobacterium*-mediated gene transfer to plant cells includes five essential steps, as follows: a) induction of the bacterial virulence system, b) generation of a T-DNA complex, c) transfer of the T-DNA from *Agrobacterium* to the nucleus of the host cell, d) integration of the T-DNA into the plant genome, and e) the expression of T-DNA genes (Gelvin, 2012; Ziemienowicz et al., 2012). To date, *Agrobacterium*-based transformation methods have proven to useful approaches to genetically modify plants of various levels, including model plants such as the barrel clover (*Medicago truncatula*) *Arabidopsis* (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabaccum*, *N. benthamiana*); cereals, such as maize (*Zea mays*), rye (*Secale cereale*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*); legume plants, such as chickpea (*Cicer arietinum*); bean (*Phaseolus* spp.); pea (*Pisum sativum*); peanut (*Arachis hypogaea*), pigeon pea (*Cajanus cajan*); alfalfa (*Medicago sativa*); soybean (*Glycine max*); clover (*Trifolium* spp.); industrial plants, such as cotton (*Gossypium hirsutum*), canola (*Brassica napus*), sunflower (*Helianthus annuus*), Indian mustard (*Brassica juncea*), *Camelina sativa* and *Brassica oleracea*; vegetable, such as tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena*), cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), root plants; cassava (*Manihot esculenta*), carrot (*Daucus carota*), sweet potato (*Ipomoea batatas*) and potato (*Solanum tuberosum*); ornamental plants, such as carnation (*Dianthus caryophyllus*), orchids (*Cymbidium* spp., *Oncidium*, *Phalaenopsis*), chrysanthemum (*Dendranthema × glandiflora* hybrid), rose (*Rosa hybrida*) and petunia (*Petunia hybrida*); tropical plants, such as *Citrus* spp., banana (*Musa* spp.), sugarcane (*Saccharum* spp.), coffee (*Coffea* spp.), pineapple (*Ananas comosus*), papaya (*Carica papaya*); grasses, such as turf grasses, perennial ryegrass (*Lolium perenne*), bentgrasses (*Argostis* spp.), bermudagrass (*Cynodon* spp.), tall fescue (*Festuca arundinacea*), switchgrass (*Panicum virgatum*); woody species, such as cork oak (*Quercus suber*), American elm (*Ulmus americana*), poplar (*Populus* spp.), eucalyptus, rubber tree (*Hevea brasiliensis*) and pine (*Pinus radiata*);

nuts and fruits, such as apple (*Malus × domestica*), American chestnut (*Castanea dentata*), strawberry (*Fragaria × ananassa*), blueberry (*Vaccinium corymbosum*), walnut (*Juglans* spp.) and grapevine (*Vitis vinifera*); and final, medicinal plants, such as hemp (*Cannabis sativa*), ginseng (*Panax ginseng*) and the opium poppy (*Papaver somniferum*) (Dunwell, 2009; Ziemienowicz, 2013).

The major concerns that have arisen regarding transgenic plants include the low efficacy of plant regeneration during tissue culture, as well as production bottlenecks, such as in the spatial and temporal aspects of transgenic expression, target production, and the high-level yield of recombinant products (Stoger et al., 2002a; Streatfield, 2007). Considering that *Agrobacterium* is a pathogen of dicotyledonous species, the efficacy of the *Agrobacterium*-based genetic transformation of monocotyledonous plants is still limited due to the low integration rate. The above-mentioned difficulties prompted researchers to create new approaches to increase transformation efficiency. An *Agrobacterium* T-DNA derived nano-complex has been introduced as a promising method for increasing the transformation efficiency of monocotyledonous plants (Ziemienowicz et al., 2012). This nano-complex was first transferred into triticale (*Triticum × Secale*) microspores. The most important nano-complexes involve *Agrobacterium* T-DNA, virulence protein VirD2 and single stranded DNA- and double stranded DNA-binding RecA protein (Chugh et al., 2010).

### Promoters

As the upstream elements of genes, promoters play an eminent role in gene expression. Promoters contain sites with core sequences to which RNA polymerase and transcription factors bind. Although constitutive promoters, such as the cauliflower mosaic virus 35S (CaMV35S) and the maize ubiquitin-1promoter, have been utilized in dicots and monocots, respectively, tissue-specific and inducible promoters provide better control of gene expression (Ko et al., 2005; Nguyen et al., 2004). Inducible promoters can be prompted to induce gene expression within selected spaces and at chosen times through the addition of the appropriate chemicals. Reportedly, applying these specific chemicals can inhibit the occurrence of the undesirable effects of gene expression on different stages of plant growth and development (Stöger et al., 2000). Another great advancement in the field of plant genetic transformation was the identification of tissue-specific promoters. These promoters control gene expression by restricting it to specific tissues, which decreases the extent of the diverse effects of transformation on the growth and development on the host plant (Stahl et al., 2002). However, the efficacy of gene expression in specific tissues depends on the availability of a promoter that induces the production of a sufficient level of protein (Ko et al., 2005; Ko et al., 2003). The level of gene expression obtained using a tissue-specific promoter is not always adequate for a molecular farming process designed to generate biotherapeutic proteins. This results could be due to the limited number of existing promoters, their activity during the regeneration process, and the unpredictability and complexity of the effect of environmental factors, all of

which can cause unwanted and unexpected pleiotropic effects (Du et al., 2003). Indeed, expressing the series of genes necessary for the production of a full-size antibody requires the application of specific promoters that control the activity of each of these genes. In addition to the problems regarding plant protein expression mentioned above, the use of adverse promoters may cause homology-based gene silencing (De Neve et al., 1999; Ko et al., 2005). Applying both a constitutive promoter and an inducible promoter is a feasible solution to the aforementioned problems. For example, the combination of the 35S promoter and the potato proteinase inhibitor II (pin2) promoter has been used to control the expression of the heavy- and light-chain antibody genes, respectively (Ko et al., 2003).

### Composition of RNA and DNA

The process for the production of a recombinant protein in transgenic plants is similar to that of normal plants, in which DNA is transcribed to produce mRNA and then, through the harmonized activities of mRNA (messenger RNA), tRNA (transfer RNAs) and rRNA (ribosomal ribonucleic acid) mRNA is translated to yield a recombinant protein (Gebauer and Hentze, 2004). The untranslated regions of mRNA (the 5' and 3' untranslated regions) have direct effects on the transcriptional and translational processes via the activity of regulator-protein complexes (Amrolia et al., 1995; Costanzo and Fox, 1995). The core sequence of an untranslated region (UTR) of a gene may function as a gene-expression repressor when it binds translation-initiation factors. As a consequence of this activity, the translation of the gene product may be down-regulated (Curie and McCormick, 1997). A motif within the 5' UTR sequence of a gene can cause the specific induction of the transcription of its mRNA (Dickey et al., 1992). In parallel, the 5'-untranslated region of tobacco mosaic virus (Mitsuhara et al., 1996) and that of alfalfa mosaic virus (AMV) RNA3 have been shown to enhance the translation efficiency of a  $\beta$ -glucuronidase (GUS) transgene and stabilize its mRNA (Datla et al., 1993), which was also reported for the luciferase (Gallie and Kobayashi, 1994) and  $\beta$ -phaseolin (Oliver et al., 1993) genes. The cytoplasmic polyadenylation element (CPE) motif at the 3' end of transcripts reportedly exhibited a regulatory function in mRNA translational events. In this regard, the above-described motif can stimulate protein translation by hastening the occurrence of polyadenylation (Kervestin and Amrani, 2004). Additionally, transgenic plants containing the untranslated leader sequence of AMV had higher levels of attacin and a recombinant lytic protein than did the plants that did not contain this sequence (Ko et al., 2000).

Thus, modifying the structural stability of mRNA and controlling RNA polyadenylation via 5' and 3' untranslated sequences of mRNAs, respectively, are useful translational-based approaches to increasing the production of recombinant proteins in transgenic plants. Differences in the codon usage of the transgene and the expression host will also affect the level of recombinant-protein expression. Codon optimization has been used to improve the level of recombinant-protein expression in

living organisms by increasing the translational efficacy of the transgene (Mechold et al., 2005).

Additionally, extensive modification of the coding region and of certain molecular factors, such as accommodating codon bias, altering the GC content, eliminating cryptic splicing sites, putative polyadenylation signals and mRNA-instability sequences and incorporating 5' and 3' regulatory sequences, are required to achieve the high-level expression of foreign genes in plants (Streatfield and Howard, 2003).

Many candidate plant species for the production of recombinant proteins have been evaluated and developed during the past 25 years based on their particular advantages and disadvantages. These candidates from the plant Kingdom includes those that can be applied in cell cultures (carrot, tobacco, and rice), leafy crops (alfalfa, tobacco, and lettuce), aquatic plants (*Lemna minor*) and seed crops (canola, soybeans, corn, and rice) (Franconi et al., 2010; Karg and Kallio, 2009).

### Tobacco

One of the most suitable platforms typically used as a green producer is tobacco (*Nicotiana tabacum*). Despite the application of tobacco in smoking, it has many unique advantages over other plant species for the production of pharmaceutically relevant proteins. Scientists believe that the role of tobacco in recombinant-protein investigations is similar to that of the white mouse in mammalian studies over the last 20 years. As a leafy species, tobacco has numerous advantages over other plants, which have encouraged researchers to focus on this plant as an undeniable alternative for recombinant protein expression. This herb is capable of producing a biomass of up to 100 ton/ha. Moreover, a well-established system for transforming tobacco that results in a high level of soluble protein exists. The potential of utilizing various strategies for the expression of proteins in a stable or transient manner using this species, as well the possibility of using chloroplast genome-based methods mediated via *Agrobacterium* or viral induction are its other advantages (Figure 2) (Karg and Kallio, 2009). Nevertheless, the high concentrations of alkaloids and nicotine in some tobacco varieties are disadvantageous to utilizing this plant for molecular farming. These drawbacks have been compensated for by breeding new cultivars such as "81V" (Ma et al., 2003). The instability of the products of tobacco is one of the disadvantages of this plant in molecular farming. Alternatively, its products can be immediately processed on farms; alternatively, the leaves can be dried or frozen before transport to processing plants (Kamenarova et al., 2005). Tobacco is not a feed or food crop, which decreases the probability of its contamination within the feed or food chains. In addition, tobacco can produce a wide variety of therapeutic immune-modulatory molecules, such as cytokines, vaccines and antibodies (Tremblay et al., 2010). A full-length monoclonal antibody (mAb) directed against a mouse catalytic IgG1(6D4) was the first antibody produced in tobacco plants (Hiatt and Pauly, 2006). Other antibodies that were generated using tobacco plants include anti-PA (protective antigen) mAb, anti-BoNT/A scFv (botulinum neurotoxin/antibody single-chain variable fragment) idiotype specific antibodies, anti-

Lewis Y mAb, H10 mAb, CO17-1A mAb, TheraCIM<sup>®</sup>, B294 mAb, B303 mAb, 2F5 mAb, R12 mAb, CaroRX<sup>™</sup>, and anti-LPS scFv, as well as antibodies targeted against different disease entities, such as anthrax (Hull et al., 2005), botulism (Almqvist et al., 2006), cancer (B-cell lymphoma) (Young et al., 2009), (breast and colon) (Brodzik et al., 2006), (broad spectrum) (Svanes et al., 2010), (colon) (Ko et al., 2005), (skin) (Rodriguez-Oroz et al., 2005), as well as hepatitis (Gleba et al., 2005), HIV (Human immunodeficiency virus) (Ward et al., 2007), rabies (Girard et al., 2006), *S. mutans* colonization (Ma et al., 1998), and *Salmonella* (Makvandi-Nejad et al., 2005). Vaccines are antigenic materials that are administered to produce immunity to diseases. To date, various types of these vaccines have been generated in tobacco and some of them have been tested in mammals. The cysteine protease Der p 1 (*Dermatophagoides pteronyssinus* allergen) (Johnston et al., 2009; Lienard et al., 2007), Der p 2 (Lienard et al., 2007), protective antigen (Koya et al., 2005), L1 major capsid protein (Lenzi et al., 2008), CTB (cholera toxin B) (Jani et al., 2004), multi-epitope vaccine (Soria-Guerra et al., 2009), VCA (viral capsid antigen) (Lee et al., 2006), VP1 (viral protein-1) (Wu et al., 2007), VP21 (Huang et al., 2009), HSP-A (heat shock protein) (Brodzik et al., 2006), UreB (urease subunit beta) (Gu and Glatz, 2007), HBsAg (Hepatitis B antigen) (Thanavala et al., 1995), Hep C (Hepatitis C) core protein (Nanou and Azzouz, 2009), HIV p24 capsid protein (Zhang et al., 2002), HIV-Nef (human immunodeficiency virus- Negative regulatory factor) (Marusic et al., 2007), F1-V, SARS-CoV-S1 protein (severe acute respiratory syndrome coronavirus) (Pogrebnyak et al., 2005), Tet-C (tetanus toxin Fragment C) (Tregoning et al., 2005), GAD65 (Mr 65,000 isoform of glutamic acid decarboxylase) (Ma et al., 2004), GAD67 (glutamic acid decarboxylase) (Ma et al., 1997), HSP-60 (Heat shock protein 60) (Tremblay et al., 2010), insulin (Lee et al., 2006) and GLP-1 (Glucagon-like peptide-1) (Brandsma et al., 2009) are some of the proteins that have been expressed in tobacco, which target diseases/disease entities such as allergy-dust mites, anthrax, cancer (cervical), cholera, DPT (diphtheria), Epstein-Barr virus, foot and mouth disease, *Helicobacter pylori*, hepatitis B/C, HIV, plague, SARS, tetanus, Type 1 diabetes and Type 2 diabetes, respectively.

Cytokines, some of which are glycoproteins, are members of a family of strong immunoregulators that are produced by different types of cells. The function of these small proteins is regulating the period and intensity of the immune response by inhibiting or stimulating the activation, proliferation, and differentiation of a variety of cells. This procedure is accomplished through regulating the secretion of Abs or other cytokines by target cells (Parkin and Cohen, 2001). Cytokines bind to cytokine receptors expressed on the membrane of the responsive target cells (Thomson and Lotze, 2003). Despite the availability of various commercial recombinant cytokinins, considering the limitations of some conventional bioproduction systems, such as low levels of expression and the lack of glycosylation of *E. coli*-derived recombinant cytokines, scientists are interested in producing such recombinant proteins using tobacco. Due to the low efficiency of *E. coli* production of a pleiotropic regulatory cytokine, such as human IL-13, which protects

against several human diseases, including Type-1 diabetes mellitus (T1DM), transgenic tobacco can be used as a reliable system for the generation of high levels of human IL-13 (Thompson and Debinski, 1999; Wang et al., 2008). Transplastomic plants are novel alternatives to nuclear transgenic plants, which are created by introducing the recombinant DNA into the genome of chloroplasts rather than the nuclear genome using gene-gun bombardment. Some of the most important accessible products that have been produced in tobacco chloroplasts are a tetanus-toxin fragment, serum albumin, and human growth hormone. However, plastids do not have the capacity to perform glycosylation; thus, chloroplast cannot be used to produce human glycoproteins (Ma et al., 2003).

### The expression of tobacco systems

The availability of various tobacco expression systems with their particular strengths is another advantage of this model plant over other plant species. Nuclear transformation is the appropriate method to achieve the long-term production of glycoproteins, such as antibodies. Alternatively, chloroplast-based expression systems allow the production of large amounts of proteins that require certain types of post-translational processing. Tobacco is amenable to several different simultaneous applications and has the potential to excel at each of them. When a product that requires rapid modification, as in the case of the above-mentioned idiotypic anti-cancer vaccine, transient expression can be anticipated to yield the proper quantities of the desired protein within a short period (Tremblay et al., 2010). Using transient protein-expression systems, researchers can produce considerable amounts of recombinant protein during a short period, which is crucial for the rapid response to a disease outbreak, as is the case when a new influenza appears, e.g., during the A/H1N1 pandemic, as well as for patient-specific cancer treatments.

### Nuclear transformation

Stable nuclear transformation involves the incorporation of an exogenous gene into the nuclear genome of a plant, which leads to the expression of the new heritable traits by the next generation of transgenic plants. This transformation method is commonly utilized for the production and accumulation of a recombinant protein in certain explants, such as the dry seeds of cereals (Horn et al., 2004).

Agrobacterial infection and biolistic delivery are the two mainstream methods used to transform an exogenous gene into the nuclear genome of plants, including tobacco. *Agrobacterium* transfers genes to dicots with great efficacy and the Agrobacterial-infection method has been improved to increase the efficacy of the genetic transformation of certain monocotyledonous plants. The best example of the success of the latter involves rice (Chan et al., 1993; Chen, 2008; Hiei et al., 1997).

Furthermore, decreased costs and simplification of the production process are the results of gene delivery leading to stable genetic integration, which frequently leads to the production of recombinant proteins with the least external input. The exogenous proteins produced due to nuclear transformation can be directed to various secretory



organelles or other organelles for standard eukaryotic post-translational modifications.

Although nuclear transformation systems are commonly employed, the low level of production of the resultant recombinant protein remains a matter of concern. Among the many strategies to address this issue, the most important are the use of 5' enhancer sequences to increase translational productivity, the use of promoters with strong tissue-specific constitutive activity, the use of a sub-cellular localization signal (Benchabane et al., 2008), the use of 3' untranslated regions that were modified to increase transcript stability and the optimization of the coding sequence of the transgene using the typical tobacco codons (Streatfield, 2007; Streatfield and Howard, 2003), the addition of fusion partners that increase protein stability/proteolysis resistance (Floss et al., 2007), and targeting proteins to the endoplasmic reticulum (ER) via the attachment of a C-terminal (K/H) DEL signal (Denecke et al., 1992). The most important factors that directly affect the performance of stable transformation are the variety and the physiology of the plant.

### Transient transformation

Transient transformation is the proper method to achieve the rapid production of vital proteins in tobacco. In contrast to the case of stable genomic integration, a large amount of recombinant protein can be produced in less than five days post-infection using the transient transformation method. Nevertheless, nuclear and transient transformation methods have similar advantages. For the production of pharmaceutical materials using viral coding sequences via *Agrobacterium tumefaciens*, transient expression is the proper method because it offers a high level of expression with the lowest input. Different strategies have been developed to increase the efficiency of transient expression. One of the successes achieved by scientists as a promising approach to directly transforming cells with a virus using *Agrobacterium tumefaciens* has decreased the energy required for genetically transforming tobacco (Gleba et al., 2005). The principle of this method to transfer specific components of the viral expression platform by mixing a dissimilar *Agrobacterium* strain harbouring portions of the viral machinery, with recombination occurring intracellularly once infection has occurred. By manipulating the viral codons and applying classic eukaryotic introns, the efficiency of gene delivery has been significantly increased. Thus, the required amount of the essential infectious *Agrobacterium* was reduced. For example, 1 litre of an overnight culture could be used to infect nearly 1000 kg of tobacco leaf tissue, yielding up to 4 kg of recombinant protein at 40% TSP (Marillonnet et al., 2005). This example demonstrates represents the scale that are presently being explored to maximize protein yield while reducing the input costs, resulting in an estimated \$1/kg of raw protein or \$50/kg of purified protein. Another alternative is to insert the viral machinery required to prevent tobacco-gene silencing via stable nuclear transformation. Applying this method will decrease the delivery requirements of the deconstructed viral vectors (Azhakanandam et al., 2007). In general, transient expression has two main problems, including the high technical requirements for induction and high risk of

accidentally spreading the infection to wild species. In addition, this method is limited to controlled conditions, such as those of a greenhouse or laboratory. Although, the latter drawback is less of a concern because the controlled environments eliminate the risks of cross-fertilization that could potentially occur when transgenic plants are grown in open fields. The agro-infiltration method that was developed by (Dillen et al., 1997) is another technique for transient expression. This method involves the infiltration of a suspension of recombinant *Agrobacterium tumefaciens* into tobacco leaf tissue, which in turn facilitates the transfer of T-DNA to a very high percentage of the cells. The method also positively affects the intensity of transient expression in transgenic crops. A method allowing transient expression in tobacco has been specifically developed as a very rapid and high-yield strategy for the production of clinical-grade bio-pharmaceuticals (Pogue et al., 2010; Vézina et al., 2009).

### Plastid transformation

Plastid transformation is an efficient alternative to nuclear transformation because has several advantages that latter method lacks. For example, despite the enormous importance of the delivery of a normal bio-containment of transgene flow by out-crossing, the transgene cannot be transferred due to the lack of chloroplasts in pollen, thereby allaying public concerns regarding genetically modified plants (Meyer et al., 2010; Pantaleoni et al., 2014). Transgenic plants subjected to homoplastomic chloroplast transformation are selected after several generations of plants have been regenerated from the gene-gun bombarded leaf explants, meaning that the plant chloroplast genome has had opportunity to incorporate the transgene. The selection of the above-mentioned bombarded leaf explants is conducted on a medium containing a either spectinomycin or streptomycin. Researchers have achieved a noticeable yield of therapeutic human and bacterial proteins, ranging from 3-6% of the total soluble proteins, using the tobacco chloroplast-transformation technique (Reddy et al., 2002; Oey et al., 2009) achieved very high-level expression of a proteinaceous antibiotic, which comprised approximately 70% of the soluble proteins, through employing a chloroplast transformation system, which is the most significant yield of recombinant protein attained using transgenic plants to date. Although plastid transformation has an enormous potential, its application remains restricted. In that regard, even though plastid transformation has been attained in plant species, such as lettuce, eggplant, soybeans and tomatoes (Bock, 2007), the plant most commonly modified using a chloroplast transformation system is tobacco, which is highly regulated and is inedible due to its high level of toxic alkaloids. Finally, whether the protein stability will change over time, even with refrigeration, is a matter of concern (Horn et al., 2004).

### Plant-cell suspension cultures

One of the best plant-based alternatives to mammalian cells for the production of biopharmaceuticals is a plant-cell suspension, which is a robust system involving a simple purification procedure and easy downstream processing

(Kim et al., 2008). Although this system requires a high level of sterility to control contamination, pharmaceuticals with a high level of purity can be produced using it (Franconi et al., 2010). Additionally, in contrast to plant-cell culture systems, plant-cell suspension systems eliminate the regeneration process, and accordingly, is a rapid procedure (Shaaltiel et al., 2007). Despite having numerous advantages over other systems, plant-cell suspension systems have been established only for a small number of plants, such as tobacco, carrot, *Arabidopsis* and rice. Moreover, due to the certain limiting factors, such as increasing proteolytic activity, which leads to a low concentration of the recombinant protein during the late stationary phase, this system is not the best method of protein expression (Obembe et al., 2011).

### **Arabidopsis**

Plants with appropriate traits have been discovered in genetic-engineering investigations. Of all the known plants in the Kingdom, *Arabidopsis* has been the main plant used for genetic transformation. Among the traits of *Arabidopsis*, the short generation period, small genome size, presence of a self-pollination mating system, ease of *in vitro* culturing, easy regeneration and *in vivo* transformation, as well as its lack of food and feed applications, are interesting characteristics that have led scientists to exploit this model plant (Koorneef and Meinke, 2010). Due to its prolific seed production and rapid propagation rate, *Arabidopsis* appears to be capable of generating a range of various products. Like other seed-producing plant bioreactors, for the production of recombinant proteins, *Arabidopsis* should be transformed during the early stages of growth, because its development toward the flowering stage may increase the risk of environmental contamination (Ruebelt et al., 2006). High levels of recombinant protein accumulation in *Arabidopsis* seeds have been achieved using a seed-specific expression cassette (Van Droogenbroeck et al., 2007).

### **Cereals and legumes**

As protein synthesis and protein storage organelles, seeds have a critical role in plant molecular farming. The most important reasons for a seed-based system being an ideal platform for molecular farming are that seeds allow the long-term storage of proteins due to having an appropriate biochemical environment, a low water content and low protease activities and that they possess biosafety and are easy to transport (Stoger et al., 2002b). The lack of phenolic components is another advantage of cereal seeds compared to other plant bioreactors, such as tobacco, that contain phenolic components in their leaves. Generally, the downstream process of removing phenol from the products is time consuming and expensive (Ma et al., 2003). As is the case for other plant bioreactors used in molecular farming, seed-based systems have some drawbacks. The seed-based transgenic plants must reach the flowering stage before the recombinant proteins can be extracted, which significantly increases the possibility of environmental contamination by the pollen of the transgenic plants. To reduce the risk of this type of environmental contamination, plant bioreactors that accumulate recombinant proteins in their vegetative

organelles are preferable to systems involving the flowering cycle (Twyman et al., 2003).

Cereals have particular characteristics that have encouraged scientists to use members of this family as model plants in molecular farming. Cereal endosperms are the most important components for researchers due to their soluble-protein content. Soluble proteins can be recognized and separated easily in cereals by displaying antibody role in molecular farming (Han et al., 2006). Among the cereals, wheat is not as an appropriate model plant because of the low efficiency of its transformation (Stevens et al., 2000). Some of the recombinant proteins that have been extracted from seed bioreactors are the most efficient vaccine antigens (Wu et al., 2007), cell-culture proteins (Nandi et al., 2002), industrial enzymes (Hood et al., 2007), therapeutic antibodies (Stöger et al., 2000) and cytokines (Zhu et al., 1994). Among the seed-based bioreactors, maize is the major viable plant that produces recombinant proteins in large amounts. Some of the remarkable features regarding this model plant compared with those of other plant systems used in molecular farming are the existence of well-established techniques for its tissue culture and transformation, its production of high levels of biomass and the ease of scaling up its use. Moreover, this plant has the ability to produce recombinant antibodies as well as protease inhibitors and enzymes for pharmaceutical/technical applications, such as aprotinin, laccase, and trypsin (Hood, 2002).

One of the initial plant molecular-farming investigations was conducted in 2003 by Xue, who used barley as a bioreactor to produce a highly active and thermo-tolerant hybrid cellulase (1,4- $\beta$ -glucanase) (Xue, 2003). Other types of recombinant proteins and materials have been produced in barley, of which serum albumin, lactoferrin, lysozyme,  $\alpha$ 1-antitrypsin and human antithrombin III are the most important examples (Stahl et al., 2002). Using rice as the host plant, Anzai and colleagues have successfully expressed human lactoferrin (Anzai et al., 2000). The recombinant single-chain Fv antibody directed against carcinoembryonic antigen that was produced in wheat and rice could be preserved for up to 4 or 5 months at room temperature without any loss in activity or of the product (Stöger et al., 2000). Although soybean and alfalfa plants produce a relatively smaller amount of green biomass compared with that of tobacco and maize, their ability to directly utilize atmospheric nitrogen through nitrogen fixation make them ideal plants for the production of recombinant antibodies and other proteins (Ma et al., 2003).

### **Vegetables and Fruits**

Notably, fruits, vegetables and leafy crops are consumed in the form of moderately processed or entirely raw foods. In addition, these groups of plant products are normally free of toxicants and are rich in nutrients, which make them particularly appropriate for the production of recombinant vaccine subunits, antibodies, and food additives for active immunotherapy (Ma et al., 2003). Potatoes have been used extensively for the production of plant-derived vaccines, which have been administered to humans in many clinical trials.

Tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), banana (*Musa acuminata colla*) and carrot (*Daucus carota* spp. *sativus*) plants have been successfully utilized for the expression of vaccine subunits. Interestingly, among these plants, tomato plants have been utilized as model genetically transformed producers of the first plant-derived rabies-vaccine component, HIV-gag and HBsAg proteins (Sala et al., 2003). The accumulation of beta-amyloid (A $\beta$ ) in the brain initiates the development of Alzheimer's disease (AD), which is a neurodegenerative disease. The accumulation of this toxic protein in the brain can lead to neuronal destruction and intensification of the disease process. Administering an antigen directed against this toxic protein might be the most useful strategy to treat AD or at least to arrest its progress. To this end, scientists have successfully expressed A $\beta$  in tomatoes (Youm et al., 2008). Moreover, thymosin  $\alpha$ 1 has been expressed in tomato fruits through *Agrobacterium*-mediated transformation, and this recombinant protein also been used to treat cancer and viral infections (Cheng et al., 2003). The potato plant, which is another member of the Solanaceae family, has attracted the attention of researchers wishing to produce plant-based recombinant proteins. Ma et al. (2003) demonstrated that the potato tuber is a suitable host for the production of diagnostic antibody-fusion proteins, human milk proteins, and other antibodies. Banana plants have been considered as green bioreactors for the production of recombinant antibodies and vaccines. The transgenic fruits of different varieties of banana commonly grown in countries in which vaccines are most desperately needed can be consumed as raw materials by both children and adults or the vaccine subunits within them can be purified (Ma et al., 2003). The specific potential of carrots for plant engineering have led to this plant becoming an ideal candidate for tissue culture and genetic transformation, as well as for plant molecular farming (Muller et al., 2003).

Celery cabbage (*Brassica rapa* var. *pekinensis*), lettuce (*Lactuca sativa*), and cauliflower (*Brassica oleracea* var. *botrytis*) are other vegetables that have been considered for plant molecular farming application, although their low regeneration rates and recombinant expression levels remain challenging (Tacket and Mason, 1999).

### Oilseeds

Plants bearing seeds that are rich in oil are known as oilseed plants. Safflower, coconut, peanut, sunflower, palm, sesame, olive, rapeseed and rice (bran) are examples of oilseed plants (Moloney et al., 2003). Safflower and rapeseed have been employed as useful sources of recombinant proteins due to their protein-production capability and the simplicity of purifying the proteins they produce. In addition to the low cost and low acreage associated with their use, the high protein yield obtained and their being self-pollinating plants are other advantages of these oilseed organisms. To produce recombinant proteins in such organisms, scientists exploited oleosin proteins, which are structural components of safflower cells. Oleosins are small structural proteins that are attached to the surface of oil bodies and subcellular organelles that store oils. Oleosin-recombinant fusion

proteins must be detached so that they can be extracted, which can be achieved using a simple procedure, namely, endoprotease digestion (Boothe et al., 1997). Safflower-derived insulin and hirudin are new-generation pharmaceutical proteins that have produced by oilseed plants (Spök et al., 2008).

### Aquatic plants

Green-cell factories are also options for plant molecular farming. Aquatic plants are promising green-cell platforms for the introduction of genes and the production of novel recombinant proteins. To this end, the chloroplast and nuclear genomes of five different microalgal species, namely, *Cylindrotheca fusiformis*, *Symbiodinium microadriaticum*, *Amphidinium carterae*, *Pheaeodactylum tricornutu* and *Chlamydomonas reinhardtii*, have been successfully transformed. Among the above-mentioned algal species, *Chlamydomonas reinhardtii* is the most suitable host for the production of edible vaccine subunits (Potvin and Zhang, 2010; Rasala et al., 2010), antibodies (Mayfield and Franklin, 2005), blood proteins (Manuell et al., 2007), industrial phytases (Yoon et al., 2011), and a growth factor within chloroplasts (Rasala et al., 2010). Their lack of toxicity, low expression levels, low cost, short growth period, high yield and capability for fresh use are the most significant advantages of employing microalgae to overcome the current obstacles in plant molecular farming (Pryer et al., 2002; Zaslavskaja et al., 2000).

Another reliable plant bioreactor for the production of pharmaceutical products is duckweed. Duckweed is a monocotyledonous plant belonging to the Lemnaceae family, which includes four major subspecies, namely, *Wolffiella*, *Spirodela*, *Lemna* and *Wolffia* (Zhang et al., 2010). Like other plant bioreactors, duckweed has many advantages for the production of recombinant proteins (Stomp, 2005). Some of specific features of this plant are that it is fast-growing, easy to harvest, safe, accumulates high levels of protein, and can produce complex proteins (Popov et al., 2006). *Agrobacterium tumefaciens* and biolistics are the main methods used to transfer genes to duckweed, such in the case for other plants. Interferon  $\alpha$ 2 (De Leede et al., 2008), avian influenza H5N1 hemagglutinin (Gu and Glatz, 2007), aprotinin (Rival et al., 2008), anti-CD20 mAb (BLX-301), human plasmin (BLX-155) (Paul and Ma, 2011), a monoclonal antibody (Cox et al., 2006) and plasminogen (Spencer et al., 2011) are some of the recombinant proteins that have been produced at various levels by duckweed (Stomp, 2005; Vunsh et al., 2007).

### Glycosylation as a modification mechanism in transgenic plants

Modifying recombinant proteins through post-translational processes plays an important role in their functions. Post-translational modifications, such as glycosylation, phosphorylation, sulphation and methylation, are vital in transgenic higher eukaryotic organisms (Gomord et al., 2004). One of the most important post-translational modifications is glycosylation. Certain aspects of this enzymatic process, in which glycans are attached to organic molecules such as proteins and lipids, are significantly different in plants and mammalian cells. The

basic N-acetyl glucosamine (GlcNAc)-mannose precursor structures added to the glycosylation sites of proteins within the endoplasmic reticulum of plant and mammalian cells are identical, but evolution of the Golgi apparatus has caused considerable variation. Glycosylation affects the basic biological functions of proteins, such as their ligand-receptor interactions, specific activity and immunogenicity. A chain of oligosaccharides can be formed via either O- or N-linked glycosylation. N-linked glycosylation occurs within the endoplasmic reticulum (ER), and the primary oligosaccharide chain is further processed during its exit from the ER and passage through the Golgi apparatus (GA) (Balen et al., 2006).

Recent reports concerning glycosylation within transgenic plants suggested that the pattern of glycosylation of an Fc antibody did not have a large effect on antibody-dependent cellular cytotoxicity (ADCC) or the stimulation of the complement cascade. However, four aspects are worth mentioning in relation to the potential effects of adding carbohydrate moieties and replacing them are the following: a) the immunogenicity of plant N-glycans per se, b) the immunogenicity of plant N-glycans in the presence of a given glycoprotein, c) probable allergies to plant N-glycans, and d) the impact of these carbohydrate moieties on pharmacokinetics (Pujol et al., 2007). A general problem is that some antibodies are not produced against compounds consisting of a large number of monosaccharides "linked glycosidically", when inoculated with plant-based enzymes. The best example of this phenomenon is the antagonistic reaction of N-glycans with horseradish peroxidase. In addition, these antibodies interact with glycoproteins from plants, insects and snails that contain the same  $\alpha(1,3)$ -fucose and  $\beta(1,2)$ -xylose glycoepitopes (Chargelegue et al., 2000). Unfortunately, the products of plant glycosylation can occasionally lead to side effects, such as allergic reactions. Because persons who are prone to pollen allergies have IgE (Ig: insoluble glycoproteins) and IgG4 reactivity to glycoepitopes, it is rational to attribute the allergenicity of plant-based glycosylated antibodies to the existence of allergenic factors, such as glycoproteins containing  $\alpha(1,3)$ -fucose and  $\beta(1,2)$ -xylose. An appropriate example is the allergenic factors of olive-tree pollens, which contain a glycoprotein with  $\alpha(1,3)$ -fucose and  $\beta(1,2)$ -xylose groups that are capable of inducing histamine release by basophils (Cárdaba et al., 2000). Therefore, it may be impossible to predict the side effects of plant-based glycosylated antibodies in humans to whom they are administered. Eventually, engineering the N-glycosylation processes of plants will enhance the efficacy of plant-made pharmaceuticals (PMPs) not only regarding the immunogenicity of N-glycans in humans but also through generating glycovariants of therapeutic proteins with a higher bioactivity levels than those produced in cultured mammalian cells (Saint-Jore-Dupas et al., 2007). In this regard, priority should be given to antibodies containing a single major N-glycan species and lacking detectable levels of plant-specific N-glycans (Cárdaba et al., 2000). Such antibodies have been shown to induce better antibody dependent cell-mediated cytotoxicity, demonstrating the potential of plants as systems for the

expression of highly demanded anti-cancer antibodies (Cox et al., 2006).

### **The effect of environmental factors on transgenic plants**

The effects of environmental factors on the health, biomass production and activity of plants are matters of concern even under normal conditions; however, these factors become more serious concerns during the post-transformational period. Indeed, genetically transformed plants are extremely susceptible to the effects of environmental factors immediately after being transferring to natural soil. Environmental condition directly affects the quality and quantity of recombinant proteins produced. The factors that may negatively affect transgenic plants are divided into biotic and abiotic stresses. In this regards, light, drought, salinity, nutritional deficits and cold have negative effects on plant products. These factors are abiotic stresses (Jamal et al., 2009). Light plays a crucial role in regulating photosynthesis and the phenological events that drive the growth and development of plants toward the flowering stage (Dahl et al., 1995). Generally, plants use light of 400- and 740-nm wavelengths to conduct photosynthesis. Light of these wavelengths is called photosynthetically active radiation. Photons of wavelengths lower and higher than this range are either impractical or destructive for photosynthesis due to their insufficient or extremely high energy levels (Zhu et al., 2008). To protect recombinant proteins against stresses, scientists try to optimize the light condition, such as the length of the day and the light intensity. Other stresses, such as drought, restrict the establishment of crops. This stress decreases the productivity and quality of plants and leads to morphological changes. Consequently, the expression of recombinant proteins may be down-regulated. Similar to the effects of other stresses, drought leads to the accumulation of reactive oxygen species (ROS) in plants, causing oxidative stress (Pastori and Foyer, 2002). Moreover, physiological traits, such as the carbon-assimilation and stomatal-conductance rates are prominent determinants of fitness under drought conditions due to their relevance to the efficiency of water-use and photosynthesis (Heschel and Riginos, 2005). Another restricting factor of plant molecular farming is salinity. Approximately one-third of the world's irrigated farms are ineffective due to the excess salt content of the soil (Munns, 2005). The adverse effects of salt on plants are manifested in two ways. Firstly, a high concentration of salt in the soil directly hampers water absorption by the roots by affecting root-soil osmotic regulation. Secondly, salt accumulation in various organs poisons plants (Munns and Tester, 2008). The two toxic ions derived from NaCl,  $\text{Na}^+$  and  $\text{Cl}^-$ , can damage plant cells through both osmotic and ionic mechanisms (Chinnusamy et al., 2005). Quantitative and qualitative changes in metabolite synthesis, as well as the occurrence of enhanced metabolic toxicity are a few of the most usual indicators of stressed plants (Karimi et al., 2005). Furthermore, salt stress alters the expression of cell-cycle progression genes through affecting mitotic cell division (Bursens et al., 2000). All of these cellular processes may be affected by altered hormonal homeostasis occurring under salt-stress conditions (Lee et

al., 2001). It has been well documented that the abscisic acid (ABA) content of plants increases under salt-stress conditions (Bray, 2002). Adaptation to saline stress is accompanied by alterations in the level of numerous metabolites, proteins, and mRNAs (García et al., 1997). A variety of genes, the expression of which is activated in response to salt stress, have been identified and have been transferred to plants (Rensink et al., 2005). Because high salinity conditions promote plant-cell dehydration (Liu et al., 2011), many of the genes that are activated by saline stress are also activated by drought. The expression of the majority of these genes is regulated by abscisic acid (ABA), a plant hormone that is generated in response to saline stress (Wilkinson and Davies, 2002).

Another important environmental factor that affects plant growth and productivity is temperature. High-temperature stress induces certain physiological, biochemical, and genetic changes in plants, including protein denaturation, lipid liquefaction, and perturbation of membrane integrity (Levitt, 1980). Among the suppositions regarding high-temperature acclimation are that several mechanisms of adaptation to high temperature involving the induction of protein synthesis or altered protein functions may exist (Turner et al., 2001). High temperatures decrease the rate of synthesis of normal cellular proteins and induce the synthesis of heat-shock proteins (Parsell and Lindquist, 1993). High temperatures are harmful to plant cells, leading to a loss of viability. In *Medicago sativa* transgenic plants, heat can inactivate the gene that encodes the phosphinothricin/N-acetyltransferase recombinant protein (Walter et al., 1992). Sometimes, transferring plants to an environment with a stressful heat level initiates the expression of members of heat-shock protein/chaperone cascades that prevent the misfolding, denaturation and aberrant aggregation of cellular proteins (Wang et al., 2004). Plants lacking temperature adaptation may be incapable of inducing structural or functional changes in their proteins. According to Stevens et al. (Stevens et al., 2000), exposure to 25 °C and high light conditions can increase the biomass and total soluble protein content of plants, whereas exposure to high light conditions and 15 °C favoured the production of a recombinant monoclonal antibody by transgenic tobacco plants.

The temperature also affects the glycosylation of recombinant proteins (antibodies) in plants. Sulphur (S) is a major component of any protein molecule; hence, its uptake and assimilation can affect the production of recombinant proteins in transgenic plants. Of course, S uptake is dependent upon a constant supply of the precursor of cysteine, O-acetylserine, which in turn, is dependent on the presence of adequate nitrogen and carbon sources (Kopriva and Rennenberg, 2004). As a good example, the combined application of S and N affects the accumulation of lipids in rapeseed (*Brassica napus* L.) via the induced increase in the rate of protein synthesis (Fazli et al., 2005). The significantly positive correlation of the antibody and total protein contents of transgenic plants allows the prediction of the fluctuating trend of antibody accumulation through monitoring changes in the amounts of total protein. The above-mentioned facts suggest that

providing balanced nutrition would enhance the production of pharmaceutical proteins by transgenic plants.

### **Large-scale transient gene transfection, climate risk-free production systems, and biosafety considerations**

Plant biotechnology typically relies on two strategies for delivery and expression of heterologous genes in plants, including a) stable genetic transformation, and b) transient expression using viral vectors (Marillonnet et al., 2005). In recent years, the technological progression in virus-based vectors has allowed plants to become a feasible platform for recombinant proteins (RPs) production, while RPs were only able to be produced from cultures of mammalian, insect, and bacteria cells, previously. The plant-based RPs are more preferable in terms of versatility, speed, cost, scalability, and safety over the current production paradigms (Chen and Lai, 2014). In spite of being a faster method, the transient approach is hampered by low contagiousity of viral vectors carrying average- or large-sized genes. Fortunately, these drawbacks have been subject to troubleshooting by developing constructs for the efficient delivery of RNA viral vectors as DNA precursors. The mentioned efforts have tended to expanding systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. As such, *Agrobacterium*-mediated delivery of the target constructs using results in gene amplification in all developed leaves of a plant simultaneously. This process is also referred to as "magnification" that can be performed on a large scale and with different plant species. The mentioned technique incorporates advantages of three biological systems consisting of: a) the transfection efficiency of *A. tumefaciens*, b) the high expression yield obtained with viral vectors, and c) the post-translational capabilities of a plant. This procedure does not entail genetic modification of plants and is faster than other current methods (Marillonnet et al., 2005).—Transient expression systems have been established to eliminate the long-time frame of generating transgenic plants, so that the transgene is not integrated into the plant genome but rather quickly directs the production of the RP while residing transiently within the plant cell. In addition to the significant acceleration of production timeline, this approach improves the recombinant proteins accumulation level by excluding the "position effect" of variable expression instigated by the random integration of transgene within the genome (Komarova et al., 2010). In another word, the climate risk-free molecular farming systems have become more achievable by conducting the transient gene transfection.

Beside all these advances achieved by the transient expression technology, some complementary strategies have been taken into consideration to limit the potential environmental and human health impacts linked to PMF. Specifically, cell cultures of transgenic plants, physical containment, dedicated land, plastid transformation, biological confinement, male sterility, gene use restriction technologies (GURTs), expression from or in roots, expression in edible parts and seeds, post-harvest inducible expression, and temporal confinement have been suggested as additional solutions to minimize the risks of PMF (Breyer et al., 2009).



### Industrialization, current status and perspectives

As plant molecular farming has come of age, there have been technological progresses on many aspects, including transformation methods, regulating gene expression, protein targeting and accumulation, as well as the use of different crops as production platforms (Twyman et al., 2003). Recently, plant molecular farming has been proposed as an example of a green development scheme in convergence with sustainable agricultural industries. Despite, the yield improvement remains as one of the most challenging issues, because the product yield has a significant impact on economic feasibility of any related project.

The advantages of transgenic plants over other expression systems make them become industrialized as economic alternatives to the conventional pharmaceuticals. Several plant-made pharmaceuticals, including the enzyme glucocerebrosidase (GCase), insulin and Interferon alfa 2b [IFN-alpha (2b)], have approached commercialization with low costs and large-scale production. Interestingly, these achievements have been attached to substantial patenting activities as well. Reportedly, there was a tangible downward trend in the number of patents filed from 2002 to 2008, and a greater number of patents were filed by public sector institutions or inventors than by the private sector (Drake and Thangaraj, 2010). The USA dominated patenting activity providing nearly 30% of inventors. Most of the patents were related to vaccine candidates (55%), followed by therapeutics and antibodies with 38 and 7%, respectively (Drake and Thangaraj, 2010).

### Conclusions

Plant molecular farming has been shown to be a promising biotechnological approach; however, because this approach is novel, its efficacy may be disputed. Methods that facilitate plant cultivation under extremely controlled conditions should be developed for the subsequent stages of this process, as we move away from aseptic plant-cell cultures to non-aseptic conditions in which plants are grown traditionally or are grown hydroponically using compost. Plant molecular farming has significant potential for the development of medicinal products. With regard to the history of plant molecular farming, the current major focus is to accelerate the improvement of plant biotechnological procedures for the generation of new products, as well as conventional products. The most important challenges in this field are identifying new plant resources and optimizing protocols for producing high levels of recombinant proteins. The cryptic medicinal plant such as *Andrographis paniculata* (Valdiani et al., 2013) can be introduced as an impending candidates (Valdiani et al., 2012), while the genetic (Valdiani et al., 2014), and proteomic (Talei et al., 2014) analyses of the herb have both performed promising horizons for being subjected to plant molecular farming.

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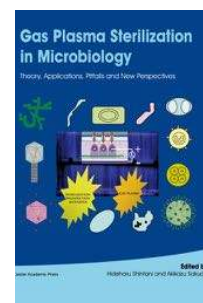
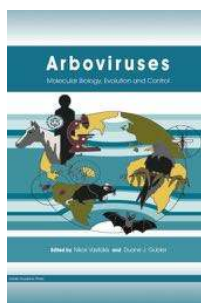
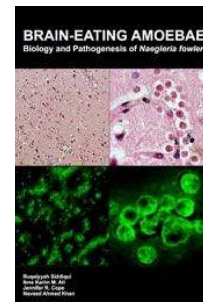
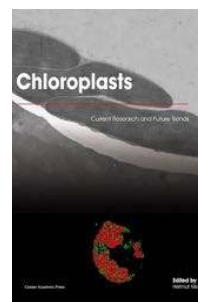
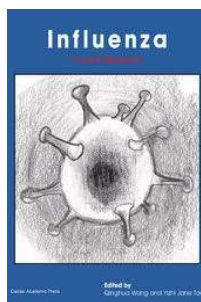
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