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Green Way of Biomedicine – How to Force Plants to Produce New Important Proteins

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1. Introduction

Recombinant proteins can be expressed in transformed cell cultures of bacteria, yeasts, molds, mammals, plants, insects, or via transgenic plants and animals. Numerous factors influence quality, functionality, yield and protein production rate, so the choice of appropriate expression system is of primary importance. During last few years, plants have become an increasingly promising and attractive platform for recombinant protein production (Basaran & Rodriguez–Cerezo, 2008). Progress in recombinant DNA technology, plant transformation and *in vitro* regeneration techniques are major reasons why plants have emerged as efficient expression systems. Plant expression systems offer significant advantages over the other expression systems (Table 1). First of all, plants have a higher eukaryote protein synthesis pathway very similar to animal cells with only minor differences in protein glycosylation. Therefore, plant biosynthesis pathway ensures correct structure even in the case of highly complex proteins. In contrast to plants, bacteria are not able to carry out most of posttranslational modifications essential for eukaryotic proteins activity. There is no risk of contamination of recombinant proteins with human or animal pathogens (HIV, hepatitis viruses, prions), bacteria endotoxins or oncogenic DNA sequences (Sharma & Sharma, 2009).

Other advantages of the plant–based expression systems include: high scalability (in the case of field cultivation), low production cost of biomass (agriculture), in some cases low upstream costs (edible vaccines, purification process can be omitted), and what is most important - the ability to produce target proteins with desired structures and biological functions (Boehm, 2007). Recombinant proteins expressed in plants can be accumulated to a high level in seed endosperm, fruit or storage organs (e.g. tubers, roots) or secreted directly to the culture media. Because plant culture media contain no exogenous proteins, the recovery of recombinant proteins from a medium is expected to be much simpler and less expensive than the recovery from homogenized biomass (Cox et al., 2009).

Features	Transgenic plants	Plants viruses	Yeast	Bacteria	Mammalian cell culture	Transgenic animals
Cost/storage	Cheap	Cheap	Cheap	Cheap	Expensive	Expensive
Distribution	Easy	Easy	Feasible	Feasible	Difficult	Difficult
Gene size	Not limited	Limited	Unknown	Unknown	Limited	Limited
Glycosylation	Correct	Correct	Incorrect	Absent	Correct	Correct
Production costs	Low	Low	Medium	Medium	High	High
Production scale	Worldwide	Worldwide	Limited	Limited	Limited	Limited
Propagation	Easy	Feasible	Easy	Easy	Hard	Feasible
Protein folding accuracy	High	High	Medium	Low	High	High
Protein homogeneity	High	Medium	Medium	Low	Medium	Low
Protein yield	High	Very high	High	Medium	Medium-high	High
Safety	High	High	Unknown	Low	Medium	High
Scale up costs	Low	Low	High	High	High	High
Therapeutic risk	Unknown	Unknown	Unknown	Yes	Yes	Yes
Time required	Medium	Low	Medium	Low	High	High

Table 1. Comparison of features of recombinant protein production in existing systems (according to Fischer and Emans 2004; worked out /modified on the basis of Demain and Vaishnav 2009).

The usage of aquatic plants e.g. *Lemnaceae* seems to be a good solution. For example Rival et al. (2008) made studies on obtaining aprotinin from *Spirodela oligorrhiza* (duckweed). Their experiments show that significant amounts of recombinant aprotinin can be produced using *Spirodela* as a plant host. Whereas Cox and co-workers (2009) expressed human monoclonal antibody (mAbs) in *Lemna minor*. The micro-alga *Chlamydomonas reinhardtii* has recently been shown as a promising platform for foreign protein production (Muto et al., 2009). This photosynthetic single-celled plant possesses several interesting features in comparison to the majority of plants as it has a rapid doubling time (ca. 10 h); its homogenous culture is easily scaled up; it has a rapid sexual cycle (ca. 2 weeks) with stable and viable haploids. All these attributes make the time of petting a final product on a large-scale much shorter in comparison to higher plants (months or years). Growth in containment bioreactors allows to control conditions of farming as well as reduces the risk of contamination and loss of algae due to pathogens. It is worth mentioning that all three genomes of *C. reinhardtii* have been fully sequenced affording strong foundation for targeted genetic manipulation (Specht et al., 2010).

Feasible storage of recombinant proteins in desiccated plant parts excludes the requirement for its immediate isolation and lowers the risk of the loss of biological function during prolonged freezing of preparations. For example, antibodies or vaccines expressed in cereal seeds remain stable at ambient temperatures for years (Stoger et al., 2002). Until recently, low accumulation levels have been the major bottleneck for plant-made recombinant protein production. However, several breakthroughs have been done during past few years allowing for high accumulation levels. Mainly through chloroplast, vacuole, ER lumen transient expression, coupled with subcellular targeting and protein fusions (Sharma and Sharma, 2009). Viral transfection and agroinfiltration are promising alternative strategies ensuring increase in yields and speeding up the development of an expression platform (Gleba et al., 2005). On the other hand, plant–based expression systems are different from the mammalian host pattern of glycosylation. The occurrence has raised concerns regarding the potential immunogenicity of plant-specific complex N-glycans (α 1,3-fucose and β 1,2-xylose residue), which are present in the heavy chains of plant-derived antibodies (Gomord and Faye 2004). The above mentioned residues have been confirmed not only to induce immune response but also to make foreign proteins undergo a conformational change making them different from the native ones which results in decrease in their biological activity. However, some achievements in humanized glycosylation or removal of enzymatic pathway generating immunogenic residues on glycoproteins have been reported. Recently it has been shown that glycoengineered moss (*Physcomitrella patens*) can synthesize proteins carrying a humanized glycosylation pattern (Decker and Reski, 2008). A few years ago *Physcomitrella patens* platform was developed and commercialized as a contained tissue culture system for recombinant protein production in photo-bioreactors [Biotech GmbH (© greenovation)]. *P. patens* has some characteristic features which make it a suitable system for foreign protein production. Firstly, it grows rapidly under photoautotrophic conditions and secondly the moss protonema can release the desired protein into the medium. The moss remains productive in the system for a period of six months, in contrast to animal cell cultures (20 days) (Decker and Reski, 2008).

Other approaches to overcome undesirable glycosylation accommodate export of foreign proteins into subcellular compartments: ER lumen, where glycosylation characteristic of plants does not take place; cytosol, where glycosylation process is not found; or recombinant protein expression export into plastids (proteins do not undergo glycosylation there). According to several studies ER targeting gives higher yield of biologically active protein than cytosol targeting (referred by Boehm, 2007).

Potential disadvantages of transgenic plants include possible contamination with pesticides, herbicides, and toxic plant metabolites. Proteolytic degradation, post/transcriptional gene silencing, position effect and transgenic recombination are other obstacles affecting stability or expression level of transgenic plants (Basaran and Rodriguez–Cerezo, 2008).

The public concern about health and environmental risk associated with transgenic plants is being considered at different levels: inherent risk of transgene leakage into non-transgene crops or naturally occurring wild type species (transgene escape through pollen); transgene spread by seed or fruit dispersal; horizontal gene transfer by asexual means; unintentional exposure of non-targeted organisms (e.g. birds, insects or soil microorganism); elicitation of allergic response/reaction in people (Basaran and Rodriguez–Cerezo, 2008). There are some strategies which allow to alleviate these problems including usage of closed culture

facilities, such as greenhouses, hydroponic or suspension bioreactors or plastid transformation (as plastids are inherited through maternal tissues in most species and the pollen does not contain chloroplasts, hence the transgene cannot be transferred) (Basaran and Rodriguez–Cerezo, 2008).

From economical point of view, plants can be an alternative system for recombinant protein production (especially biopharmaceutical) in comparison to those exploiting mammalian or bacterial cell cultures. In this system a desired foreign protein can be produced at 2-10% of the cost of microbial fermentation system and at 0.1% of mammalian cell cultures, although it depends on the protein of interest, product field and a plant used. In general, the recombinant protein yields up to 1.5% of the total soluble protein (TSP). For example the content of antibodies does not exceed 0.35%-2% and vaccines- 0.01-0.4% of TSP (Basaran and Rodriguez–Cerezo, 2008). On the other hand, phytase from *A. niger* was obtained at the level 14% of the total tobacco soluble protein, but hirudin from *H. medicinalis* at 1% of canola seed weight and GUS from *E. coli* was produced in corn at 0.7% of TSP (Demain and Vaishnav 2009).

2. Expression strategies

Gene expression and synthesis of proteins is a complex multi-step process. For efficient expression of recombinant proteins in plants, it is essential to optimize every step of the process for the plant machinery. This includes the methods of plant transformation, the choice of a transgene promoter, improvement of transcript stability and the efficiency of its translation. After translation, the protein needs to be accumulated in plant cells or effectively secreted.

2.1 Stable nuclear transformation

The first step in plant transformation consists in the entrance of a desired genomic sequence into a plant cell. Stable nuclear transformation is caused by integration of the recombinant DNA in the nuclear genome. DNA can be transferred into the nuclear genome by either direct (e.g. biolistics) or indirect (e.g. *Agrobacterium*) methods, it depends on the plant species and the type of tissue (Thanavala et al., 2006).

In the stable nuclear transformation whole plants can be regenerated, eventually producing a seed stock or a plant tissue maintained in an aseptic culture. The advantage of this system is that the transgene is heritable, permitting the establishment of a seed stock for future use. Establishment and characterization of stable transgenic lines can be costly and time consuming. Large numbers of transgenic lines need to be screened and analyzed before a single optimal line can be selected for protein production (Ling et al., 2010). Other disadvantages are gene silencing and position effects.

Nuclear transformation has been employed and extensively studied in many plant species, however, it generally results in low expression of soluble foreign proteins (Yap & Smith, 2010).

Recombinant proteins can be targeted to different subcellular compartments in plant cells, such as cytostol, apoplast, endoplasmic reticulum, vacuole or chloroplast.

2.2 Transplastomics

Using particle bombardment or polyethylene glycol (PEG) treatment, DNA can be targeted into the chloroplast genome (Yusibov & Rabindran, 2008). Each cell contains a large number of plastids, ~100 chloroplasts per cell, and each of them contains about 100 genomes. Transplastomic lines vs. nuclear ones have significantly greater yield of foreign proteins (1-20% TSP) due to the high number of copies of the chloroplast genome and they offer major advantage in terms of transgene containment, as chloroplast genomes are predominantly maternally inherited, limiting out-crossing of the transgenic pollen. No transcriptional or post-transcriptional silencing effects have been observed in chloroplast transformation (Yap & Smith, 2010). Chloroplasts also support operon based on transgene allowing the expression of multiple proteins from a single transcript. There are two disadvantages of the chloroplast system – first: chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops, second: lack of some of the eukaryotic machinery for post-translational modification (Yusibov & Rabindran, 2008).

Gene integration in the plastid genome occurs by means of two homologous recombinant events mediated by a bacterial-like Rec A based system. Vectors include two 'targeting' regions flanking the selectable marker gene and a cloning site for insertion of the gene of interest. The targeting regions are between 1 and 2 kb in size and are plastid DNA sequences able to direct transgenic integration into plastome intergenic regions. Integration by homologues recombination in a preselected genome region enables insertion of only transgenic sequences and prevents uncontrollable variation in the expression of transgene. Strong promoters for plastid encoded polymerase (PEP) from the *rrn* operon and the *psbA* gene are used. Rregulatory sequences at the 5'-terminus must include a 5' untranslated region (UTR). Plastid transgene expression can be also achieved with the use of the T7 phage promoter and nuclear-encoded, plastid imported T7 RNA polymerase. In some cases protein accumulation was enhanced by translational fusion of a plastid gene N-terminal sequence with the protein of interest by including sequences downstream of the ATG start codon (downstream box) in the transgene 5'cassette that resulted in improved translation and/or protein stability. The 3'cassettes derived from 3'UTR of plastid genes generally function as inefficient terminators of transcription, but are important for plastid transcripts stability (Cardi et al., 2010).

2.3 Optimization of expression level

Increasing the transcription rate of stably transformed gene sequences is the most direct and efficient approach to increase protein expression. This is mainly achieved with the use of a strong constitutive or inducible promoter. Constitutive promoters directly drive the expression in all plant tissues and are independent of the production host developmental stage. The best known and most widely used constitutive promoter in plant biotechnology is derived from *Cauliflower Mosaic Virus (CAMV35S)*. It is more effective in dicots than monocots. Alternative constitutive promoters frequently used in plant cell transformation are the *ubiquitin* promoter, histone *H2B* promoter and the (*ocs*)*3mas* promoter (Hellwig et al., 2004). The *ubiquitin* promoter, isolated from a variety of plants including maize, *Arabidopsis*, potato, sunflower, tobacco and rice, has been frequently used to express biopharmaceuticals in plant cells. The (*ocs*)*3mas* promoter, constructed from octopine synthase (*osc*) and

mannopine synthetase (*mas*) agrobacterial promoter sequences , was used for the expression of *Hepatitis B* antigen in a soybean cell culture (Smith et al., 2002). Other constitutive promoters used for expression of foreign genes in transgenic plants include: tobacco cryptic constitutive promoter (Menassa et al., 2004), Mac promoter which is a hybrid mannopine synthetase promoter and cauliflower mosaic virus 35S promoter enhancer region (Dai et al., 2000), rice actin promoter (Huang et al., 2006), banana actin promoter (Herman et al., 2001), C1 promoter of cotton leaf curl Multan virus (Xie et al., 2003), nopaline synthase promoter (Stefanov et al., 1991).

Inducible promoters allow external regulation by chemical stimuli such as alcohol, steroids, salts, sucrose or environmental factors such as temperature, light, oxidative stress and wounding. Inducible expression is advantageous as this allows protein production to be separated from cell growth. The use of chemical inducible promoters in combination with the chemical responsive transcription factor can further restrict the target transgene expression to specific organs, tissues or even cell types (Zuo & Chua, 2000). The examples of inducible promoters and synthetic transcription activators are: the rice α-amylase 3D (*RAmy3D*) promoter, which is induced by sucrose starvation; the oxidative stress-inducible a peroxidase (*SWAPA2*); an estradiol-inducible chimeric XVE transcription activator and dexamethasone-inducible pOp/4v transcription activator (Xu et al., 2011), hydroxyl-3-methylglutaryl CoA reductase 2 promoter, which is inducible by mechanical stress (Cramer et al., 1996).

Tissue-specific promoters control gene expression in a tissue or in a developmental stage specific way. The transgen driven by such a promoter is expressed in a specific tissue leaving all the other tissues unaffected. It helps to force transgene expression in storage organs like seeds, tubers or fruits. Several of such promoters were tested: tuber specific patatin promoter (Jefferson et al., 1990), fruit specific E8 promoter (Jiang et al., 2007), arcelin promoter (Osborn et al., 1988), maize globulin 1 promoter (Rusell & Fromm, 1997), 7s globulin promoter (Fogher, 2000), rice glutelin promoter (Wu et al., 1988) and soybean P-conglycinin subunit promoter (Chen et al., 1986).

The optimization of promoters activity can be further improved by means of engineered DNA elements - enhancers, activators or repressors located up or downstream of the core promoter. Enhancers are shown to increase gene expression when placed proximally to the promoter, they bind activator proteins and promote RNA polymerase II placement at the TATA box. Transcription is also enhanced with flanking the transgene by nuclear scaffold/matrix attachment regions (S/MARs) important for structural organization of eukaryotic chromatin (Halweg et al., 2005).

The translational efficiency of a transgene is determined by proper processing (capping, splicing, polyadenylation, nuclear export) and mRNA stability. The 5′ and 3′ untranslated region (UTR) of the plant mRNA plays crucial roles in its processing (Cowen et al., 2007). The 5′-UTR is very important for 5′ capping and enables translation initiation, the 3′-UTR is indispensable in transcript polyadenylation which in turn influences the stability of mRNA (Chan and Yu, 1998). These untranslated sequences can be manipulated for the optimization of protein expression.

As the protein is synthesized, it undergoes several modifications before final delivery to its cellular destination. These modifications include enzyme involving glycosylation,

phosphorylation, methylation, ADP-ribosylation, oxidation, acylation, proteolytic cleavage and non-enzymatic modifications like deamidation, glycation, racemization and spontaneous changes in protein conformation (Gomord & Faye, 2004). Post-translational proteolysis can be effectively minimized by targeting the foreign proteins to sub-cellular compartments such as the endoplasmic reticulum (ER). Proteolysis is more likely to occur in the apoplast and cytosol. ER retrieval signal (e.g. KDEL, HDEL) retains the expressed protein in the ER lumen and has been used to improve foreign protein stability. The ER contains many molecular chaperones facilitating nascent proteins folding or assembly and it is regarded as an ideal compartment for accumulating many classes of foreign proteins (Nuttal et al., 2002).

Other strategies for proteolytic degradation reduction are: co-expression of recombinant protein and protease inhibitors, co-expression of protein co-factors or subunits, knockout mutations in the genes encoding specific proteolytic enzymes.

The recent advent of highly efficient transient expression systems has completely changed the concept and revolutionized plant made pharmaceutical research. Transient transformation implies the expression of foreign DNA which cannot be inherited but is still transcribed within the host cell in a transient manner. Transient gene expression provides a rapid alternative to the time consuming stable transformation methods. This approach uses the plant hosts - *Arabidopsis thaliana*, *Nicotiana tabacum*, *Nicotiana benthamina*, *Lactuca sativa*. Transient expression of recombinant proteins in plants is performed by the use of engineered plant viruses and/or *Agrobacterium* mediated DNA transfer (agroinfection/agroinfiltration). Fast and high level expression is the major advantage of the transient expression systems. Full expression of a gene of interest in agroinjected leaves may be achieved in 3-4 days after infiltration with *Agrobacteria*. This system is simple and experimental procedures do not require expensive supplies and equipment. Leaves of greenhouse grown plants are infiltrated using a syringe without a needle, vacuum infiltration or the wound and agrospray inoculation method (Medrano et al., 2009). Supplementation of the infiltration media with Silwet L-77, Tween-20, or Triton X-100 improves the efficiency of transformation. In the transient expression system one can use different virus types: Tobamoviruses, Potexviruses, Potyviruses, Bromoviruses, Comoviruses and Gemniviruses. Prolific production of any given protein using the plant virus approach results from the fact that a virus can infect a plant systemically by moving in its symplast. The *Agrobacterium* based method involves the injection or vacuum infiltration of whole plants or their parts with a suspension of bacteria harboring the construct of interest (Gómez et al., 2009). *Agrobacterium* delivered plant viral vectors use the RNA polymerase II mediated nuclear export route including 5' end capping, splicing and 3' end formation. Plant RNA viruses replicate in the cytoplasm and are not adapted to nuclear splicing machinery which recognizes and removes cryptic introns from viral RNA leading to its degradation. The *Agrobacterium* delivered so called 'first generation' TMV and PVX vectors have low production capacity and require coinjection of a plasmid encoding gene silencing suppressor such as tombusvirus p19 or potyvirus P1/HC-Pro (Komarova et al., 2010).

A major breakthrough in viral expression strategies was facilitated by the recent advent of deconstructed virus vectors. Originally reported for the TMV-based magnICON system developed by ICON Genetics GmbH merges advantages of *Agrobacterium*-mediated DNA

delivery and upgraded TMV based vectors where putative cryptic splice sites were removed and multiple plant introns inserted. Thus the basic idea is to amplify the foreign gene delivered by *Agrobacterium tumefaciens* to multiple areas of the plant allowing the virus to replicate and spread. In this process, bacteria start initial infection delivering the T-DNA encoded viral replicon to the nuclei of a large number of cells. Then, the transcripts are transported to the cytoplasm where the viral RNA amplification renders high yields of the desired protein (Gleba et al., 2005).

In conclusion, the two major strategies for expressing proteins in whole plants are transient expression with viral vectors and stable transformation where transgenes are targeted to either the nuclear or chloroplast genome. Stable transformation offers the advantage that protein production is scalable to large field production methods. However, this can be offset by low expression levels and the long time required for creating expressor lines stable across multiple generations. Today's most promising direction in the referred field is emerging from synthesis of genetically engineered agrobacteria, viruses and plants in one precisely tailored system where synthetic and system biology meet each other.

3. Overview of plant-derived medical recombinant proteins

3.1 Plant derived antibodies

Over the last few decades, medical biotechnology has led to major advances in diagnosis and therapy. At present most diseases can be detected at an early stage, and their treatment is more specific and potent. Biotechnological methods allow to identify the molecular mechanisms of a disease facilitating development of new diagnostic techniques and speeding up development of novel molecularly targeted drugs. One of the therapeutic strategies in the treatment of many diseases is the use of antibodies. Antibodies are a class of topographically homologous multidomain glycoproteins produced by the immune system and they display a remarkably diverse range of binding specificities. Since the first production of monoclonal antibodies by Kohler and Milstein in 1975 they have become an extremely important and valuable tool in medicine (Yarmush et al., 2003).

Constantly increasing demand for new and safe monoclonal antibodies forces development of high-performance production systems. Since the first report on antibody production in *N. tabacum* plants (Hiatt et al., 1989), plantibodies have been produced in various plant systems (Table 2).

Product	Disease/Pathogen	Plant	Promoter	Expression level	Organ	Reference
Human anti-rabies monoclonal antibody	Rabies	Tobacco	CaMV 35S promoter with duplicated upstream B domains	0.07% TSP	Leaves	Ko et al., 2003
Human monoclonal antibody	Hepatitis-B virus	Tobacco	CaMV 35S promoter with the omega sequence	0.2-0.6% TSP	Suspension cell cultures	Yano et al., 2004

Product	Disease/Pathogen	Plant	Promoter	Expression level	Organ	Reference
Full-length monoclonal mouse IgG1 (MGR48)	-	Tobacco	CaMV 35S, TR2' promotor	30–60 mg of fresh weight	Leaves	Stevens et al., 2000
Human-derived, monoclonal antibody	Anthrax	Tobacco	CaMV35S	-	Leaves	Hull et al., 2005
Anti-*Salmonella enterica* single-chain variable fragment (scFv) antibody	*Salmonella enterica*	Tobacco	EntCUP4, single and double-enhancer versions CaMV 35S	41.7 ug of scFv/g leaf tissue	Leaves	Makvandi-Nejad et al., 2005
Human anti-rabies virus monoclonal antibody	Rabies	Tobacco	CaMV 35S with duplicated upstream B domains (Ca2p), (Pin2p)	30 ug/g of cell dry weight	Cell suspension culture	Girard et al., 2006
BoNT antidotes	Botulinum neurotoxins (BoNTs)	Tobacco	CaMV35S	20-40 mg/kg	Leaves	Almquist et al., 2006
TheraCIM recombinant humanized antibody	Skin cancer	Tobacco	CaMV35S/ Agroinfiltration	1.2 mg/kg of leaves	Leaves	Rodríguez et al., 2005
Human monoclonal antibody 2F5	Activity against HIV-1	Tobacco	duplicated CaMV35S	2.9 ug/g fresh weight	Cell suspension	Sack et al., 2007
mAb BR55-2 (IgG2a)	Carcinomas, particularly breast and colorectal cancers	Tobacco	CaMV 35S	30 mg kg of fresh leaves	Leaves	Brodzik et al., 2006
LO-BM2, a therapeutic IgG antibody	Possible tool to prevent graft rejection	Tobacco	En2pPMA4	99 ug in the cell extract of a 100-ml culture, 12.81 ug. medium-associated antibody	Leaf and cell suspension culture	De Muynck et al., 2009
Monoclonal antibody H10 (mAb H10)	Tumour-associated antigen tenascin-C (TNC)	Tobacco	CaMV 35S with omega translational enhancer sequence from (TMV)	50–100 mg/kg fresh plant tissue	Leaves	Villani et al., 2009

Table 2. Plant derived antibodies.

3.2 Plant derived vaccines

Plants can be used to produce inexpensive and highly immunogenic vaccines. It is connected with heterologous expression of antigens. These are further purified to formulate injectable vaccine or are applied as edible vaccines. The latter idea is a very attractive alternative to injection, mostly because of low costs (no need for protein purification) and comfort of administration. However, there are some essential conditions which have to be satisfied. First of all, plants used for oral vaccine production should produce edible parts that can be consumed uncooked (antigens are often heat sensitive). Besides, these parts should be rich in protein because the antigen protein will constitute only a minor portion (0.01-0.4%) of TSP. Seeds seems to be a good choice because of antigen extended stability, even at ambient storage temperatures. As many studies revealed, vaccine antigens present in plant tissues were resistant to digestion in the gastrointestinal tract, on the other hand during this process they were release to elicite both mucosal and systemic immune responses (Sharma and Sood, 2011). Current progress in the matter is summarized in Table 3.

Vaccines	Disease	Plant	Promoter	Expression level	Organ	References
Subunit HAC1 and HAI-05	H1N1, H5N1 influenza	Tobacco	Not reported	HAC1 90 mg/ and HAI-05 50 mg/kg of plant biomass	Leaves	Shoji et al., 2011
VP1-capsid protein	FMDV (Foot and Mouth Disease Virus)	Tobacco	*psbA*	51% TSP	Leaves (Chloroplasts)	Lentz et al., 2010
TonB protein	Immunization against *Helicobacter infections*	*A. thaliana*	CaMV 35S	0.05% TSP	Entirely plant	Kalbina et al., 2010
Mycobacterial antigens Ag85B	Vaccine against tuberculosis	Tobacco	CaMV 35S	4 % TSP	Leaves	Floss et al., 2010
Surface protein 4/5 (PyMSP4/5)	Plasmodium	Tobacco	MagnICON® viral vector system	10% TSP or 1–2 mg/g of fresh weight	Leaves	Webster et al. 2009
TetC and PTX S1 antigens	DTP (diphtheria–tetanus–pertussis)	Tobacco Daucus carrota	CaMV 35S	Not reported	Leaves; Hypocotyls	Brodzik et al., 2009
HN glycoprotein	Newcastle Disease Virus (NDV)	Tobacco	P-RbcS	3µg of HN protein per mg of total leaf protein	Leaves	Gómeza et al., 2009
HBsAg	HBV (hepatitis B virus)	*Lactuca sativa*	CaMV 35S	Not reported	Shoots	Marcondes & Hansen, 2008

Vaccines	Disease	Plant	Promoter	Expression level	Organ	References
HPV-16 L1 protein	HPV (Human Papilloma Virus)	Tobacco	*psbA* promoter	24 % TSP	Leaves	Fernández-San Millán et al. 2008
16 E7 oncoprotein	HPV	Tomato; Potato	CaMV 35S	0.5 % of the cell protein-potato	Potato protoplast; leaves	Briza et al., 2007
G protein	Rabies virus	*Daucus carotta*	CaMV 35S	0.2–1.4% (TSP)	Carrot roots	Royas-Anaya et al., 2009
Capsid protein VP6	Rotavirus	Potato	P2	0.01%	Leaves, tubers	Yu & Landgridge, 2003

Table 3. Plant derived vaccines.

3.3 Plant derived biopharmaceuticals

Plants can be used to produce inexpensive biopharmaceuticals (Table 4).

Biopharmaceutical	Potential application	Plant	Promoter	Expression level	References
IL-10	Inflammatory and autoimmune diseases	Rice seeds	Glutelin B-1 promoter	2 mg pure IL-10	Fujiwara et al., 2010
Human transfferin	Receptor-mediated endocytosis pathway	Rice seeds	Glutelin 1 G-1 promoter	1% seed dry weight	Zhang et al., 2010
Glutamic acid decarboxylase (GAD65)	Autoimmune T1DM	Tobacco leaves	CaMV 35S	2.2% total soluble protein	Avesani et al., 2010
hGH, somatotropin	Growth hormone-treatment of dwarfism	*N. benthamiana*	CaMV 35S	60 mg per kilogram offresh tissue; 7%	Rabindran et. al., 2009;
Human erythropoietin (EPO)	Anemia, Renal failure	*N. tabacum*	CaMV 35S	0.05% of total soluble protein	Conley et al., 2009
Human serum albumin (HSA)	Deficiences	Tobacco, potato	Prrn; B33	11.1%TSP% (tobacco chloroplasts); 0.2%TSP (potato tuber)	Faran et al., 2002
Human lactoferrin (hLF)	Anti-inflammatory and immuno-modulation effects	Potato	Tandem promoter: P2& CaMV 35S	0.10% TSP	Chong et al., 2000
Enkephalins	Painkiller	Cress, *A. thaliana*	--------------	0.10% seed protein	Daniell et al., 2001
Staphylokinase	Thrombolytic factor	*A. thaliana*	CaMV 35S	not reported	Wiktorek-Smagur et al., 2011

Table 4. Plant derived biopharmaceuticals.

3.4 Nutraceutical and non-pharmaceutical plant derived proteins

Antimicrobial nutraceutics, such as human lactoferrin and lysozymes, have now been successfully produced in several crops (Stefanova et al., 2008), and are commercially available (Table 5). Cobento Biotechnology (Denmark) has recently received approval for its *Arabidopsis* derived human intrinsic factor which is used against vitamin B12 deficiency and it is now commercially available as Coban. Other nutraceutical products are listed in Table 5.

Trypsin is a proteolytic enzyme that is used in a variety of commercial applications, including processing of some biopharmaceuticals (Sharma & Sharma, 2009). In 2004, the first plant derived recombinant protein product (bovine sequence trypsin; trade name – trypZean) developed in corn plant (Prodi Gene, USA) was commercialized. Avidin, a glycoprotein found in avian, reptilian and amphibian egg white, is primarily used as a diagnostic reagent. The plant optimized avidin coding sequence was expressed in corn and now it is available on the market. β-glucuronidase, peroxidase, laccase, cellulase, aprotinin were also developed and marketed (Basaran & Rodrigez-Cerezo, 2008).

Spider silk proteins, elastin and collagen, have been expressed in transgenic plants (Scheller et al., 2004). These are promising biomaterials for regenerative medicine.

Product name	Company name	Plant	Commercial name	Source
Avidin	Prodigene	Corn	Avidin	Obembe at al., 2011
β-glucuoronidase	Prodigene	Corn	GUS	Obembe at al., 2011
Trypsin	Prodigene	Corn	TrypZean	Obembe at al., 2011
Recombinant human lactoferrin	Meristem Therapeutic, Ventria Bioscience	Corn, Rice	Lacromin	http://www.meristemthera-peutics.com
Recombinant human lysozyme	Ventria Bioscience	Rice	Lysobac	http://www.ventria.com
Aprotinin	Prodigene	Corn, Tobacco	AproliZean	Obembe at al., 2011
Recombinant lipase	Meristem Therapeutic	Corn	Merispase	http://www.meristemtherapeutics.com
Recombinant human intrinsic factor	Cobento Biotech AS	*Arabidopsis*	Coban	http://www.cobento.dk
Human growth factors	ORF Genetics	Barley	ISOkine™	http://www.orfgenetics.com
Food additive for shrimps	SemBioSys	Safflower	Immuno-spherte	http://www.sembiosys.com

Table 5. Transgenic plants based on products commercially available in the market.

4. Recombinant protein purification

4.1 Affinity chromatography

Isolation and purification of a biologically active protein from a crude lysate is often difficult and costly. Simple, cheap and more efficient strategies of its purification on the laboratory and industrial scale are thus on great demand. One of the numerous approaches in this field is an affinity tags system easily applicable for recombinant protein purification by affinity chromatography. The term 'affinity chromatography' was introduced in 1968 by Pedro Cuatrecasas, Meir Wilchek, and Christian B. Anfinsen (1968). Now it is the method of choice (Kabir et al., 2010). Affinity chromatography is based on specific interaction between two molecules in order to isolate the protein of interest from a pool of unwanted proteins and other contaminants. For this purpose a fusion protein is created. A short fragment of DNA can be ligated to the 5 ' or 3' - terminus of the target gene. This peptide or protein coding sequence (so called tag), which is translated in frame with protein of interest exhibits a characteristic property, strong and selective binding to the molecules immobilized on the solid matrices (Fong et al., 2010). Purification process is effective and simple. During passage of the cell extract containing the fusion protein and contaminants through an appropriate column the tagged protein is retained, while all the others migrate freely through the column (Fig. 1).

In the next step, the bound protein is eluted by a change in buffer composition /parameters (i.e. competitors, chelators, pH, ionic strength or temperature). Affinity tags are divided into three main classes according to their properties and the properties of molecules that interact with them: 1) tags, binding to small molecule ligands linked to a solid support (i.e. HIS-tag), 2) protein tags binding to a macromolecular partner immobilized on chromatography support (i.e. CBP-tag), 3) the protein-binding partner attached to the resin in an antibody which recognizes a specific peptide epitope in a recombinant protein (i.e. FLAG-tag) (Lichty et al., 2005, Arnau et al., 2006, Waugh et al., 2005). To date large number of gene fusion tags has been described, the most commonly used ones are presented in Table 6.

Tag	Comments	References
His-tag	Purification by interaction between immobilized metal ions and chelating amino acids	Valdez-Ortiz et al., 2005, Vaquero et al., 2002
FLAG	Purification based on binding the FLAG peptide to antibodies	Brodzik et al., 2009, Zhou and Li., 2005
Strep-tag II	Strong specific interaction between Streptag and strep-Tactin (streptavidin derivate) immobilised on resin	Witte et al., 2004

Table 6. Some examples of affinity tags commonly used for protein purification.
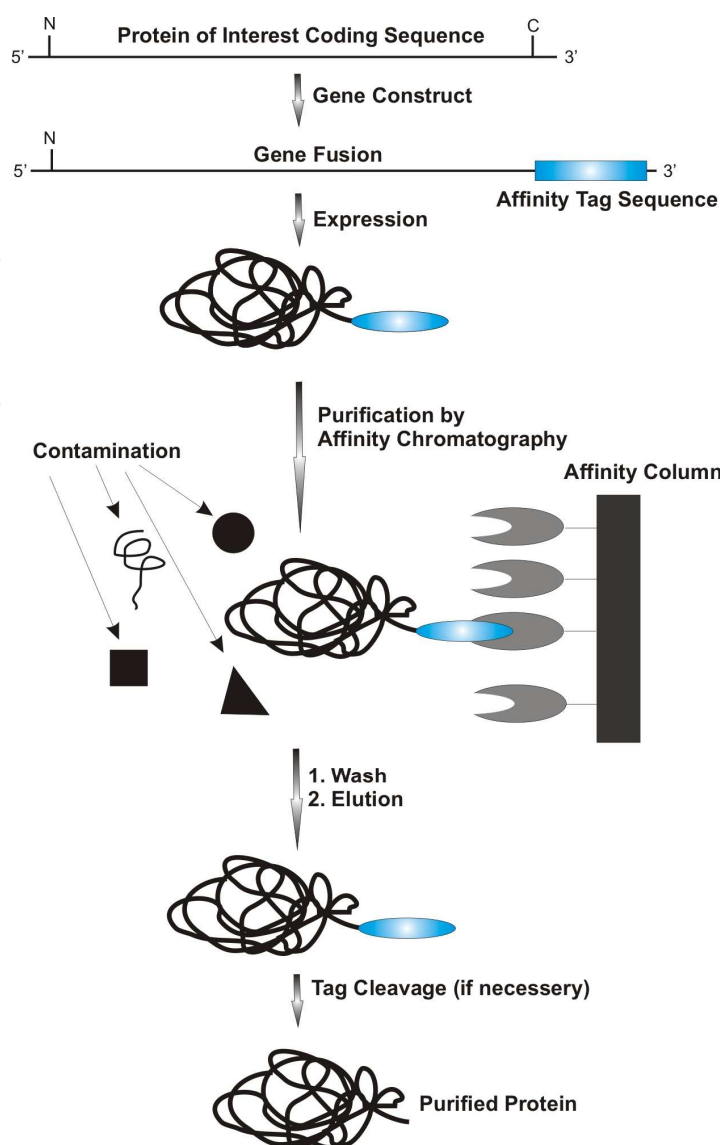
Fig. 1. Schematic representation of the recombinant protein purification process by affinity chromatography (Hearn & Acosta, 2001, modified).

4.2 Elastin-like polypeptides in recombinant protein purification

While affinity chromatography is used for purification of a broad spectrum of recombinant proteins it is not free from drawbacks. The main limitations associated with the use of this method are: 1) high cost of chromatography packing materials, 2) volume-limited sample throughput, 3) dilution of the protein product in elution buffer, 4) additional concentration step may cause loss in protein yield (Chow et al., 2008). Taking into account the above, there is a need to introduce new alternative methods for purification of recombinant proteins.

One of the possible solutions is application of non-chromatographic purification tags. Elimination of resins allows us to reduce some of the aforementioned problems.

Elastin-like polypeptides (ELP), artificial polymers containing Val-Pro-Gly-Xaa-Gly pentapeptide repeats, are an example of such tags. Such repeats occur naturally in the

hydrophobic domain of human tropoelastin (soluble precursor of elastin) and they play an important role in the process of elastin formation (Mithieux & Weiss 2005, Valiaev et al., 2008). Xaa (so called guest residue) in the ELP sequence can contain any amino acid except for proline (Meyer & Chilkoti, 1999). Occurrence of proline at these positions eliminates distinctive and very useful properties of these polymers (Trabbic-Carlson et al., 2004). Literature classification of ELP is based on the type and number of amino acids present in the guest residue positions (Meyer & Chilkoti 2004).

Elastin-like polypeptides belong to one of the three classes of thermosensitive biopolymers (Mackay and Chilkoti, 2008) whose properties are changed under the influence of moderate temperature differences. Aqueous solutions of ELP exhibit lower critical solution temperature (LCST) which causes that the above phase transition temperature (T_t) ELP pass from soluble to an insoluble form (Ge et al., 2006) in a narrow temperature range (~ 2 ° C) (Ge and Filipe, 2006). This is a reversible process called coacervation. In solutions with temperature below T_t , free polymer chains remain in a disordered soluble form. The opposite occurs in solutions with temperatures above T_t, when the polymer chains have more ordered structure (called β-helix), stabilized by hydrophobic interactions (Rodriguez-Cabello et al., 2007) that increase association of polymer chains (Serrano et al., 2007). This process is reversible. The fact that ELP –protein fusions are prone to reversible transition is of great importance (Kim et al., 2004). The process of ELP-tagged protein purification involves increasing ionic strength and/or temperature of the cell lysate to induce ELP-fusion protein aggregation (Fig. 2). Next sample centrifugation/filtration separates the ELP fusion protein from contaminants. After resolubilization of an ELP fusion, another centrifugation/filtration removes denatured and aggregated biomolecules. This process called Inverse Transition Cycling (ITC) can be repeated to achieve the required purity of the product (Floss, Schallau et al., 2010).
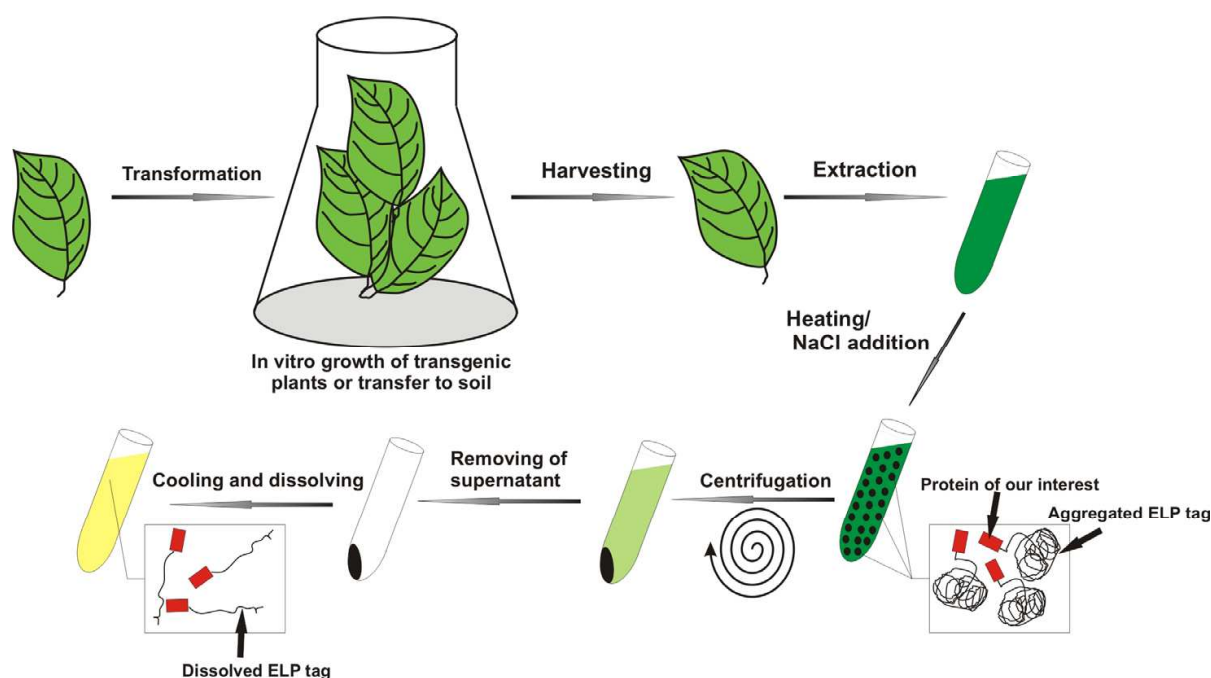

Fig. 2. Purification of ELPylated target proteins from plants using ITC (Floss et al., 2010 modified.

Purification of proteins using elastin-like polypeptides has several advantages over the traditional chromatographic methods: 1) purification of proteins with ELP tags by ITC appears to be universal for soluble recombinant proteins, 2) chromatography beads are not required, which significantly reduces the costs, 3) final concentration step is not required (Chow et al., 2008).

4.3 Application of ELP to the process of production and purification of recombinant proteins in transgenic plants

Scheller and co-workers (2004) achieved efficient and stable expression of spider's silk-ELP fusion protein in the ER of transgenic tobacco and potato. Application of ITC allowed them to obtain 80mg pure recombinant protein from 1kg tobacco leaf material. Purified biopolymer was tested as a potential component used for the cultivation of anchorage-dependent CHO-K1 cells and human chondrocytes. The most common coating substances such as collagen, fibronectin and laminin are derived from animal sources, so there is a risk of contamination of cell cultures by viruses or prions which is essentially undesirable in the case of medical applications. What is more, production of this fusion protein in plants is less costly. Lin and associates (2006) obtained active soluble glycoprotein 130 which seems to be potent drug in Crohn's disease, rheumatoid arthritis and colon cancer therapy. This work a presents creation and expression of mini-gp130-ELP. A fusion protein containing Ig-like domain and cytokine binding module of gp 130 fused to 100 repeats of ELP was expressed in tobacco leaves (ER retention). Inverse transition cycling (ITC) purification resulted in 141 μg of active mini-gp130-ELP per 1g of leaf fresh weight. Floss and co-workers (2010) demonstrated the ability of genetically engineered tobacco to produce mycobacterial antigens Ag85B and ESAT-6 as the vaccine against tuberculosis. In this work Ag85B-ELP and ESAT-6-ELP (TBAg) fusions were created, purified by inverse transition cycling and tested on animals. Production of this TBAg-ELP fusion proteins reached 4% of the tobacco leaf total soluble proteins (TSP) for the best producer plants. Further testing of the vaccine showed mycobacterium-specific immune response with no side effects in an animal model. What is more, this study also confirmed that ELP had no immunomodulating activity. Joensuu and co-workers (2009) demonstrated ELP application in production of antibodies for Foot-and-mouth disease virus (FMDV) therapy. Single chain variable antibody fragment (scFv) recognizing FMDV coat protein VP1 was expressed in transgenic tobacco plants. To recover the fusion protein in the active form the plants, ITC was performed. Finally, the authors demonstrated that scFv expressed in plants were able to bind FMDV.

It has been shown for spider silk proteins (Scheller et al., 2004), murine interleukin-4, human interleukin-10 (Patel et al., 2007) and anti-HIV type 1 antibodies (Floss et al., 2008, Floss et al., 2009) that the ELP fusion significantly enhances accumulation of recombinant proteins produced in plants. So far the mechanism of that phenomenon is not known.

5. Status of plant-derived biopharmaceuticals in clinical development

At present some non-pharmaceutical products from plants are on the market (Basaran and Rodriguez-Cerezo, 2008). Although no plant made pharmaceutical (PMP) has been commercialized as a human drug, several PMPs are at the late stage of development and some have already received regulatory approval, including a vaccine and several nutraceuticals (Table,7, 8, 9).

Antibodies	Target	Plant	Clinical trial status	Company	Source
DoxoRx	Side-effects of cancer therapy	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
RhinoRX	Common cold	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
IgG (ICAM1)	Common cold	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
CaroRX	Dental caries	Tobacco	EU approved as medical advice	Planet Biotechnology,	http://www.planet biotechnology.com

Table 7. Plant derived antibodies in clinical phages of development.

Antigen or vaccine	Disease	Plant	Clinical trial status	Company	Source
Hepatitis B antigen	Hepatitis B	Lettuce	Phase I	Thomas Jefferson University	Streatfield, 2006
Hepatitis B antigen	Hepatitis B	Potato	Phase II	Arizona State University	Streatfield, 2006
Fusion proteins	Rabies	Spinach	Phase I	Thomas Jefferson University	http://www.labome.org
Heat labile toxin B subunit of *E.coli*	Diarrhea	Potato	Phase I	ProdiGene	Tacket, 2005
Capsid protein Norwalk virus	Diarrhea	Potato	Phase I	Arizona State University	Khalsa et al., 2004
Vibrio cholerae	Cholera	Potato	Phase I	Arizona State University	Tacket, 2005
HN protein of Newcastle disease virus	Newcastle disease (Poultry)	Tobacco	USDA Approved	Dow Agro Sciences	http://www.dowagro.com
Viral vaccine mixture	Diseases of horses, dogs	Tobacco	Phase I	Dow Agro Sciences	http://www.dowagro.com
Poultry vaccine	Coccidiosis infection	Canola	Phase II	Guardian Bioscence	Basaran & Rodrigez-Cerezo, 2008
Gastroenteritis virus (TGFV) capsid protein	Piglet gastroenteritis	Maize	Phase I	ProdiGene	Basaran & Rodrigez-Cerezo, 2008
H5N1 vaccine candidate	H5N1 pandemic influenza	Tobacco	Phase I	Medicago	http://www.medicago.com

Table 8. Plant derived vaccines in clinical phages of development.

Therapeutic humans protein	Disease	Plant	Clinical trial status	Company	Source
α-Galactosidase	Fabry disease	Tobacco	Phase I	Planet Biotechnology	http://www.planet biotechnology.com
Lactoferon	Hepatitis C	Duckweed	Phase II	Biolex	http://www.biolex.com
Fibrinolytic drug	Blood clot	Duckweed	Phase I	Biolex	http://www.biolex.com
Human glucocerebrosidase	Gaucher's disease	Carrot	Waiting USDA's approval	Prostalix Biotherapeutic	http://www.prostalix.com
Insulin	Diabetes	Safflower	Phase III	SemBioSys	http://www.sembiosysys.com
Apolipoprotein	Cardio vascular	Safflower	Phase I	SemBioSys	http://www.sembiosysys.com

Table 9. Plant derived pharmaceuticals in clinical phages of development.

In 2006 the world's first plant made vaccine candidate for Newcastle disease in chickens, produced in a suspension cultured tobacco cell line by Dow Agro Science, was registered and approved by the US Department of Agriculture (USDA) – the final authority for veterinary vaccines. In addition, two plant made pharmaceuticals are moving through Phase II and Phase III human clinical trials. Biolex's product candidate, Locteron®, is in Phase IIb clinical testing for the treatment of chronic hepatitis CA. This company uses two genera, *Lemna* and *Spirodela*, as a platform for production of their biopharmaceuticals. The positive outcome of Phase III trials of Protalix's glucocerebrosidase (UPLYSO®) for the treatment of Gaucher's disease which is now waiting for USDA's approval is another positive example. The successful completion of Phase III trial that concerned SemBioSys insulin bioequivalent of the commercial standard represents an important landmark in the plant made pharmaceuticals scenario and, most likely, in the next few years recombinant human insulin produced in safflower will become commercially available for diabetic people.

Medicago Inc. of Canada was invited to the sixth WHO meeting about evaluation of pandemic influenza prototype vaccines in clinical trials. One of the purposes of this meeting was to make recommendations on research activities that will contribute to the development of effective pandemic vaccines. Medicago has recently reported positive results from a Phase I human clinical trial with its H5N1 avian influenza vaccine candidate (a VLP based vaccine produced with a transient expression system). The vaccine was found to be safe, well tolerated and it also induced a solid immune response. Based on these results, Medicago will process with Phase II clinical trial with the first plant made influenza vaccine (Franconi et al., 2010). These examples will pave the way to easy public acceptance of transgenic plants as new production platforms for human therapeuticals.

6. Concluding remarks

Biopharming is still a relatively new field in plant science but in the coming years it may become the premier expression system for a wide variety of new biopharmaceuticals. The use of plants as factories for the synthesis of therapeutic protein molecules will undoubtedly develop. Since the first development of a genetically modified plant in 1984, numerous comprehensive review articles have been published demonstrating the tremendous potential of plants for pharmaceutical production. As it has been clearly shown plants are no

longer considered only in terms of diet or beauty. The proteins targeted for biopharmaceutical technology form three broad categories: antibodies, vaccines, and other therapeutics. Plant bioreactors represent an attractive alternative for their synthesis requiring the lowest capital investment of all tested production systems. The events of heterologous proteins in planta production were rapidly followed with development/improvement of significant technologies (e.g. DNA delivery systems, selection methods). At present a number of promoters with tissue-specific activity or sub-cellular targeting sites that offer protein stability are known and many are still under intense study. Obviously, the construction of a transgenic plant synthesizing a functional therapeutic is a multidisciplinary process and the society of biotechnologists takes a keen interest in its success. However, over the past years various plant expression platforms have been tested and it is evident that further development and improvement are needed for more effective molecular farming. Apart from continuously increasing transgene yields efforts will need to ensure that plant-derived biopharmaceuticals would meet the same safety and efficacy standards as products of non-plant origin. There is no doubt that sooner or later the scientific limitations of molecular farming will be overcome, especially when numerous therapeutics and plant platforms are developed by many laboratories and companies. Thus, this is the regulatory requirements and public acceptance which are the greatest challenge of modern plant biotechnology. Of course, molecular farming raises less objection than technologies using genetically modified animals, but still the existing or proposed regulations remain based on public fears rather than on scientific facts.

In conclusion, "the molecular farming industry" means a natural advance in drug production technology. The dynamics of optimization and improvement of plant expression platforms illustrates its potential and tremendous scientific background. The possible success in this field will have to face the question of public acceptance. Thus, the scientists should send the clear massage to the public opinion that molecular farming is a strictly controlled technology that has strong benefits. And that probably will be more difficult than the construction of functional bioreactor itself.

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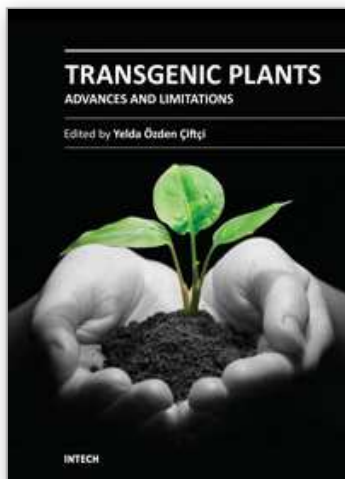
Transgenic Plants - Advances and Limitations

Edited by PhD. Yelda Ozden Çiftçi

Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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