

Transgenic Plants as Biofactories for the Production of Biopharmaceuticals: A Case Study of Human Placental Lactogen

Iratxe Urreta and Sonia Castañón
*Neiker-Tecnalia, Vitoria Gasteiz
Spain*

1. Introduction

Throughout human evolution, plants have provided us with food, fibers to produce clothes, and medicines to treat different kind of diseases. In fact, we could say that plants represent the “chemists” of the antiquity. Examples of important therapeutic molecules obtained from plants are morphine, atropine, ephedrine, codeine, and digitalin and so on (Farnsworth et al., 1985). Plants have always been a common source of medicaments, either in the form of traditional preparations or as pure active principles. For many people worldwide, natural plant-based remedies are used to treat both acute and chronic health problems. Indeed, about 40% of the drugs prescribed in the USA and Europe come from active compounds found in plants (Rates, 2001; Sivakumar, 2006). The chemical synthesis of these plant-derived compounds led to the production of pharmaceuticals at an industrial level, allowing the development of medicament industry. These active compounds from plants have been basically small molecules. Aspirin, one of the most famous medicaments, was developed as an analogous of the salicylic acid extracted from willow bark in the 19th century (Knäblein, 2005). Moreover, the development of more sophisticated extraction and purification procedures allowed the extraction of alkaloids (morphine) and other kind of molecules from plants and mammals (Liénard et al., 2007). However, chemical synthesis has some limitations to produce complex therapeutic molecules such as antibodies. So, many therapeutic molecules have to be isolated and purified from living material (e.g. from blood plasma), which involves a high risk of transmission of pathogens to the manufactured product (Engelhard, 2007).

The emergence of genetic engineering in the early 1970s has made possible a new way to produce pharmaceuticals outside their natural host. The term “biopharmaceutical” appears to be originated in the 1980s and is used to refer to therapeutic proteins produced by modern biotechnological techniques (Walsh, 2005a). Genetic transformation techniques have allowed the scientist to transform living organisms like bacteria, yeast, animal cells and plants into production “biofactories”. These organisms are forced to produce customized therapeutics making possible the treatment of diseases like genetic disorders, AIDS, and diabetes. Unlike chemically produced therapeutics, biopharmaceuticals are our own molecules and hence more compatible with biological systems (Rai & Padh, 2001).

Biopharmaceuticals represent the fastest growing sector within pharmaceutical industry, with worldwide sales of \$94 billion from the total market of \$600 billion in 2007. In 2009, biopharmaceuticals recorded global sales of \$99 billion (\$61 billion for therapeutic proteins and \$38 billion for monoclonal antibody (mAb)-based products (Walsh, 2010), and these numbers are expected to reach sales of \$125 billion by the year 2015 (Xu et al., 2011). Regarding the most lucrative molecules, mAb-based products indicated for treating cancer are the bestsellers of biopharmaceuticals. The next most lucrative group is represented by insulin and insulin analogs, generating \$13.3 billion in sales, followed by EPO-based products whose sales stands at \$9.5 billion (Walsh, 2010).

The biopharmaceutical industry relies basically in non-human mammalian cell lines like CHO cells, and bacterial systems. In mammalian cell lines human proteins are correctly processed and modified, but the scalability is limited, maintenance of bioreactors is expensive, and there is a risk of contamination of the product with human pathogens (Daniell et al., 2001; Twyman et al., 2005). *Escherichia coli* was the pioneer expression system and in 1977 it was used for the successful expression of somatostatin (growth hormone-inhibiting hormone). *E. coli* is widely used because it is the most cost-effective production system, allowing the large-scale production of proteins (Rai & Padh, 2001). Insulin produced in *E. coli*, also known as "humulin", received the marketing authorization in 1982 which represented the beginning of the biopharmaceutical industry (Walsh, 2005b). However, the prokaryotic nature of this organism limits the complexity of the proteins that can be correctly processed, and the appearance of inclusion bodies increases the cost of the product (Boehm, 2007). Yeast-based systems are able to grow in well-defined simple media, in large-scale bioreactors and at high cell-densities (Gerngross, 2004). Therefore interferon was expressed in yeast (1981). However, the low product yields, inefficient protein secretion and hyperglycosylation of proteins (addition of large number of mannose residues) are common in problems in this system. Transgenic animals allow the scaling-up of protein production but they require long generation times, and have technical difficulties. Moreover, ethical aspects also limit this system (Hunter et al., 2005). Insect cell-based systems provide a eukaryotic expression system readily amenable to scale-up, but they are inefficient in proteolytic cleavage of proteins as well as for glycosylation (Rai & Padh, 2001). Nevertheless, β interferon and factor IX were expressed in insect cells (1983) and transgenic animals (1988), respectively (Desai et al., 2010).

Genetic engineering techniques have made it possible to produce therapeutic molecules using different host system. But what are the ideal characteristics that should satisfy an expression system to be considered a good system? It is important to (1) produce proteins with the correct conformation and biologically active, (2) allow good productivities, (3) have minor economic costs associated to manufacturing and purification, and (4) be recognized as safe system by the society.

Despite the variety of expression systems available for biopharmaceutical industry, none of them meets completely all the requirements for a production system to be able to produce any kind of therapeutic protein. There is no ideal or universal expression system. All of them have some advantages and some limitations, so the choice for the best system should be done by evaluating biological as well as economic aspects, and bearing in mind the purpose of the product of interest.

2. Transgenic plants as production platform for biopharmaceuticals

The total global market for biopharmaceuticals is projected to grow at between 7% and 15% annually over the next several years, with mAb-based approvals continuing to dominate (Walsh, 2010). Efforts on human genome project have identified new proteins with therapeutic potential and the growing number of candidates in clinical trials will be in the market in the next years. So it just keeps getting more apparent that the traditional systems used by the industry so far will not be able to meet the growing demand and will represent a bottleneck in bringing therapeutic proteins to the society. Pharmaceutical industry requires a production platform able to provide enough quantity of safe and high-quality products at the lowest cost. Transgenic plants are becoming one of the most interesting alternative systems for biopharmaceutical production as they offer many advantages over traditional systems.

One of the most important benefits of transgenic plants is the capacity to obtain high production volumes at relatively low cost, due to their flexibility in terms of scale-up (Sparrow et al., 2007). It has been estimated that the cost associated with the production of 300 kg of a secretory antibody in maize or tobacco plants is 0.5-2 million \$ per year, while the cost increases to 6-7 million per year using transgenic goat or mammalian cell cultures (Gerlach et al., 2010). Transgenic plants allow the increase or decrease of cultivated area depending on the market demand. Moreover, the availability of harvesting, storage and transport infrastructures turns plants into a very efficient production system (Kusnadi et al., 1997).

As eukaryotic organisms, protein synthesis pathways are highly conserved among plants and animals, so plant cells are able to correctly fold and assemble proteins as well as to perform post-translational modifications required for protein activity and stability (Kamenarova et al., 2005). This is demonstrated by the ability of plant cells to produce various types of antibodies, such as IgGs and IgAs, which are complex proteins requiring the assembly of various polypeptide chains through disulphide bonds (Twyman et al., 2003). Apart from cultured mammalian cells, only plants are able to assemble the light and heavy chains of antibodies (Gomord et al., 2004). For this reason, amino acid sequences of human proteins expressed in plant cells are usually the same as native counterparts, which ensures the quality of products produced in plants (Schillberg & Twyman, 2007). Moreover, plant cells have a very high ratio of biologically active protein (92% of total protein in tobacco BY-2 cells) when compared to *E. coli* (12%) and *P. pastoris* (40%) (Boehm, 2007). Proteins produced in plant cells are less likely to be contaminated with human or animal pathogens and hence product safety can be guaranteed.

The diversity of plant host makes possible the production of oral vaccines in edible parts of the plant, eliminating the need of cold-chain and economic costs associated with purification processes, and thus being more amenable for developing countries. Moreover, tissues like seeds, tubers or fruits allow the storage and stability of biopharmaceuticals produced in plants (Desai et al., 2010).

One of the major challenges of plant systems, besides low protein expression level which will be discussed below is the inability of plants, as in other eukaryotic systems, to perfectly reproduce human-type glycosylation on biopharmaceuticals. Glycosylation is the most widespread post-translational modification with more than half of the human

biopharmaceuticals being glycoproteins. Glycosylation affects their function, plasma half-life and/or biological activity (Saint-Jore-Dupas et al., 2007). Main differences between plants and mammal cells are relative to modification of glycans in Golgi apparatus. Plants do not have sialic acid residues present in human glycoproteins, and add α (1,3)-fucose and β (1,2)-xylose residues which are responsible of immunogenic response in human therapy (Chen et al., 2005). However, there are some strategies available to “humanize” recombinant glycoprotein produced in plants. The first one is based on endoplasmatic reticulum (ER) retention of proteins in their biosynthetic pathway bypassing Golgi apparatus. This can be done through the addition on KDEL sequence in the recombinant protein (Gomord & Faye, 2004). The second one relies on inactivation or down-regulation of plant xylosyltransferase and fucosyltransferase using RNA interference technology. Alternatively it is possible to “humanize” glycoproteins co-expressing human glycosyltransferases (Gomord et al., 2004) or by *in vitro* galactosylation of recombinant proteins using mammalian enzymes (Bardor et al., 2003).

Another future challenge for plants is the concern about biosafety and regulatory issues. General public is very reluctant to transgenic crops, especially since the appearance of those resistant to pests or pesticides, and unfortunately transgenic plants for biopharmaceutical production must suffer the same rejection. The main biosafety problems are related to risks associated to human/animal health and to the environment, so it is very important to evaluate and manage carefully those risks through risk assessments (Peterson & Arntzen, 2004). There are many strategies available to avoid those problems: apart from delimitation of physic barriers and strict agricultural management (crop destruction, field cleaning, and crop rotation) to avoid mixing of modified and food/feed crops, the most obvious alternative is the use of non-food/feed crops (tobacco, *Arabidopsis thaliana*, *Phiscomitrella patens*, *Lemna* sp.), or the use of greenhouses/glasshouses where crops are confined and controlled. Biological containment provides a natural and additional barrier to gene flow. Production of biopharmaceuticals in self-pollinating species (rice, wheat, and pea) or engineering other species to make them cleistogamic (self-pollination before flower opening) is interesting for gene containment (Commandeur et al., 2003).

Biotechnological tools have allowed the development of different strategies for gene and protein containment. Chloroplast transformation technology is a very attractive approach as chloroplast genome is maternally inherited in most crops, and gene spread via pollen occurs at low frequency (Svab & Maliga, 2007). The use of male-sterile plants (Gils et al., 2008) or “terminator” technology (also known as GURTs, “Genetic use restriction technologies), which is based on a repressible system to produce plants with non-viable seeds unless the plants are exposed to specific activators (Lee & Natesan, 2006), have also been used to contain transgenes. Other strategies include organ- or tissue-specific expression of transgenes, ER-retention of proteins and inducible promoters (Obembe et al., 2010).

It is clear that taking into account the previously reported concerns regarding health and environmental risks, transgenic plants producing biopharmaceuticals must be regulated strictly, and in fact they are. However, there is a need for the harmonization of international regulation of plants producing biopharmaceuticals in European Union and USA (Gerlach et al., 2010) and to unify the criterions. Plants as production system for biopharmaceuticals comply not only with the strict regulatory requirements covering other GM crops, but also the regulations set out by agencies that oversee the production of pharmaceuticals (Sparrow et al., 2007).

Plants represent a very versatile and plastic expression system to produce biopharmaceutical proteins, because they offer a great variety of strategies. Depending on production needs, product quality, or compliance with certain legal requirements, we can choose between different plant-based systems without the need to use bacteria or animal cells.

The most used strategy for protein production is that based on whole plants. Most biopharmaceuticals are produced in nuclear transformed plants, which are obtained mainly by using soil pathogen *Agrobacterium tumefaciens*, and allow the stable expression of transgenes with the required post-translational modifications. Genetic transformation of chloroplast to obtain transplastomic plants constitutes a promising alternative strategy for protein production due to the important advantages over nuclear transformation. Proteins can achieve high expression levels due to high copy-number of transgene per cell. It has been reported an expression level of up to 31% of total soluble protein (TSP) for an animal vaccine produced in transplastomic tobacco plants (Molina et al., 2004). Chloroplast transformation is based on homologous recombination events so there is no gene silencing (Maliga, 2003). It is possible to integrate multiple genes in operons and, as previously mentioned, this system offers transgene containment through maternal inheritance (Bock & Khan, 2004). However, in the chloroplast it is not possible to carry out post-translational modifications present in the ER limiting the range of proteins that can be produced in this system. Against stable nuclear or chloroplast transformation, plants can be transiently transformed by agroinfiltration with *A. tumefaciens*, biolistic methods and viral vectors. This strategy is very useful for transformation construct verification and to test functionality of recombinant proteins (Fischer et al., 2004).

2.1 Plant cell cultures

As an alternative to whole plants, biopharmaceuticals can be produced using plant cell cultures. Plant cell culture is a very interesting and promising alternative system and has been used for almost two decades for protein production, as well as for secondary metabolite production.

Suspended cell cultures are derived from callus, which are unorganized and generally undifferentiated cell aggregates derived from plant tissues cultured in solid media supplemented with growth regulators. Calli are suspended in liquid media to form a homogeneous suspension. Transgenic suspension cultures can be obtained from wild type callus or suspensions transformed by *A. tumefaciens* or biolistic methods, depending on plant species, but also from transgenic plant tissue such as leaf or stem. With the second approach there is no need for further genetic manipulation like transformed tissue selection and line screening (Hellwig et al., 2004).

Since 1990, when Sijmons et al. reported the expression of human serum albumin in transgenic tobacco suspension cells, a diverse array of biopharmaceuticals have been produced using suspended cell cultures. Huang & McDonald (2009) reported an extensive list of recombinant proteins produced in this system, including human erythropoietin, human granulocyte-macrophage colony-stimulating factor (hGM-CSF), human interleukins, hepatitis B surface antigen, and many types of antibodies. Although productivity of plant cell cultures can vary considerably from 0.5 µg/L to 200 mg/L (Hellwig et al., 2004), many proteins have been produced at high yields (>10 mg/L) (Xu et

al., 2011). Most of biopharmaceutical proteins have been expressed in tobacco cells like well characterized BY-2 and NT-1 cell lines, because these host lines are fast-growing, well synchronized and susceptible to *Agrobacterium* mediated transformation (Nagata & Kumagai, 1999). Other host plants used include rice, an emerging host specie (Kim et al., 2008), sweet potato (Min et al., 2006), tomato (Kwon et al., 2003), and carrot (Shaaltiel et al., 2007).

Suspended cell cultures offer many advantages over whole plant systems, and perhaps one of the most important is that *in vitro* plant cultures, as grown in confined and sterile conditions, avoid the political resistance to release of genetically modified plants to the field. Moreover, confined culture eliminates problems related to weather conditions, soil quality, season, plagues, and contamination with agrochemicals and fertilizers that affect field grown plants (Weathers et al., 2010). This is an ideal characteristic for production of high purity biopharmaceuticals. Whole plant systems require longer period of time to be productive (sowing, growing, harvesting) while proteins could be manufactured in days or weeks on a time-scale compatible with that of market demands (Doran, 2000). Growth in bioreactors not only allows the reduction in production cycles, but also the scalability and the tight control of growth parameters (temperature, O₂ supply, agitation, pH), ensuring product quality and batch-to-batch consistency, as well as product traceability (Ma et al., 2005). These features make the system amenable to good manufacturing processes (GMP), facilitating compliance with regulatory and environmental requirements (Spök et al., 2008). Bioreactor technology provides a great variety of culture modes and bioreactor types to increase recombinant protein production. Large-scale culturing can be done in standard stirred-tank bioreactor, pneumatic bioreactor (bubble column or air-lift), wave bioreactor, membrane bioreactor, hollow fiber bioreactor or miniature bioreactor (Huang & McDonald, 2009). Regarding to operation modes, we can choose between batch culture, fed-batch culture, continuous and semi-continuous culture, and perfusion culture (Xu et al., 2011). Choosing a suitable bioreactor type and operation mode should include adequate oxygen mass transfer to cells, low shear stress to cells, and proper nutrient supply to cells and product removal from cells (Huang & McDonald, 2009).

The potential of suspended cells to secrete biologically active proteins into the culture medium has a big impact on downstream processing costs. Cells are grown in relative simple and synthetic protein free media which facilitates the recovery of proteins (Hellwig, 2004). Protein secretion is determined by the presence of signal peptide and protein size to pass through wall pores. Due to their undifferentiated nature, callus cells lack functional plasmodesmata, and as grown in suspended form, there is minimal cell-to-cell communication (Su, 2006). All those features can reduce post-transcriptional gene silencing (PTGS) because signal transmission between cells is avoided (Doran, 2000). In conclusion, plant cell suspension cultures integrate many of the advantages of whole plant systems with those of microorganisms and mammalian cell cultures.

In contrast to suspended cells, plant cells can also be immobilized for biopharmaceutical production. This system is based on the immobilization of cells using encapsulating gels, such as alginate, which protect cells against mechanical damages. Immobilization also facilitates the re-use of cells in continuous or semi-continuous culture, allowing higher inoculums than standard methodologies (James, 2001).

Differentiated organs such as hairy roots and shooty teratomas have also been developed for protein production. Hairy root cultures have been used for decades to produce secondary metabolites such as resveratrol (Medina-Bolivar et al., 2007). To generate hairy roots, wounded transgenic host plant is co-cultivated with *Agrobacterium rhizogenes* which transfers *rol* and *aux* genes which are responsible of root phenotype and induction (Sivakumar, 2006). Hairy root cultures, grown in hormone-free medium, are genetically stable over time and allow uniform expression of proteins at high level in relatively short periods of time (Franconi et al., 2010). Secretion of proteins is also allowed in root cultures which can be cultured in bioreactors or in hydroponic tanks from where they take water and nutrients while releasing proteins continuously (Knäblein, 2005). Shooty teratomas are generated from transgenic seedlings co-cultured with *A. tumefaciens* strain T37. After co-culture and *Agrobacterium* elimination, shoots are cultured in liquid medium (Sharp & Doran, 2001).

2.2 Emerging plant-based systems

In the last decade alternative novel expression systems have been developed for the production of biopharmaceuticals. We would like to highlight the potential of novel systems such as mosses, algae and aquatic plants which can be cultured in contained conditions in bioreactors.

Mosses as multicellular eukaryotic organisms can produce biologically active therapeutic proteins. *Physcomitrella patens* is the model organism which has been studied for a long time. It can be cultured during its complete lifecycle, but when cultured in liquid medium vegetative growth is favored (Franconi et al., 2010). The fact that *P. patens* is grown in small plant fragments, and not as protoplast, provides genetic stability avoiding somaclonal variation (Knäblein, 2005). The moss is photoautotrophic and only requires inorganic salts, water and CO₂ for growth, so it is easily cultured in stirred glass tanks or tubular photobioreactors. The most interesting feature of *P. patens* is its high frequency of homologous recombination which facilitates the precise knockout of genes (Decker & Reski, 2007). In this context, genes for $\alpha(1,3)$ -fucosyltransferase and $\beta(1,2)$ -xylosyltransferase were disrupted by homologous recombination to obtain double knockout clones of *P. patens* with no allergenic N-glycans (Koprivova et al., 2004). The potential of this species for biopharmaceutical production gave rise to the company Greenovation in 1999. Since 2001, the company is developing the “bryotechnology”, using double-knockout strain of *P. patens* cultured in photobioreactors. Currently this company is producing a variety of biopharmaceuticals such as growth factor (VEGF), serum proteins (HSA), peptide hormones (EPO), enzymes (phosphatase), vaccines and a wide range of oncology mAb's in 100L tubular bioreactors and 200L disposable bag systems (www.greenovation.com).

Microalgae represent a diverse group of prokaryotic (cyanobacteria) and eukaryotic photosynthetic microorganisms that are found in marine and freshwater environments. Microalgae combine simple and inexpensive growth requirements and ability for post-transcriptional processing of proteins, with the rapid growth rate and potential for high-density culture (Walker et al., 2005). Most green algae are classified as generally regarded as safe (GRAS), making processing of expressed products more amenable to regulatory issues (Potvin & Zhang, 2010), and offering a safe platform for vaccine production. Microalgae can be grown in contained bioreactors in a matter of weeks from initial transformation event to

large-scale protein production, and as single cell type culture, there should be less variation in protein accumulation, making downstream processing more uniform (Specht et al., 2010). However, light utilization and distribution in the bioreactor represents a limiting factor for cell growth which requires careful design of bioreactors. In this regard, heterotrophic growth using glucose as carbon source should be advantageous for well established “dark” bioreactors allowing high cell density (Franconi et al., 2010). *Chlamydomonas reinhardtii* is the most successfully used microalgae, because it is genetically well characterized with all three genomes (the nuclear, chloroplast and mitochondrial) sequenced, and genetic transformation methods are well established (Rasala & Mayfield, 2011). Proteins such as human antibodies (Mayfield & Franlikn, 2005), human glutamic acid decarboxylase 65 (hGAD65) (Wang et al., 2008), domain 14 of fibronectin (14FN3), VEGF and HMGB1 (Rasala & Mayfield, 2011) have been produced in *C. reinhardtii*.

Finally we would like to pay attention to aquatic higher plants from *Lemnaceae* family such as those from *Lemna*, *Spirodela* and *Wolffia* genus. These edible plants are safe, fast-growing, and easy to grow and harvest species amenable to genetic transformation using *A. tumefaciens* or biolistic method (Weathers et al., 2010). *Lemna minor* has been used to produce mAb's fused with an RNA interference construct targeting the expression of endogenous genes $\alpha(1,3)$ -fucosyltransferase and $\beta(1,2)$ -xylosyltransferase to obtain an antibody without plant specific N-glycans (Cox et al., 2006). Many therapeutic proteins have been expressed in this system, mainly by Biolex Company and its subsidiary LemnaGene who produce interferon for hepatitis C treatment, plasmin for thrombosis treatment and anti-CD20 antibody optimized for the treatment of non-Hodgkin's B-cell lymphoma (www.biolex.com). At the present time, *Spirodela oligorrhiza*, with an expression level for GFP protein of 25% of TSP represents the best expressing system for nuclear transformation in higher plants (Franconi et al., 2010).

2.3 Factors influencing therapeutic protein production in plants

Many authors agree that one of the most important limitations of transgenic plants is their low expression level of recombinant proteins. Increasing the amount of recombinant protein is crucial for the system to be economically viable. The yield of recombinant proteins produced in plants depends on many factors: the intrinsic limitations of host plant, limitations imposed by transgene expression and protein stability, which are optimized by careful design of expression vector, and downstream processing. Finally, we would like to analyze the environmental factors affecting transgenic plants in open field.

2.3.1 Choice of host plant

The range of plant species amenable to genetic transformation is very wide so there is no an ideal host for molecular farming. There are many factors needed to be taken into account when choosing the host plant: from production factors such as infrastructure availability, storage and distribution cost, or plant productivity, to factors affecting environment and human health and food safety (Schillberg & Twyman, 2007).

Usually host plants are divided into food crops and non-food crops. Tobacco is the most used specie among non-food crops due to its easy of genetic transformation (both nuclear and chloroplast genomes), high biomass yield and seed production, and ability to produce many therapeutic proteins (antibodies, vaccines, cytokines, serum and blood proteins,

hormones). Its widespread use has led to the production of low-alkaloid cultivars suitable for oral delivery of vaccines (Tremblay et al., 2010). It has also been reported that the expression of biologically active GM-CSF in commercial sugarcane which is propagated vegetatively from stem pieces offering a “secure” platform for production of recombinant proteins (Wang et al., 2005). Food-crops include seed-crops, vegetables and fruits. Species such as cereals, rice, maize, soybean, or barley allow the expression of recombinant proteins in the seed which enables the long-term storage and the containment of proteins avoiding the exposure of non-target organisms. However seed-crops must go through a flowering cycle to produce seeds, so it is very important to control pollen transfer (Sparrow et al., 2007). Oilseed crops are useful for protein production because the fusion of recombinant proteins to the endogenous protein oleosin allow the easy recovery of recombinant proteins (Stoger et al., 2005). Vegetable crops include potato, worldwide cultivated specie with a very well-developed agricultural infrastructure. Tuber has been used mainly for oral vaccine production using tissue-specific promoter patatin. This vegetative organ offers stability to the recombinant protein. Carrot, alfalfa, lettuce and spinach have also been used for vaccine production because can be consumed raw, because can be grown again after leaf harvest, or because are very easy to scale-up (Sparrow et al., 2007). Fruits such as tomato and banana, more palatable than potatoes for raw consumption, are suitable for oral vaccines because of high biomass yield (tomatoes) and easy distribution (bananas) (Kamenarova et al., 2005).

2.3.2 Design of the expression vector

The expression vector is the vehicle to integrate the gene of interest into the plant genome. As stated before, the final yield of recombinant protein depends on many factors, and most of them can be addressed through inclusion of suitable regulatory sequences in the vector. In this context we can control factors that affect transcription, translation as well as protein accumulation. Figure 1 shows the schematic representation of a general expression vector showing potential regulatory sequences.

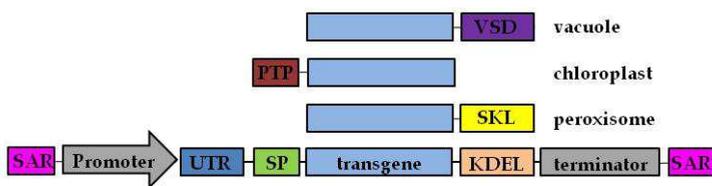


Fig. 1. Schematic diagram of plant expression vector showing the main regulatory sequences. SAR, scaffold attachment region; UTR, untranslated region; SP, signal peptide; KDEL, endoplasmic retention signal; SKL, peroxisomes target sequence serine-lysine-leucine; PTP, plastid transit peptide; VSD, vacuole sorting determinant.

2.3.2.1 Promoter

This is one of the most important factors affecting transcription of genes because it is responsible of when, where and how does the gene transcribe. Promoters can be from plant or viral origin and usually are divided into constitutive, tissue-specific and inducible categories. Constitutive promoters drive the continuous expression of the transgene in all plant tissues. The best known and most used promoter is derived from the cauliflower mosaic virus (CaMV35S), a strong promoter mainly used for dicot species (Xu et al., 2011).

The duplication of an enhancer region located in the 5' region of CaMV35S promoter allowed the development of the strongest version commonly known as double CaMV35S (2xCaMV35S) (Kay et al., 1987). For monocot species, rice actin gene or bean arcelin gene promoters are most used. The choice of promoter also depends on the nature of recombinant protein: when the protein of interest is toxic for the host plant, tissue-specific expression could be a helpful strategy (Desai et al., 2010). This type of promoter allows the control and restricted expression of transgenes to specific tissues such as leaf (small subunit of Rubisco), seeds (maize globulin-1, barley D-hordein, rice glutelin), and storage organs (tuber patatin). Inducible promoters allow not only the tight control of gene expression, but also the quantity of expression level. Induction can be regulated through chemical stimuli (steroid, sucrose, salt) and/or environmental factors (light, temperature, wounding). In this kind of promoters it is very important to specify between promoter and inductor, the rapid response upon induction, and the safety of the inducer for plant (Corrado & Karali, 2009).

2.3.2.2 5' UTR sequences

The UTR sequences are located in the upstream region of ORF and are related to the efficiency of initiation of translation. In eukaryotes this process is thought to follow the scanning mechanism conducted by small subunit of ribosome from 5' cap of mRNA through the untranslated leader until the first start codon (AUG) is found (Kermode, 2006). Including 5' UTR sequences from Alfalfa mosaic virus (AMV), Tobacco mosaic virus (TMV), *Chalcone synthase* (CHS), or *Alcohol dehydrogenase* (NtADH) have been successfully used to enhance the translation efficiency, allowing transgene levels 30 to 100-fold higher (Sato et al., 2004; Schiermeyer et al., 2005).

2.3.2.3 Terminator sequences

Also known as polyadenylation signals, these sequences are very important for RNA stabilization because they are responsible for the correct processing of RNA after stop codon (Desai et al., 2010). The most commonly used terminator sequences are derived from the *A. tumefaciens nopaline synthase* gene which has been used successfully in both dicot and monocot plants.

2.3.2.4 Codon optimization of transgenes

Many of the transgenes introduced in plants for biopharmaceutical production come from humans. Codon usage between divergent species is often very different and will affect the expression level of recombinant proteins (Lessard et al., 2002). Based on available data of codon usage for a given host plant, transgene codons with the lowest usage frequency are modified by changing the nucleotide sequence without changing the amino acid sequence (Desai et al., 2010).

2.3.2.5 SAR/MAR sequences

The inclusion of sequences containing scaffold/matrix attachment regions (SAR/MAR) flanking the expression cassette has been used as a mechanism to increase transgene expression. SAR/MAR are AT-rich DNA sequences of 300-500 bp-long which interact with nuclear scaffolds organizing the structure of the genome (Allen et al., 2000). These sequences facilitate transcription of genes by changing the chromatin topology into less condensed regions (Kermode, 2006). With the use of SAR/MAR sequences it is possible to increase transgene expression levels through stabilization of the expression in progeny and reduction of expression variability (Ulker et al., 1999).

2.3.2.6 Subcellular targeting

Specific targeting of recombinant proteins to plant organelles not only guarantees the correct post-translational modifications required, but also allows the enhancement of protein stabilization, minimizing proteolytic degradation and facilitating downstream processing such as purification (Kermode, 2006). Eukaryotic preproteins synthesized with an N-terminal signal peptide are targeted to the secretory pathway. In the absence of signal peptide, recombinant protein is accumulated into the cytosol. In most cases this is not an appropriate compartment for recombinant proteins due to its hydrolytic activity and its negative redox potential which is unfavorable for protein folding (Benchabane et al., 2008).

In the case of proteins with signal peptide and no further targeting, expressed proteins are secreted from the ER to the apoplast. This compartment has been implicated in the production of proteolytic fragments in several transgenic plant systems due to proteases found in the secretory pathway between the ER and Golgi (Doran, 2006).

One of the most used compartments within plant cells for subcellular targeting is the ER. This organelle has many advantages for recombinant protein accumulation due to its specific characteristics. ER has very low hydrolytic activity and can tolerate unusually high accumulation of proteins without compromising plant development and reproduction because of its plasticity to become a reservoir of protein and oil bodies (Vitale & Pedrazzini, 2005). Recombinant proteins targeted to ER are protected from proteolytic degradation and correct folding and disulphide bond formation are allowed (Benchabane et al., 2008). Inclusion of KDEL/HDEL tetrapeptide in the C-terminal region of the protein ensures ER-retention. Targeting recombinant proteins into the ER has led to an increase in expression levels from 4.5 to 100-fold (Fiedler et al., 1997; Schouten et al., 1996; Torres et al., 1999).

Another suitable cell compartment for recombinant proteins accumulation is the vacuole. Important functions of this organelle include control of cell turgor, turnover of macromolecules, sequestration of toxic compounds, and finally, storage of high-energy compounds (Benchabane et al., 2008). From two types of vacuoles in plants, only protein storage vacuoles which are very abundant in seeds are suitable for recombinant proteins because of their mild environment (Stoger et al., 2005).

With chloroplast transformation, it is possible to target the recombinant protein to this organelle through the suitable targeting signals. The chloroplast-transit peptide of potato *rbcs1* gene allowed the expression of human papillomavirus type 16 L1 (HPV-16 L1) protein into chloroplast of *Nicotiana benthamiana*, obtaining expression levels of up to 11% of TSP (Maclean et al., 2007). Moreover, chloroplast targeting can avoid the toxic effect of recombinant protein in the cytosol (Gils et al., 2005; Hühns et al., 2008).

It has also been reported that the subcellular targeting to mitochondria (Menassa et al., 2004) and chloroplast and peroxisomes at the same time, it is possible to accumulate the recombinant protein 160% of that in chloroplasts alone and 240% of that in peroxisomes alone (Hyunjong et al., 2006).

2.3.2.7 Protein/tag fusions

Expression of the recombinant protein attached to a protein with enhanced stability may prove to be useful to enhance fusion partner. In plants, several authors have reported the suitability of engineering fusion proteins to improve expression levels (Wirth et al., 2006; Xu

et al., 2010). Many fusion proteins can also serve as purification tags due to their specific binding features or easy of recovery, but those will be discussed in the next section.

2.3.3 Downstream processing

The downstream processing of recombinant proteins includes protein extraction, purification and characterization, a process that may represent 80% or more of total production costs (Twyman et al., 2003). Purification strategies may be based on standard chromatographic techniques, but affinity purification using His-tag, protein A, or glutathione S-transferase (GST) is an alternative approach widely used by researchers (Arнау et al., 2006). We have successfully used His-tag to purify human placental lactogen (hPL) from tobacco leaf tissue (Urreta et al., 2010).

Fusion of recombinant proteins to endogenous proteins such as oleosin or γ -zein is also very attractive for therapeutic protein production and is gaining more attention for commercial issues. Oleosin fusion platform has been developed by SemBioSys Inc. (www.sembiosys.com) and allows recombinant protein to be targeted to the oil bodies in rapeseed and safflower. The purification is based on separation of oil bodies by simple and inexpensive schemes (Schilberg & Twyman, 2007). Similarly, fusion of recombinant protein to γ -zein, a prolamin of maize, induces protein body formation and high accumulation of foreign protein within ER. Protein bodies are insoluble and easily purified by centrifugation (Vitale & Pedrazzini, 2005). An alternative emerging purification strategy relies on elastin like polypeptide (ELP) fusion to recombinant protein (Floss et al., 2009).

2.3.5 Environmental factors

Plants, as grown in the field or in the glasshouse, are subjected to environmental factors. Those factors influence their health condition, and subsequently, the quality and the quantity of recombinant protein. The stability of foreign protein in plants grown in the field is very important because it determines the homogeneity of the product. The most important environmental factors are light, temperature, soil (nutrients), water and insect attacks (Jamal et al., 2009). Most of these factors can be controlled to greater or lesser extent for plants cultivated in the glasshouse. However, when transgenic plants are grown in the field, light and temperature became the critical factors. Light is crucial for plants as energy source and temperature also affects plant growth and productivity being especially dangerous at high temperatures. The analysis of the influence of plant's physiology on recombinant protein accumulation is crucial to plan the best strategy for plant harvesting (Conley et al., 2010).

Although protein production in plants has long been investigated, the most of the reported works are based on plants grown in glasshouses under controlled conditions. Arlen et al. (2007) reported that the field production of chloroplast derived interferon (IFN) from tobacco plants. They cultivated 0.26 acre containing 7369 plants and obtained 107.7 kg of biomass from a single harvest. This biomass contained approximately 87 g IFN (0.8 mg g^{-1}). It must be noted that tobacco plants can be harvested 4-6 times within a grow season from a 1 acre. They also tested light and temperature influence on glasshouse grown plants revealing the importance of leaf maturity and illumination, and most importantly, they observed higher IFN yield in those plants ($1-3 \text{ mg g}^{-1}$ fresh weigh).

3. Case study: Production of human placental lactogen in transgenic plants

Human placental lactogen (hPL), or chorionic somatomotropin, is a 22 kDa peptidic hormone secreted by the placenta during pregnancy (Barrera-Saldaña et al. 1982). This protein is involved in the adaptation of islets of Langerhans to pregnancy through the regulation of beta (β) cell mass and function (Brelje et al. 1993). The capacity of hPL to improve β cell function, proliferation and survival *in vitro* and *in vivo* (Vasavada et al. 2000) makes possible its use as therapeutic protein for Langerhans islet transplantation to patients with type 1 diabetes. The transplantation of pancreatic tissue has become an interesting alternative treatment for diabetes, allowing the independence of patients to insulin injection. Unfortunately, the low availability of pancreatic tissue and great prevalence of the disease limits the potential of this treatment. Therefore, hPL together with other peptidic growth factors (HGF and PTHrP) belongs to the short list of proteins capable of improving the critical beta cell parameters, namely function, proliferation and survival, to improve tissue availability for islet transplantation (Fujinaka et al. 2007). Currently, the commercially available hPL protein is purified from human placenta which increases not only the risk of human pathogen propagation but also increases the cost of the protein in the market. This hormone has also been expressed in *E. coli* as inclusion bodies, which required the solubilization and refolding of the protein (Lan et al., 2006), making the process and the product more expensive. Due to the potential of hPL protein for type 1 diabetes treatment, we analyzed the suitability of potato plants as an alternative production system for hPL protein. In our laboratory, we thought that due to the potential of PL as a candidate for the treatment of type-1 diabetes, it would be very interesting to investigate alternative production strategies for the safe and easy production of this protein at low cost. Of course, plants represent for us the better production platform for all the reasons previously exposed and discussed, as well as for our experience on therapeutic protein production in plants.

We have successfully expressed hPL protein in *Nicotiana tabacum* cv. Xanthi plants reaching expression levels of up to 1% of TSP (Urreta et al., 2010). *In vitro* bioassays using the rat insulinoma (INS-1) cell line showed that recombinant protein was able to induce cell proliferation, demonstrating that plant cells can produce the biologically active hPL protein. Due to the difficulty to evaluate the relative performance of different crops for commercial production of therapeutic proteins which requires the production of the same protein in different host plant (Schilberg & Twyman, 2007), we attempted to produce hPL protein in *Solanum tuberosum*. We chose potato because it is easily transformed and widely used for molecular farming. Potato is an important crop worldwide and in our region, Álava (Spain). Indeed, our research institute has a long experience in the cultivation and improvement of this crop, being the reference germplasm bank in Spain.

To achieve our objective, the cDNA encoding hPL gene was obtained by RT-PCR from human placenta mRNA (Clontech) as previously described (Urreta et al., 2010). Briefly, the amplified sequence of 654-bp was ligated into pGJ2750 binary vector (kindly provided by Max Planck Institute, Köln, Germany) to obtain pNEKhPL1 expression vector where hPL gene is regulated by the promoter and terminator of CaMV 35S gene. The expression vector was introduced into *Agrobacterium tumefaciens* EHA105 strain and co-cultivated with *Solanum tuberosum* cv. Désirée leaf disks following the protocol described by Dietze et al. (1995). The regenerated shoots (Figure 2A) were subcultured in MS medium (Murashige & Skoog 1962) supplemented with kanamycin (100 mg L⁻¹) at 22°C, under 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photon flux and a 16-h photoperiod in a growth chamber. Most of the regenerated putative

transgenic plants had the same phenotype as the non transformed wild type plants (Figure 2, B-C). Integration of hPL and *neomycin phosphotransferase (nptII)* genes in the genome of regenerated plants was verified by polymerase chain reaction (PCR) using specific primers. All transgenic plants screened by PCR were positive (Figure 2, D-E), ensuring the correct integration of T-DNA in the genome of plants. Further molecular characterization of recombinant hPL protein was assessed by western blot. The proteins were separated on 12% polyacrylamide gels under reducing conditions and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked for 1 hour in PBS with 0.05% (v/v) Tween-20 (PBS-T) and 5% non-fat dry milk, and incubated with polyclonal anti-hPL antibody (RB9067, NeoMarkers, Fremont, CA, USA). Blots were incubated with Anti-Rabbit IgG secondary antibody conjugated with alkaline phosphatase (Sigma-Aldrich, St Louis, MO, USA). Antibody binding was detected with NBT/BCIP (Sigma). The recombinant protein was detected in leaf protein extracts of transgenic plants cultivated in the greenhouse, which showed the expected molecular weight of 22 kDa (Figure 2F) demonstrating that plant cells are able to process signal peptides from human origin.

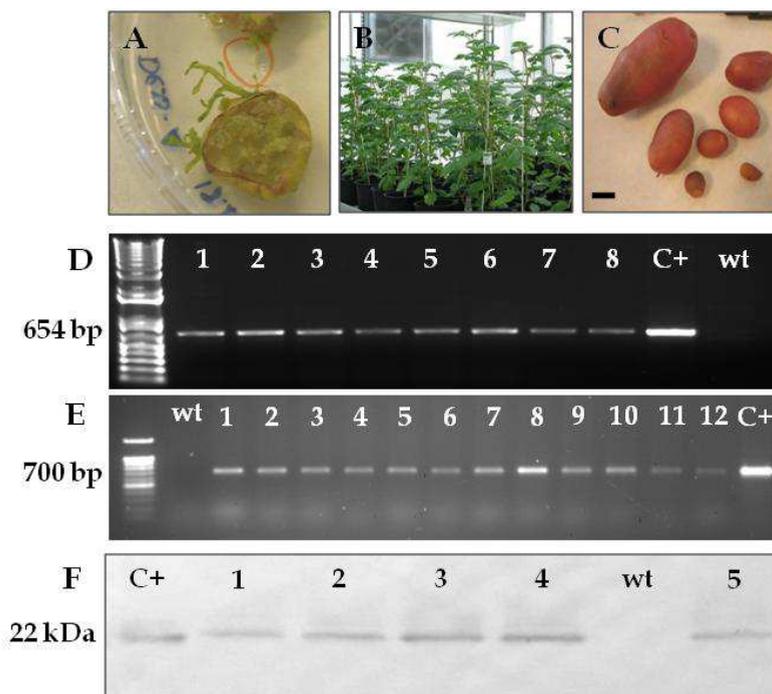


Fig. 2. Phenotypic and molecular characterization of transgenic potato plants. A, regenerated shoots obtained from leaf tissue co-cultured with *A. tumefaciens*. B, phenotype of potato plants cultivated in the glasshouse. C, tubers from transgenic lines (scale 1:2 cm). D and E, PCR amplification of hPL (D) and *nptII* (E) genes in the genome of regenerated plants. 1-12 independent lines; c+, pNEKhPL1 vector; wt, non transformed plant. F, western blot of transgenic plants cultivated in the glasshouse. C+, 10 ng of commercial hPL protein (NeoMarkers); 1-5, 20 μ g of TSP from different transgenic lines.

The amount of hPL protein was determined by sandwich enzyme linked immunosorbent assay (ELISA) in leaf tissue of plants grown *in vitro* and cultivated in the greenhouse. Maxisorp 96-well microtiter plates were coated with polyclonal anti-hPL antibody (NeoMarkers) overnight at 4°C. The wells were blocked with 1.5% horse serum in PBS-T and then samples as well as serial dilutions of commercially available hPL protein were added in PBS for 1 hour at 37°C. Monoclonal anti-HPL antibody (MCA322, Serotec, Oxford, UK) and alkaline-phosphatase conjugated goat anti-mouse IgG antibody (Sigma) were coated subsequently to finally detect color development using 4-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was measured at 405 nm in a microplate reader (Multiskan RC, Labsystems, Helsinki, Finland) and TSP content was determined by the Bio-Rad protein assay. The results showed that plants cultivated in the greenhouse had 2.4-fold higher expression levels than those grown *in vitro* (Table 1). The lower protein levels detected in plants grown *in vitro* could be due to the stressful conditions of culture conditions. It has been described that the artificial medium and the high air humidity and low gas exchange of this culture type could induce disturbances in plant development (Kadlecěk et al. 2001) which possibly limit the production of endogenous and foreign protein levels. Using the expression vector pNEKhPL1, the expression levels reached a maximum of 0.21% of TSP. These expression values are similar to those reported by other authors using vectors similar to pNEKhPL1 (Castañón et al., 2002; Kim et al., 2003; Mason et al., 1998; Ritcher et al., 2000).

Culture condition/tissue	N° of plants analyzed	Expression level (mean value±s.e.)	Expression range
<i>In vitro</i>	24	0.25±0.03	0.03-0.63
Glasshouse	24	0.62±0.13	0.06-2.12
Tuber	16	1.34±0.13	0.69-1.87
callus	20	4.46±0.26	2.77-7.41

Table 1. Yield of the recombinant human placental lactogen protein expressed in potato plants grown under different conditions and in different organ/tissues. The expression levels were measured by enzyme-linked immunosorbent assay (ELISA) and are represented as recombinant protein per total soluble protein content (ng hPL μg^{-1} PTS).

Compared to the levels obtained in tobacco plants (Urreta et al. 2010), those reached in potato plants are much lower. However it must be taken into account that pNEKhPL1 expression vector is the simplest version of a plant expression vector; with no ER-retention signal, SAR sequence, nor enhanced 35S promoter included in pNEKhPL2 vector used in tobacco plants (Urreta et al., 2010). This difference in expression levels highlights the importance of the design of expression vector including regulatory sequences suitable to achieve our goals.

Regarding to the influence of host plant, in our laboratory we also transformed tobacco plants with pNEKhPL1 expression vector, obtaining a maximum expression level 2-fold higher than that obtained in potato (data not shown). So in the case of hPL production in plants, tobacco seems to be more suitable than potato with allowing higher expression levels in leaf tissue of plants cultivated in the greenhouse.

Because of the constitutive nature of CaMV35S promoter, we also analysed the expression level of recombinant hPL in tubers from 16 transgenic plants. We obtained elevated levels of recombinant protein in this tissue, with a mean value 2-fold higher than that of leaf tissue

(Table 1). The maximum hPL expression level was 0.18% of TSP, slightly lower than the maximum in leaf tissue. These results are in the range of expression levels reported by other Authors in potato tubers using CaMV35S promoter (Mason et al., 1998; Zhou et al., 2003; Bielmet et al., 2003). Although CaMV35S promoter is not tuber specific, the expression levels obtained are also similar to those reported by Mason et al. (1996) and Castañón et al. (2002) using tuber specific promoters such as patatin. It is also noteworthy that from the tubers tested, 4 of them did not show detectable levels of hPL.

In order to assess the suitability of hPL production in plant cell cultures we induced the formation of callus from leaf tissue of transgenic plants cultivated *in vitro* (Figure 3, A). Leaf explants were cultured in MS medium supplemented with casein hydrolysate (0.2% w/v), 2,4-D (5 mg L⁻¹) and kinetin (0.2 mg L⁻¹), and incubated at 23°C in the dark. Friable callus developed in 3-4 weeks (Figure 3, B) which were analysed by western blot. Recombinant protein was detected at 22 kDa, as expected, but not in all lines tested (Figure 3, C).

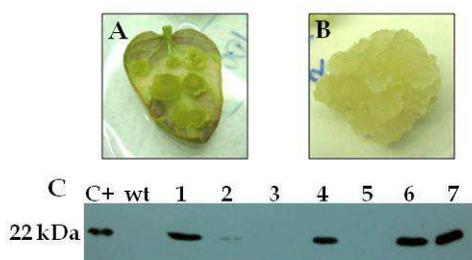


Fig. 3. Induction and characterization of callus tissue. A, induction of callus from leaf tissue cultured in MS medium supplemented with 2,4-D and kinetin. B, isolated callus line from leaf tissue and subcultured to fresh medium. C, western blot of callus protein extracts (10 µg). c+, commercial hPL protein (30 ng) (NeoMarkers); 1-7, transgenic lines; wt, callus induced from leaf explants of non transformed potato plants.

Although those calli were kanamycin-resistant, they did not express hPL protein. This could be as a result of promoter methylation during callus induction process as observed by other Authors (De Carvalho et al., 1992; Fojtova et al., 2003). The hPL protein levels were measured by ELISA. Results showed that a mean value 7-fold higher than that of leaf tissue of plants grown in the glasshouse, reaching a maximum level of 0.74% of TSP (Table 1). The expression of hPL protein in callus is favored because of the lower TSP content in this tissue. Data presented in this work represents a good starting point to further analyze the suitability of cell suspensions as an alternative plant-based expression system for hPL protein production. As previously discussed, plant cell cultures offer many advantages over whole plants for the production of human therapeutics, like rapid growth and easier purification strategies when the protein is secreted to the culture media. The expression vector pNEKhPL1 allows the targeting of hPL to the apoplast and hence to the culture medium. Moreover, using bioreactors we can avoid the release of genetically modified organisms to the field, and improve protein yields through the control of growth parameters allowing a homogeneous batch to batch production.

Finally, to further characterize the transgenic potato plants expressing the hPL protein, we analyzed the stability of the recombinant protein in the second generation (vegetative) of

four transgenic lines. Recombinant hPL levels were measured by ELISA, showing that one of the lines lost the expression of hPL and in the remaining lines the levels ranged from 0.2 to 0.38 ng μg^{-1} of TSP. These levels are higher than those of their counterpart grown *in vitro*, and similar to that of first generation cultivated in the glasshouse.

All the results obtained in this work suggest that although hPL protein is correctly produced in potato leaf tissue, tubers accumulate higher mean protein levels, allowing the storage of the protein. Further investigation will be necessary to analyze the stability of the protein in long term storage and the influence of temperature conditions needed to maintain its integrity. Although potato plants show lower expression levels than tobacco plants transformed with the same expression vector, expression levels in callus tissue opens the possibility for the production of hPL protein using plant cell cultures.

4. Conclusion

Transgenic plants represent an attractive production platform for therapeutic proteins due to all of the previously mentioned features. However, the commercial production of plant-made proteins is still limited, as compared to other expression systems currently available. Twenty two years after the first antibody produced in plants, the great research efforts of public and private institutions have lead to hundreds of plant-made protein publications and patents. Plant-based systems have demonstrated their suitability for protein production, ensuring their capacity for correct production of many types of therapeutic proteins and their great versatility to allow the production of proteins in different conditions. Many companies are exploiting plant systems for protein production (SemBiosys, Protalix, Dow Agrosicences, Méristem Therapeutics) but the pharmaceutical industry still shows reluctance to integrate plant systems in their production strategy. The lower protein levels obtained in plant systems can be greatly improved through the careful design of expression vectors and the choice of host plant. On the other hand, there is no biosafety and/or regulatory issue that can't be solved. We hope that all the efforts made until now in the research field will be rewarded with a greater number of therapeutic proteins produced in plants in the market, for the benefit of society.

5. References

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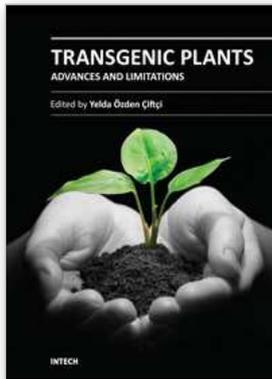
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Phone: +86-21-62489820
Fax: +86-21-62489821