

## Plant-based Expression of Biopharmaceuticals

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## Keywords

### GMP

Good Manufacturing Practice (GMP) was established by WHO in 1968 to guarantee the optimum degree of quality during production and processing of pharmaceuticals (cGMP means under the current regulations of the authorities).

### Transgenic

Organisms that have externally introduced foreign DNA/genes stably integrated into their genome to, for example, produce desired substances like human insulin.

### Plant-based Expression

Transgenic plants can be genetically modified with a gene of interest to produce a biopharmaceutical of interest.

### Glycosylation

It is the addition of polysaccharides to a certain molecule such as a protein. The majority of proteins are synthesized in the rough endoplasmic reticulum (ER) where they undergo glycosylation.

### Bioreactor

It is a vessel in which a (bio)chemical process that involves organisms or biochemically active substances (e.g. enzymes) derived from such organisms is carried out.

■ Biopharmaceuticals are currently the mainstay products of the biotechnology market and represent the fastest growing and, in many ways, the most exciting sector within the pharmaceutical industry. The term “biopharmaceutical” was originated in the 1980s, when a general consensus evolved that it represented a class of therapeutics produced by means of modern biotechnologies. Already a quarter of a century ago, “humulin” (recombinant human insulin, produced in *E. coli* and developed by Genentech in collaboration with Eli Lilly) was approved and received marketing authorization in the United States of America in 1982. Since then the market for biopharmaceuticals has been steadily growing and currently nearly 150 biopharmaceuticals have gained approval for general human use (EU and USA). Over this period it became obvious that production capacities for biopharmaceuticals with “conventional” bioreactors would be a bottleneck and that worldwide fermentation capacities are limited. One exciting solution to these “capacity crunches” is the use of transgenic plants to produce biopharmaceuticals. This article describes different plant expression systems, their advantages and limitations, and concludes by considering some of the innovations and trends likely to influence the future of plant-based biopharmaceuticals.

## 1 Introduction

Biopharmaceuticals, which are large molecules produced by living cells, are currently the mainstay products of the biotechnology industry. Indeed, biologics such as Genentech's (Vacaville, CA, USA) human growth factor somatotropin or Amgen's (Thousand Oaks, CA, USA) recombinant erythropoietin (EPO) have shown that biopharmaceuticals can benefit a huge number of patients and also generate big profits for these companies at the same time. The single most lucrative product is EPO and combined sales of the recombinant EPO products "Procrit" (Ortho biotech) and "Epogen" (Amgen) have reportedly surpassed the \$6.5 billion mark. But it has also become obvious over the last couple of years that current fermentation capacities will not be sufficient to manufacture all biopharmaceuticals (in the market already or in development), because the market and demand for biologics is continuously and very rapidly growing; for antibodies alone (with at least 10 monoclonal antibodies approved and being marketed), the revenues are predicted to expand to US\$3 billion in 2002 and US\$8 billion in 2008. The 10 monoclonal antibodies on the market consume more than 75% of the industry's manufacturing capability. And there are up to 60 more that are expected to reach the market in the next six or seven years. Altogether, there are about 1200 protein-based products in the pipeline with a 20% growth rate and the market for current and late stage (Phase III) is estimated to be US\$42 billion in 2005 and even US\$100 billion in 2010. But, there are obvious limitations of large-scale manufacturing resources and production capacities – and pharmaceutical companies

are competing (see ref Knäblein (2004), review).

To circumvent this capacity crunch, it is necessary to look into other technologies rather than the established ones, like, for example, *Escherichia coli* or CHO (Chinese hamster ovary) cell expression. One solution to avoid these limitations could be the use of transgenic plants to express recombinant proteins at low cost, in GMP (good manufacturing practice) quality greenhouses (with purification and fill finish in conventional facilities). Plants therefore provide an economically sound source of recombinant proteins, such as industrial enzymes, and biopharmaceuticals. Furthermore, using the existing infrastructure for crop cultivation, processing, and storage will reduce the amount of capital investment required for commercial production. For example, it was estimated that the production costs of recombinant proteins in plants could be between 10 and 50 times lower than those for producing the same protein in *E. coli* and Alan Dove describes a factor of thousand for cost of protein (US dollar per gram of raw material) expressed in, for example, CHO cells compared to transgenic plants. So, at the dawn of this new millennium, a solution is imminent to circumvent expression capacity crunches and to supply mankind with the medicines we need. Providing the right amounts of biopharmaceuticals can now be achieved by applying our knowledge of modern life sciences to systems that were on this planet long time before us – plants.

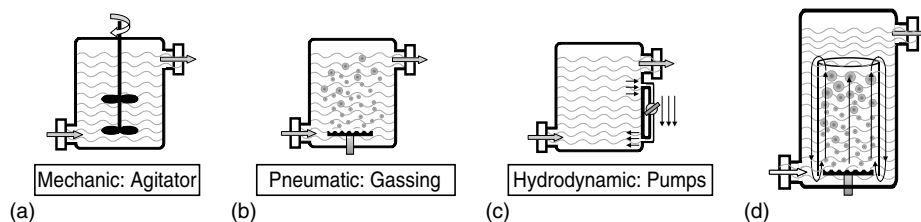
## 2 Alternative Expression Systems

Currently, CHO cells are the most widely used technology in biomanufacturing because they are capable of expressing eukaryotic proteins (processing, folding,

and posttranslational modifications) that cannot be provided by *E. coli*. A long track record exists for CHO cells, but unfortunately they bring some problems along when it comes to scaling up production. Transport of oxygen (and other gases) and nutrients is critical for the fermentation process, as well as the fact that heat must diffuse evenly to all cultured cells. According to the Michaelis–Menten equation, the growth rate depends on the oxygen/nutrient supply; therefore, good mixing and aeration are a prerequisite for the biomanufacturing process and are usually achieved by different fermentation modes (see Fig. 1). But the laws of physics set strict limits on the size of bioreactors. For example, an agitator achieves good heat flow and aeration, but with increased fermenter size, shear forces also increase and disrupt the cells – and building parallel lines of bioreactors multiplies the costs linearly. A 10 000-L bioreactor costs between US\$ 250 000 to 500 000 and takes five years to build (conceptual planning, engineering, construction, validation, etc.). An error in estimating demand for, or inaccurately predicting the approval of, a new drug can be incredibly costly. To compound the problem, regulators in the United States and Europe demand that drugs have to be produced for the market in the same system used to





produce them for the final round of clinical trials, in order to guarantee bioequivalence (e.g. toxicity, bioavailability, pharmacokinetics, and pharmacodynamics) of the molecule. So, companies have to choose between launching a product manufactured at a smaller development facility (and struggling to meet market demands) or building larger, dedicated facilities for a drug that might never be approved!

Therefore, alternative technologies are used for the expression of biopharmaceuticals, some of them also at lower costs involved (see Fig. 2). One such alternative is the creation of transgenic animals (“pharming”), but this suffers from the disadvantage that it requires a long time to establish such animals (approximately 2 years). In addition to that, some of the human biopharmaceuticals could be detrimental to the mammal’s health, when expressed in the mammary glands. This is why ethical debates sometimes arise from the use of transgenic mammals for production of biopharmaceuticals. Although there are no ethical concerns involved with plants, there are societal ones that will be addressed later. Another expression system (see Fig. 2) utilizes transgenic chicken. The eggs, from which the proteins are harvested, are natural protein-production systems. But production of transgenic birds is still



**Fig. 1** Different fermentation modes for bioreactors. In order to achieve best aeration and mixing and to avoid high shear forces, different fermentation modes are applied. (a) mechanical, (b) pneumatical, (c) hydrodynamic pumps,

(d) airlift reactor. Source: Knäblein J. (2002) *Transport Processes in Bioreactors and Modern Fermentation Technologies*, Lecture at University of Applied Sciences, Emden, Germany.

				
Major technology companies	Mammalian (CHO) cells Amgen (Thousand Oaks, CA) Genentech (S. San Francisco, CA) other current biologics manufacturers: Cruceel (Leiden, Netherlands) uses human cells	Transgenic mammal milk GTC Biotherapeutics (Framingham, MA) PPL Therapeutics (Edinburgh, UK) BioProtein (Paris, France)	Transgenic chicken eggs Avigenics (Athens, GA) Origen Therapeutics (Burlingame, CA) TranXenoGen (shrewsbury, MA) Viragen (Plantation, FL) GeneWorks (Ann Arbor, MI) Vivalis (Nantes, France)	Transgenic plants Croptech (Blacksburg, VA) Epicyte (San Diego, CA) Large Scale Biology (Owensboro, KY) Meristem Therapeutics (Clermont-Ferrand, France) Prodigene (College Station, TX)
Estimated cost (cost/g raw material)*	\$150	\$1–\$2	\$1–\$2	\$0.05
*Company estimates				

**Fig. 2** Companies and technologies in biomanufacturing. A comparison of different expression systems shows the big differences in terms of costs, ranging from 150 US\$ per gram

for CHO cells to 0.05 US\$ per gram for transgenic plants. Source: Dove, A. (2002) Uncorking the biomanufacturing bottleneck, *Nat. Biotechnol.* **20**, 777–779.

several years behind transgenic mammal technology. Intensive animal housing constraints also make them more susceptible to disease (e.g. Asia 1997 or Europe 2003: killing of huge flocks with thousands of chicken suffering from fowl pest). In the light of development time, experience, costs, and ethical issues, plants are therefore the favored technology, since such systems usually have short gene-to-protein times (weeks), some are already well established, and as mentioned before, the involved costs are comparatively low. This low cost of goods sold (COGS) for plant-derived proteins is mainly due to low capital costs: greenhouse costs are only US\$ 10 per m<sup>2</sup> versus US\$ 1000 per m<sup>2</sup> for mammalian cells.

### 3 History of Plant Expression

Plants have been a source of medicinal products throughout human evolution. These active pharmaceutical compounds

have been primarily small molecules, however. One of the most popular examples is aspirin (acetylsalicylic acid) to relieve pain and reduce fever. A French pharmacist first isolated natural salicin (a chemical relative of the compound used to make aspirin) from white willow bark in 1829. Advances in genetic engineering are now allowing for the production of therapeutic proteins (as opposed to small molecules) in plant tissues. Expression of recombinant proteins in plants has been well documented since the 1970s and has slowly gained credibility in the biotechnology industry and regulatory agencies. The first proof of concept has been the incorporation of insect and pest resistance into grains. For example, “Bt corn” contains genes from *Bacillus thuringensis* and is currently being grown commercially. Genetic engineering techniques are now available for the manipulation of almost all commercially valuable plants. Easy transformation and cultivation make plants suitable

for production of virtually any recombinant protein.

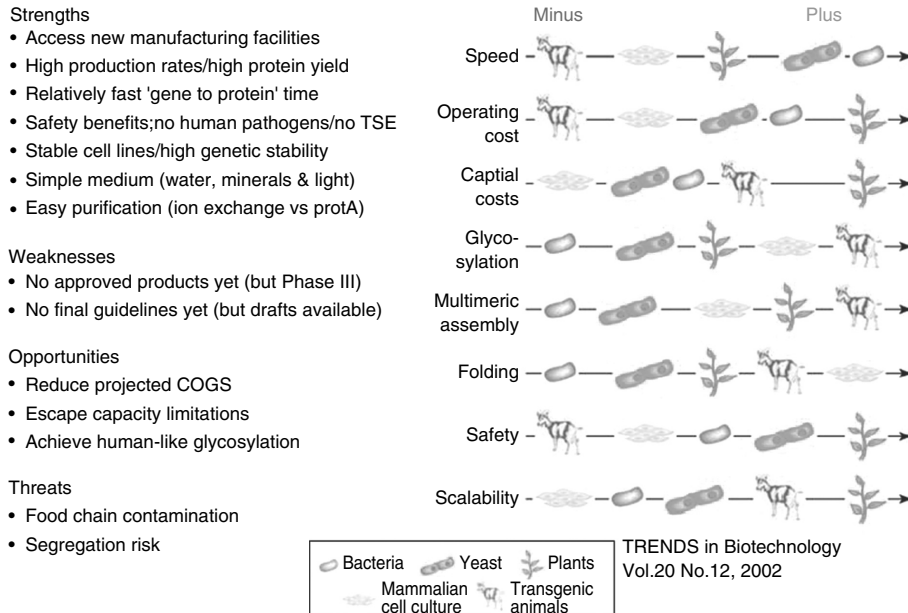
Plants have a number of advantages over microbial expression systems, but one of them is of outmost importance: they can produce eukaryotic proteins in their native form, as they are capable of carrying out posttranslational modifications required for the biological activity of many such proteins (see Fischer Schillberg (2004), books). These modifications can be acetylation, phosphorylation, and glycosylation, as well as others. Per se, there is no restriction to the kind of proteins that can be expressed in plants: vaccines (e.g. pertussis or tetanus toxins), serum proteins (e.g. albumin), growth factors (e.g. vascular endothelial growth factor (VEGF), erythropoietin), or enzymes (e.g. urokinase, glucose oxidase, or glucocerebrosidase). However, enzymes sometimes have very complex cofactors, which are essential for their catalytic mode of action, but cannot be supplied by most expression systems. This is why, for the expression of some enzymes, expression systems with special features and characteristics need to be developed. Another very important class of proteins is the antibodies (e.g. scFv, Fab, IgG, or IgA). More than 100 antibodies are currently used in clinical trials as therapeutics, drug delivery vehicles, in diagnostics and imaging, and in drug discovery research for both screening and validation of targets. Again, plants are considered as the system of choice for the production of antibodies (“plantibodies”) in bulk amounts at low costs. Since the initial demonstration that transgenic tobacco (*Nicotiana tabacum*) is able to produce functional IgG1 from mouse, full-length antibodies, hybrid antibodies, antibody fragments (Fab), and single-chain variable fragments (scFv) have been expressed in higher plants for a number of

purposes. These antibodies can serve in health care and medicinal applications, either directly by using the plant as a food ingredient or as a pharmaceutical or diagnostic reagent after purification from the plant material. In addition, antibodies may improve plant performance, for example, by controlling plant disease or by modifying regulatory and metabolic pathways.

## 4 Current Status of Plant-based Expression

### 4.1 SWOT Analysis Reveals a Ripe Market for Plant Expression Systems

When I analyzed the different expression systems regarding their strengths, weaknesses, opportunities, and threats (SWOT), the advantages of plants and their potential to circumvent the worldwide capacity limitations for protein production became quite obvious (see Fig. 3). Comparison of transgenic animals, mammalian cell culture, plant expression systems, yeast, and bacteria shows certain advantages for each of the systems. In the order in which the systems were just mentioned, we can compare them in terms of their development time (speed). Transgenic animals have the longest cycle time (18 months to develop a goat), followed by mammalian cell culture, plants, yeast, and bacteria (one day to transform *E. coli*). If one looks at operating and capital costs, safety, and scalability, the data show that plants are beneficial: therefore, in the comparison (see Fig. 3), they are shown on the right-hand side already. But even for glycosylation, multimeric assembly and folding (where plants are not shown on the right-hand side, meaning other systems are advantageous), some plant expression systems are moving in



**Fig. 3** SWOT analysis of plant expression systems. Plant expression systems have a lot of advantages (plus) over other systems and are therefore mostly shown on the right-hand side of the picture (Raskin, I., Fridlender, B., et al. (2002) Plants and human health in the twenty-first century, *Trends Biotechnol.* **20**, 522–531). Herein different systems (transgenic animals, mammalian cell culture, plants, yeast, and bacteria) are compared in terms of speed (how quickly they can be developed), operating and capital costs, and so on, and plants are obviously advantageous. Even for glycosylation,

assembly, and folding, where plants are not shown on the right-hand side (meaning other systems are advantageous), some plant expression systems are moving in that direction (as will be shown exemplarily in the section on moss). Also the weaknesses and threats can be dealt with, using the appropriate plant expression system. Source: Knäblein J. (2003) *Biotech: A New Era In The New Millennium – From Plant Fermentation To Plant Expression Of Biopharmaceuticals*, PDA International Congress, Prague, Czech Republic.

that direction. An example of this is the moss system from the company greenovation Biotech GmbH (Freiburg, Germany), which will be discussed in detail in the example section. This system performs proper folding and assembly of even such complex proteins like the homodimeric VEGF. Even the sugar pattern could successfully be reengineered from plant to humanlike glycosylation.

In addition to the potential of performing human glycosylation, plants also enjoy the distinct advantage of not harboring

any pathogens, which are known to harm animal cells (as opposed to animal cell cultures and products), nor do the products contain any microbial toxins, TSE (Transmissible Spongiform Encephalopathies), prions, or oncogenic sequences. In fact, humans are exposed to a large, constant dose of living plant viruses in the diet without any known effects/illnesses. Plant production of protein therapeutics also has advantages with regard to their scale and speed of production. Plants can be grown in ton quantities (using

existing plant/crop technology, like commercial greenhouses), be extracted with industrial-scale equipment, and produce kilogram-size yields from a single plot of cultivation. These economies of scale are expected to reduce the cost of production of pure pharmaceutical-grade therapeutics by more than 2 orders of magnitude versus current bacterial fermentation or cell culture reactor systems (plus raw material COGS are estimated to be as low as 10% of conventional cell culture expenses).

Although a growing list of heterologous proteins were successfully produced in a number of plant expression systems with their manifold advantages, there are also obvious downsides. One weakness is that no product has been approved for the market yet (but will be soon, since some are in Phase III clinical trials already, see Table 1). The other weakness is that no final regulatory guidelines exist. But as mentioned before, regulatory authorities (Food and Drug Administration (FDA), European Medicine Evaluation Agency (EMA), and Biotechnology Regulatory Service (BRS) and the Biotechnology Industry Organization (BIO) have drafted guidelines on plant-derived biopharmaceuticals (see Table 2) and have asked the community for comments. The FDA has also issued several PTC (Points To Consider) guidelines about plant-based biologics, and review of the July 2002 PTC confirms that the FDA supports this field and highlights the benefits of plant expression systems – including the absence of any pathogens to man from plant extracts. The main concerns of using plant expression systems are societal ones about environmental impacts, segregation risk, and contamination of the food chain. But these threats can be dealt with, using nonedible plants (nonfood, nonfeed),

applying advanced containment technologies (GMP greenhouses, bioreactors) and avoiding open-field production.

Owing to the obvious strengths of plant expression systems, there has been explosive growth in the number of start-up companies. Since the 1990s, a number of promising plant expression systems have been developed, and in response to this “blooming field” big pharmaceutical companies have become more interested. Now, the plant expression field is “ripe” for strategic alliances, and, in fact, the last year has seen several major biotech companies begin partnerships with such plant companies. The selection of several such partnerships shown in Table 1 clearly demonstrates that, in general, there has been sufficient experimentation with various crops to provide the overall proof of concept that transgenic plants can produce biopharmaceuticals. However, and this can be seen in the table as well, the commercial production of biopharmaceuticals in transgenic plants is still in the early stages of development and yet the most advanced products are in Phase III clinical development.

#### 4.2

##### **Risk Assessment and Contingency Measures**

For a number of reasons, including the knowledge base developed on genetically modifying its genome, industrial processes for extracting fractionated products and the potential for large-scale production, the preferred plant expression system has been corn. However, the use of corn touches on a potential risk: some environmental activist groups and trade associations are concerned about the effect on the environment and possible contamination of the food supply. These issues

Tab. 1 Plant-derived biopharmaceuticals in clinical trials.

<b>Company</b>	<b>Partner</b>	<b>Protein/indication</b>	<b>Host</b>	<b>Stage</b>
Monsanto	Guy's Hospital London	Anticaries antibody	Corn	Phase III
Large Scale Biology	Own product	scFv (non-Hodgkin)	Tobacco	Phase IIIs
Meristem Therapeutics	Solvay Pharmaceuticals	Gastric lipase	Corn	Phase II
Large Scale Biology	ProdiGene, Plant Bioscience	Anti-ideotype antibody	Tobacco	Phase I
Monsanto	NeoRx	Antitumor antibody	Corn	Phase I
ProdiGene	Own product	TGEV vaccine	Corn	Phase I
Epicyte Pharmaceutical	Dow, Centocor	Anti-HSV antibody	Corn	Phase I
Crop Tech	Immunex	Enbrel (arthritis)	Tobacco	Preclinical
Crop Tech	Amgen	Therapeutic antibodies	Tobacco	Preclinical
AltaGen Bioscience Inc.	U.S. Army 3 + biotechs	Antibodies	Potato	Preclinical
Meristem Therapeutics	CNRS	Human lactoferrin	Corn	Preclinical
MPB Cologne GmbH	Aventis CropScience	Confidential	Potato	Preclinical

Tab. 2 Drafted guidelines on plant-derived biopharmaceuticals.

<b>Agency</b>	<b>Guideline</b>	<b>Status</b>
<b>BRS</b> (Biotechnology Regulatory Services)	"Case study on plant-derived biologics" for Office of Science and Technology Policy/Council on Environmental Quality	Released: Mar 5, 2001
<b>BIO</b> (Biotechnology Industry Organization)	"Reference Document for Confinement and Development of Plant-made Pharmaceuticals in the United States"	Released: May 17, 2002
<b>BIO</b> (Biotechnology Industry Organization)	"BIO Position on Geographic Restrictions for Plant-made Pharmaceuticals and Industrials"	Released: Oct 22, 2002
<b>EMEA</b> (European Medicine Evaluation Agency)	"Concept Paper on the Development of a Committee for Proprietary Medicinal Products (CPMP) Points to Consider on the Use of Transgenic Plants in the Manufacture of Biological Medicinal Products for Human Use"	Released: Mar 01, 2001
<b>FDA</b> (Food and Drug Administration)	"Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals"	Issued: Sep 6, 2002
<b>EMEA</b> (European Medicine Evaluation Agency)	"Points To Consider Quality Aspects of Medicinal Products containing active substances produced by stable transgene expression in higher plants"	Issued: Mar 13, 2002

are reflected in the regulatory guidelines and have been the driving force to investigate other plants as well. While many mature and larger companies have been working in this area for many years, there are a number of newcomers that are developing expertise as well. These smaller companies are reacting to the concerns by looking at the use of nonedible plants that can be readily raised in greenhouses. All potential risks have to be assessed and contingency measures need to be established. Understanding the underlying issues is mandatory to make sophisticated decisions about the science and subsequently on the development of appropriate plant expression systems for production of biopharmaceuticals.

Ongoing public fears from the food industry and the public, particularly in Europe (“Franken Food”) could have spillover effects on plant-derived pharmaceuticals. Mistakes and misunderstandings have already cost the genetically enhanced grain industry hundreds of millions of dollars. The only way to prevent plant expression systems from suffering the same dilemma is to provide the public with appropriate information on emerging discoveries and newly developed production systems for biopharmaceuticals. Real and theoretical risks involve the spread of engineered genes into wild plants, animals, and bacteria (horizontal transmission). For example, if herbicide resistance was transmitted to weeds, or antibiotic resistance was to be transmitted to bacteria, superpathogens could result. If these genetic alterations were transmitted to their progeny (vertical transmission), an explosion of the pathogens could cause extensive harm. An example of this occurred several years ago, when it was feared that pest-resistant genes had been transmitted from Bt corn to milkweed – leading

to the widespread death of Monarch butterflies. Although this was eventually not found to be the case, the public outcry over the incident was a wake-up call to the possible dangers of transgenic food technology. To avoid the same bad perception for biopharmaceuticals expressed in plants, there is the need for thorough risk assessment and contingency planning. One method is the employment of all feasible safety strategies to prevent spreading of engineered DNA (genetic drift), like a basic containment in a greenhouse environment. Although no practical shelter can totally eradicate insect and rodent intrusion, this type of isolation is very effective for self-pollinators and those plants with small pollen dispersal patterns. The use of species-specific, fragile, or poorly transmissible viral vectors is another strategy. Tobacco mosaic virus (TMV), for example, usually only infects a tobacco host.

It requires an injury of the plant to gain entry and cause infection. Destruction of a field of TMV-transformed tobacco requires only plowing under or application of a herbicide. These factors prevent both horizontal and vertical transmission. In addition, there is no known incidence of plant viruses infecting animal or bacterial cells. Another approach is to avoid stable transgenic germplines and therefore most uses of transforming viruses do not involve the incorporation of genes into the plant cell nucleus. By definition, it is almost impossible for these genes to be transmitted vertically through pollen or seed. The engineered protein product is produced only by the infected generation of plants. Another effective way to reduce the risk of genetic drift is the use of plants that do not reproduce without human aid. The modern corn plant cannot reproduce without cultivation and

the purposeful planting of its seeds. If a plant may sprout from grain, it still needs to survive the wintering-over process and gain access to the proper planting depth. This extinction process is so rapid, however, that the errant loss of an ear of corn is very unlikely to grow a new plant. Another very well-known example of self-limited reproduction is the modern banana. It propagates almost exclusively through vegetative cloning (i.e. via cuttings).

Pollination is the natural way for most plants to spread their genetic information, make up new plants, and to deliver their offspring in other locations. The use of plants with limited range of pollen dispersal and limited contact with compatible wild hosts therefore is also very effective to prevent genetic drift. Corn, for example, has pollen, which survives for only 10 to 30 min and, hence, has an effective fertilizing radius of less than 500 m. In North America, it has no wild-type relatives with which it could cross-pollinate. In addition to being spatially isolated from nearby cornfields, transgenic corn can be “temporally isolated” by being planted at least 21 days earlier or 21 days later than the surrounding corn, to ensure that the fields are not producing flowers at the same time. Under recent USDA (U.S. Department of Agriculture) regulations, the field must also be planted with equipment dedicated to the genetically modified crop. For soybeans, the situation is different, since they are virtually 100% self-fertilizers and can be planted in very close proximity to other plants without fear of horizontal spread. Another option is the design of transgenic plants that have only sterile pollen or – more or less only applicable for greenhouses – completely prevent cross-pollination by covering the individual plants. One public fear regards

spreading antibiotic resistance from one (transgenic donor) plant to other wild-type plants or bacteria in the environment. Although prokaryotic promoters for antibiotic resistance are sometimes used in the fabrication and selection of transgenic constructs, once a transgene has been stably incorporated into the plant genome, it is under the control of plant (eukaryotic) promoter elements. Hence, antibiotic-resistance genes are unable to pass from genetically altered plants into bacteria and remain functional. As stated earlier, another common fear is the creation of a “super bug.” The chance of creating a supervirulent virus or bacterium from genetic engineering is unlikely, because the construction of expression cassettes from viral or bacterial genomes involves the removal of the majority of genes responsible for the normal function of these organisms. Even if a resultant organism is somewhat functional, it cannot compete for long in nature with normal, wild-type bacteria of the same species.

As one can see from the aforementioned safety strategies, considerable effort is put into the reduction of any potential risk from the transgenic plant for the environment. In general, the scientific risk can be kept at a minimum, if common sense is applied – in accordance with Thomas Huxley (1825–1895) that “Science is simply common sense at its best.” For example, protein toxins (for vaccine production) should never be grown in food plants.

Additionally, the following can be employed as a kind of risk management to prevent the inappropriate or unsafe use of genetically engineered plants:

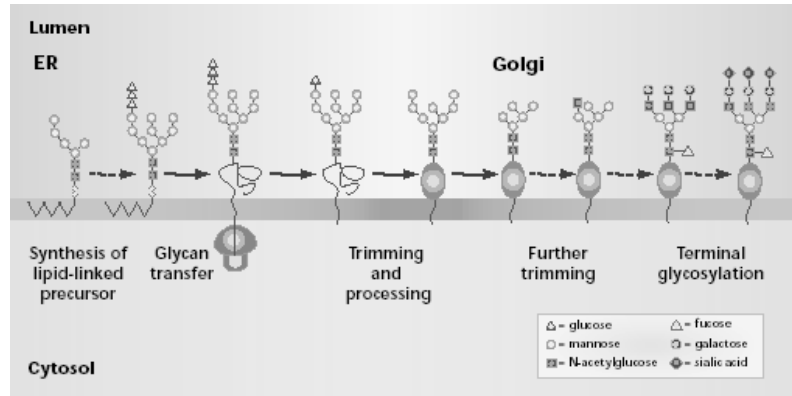
- An easily recognized phenotypic characteristic can be coexpressed in an

engineered product (e.g. tomatoes that contain a therapeutic protein can be selected to grow in a colorless variety of fruit).

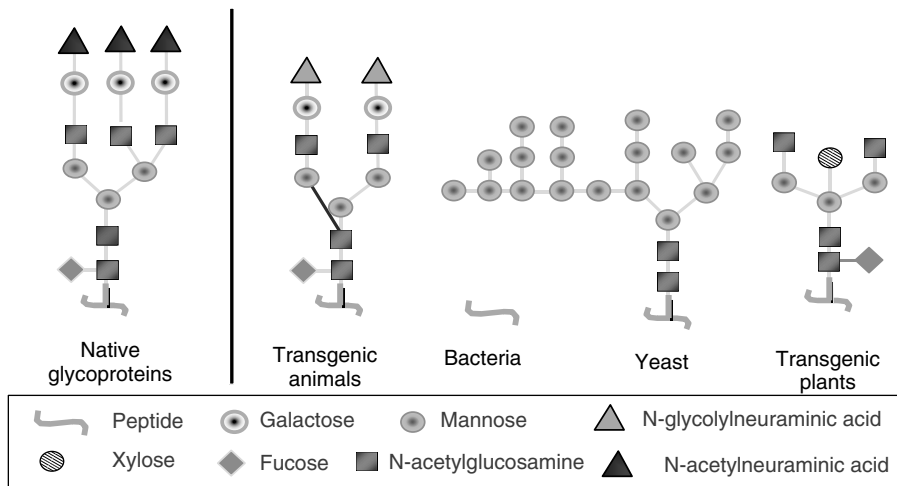
- Protein expression can be induced only after harvesting or fruit ripening. For example, CropTech's (Blacksburg, VA, USA) inducible expression system in tobacco, MeGA-PharM, leads to very efficient induction upon leaf injury (harvest) and needs no chemical inducers. This system possesses a fast induction response and protein synthesis rate, and thus leads to high expression levels with no aged product in the field (no environmental damage accumulation).
- Potentially antigenic or immunomodulatory products can be induced to grow in, or not to grow in, a certain plant tissue (e.g. root, leaf/stem, seed, or pollen). In this way, for example, farmers can be protected from harmful airborne pollen or seed dusts.
- Although no absolute system can prevent vandalism or theft of the transgenic plants, a very effective, cheap solution has been used quietly for many years now in the United States. Plots of these modified plants are being grown with absolutely no indication that they are different from a routine crop. In the Midwest, for example, finding a transgenic corn plot among the millions of acres of concurrently growing grain is virtually impossible. The only question here is, if this approach really helps facilitating a fair and an open discussion with the public. Asking the same question for the EU is not relevant: owing to labeling requirements, this approach would not be feasible, as, in general, it is much more difficult to perform open-field studies with transgenic plants.

## 5 The Way Forward: Moving Plants to Humanlike Glycosylation

As discussed earlier, plant production of therapeutic proteins has many advantages over bacterial systems. One very important feature of plant cells is their capability of carrying out posttranslational modifications. Since they are eukaryotes (i.e. have a nucleus), plants produce proteins through an ER (endoplasmatic reticulum) pathway, adding sugar residues also to the protein – a process called *glycosylation*. These carbohydrates help determine the three-dimensional structures of proteins, which are inherently linked to their function and their efficacy as therapeutics. This glycosylation also affects protein bioavailability and breakdown of the biopharmaceutical; for example, proteins lacking terminal sialic acid residues on their sugar groups are often targeted by the immune system and are rapidly degraded. The glycosylation process begins by targeting the protein to the ER. During translation of mRNA (messenger RNA) into protein, the ribosome is attached to the ER, and the nascent protein fed into the lumen of the ER as translation proceeds. Here, one set of glycosylation enzymes attaches carbohydrates to specific amino acids of the protein. Other glycosylation enzymes either delete or add more sugars to the core structures. This glycosylation process continues into the Golgi apparatus, which sorts the new proteins, and distributes them to their final destinations in the cell (see Fig. 4). Bacteria lack this ability and therefore cannot be used to synthesize proteins that require glycosylation for activity. Although plants have a somewhat different system of protein glycosylation from mammalian cells, the differences usually prove not to be a problem. Some proteins, however, require



**Fig. 4** The glycosylation pathway via ER and Golgi apparatus. In the cytosol, carbohydrates are attached to a lipid precursor, which is then transported to the lumen of the ER to finish core glycosylation. This glycan is now attached to the nascent, folding polypeptide chain (which is synthesized by ribosomes attached to the cytosolic side of the ER from where it translocates into the lumen) and subsequently trimmed and processed before it is folded and moved to the Golgi apparatus. Capping of the oligosaccharide branches with sialic acid and fucose is the final step on the way to a mature glycoprotein. Source: Dove, A. (2001) The bittersweet promise of glycobiology, *Nat. Biotechnol.* **19**, 913–917.



**Fig. 5** Engineering plants to humanlike glycosylation. The first step to achieve humanlike glycosylation in plants is to eliminate the plant glycosylation pattern, that is, the attachment of  $\beta$  1–2 linked xylosyl- and  $\alpha$  1–3 linked fucosyl sugars to the protein. Because these two residues have allergenic potential, the corresponding enzymes Xylosyl- and Fucosyl Transferase are knocked out. In case galactose is relevant for the final product, Galactosyl Transferase is inserted into the host genome. Galactose is available in the organism so that this single gene insertion is sufficient to ensure galactosylation. Source: Knäblein J. (2003) *Biotech: A New Era In The New Millennium – Biopharmaceutical drugs manufactured in novel expression systems*, DEHEMA-Jahrestagung der Biotechnologen, Munich, Germany, 21.

humanlike glycosylation (see Fig. 5) – they must have specific sugar structures attached to the correct sites on the molecule to be maximally effective. Therefore, some efforts are being made in modifying host plants in such a way that they provide the protein with human glycosylation patterns. One example of modifying a plant expression system in this way is the transgenic moss, which will be discussed in the next section.

## 6 Three Promising Examples: Tobacco (Rhizosecretion, Transfection) and Moss (Glycosylation)

To further elaborate on improving glycosylation and downstream processing, three interesting plant expression systems will be discussed. All systems share the advantage of utilizing nonedible plants (nonfood and nonfeed) and can be kept in either a greenhouse or a fermenter to avoid any segregation risk. Another obvious advantage is secretion of the protein into the medium so that no grinding or extraction is required. This is very important in light of downstream processing: protein purification is often as expensive as the biomanufacturing and should never be underestimated in the total COGS equation.

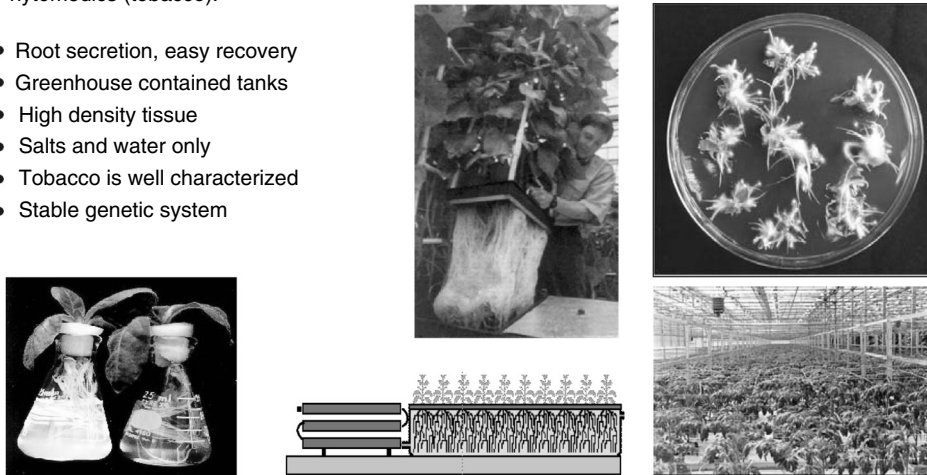
### 6.1 Harnessing Tobacco Roots to Secrete Proteins

Phytomedics (Dayton, NJ, USA) uses tobacco plants as an expression system for biopharmaceuticals. Besides the advantage of being well characterized and used in agriculture for some time, tobacco has a stable genetic system, provides high-density tissue (high protein production),

needs only simple medium, and can be kept in a greenhouse (see Fig. 6). Optimized antibody expression can be rapidly verified using transient expression assays (short development time) in the plants before creation of transgenic suspension cells or stable plant lines (longer development time). Different vector systems, harboring targeting signals for subcellular compartments, are constructed in parallel and used for transient expression. Applying this screening approach, high expressing cell lines can rapidly be identified. For example, transgenic tobacco plants, transformed with an expression cassette containing the GFP (Green Fluorescent Protein) gene fused to an *aps* (amplification-promoting sequence), had greater levels of corresponding mRNAs and expressed proteins compared to transformants lacking *aps*. Usually, downstream processing (isolation/extraction and purification of the target protein) is limiting for such a system, for example, if the protein has to be isolated from biochemically complex plant tissues (e.g. leaves), this can be a laborious and expensive process and a major obstacle to large-scale protein manufacturing. To overcome this problem, secretion-based systems utilizing transgenic plant cells or plant organs aseptically cultivated *in vitro* would be one solution. However, *in vitro* systems can be expensive, slow growing, unstable, and relatively low yielding. This is why another interesting route was followed. Secretion of molecules is a basic function of plant cells and organs in plants, and is especially developed in plant roots. In order to take up nutrients from the soil, interact with other soil organisms, and defend themselves against numerous pathogens, plant roots have evolved sophisticated mechanisms based on the secretion of different biochemicals (including proteins

Phytomedics (tobacco):

- Root secretion, easy recovery
- Greenhouse contained tanks
- High density tissue
- Salts and water only
- Tobacco is well characterized
- Stable genetic system



**Fig. 6** Secretion of the biopharmaceuticals via tobacco roots. The tobacco plants are genetically modified in such a way that the protein is secreted via the roots into the medium ("rhizosecretion"). In this example, the tobacco plant takes up nutrients and water from the medium and releases GFP (Green Fluorescent Protein). Examination of root cultivation medium by its exposure to near ultraviolet-illumination reveals the bright green-blue fluorescence characteristics of GFP in the hydroponic medium

(left flask in panel lower left edge). The picture also shows a schematic drawing of the hydroponic tank, as well as tobacco plants at different growth stages, for example, callus, fully grown, and greenhouse plantation. Source: Knäblein J. (2003) *Biotech: A New Era in the New Millennium – Biopharmaceutical Drugs Manufactured in Novel Expression Systems*, DECHEMA-Jahrestagung der Biotechnologen, Munich, Germany, 21. (See color plate. p. xxv)

like toxins) into their neighborhood (rhizosphere). In fact, Borisjuk and coworkers could demonstrate that root secretion can be successfully exploited for the continuous production of recombinant proteins in a process termed "*rhizosecretion*." Here, an endoplasmic reticulum signal peptide is fused to the recombinant protein, which is then continuously secreted from the roots into a simple hydroponic medium (based on the natural secretion from roots of the intact plants). The roots of the tobacco plant are sitting in a hydroponic tank (see Fig. 6), taking up water and nutrients and continuously releasing the biopharmaceutical. By this elegant set up, downstream processing becomes easy and cost-effective, and also offers the advantage

of continuous protein production that integrates the biosynthetic potential of a plant over its lifetime and might lead to higher protein yields than single-harvest and extraction methods. Rhizosecretion is demonstrated in Fig. 6, showing a transgenic tobacco plant expressing GFP and releasing it into the medium.

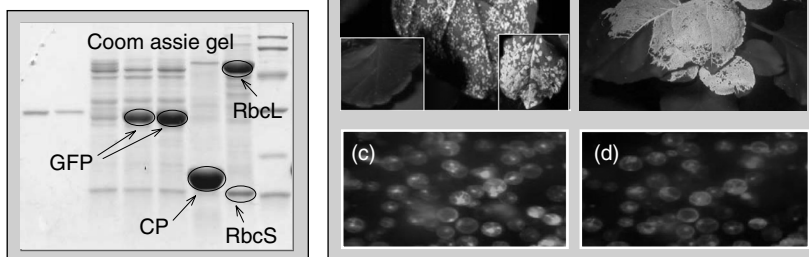
## 6.2

### High Protein Yields Utilizing Viral Transfection

ICON Genetics (Halle, Germany) has developed a protein-production system that relies on rapid multiplication of viral vectors in an infected tobacco plant (see Fig. 7). Viral transfection systems offer

ICON Genetics (tobacco):

- Viral transfection
- Fast development
- High protein yields
- Coexpression of genes



**Fig. 7** Viral transfection of tobacco plants. This new generation platform for fast (1 to 2 weeks), high-yield (up to  $5 \text{ g kg}^{-1}$  fresh leaf weight) production of biopharmaceuticals is based on proviral gene amplification in a nonfood host. Antibodies, antigens, interferons, hormones, and enzymes could successfully be expressed with this system. The picture shows development of initial symptoms on a tobacco following the Agrobacterium-mediated infection with viral vector components that contain a GFP gene (a); this development eventually leads to a systemic spread of the virus, literally converting the plant into a sack full of protein of interest within two weeks (b). The system allows to coexpress two proteins in the same cell, a feature that allows expression of complex proteins such as full-length monoclonal antibodies. Panels (c) and (d) show the same microscope section with the same cells, expressing Green Fluorescent Protein (c) and Red Fluorescent Protein (d) at the same time. The yield and total protein concentration achievable are illustrated by a Coomassie gel with proteins in the system: GFP (protein of interest), CP (coat protein from wild-type virus), RbcS and RbcL (small and large subunit of ribulose-1,5-bisphosphate carboxylase). Source: Knäblein J. (2003) *Biotech: A New Era in the New Millennium – Biopharmaceutical Drugs Manufactured in Novel Expression Systems*, DECHEMA-Jahrestagung der Biotechnologen, Munich, Germany, 21. (See color plate. p. xxv)

a number of advantages, such as very rapid (1 to 2 week) expression time, possibility of generating initial milligram quantities within weeks, high expression levels, and so on. However, the existing viral vectors, such as TMV-based vectors used by, for example, Large Scale Biology Corp. (Vacaville, CA, USA) for production of single-chain antibodies for treatment of non-Hodgkin lymphoma (currently in Phase III clinical trials, see Table 1), had numerous shortcomings, such as inability to express genes larger than 1 kb, inability to coexpress two or more proteins (a prerequisite for production of

monoclonal antibodies, because they consist of the light and heavy chains, which are expressed independently and are subsequently assembled), low expression level in systemically infected leaves, and so on. ICON has solved many of these problems by designing a process that starts with an assembly of one or more viral vectors inside a plant after treating the leaves with agrobacteria, which deliver the necessary viral vector components. ICON's proviral vectors provide advantages of fast and high-yield amplification processes in a plant cell, simple and inexpensive assembly of expression cassettes *in planta*, and

full control of the process. The robustness of highly standardized protocols allows the use of inherently the same safe protocols for both laboratory-scale as well as industrial production processes. In this system, the plant is modified transiently rather than genetically and reaches the speed and yield of microbial systems while enjoying posttranslational capabilities of plant cells. De- and reconstructing of the virus adds some safety features and also increases efficiency. There is no “physiology conflict,” because the “growth phase” is separated from the “production phase,” so that no competition occurs for nutrients and other components required for growth and also for expression of the biopharmaceutical at the same time.

This transfection-based platform allows the production of proteins in a plant host at a cost of US\$1 to 10 per gram of crude protein. The platform is essentially free from limitations (gene insert size limit, inability to express more than one gene) of current viral vector-based platforms. The expression levels reach 5 g per kilogram of fresh leaf tissue (or some 50% of total cellular protein!) in 5 to 14 days after inoculation. Since the virus process (in addition to superhigh production of its own proteins, including the protein of interest) leads to the shutoff of the other cellular protein synthesis, the amount of protein of interest in the initial extract is extremely high (Fig. 7). It thus results in reduced costs of downstream processing. Milligram quantities can be produced within two weeks, gram quantities in 4 to 6 months, and the production system is inherently scalable. A number of high-value proteins have been successfully expressed, including antibodies, antigens, interferons, hormones, and enzymes (see Klimyuk, Marillonnet, Knäblein, McCaman, Gleba (2005), books).

### 6.3

#### Simple Moss Performs Complex Glycosylation

Greenovation Biotech GmbH (Freiburg, Germany) has established an innovative production system for human proteins. The system produces pharmacologically active proteins in a bioreactor, utilizing a moss (*Physcomitrella patens*) cell culture system with unique properties (see Fig. 8). It was stated before that posttranslational modifications for some proteins are crucial to gain complete pharmacological activity. Since moss is the only known plant system that shows a high frequency of homologous recombination, this is a highly attractive tool for production strain design. By establishing stable integration of foreign genes (gene knock-out and new transgene insertion) into the plant genome, it can be programmed to produce proteins with modified glycosylation patterns that are identical to animal cells. The moss is photoautotrophic and therefore only requires simple media for growth, which consist essentially of water and minerals. This reduces costs and also accounts for significantly lower infectious and contamination risks, but in addition to this, the system has some more advantages:

- The transient system allows production of quantities for a feasibility study within weeks – production of a stable expression strain takes 4 to 6 months.
- On the basis of transient expression data, the yield of stable production lines is expected to reach 30 mg L<sup>-1</sup> per day. This corresponds to the yield of a typical fed-batch culture over 20 days of 600 mg L<sup>-1</sup>.
- Bacterial fermentation usually requires addition of antibiotics (serving as selection marker and to avoid loss of the

Greenovation (moss system):

- Simple medium (photoautotrophic plant needs only water and minerals)
- Robust expression system (good expression levels from 15 to 25°C)
- Secretion into medium via human leader sequence (broad pH range: 4-8)
- Easy purification from low salt medium via ion exchange
- Easy genetic modifications to cell lines
- Stable cell lines / high genetic stability
- Codon usage like human (no changes required)
- Inexpensive bioreactors from the shelf
- Nonfood plant (no segregation risk)
- Good progress on genetic modification of glycosylation pathways (plant to human)



**Fig. 8** Greenovation use a fully contained moss bioreactor. This company has established an innovative production system for human proteins. The system produces pharmacologically active proteins in a bioreactor, utilizing a moss (*Physcomitrella patens*) cell culture system with unique properties. Source: Knäblein J. (2003) *Biotech: A New Era in the New Millennium – Biopharmaceutical drugs Manufactured in Novel Expression Systems*, DECHEMA-Jahrestagung der Biotechnologen, Munich, Germany, 21.

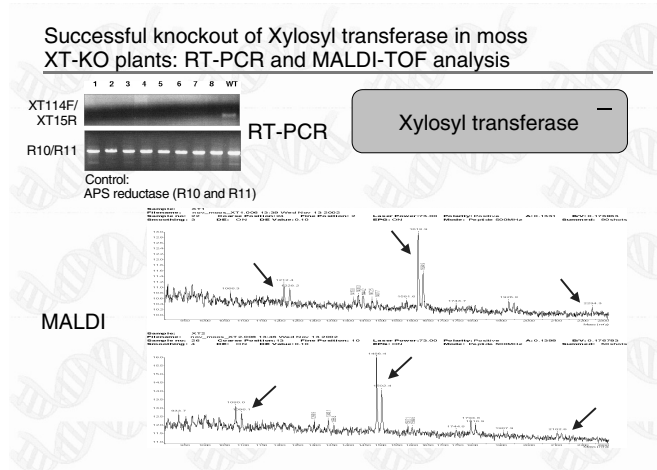
expression vector). For moss cultivation, no antibiotics are needed – this avoids the risk of traces of antibiotics having a significant allergenic potential in the finished product.

- Genetic stability is provided by the fact that the moss is grown in small plant fragments and not as protoplasts or tissue cultures avoiding somaclonal variation.
- As a contained system, the moss bioreactor can be standardized and validated according to GMP standards mandatory in the pharmaceutical industry.
- Excretion into the simple medium is another major feature of the moss bioreactor, which greatly facilitates downstream processing.

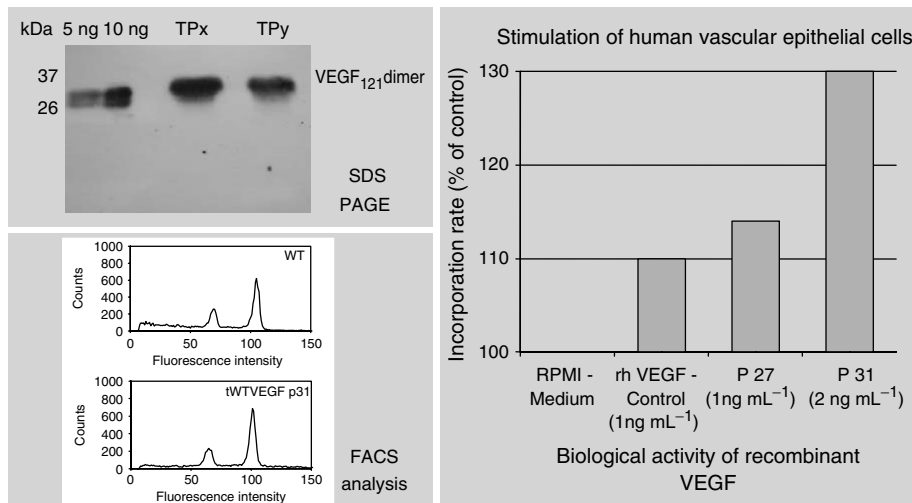
As discussed in detail, the first step to get humanlike glycosylation in plants is

to eliminate the plant glycosylation, for example, the attachment of  $\beta$ -1-2-linked xylosyl and  $\alpha$ -1-3-linked fucosyl sugars to the protein, because these two residues have allergenic potential. Greenovation was able to knockout the relevant glycosylation enzymes xylosyl transferase and fucosyl transferase, which was confirmed by RT-PCR (reverse transcriptase PCR). And indeed, xylosyl and fucosyl residues were completely removed from the glycosylation pattern of the expressed protein as confirmed by MALDI-TOF (matrix assisted laser desorption ionization time of flight) mass spectroscopy analysis (see Fig. 9).

A very challenging protein to express is VEGF because this homodimer consists of two identical monomers linked via a disulfide bond. To produce VEGF in



**Fig. 9** Knockout of Xylosyl Transferase in moss. To avoid undesired glycosylation, greenovation knocked out the Xylosyl and Fucosyl Transferase, as confirmed by RT-PCR. MALDI-TOF results show that indeed, xylosyl- and fucosyl-residues were completely removed from the glycosylation pattern of the expressed protein (data for knockout of Fucosyl Transferase not shown). Source: Knäblein J. (2003) *Biotech: A New Era in the New Millennium – Biopharmaceutical Drugs Manufactured in Novel Expression Systems*, DECHEMA-Jahrestagung der Biotechnologen, Munich, Germany, 21.

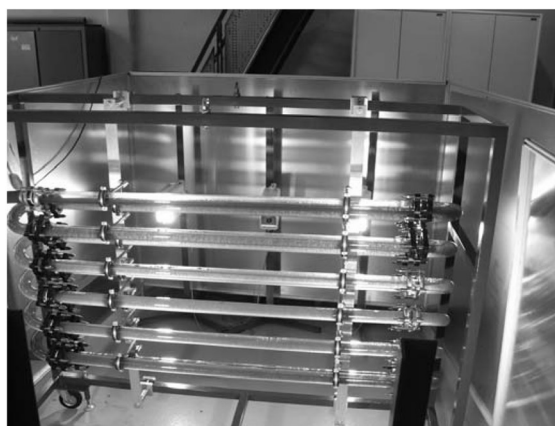


**Fig. 10** Greenovation could successfully express the biopharmaceutical VEGF. This growth factor is a very complex protein consisting of two identical monomers linked via a disulfide-bond. To produce VEGF in an active form, the monomers need to be expressed to the right level, correctly folded, assembled, and

linked via the disulfide-bond. The analytical assays clearly show that expression in moss yielded completely active VEGF. Source: Knäblein J. (2003) *Biotech: A New Era in the New Millennium – from Plant Fermentation to Plant Expression of Biopharmaceuticals*, PDA International Congress, Prague, Czech Republic.



30 L pilot reactor for moss



Two weeks after incubation

**Fig. 11** Scaling of photobioreactors up to several 1000 L. The moss bioreactor is based on the cultivation of *Physcomitrella patens* in a fermenter. The moss protonema is grown under photoautotrophic conditions in a medium that consists essentially of water and minerals. Light and carbon dioxide serve as the only energy and carbon sources. Cultivation in suspension allows

scaling of the photobioreactors up to several 1000 L. Adaptation of existing technology for large-scale cultivation of algae is done in cooperation with the Technical University of Karlsruhe. Source: greenovation Biotech GmbH (Freiburg, Germany) and Professor C. Posten, Technical University (Karlsruhe, Germany).

an active form, the following need to be provided:

- Monomers need to be expressed to the right level.
- Monomers need to be correctly folded.
- Homodimer needs to be correctly assembled and linked via a disulfide bond.
- Complex protein needs to be secreted in its active form.

And in fact, all this could be achieved with the transgenic moss system as shown in Fig. 10. These results are very promising because they demonstrate that this system is capable of expressing even very complex proteins. In addition to that, the moss system adds no plant-specific sugars to the protein – a major step toward humanlike glycosylation. Furthermore, moss is a robust expression system leading to high yields at 15 to 25 °C and the pH can be adjusted from 4 to 8 depending on

the optimum for the protein of interest. Adapting existing technology for large-scale cultivation of algae, fermentation of moss in suspension culture allows scaling of the photobioreactors up to several 1000 L (see Fig. 11). Finally, the medium is inexpensive, since only water and minerals are sufficient.

## 7 Other Systems Used for Plant Expression

Several different plants have been used for the expression of proteins in plants. All these systems have certain advantages regarding edibility, growth rate, scalability, gene-to-protein time, yield, downstream processing, ease of use, and so on, which I will not discuss in further detail here. A selection of different expression systems is listed:

<i>Alfalfa</i>	Ethiopian mustard	Potatoes
<i>Arabidopsis</i>	<i>Lemna</i>	Rice
Banana	Maize	Soybean
Cauliflower	Moss	Tomatoes
Corn	Oilseeds	Wheat

Some of these systems have been used for research on the basis of their ease of transformation, well-known characterization, and ease to work with. However, they are not necessarily appropriate for commercial production. Which crop is ultimately used for full-scale commercial production will depend on a number of factors including

- time to develop an appropriate system (gene-to-protein);
- section of the plant expressing the product/possible secretion;
- cost and potential waste products from extraction;
- “aged” product/ease of storage;
- long-term stability of the storage tissue;
- quantities of protein needed (scale of production).

Depending on the genetic complexity and ease of manipulation, the development time to produce an appropriate transgenic plant for milligram production of the desired protein can vary from 10 to 12 months in corn as compared to only weeks in moss. Estimates for full GMP production in corn are 30 to 36 months and approximately 12 months for moss. Expression of the protein in various tissues of the plant can result in a great variation in yield. Expression in the seed can often lead to higher yields than in the leafy portion of the plant. This is another explanation for the high interest in using corn, which has a relatively high seed-to-leaf ratio. Extraction from leaf can be costly as it contains a

high percentage of water, which could result in unavoidable proteolysis during the process. Proteins stored in seeds can be desiccated and remain intact for long periods of time. The purification and extraction of the protein is likely to be done by adaptations of current processes for the extraction and/or fractionation. For these reasons, it is anticipated that large-scale commercial production of recombinant proteins will involve grain and oilseed crops such as maize, rice, wheat, and soybeans. On the basis of permits for open-air test plots issued by the USDA for pharmaceutical proteins and industrial biochemicals, corn is the crop of choice for production with 73% of the permits issued. The other major crops are soybeans (12%), tobacco (10%), and rice (5%).

In general, the use of smaller plants that can be grown in greenhouses is an effective way of producing the biopharmaceuticals and alleviating concerns from environmental activist groups that the transgenic plant might be harmful to the environment (food chain, segregation risk, genetic drift, etc.).

## 8 Analytical Characterization

Validated bioanalytical assays are essential and have to be developed to characterize the biopharmaceuticals during the production process (e.g. in-process control) and to release the final product for use as a drug in humans. These assays are applied to determine characteristics such as purity/impurities, identity, quantity, stability, specificity, and potency of the recombinant protein during drug development. Since the very diverse functions of different proteins heavily depend on their structure, one very valuable parameter in

protein characterization is the elucidation of their three-dimensional structure. Although over the last couple of years a lot of effort was put into a method for improving the elucidation of protein structures (during my PhD thesis, I was also working in this fascinating field together with my boss Professor Robert Huber, Nobel Prize Laureate in 1988, “for the determination of the three-dimensional structure of a photosynthetic reaction centre”), it is still very time consuming to solve the 3-D structure of larger proteins. This is why despite the high degree of information that can be obtained from the protein structure, this approach cannot be applied on a routine basis. Therefore, tremendous efforts are put into the development of other assays to guarantee that a potent biopharmaceutical drug is indeed ready for use in humans.

## 9

### Conclusion and Outlook

The production of protein therapeutics from transgenic plants is becoming a reality. The numerous benefits offered by plants (low cost of cultivation, high biomass production, relatively fast gene-to-protein time, low capital and operating costs, excellent scalability, eukaryotic post-translational modifications, low risk of human pathogens, lack of endotoxins, as well as high protein yields) virtually guarantee that plant-derived proteins will become more and more common for therapeutic uses. Taking advantage of plant expression systems, the availability of cheap protein-based vaccines in underdeveloped countries of the world is possible in the near future. The cost of very expensive hormone therapies (erythropoietin, human growth hormone, etc.)

could fall dramatically within the next decade because of the use of, for example, plant expression systems. Fears about the risks of the plant expression technology are real and well founded, but with a detailed understanding of the technology, it is possible to proactively address these safety issues and create a plant expression industry almost free of mishaps. For this purpose, the entire set up, consisting of the specific plant expression system and the protein being produced, needs to be analyzed and its potential risks assessed on a case-by-case basis. As plant-derived therapeutics begin to demonstrate widespread, tangible benefits to the population and as the plant expression industry develops a longer safety track record, public acceptance of the technology is likely to improve continuously. Plants are by far the most abundant and cost-effective renewable resource uniquely adapted to complex biochemical synthesis. The increasing cost of energy and chemical raw materials, combined with the environmental concerns associated with conventional pharmaceutical manufacturing, will make plants even more compatible in the future. With the words of Max Planck (1858–1947) “How far advanced Man’s scientific knowledge may be, when confronted with Nature’s immeasurable richness and capacity for constant renewal, he will be like a marveling child and must always be prepared for new surprises,” we will definitely discover more fascinating features of plant expression systems. But there is no need to wait: combining the advantages of some technologies that we already have in hand could lead to the ultimate plant expression system. This is what we should focus on, because, then, at the dawn of this new millennium, this would for the first time yield large-enough amounts of biopharmaceuticals to treat everybody on our planet!

## Acknowledgments

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See also *Bioprocess Engineering; Expression Systems for DNA Processes; Plant Gene Expression, Regulation of*.

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