

Bioproduction of Therapeutic Proteins in the 21st Century and the Role of Plants and Plant Cells as Production Platforms

ROBERT BOEHM

Institute of Plant Molecular Physiology and Biotechnology (IMBIO), University of Bonn, Bonn, Germany

ABSTRACT: In the last decade, the technique to genetically modify crop plants has gained more and more interest in terms of bioproduction of heterologous proteins. Plants have been discovered as a possible source for large amounts of cost effective recombinant protein. Main application fields are therapeutics for use in animal and human health, diagnostics, and technical enzymes. This review is focused on the recent progress in this field of molecular farming. After a comparison with hitherto established protein production systems, the advantages of plants as an alternative production system are discussed. An overview about the different host plants and possible expression strategies is given and the progress in commercialization of the techniques is highlighted. Finally, the role of plant cell cultures for the production of recombinant proteins is discussed.

KEYWORDS: bioproduction; gene expression; molecular farming; recombinant protein; transgenic plants

INTRODUCTION

Plants have been used for human benefit since the dawn of the human race, supplying the growing population with food, fibers, wood, and therapeutics. Thus, plants were, besides animals, the basic bioproduction system for important bulk substances. Especially in the field of pharmaceuticals, plants offer a huge variety of secondary metabolites with many therapeutic effects. These substances, evolutionarily developed by plants to protect themselves against pathogens and predators or to attract pollinators,¹ exhibit wound-healing, anti-inflammatory, antimicrobial, or psychoactive properties. They can be used

Address for correspondence: Robert Boehm, Institute of Plant Molecular Physiology and Biotechnology (IMBIO), Karlrobert Kreiten-Str. 13, 53115 Bonn, University of Bonn, Germany. Voice: +49-228-732156; fax +49-228-731696.
r.boehm@uni-bonn.de

Ann. N.Y. Acad. Sci. 1102: 121–134 (2007). © 2007 New York Academy of Sciences.
doi: 10.1196/annals.1408.009

to protect and maintain human and animal health. Modern biotechnological methods to produce these therapeutics in a controlled and predictable manner encompass cell cultures initiated from the respective medicinal plants. This sometimes is the only possibility for the large-scale production of these substances since the original plant species often are difficult to cultivate and are rare in nature. Successful examples for this kind of bioproduction are the alkaloids,² paclitaxel,³ and shikonin.⁴

With the beginning of modern gene technology, it also became feasible to alter the plant genome by the addition or replacement of specific genes and their surrounding regulatory DNA sequences, for example, for tobacco⁵ or sunflower.⁶ This in the first instance was used to improve or alter the secondary products of therapeutic value that already were produced by plant cell cultures, for example, on tropan alkaloid or nikotin biosynthesis.^{7,8} At the same time, first reports could demonstrate that plant cells are also able to express antibodies in a correct and biologically active way.^{9,10} This opened up a completely new application field for transgenic plants. Here, the improvement of plant-derived substances or the plants themselves are no longer at the center of interest but the use of plants as a sole production tool for recombinant proteins of virtually any origin is of interest. These proteins can be purified after the plant harvest and applied for human benefit.

This review focuses on the current state of the use of intact plants and plant cell cultures as protein production systems for human and animal health in research and commercial applications. It highlights the potential that plants offer as a serious alternative to contribute to current protein production facilities in the future. Additionally, the use of plant cell cultures for this purpose is discussed.

ESTABLISHED PROTEIN PRODUCTION SYSTEMS

In the last decade, protein-based therapeutics have attracted more and more attention for the application in diagnosis and therapy. This includes mainly antibodies and antibody derivatives but also many serum-derived proteins (cytokines, growth hormones, interleukines, interferons, etc.). To date, 94 proteins of human therapeutic value have entered the market.¹¹ All these biologics are produced recombinantly with the state-of-the-art *in vitro* production systems: bacterial fermentation (*Escherichia coli*), yeast cell cultures (*Saccharomyces cerevisiae*, *Pichia pastoris*), or mammalian cell cultures. These production platforms, though very sophisticated and well characterized, still bear some disadvantages that are inherent in the respective production systems. Criteria for the assessment of the different systems are of biologic (yield of biologically active protein, posttranslational modifications, etc.) as well as economic nature (overall production costs, time to market, etc.).

To date, the bacterial fermentation is the best-characterized and most cost-effective production system. However, because of the procaryotic nature of

the organisms, a eucaryotic type of protein processing is poorly developed and the production is limited to simple structures (peptides, small proteins) where posttranslational modifications are absent or unimportant for the biologic activity. Moreover, high protein accumulation often results in aggregation (inclusion bodies), thus, laborious renaturation steps are needed to recover the proteins in their biologically active form. The overall yield, however, remains relatively low, ranging from 100 to 1,500 mg/L.¹² Another point to consider is the bacterial toxins that have to be eliminated during downstream processing. Yeasts as eucaryotic organisms avoid some of these disadvantages, but the practical experiences show a high amount of product loss due to degradation of the target protein in the medium. Moreover, especially *S. cerevisiae* tends to hyperglycosylate the produced proteins what results in N-glycans of high-mannose structure, which are totally different from the mammalian or human structures of N-glycans. These critical points can be improved by using *P. pastoris* as expression system.¹³ Here, a protein yield up to 6.4 g/L could be obtained,¹⁴ although the average protein yield is 100–200 mg/L.¹³ Despite these limitations, 43% of the current biologics on the market are produced in bacteria and yeasts. The majority of the biologics (57%), however, is produced in different mammalian cell cultures (e.g., NS0, BHK, CHO). As cell types most similar to their human counterpart, these systems offer the highest yield of functional recombinant protein with correct N-glycosylation and other posttranslational modifications. The yield typically ranges between 1 and 3 g/L.¹² This “golden standard,” however, requires expensive infrastructure and media components. Additionally, the risk of viral or oncogenic contamination is given. This leads to reasonably high overall production costs, ranging from 300 to 10,000 USD/g, depending of the quantity of the protein produced.¹⁵

FUTURE QUANTITY DEMAND OF THERAPEUTIC PROTEINS

The human genome project has also boosted the identification of target proteins of therapeutic potential. Thus, the pipeline of protein-based therapeutics in preclinic and clinical trials at the moment by far exceeds the amount of such kind of therapeutics on the market.¹¹ Within this target pipeline, the portion of monoclonal antibodies will enlarge from today's 25% of all biologics to nearly 62%, mainly based in the therapeutic field of cancer treatment, inflammatory diseases, and anti-infective therapy.¹¹ After these drug candidates have been approved for the market, the quantity demand for each will range from hundreds of kilograms to several metric tons of highly pure protein per year. This demand exceeds today's industrial production capacities and even after increase of manufacturing capacities, this issue is regarded as a future bottleneck in bringing protein-based therapeutics to the market.¹⁶ Even when this capacity problem is solved using the established production systems, the high investment and production costs will put reasonable financing pressure on the

national health systems. From that point of view, new technologies for large-scale and cost-effective biomanufacturing of therapeutic proteins are highly appreciated.

WHY PLANTS AS PRODUCTION PLATFORM?

Since the early days of expression of antibodies in plants,^{9,10} many feasibility studies of the production of different therapeutic proteins have been performed in plants^{17,18} and plant cell cultures.^{19,20} This includes IgG,²¹ acetylcholinesterase,²² α -interferon,²³ human α -1-antitrypsin,²⁴ or secretory IgA,²⁵ which cannot be produced in any other production system so far. Plant cells as eucaryotic systems possess all the features for the biologically active generation of complex therapeutic proteins. This results in a very high ratio of biologically active recombinant protein. In a comparative study of the expression of anti-CEA scFv, tobacco BY-2 cells showed by far the highest amount of functional protein (92%) of total recombinant protein (TP), compared to *E. coli* (12% TP) and *P. pastoris* (40% TP).¹²

Using open-field cultivation, transgenic plants have superior advantages over all established expression systems in terms of capacity, flexibility, scalability, and production costs. If transgenic seed material can be supplied to the market in sufficient quantities, farming will rely on the well-established, low-cost agricultural infrastructure. The acreage of planted transgenic plants can be adopted to meet altered quantity demands from one year to another without additional costs. Taking into account an average protein yield of 0.5–1 g per kg fresh weight, Fischer and coworkers²⁶ calculate that 1 hectare of tobacco plants would result in 100 tons of fresh material per year. From this, 50 kg of sIgA or 100 kg of recombinant glucocerebrosidase can be harvested. Thus, the relatively low protein yield, which is a general feature of transgenic plants compared to other expression systems, can be compensated easily by the huge quantity of generated biomass. Moreover, the downstream processing costs may also be diminished because there is no need to downgrade any bacterial toxins or human pathogenic particles (viruses, toxins, prions) existing in animal production systems. Taking these unique features into account, the costs for the resulting proteins have been calculated to be below 50 USD per gram.²⁷

CHOOSING A HOST PLANT FOR THE GENE EXPRESSION

For the production of valuable secondary metabolites, one is restricted to the plant species where this specific and complex biosynthetic pathway can be found and exploited. This entire pathway cannot be transferred to another plant. In contrast, proteins are encoded by only one or very few genes that can be inserted and expressed in virtually any host plant. This gives much more

freedom to operate, but, on the other hand, leads to a vast variety of possible host plants and expression strategies for the production of recombinant proteins. There is not one clear advantageous, leading plant expression system, although the well-established crop species are preferably used, including tobacco, rice, and corn.^{15,18} Depending on the biologic and economic requirements of one specific target protein, one is free to choose the most suitable plant expression system. There are two basic strategies for the expression: transient expression or stably transformed plants. Transient expression has some advantages since this technique does not result in transgenic plants with its ecologic or regulatory concerns. Moreover, a reasonable amount of protein can be obtained within few days after infection.²⁸ The transient expression can be achieved using infiltration of whole plants or plant parts (e.g., leaves) with *Agrobacterium tumefaciens*.^{29,30} This results in lab-scale quantities of protein in the milligram range for feasibility or preclinical studies. In another transient expression strategy, the target gene is fused to the coat protein gene of plant viruses, for example, tobacco mosaic virus, and the thus altered virus is used for plant infection.²⁸ This technique is used commercially for large-scale protein production in open-field plants, for example, by Large Scale Biology's Geneware® technology³¹ and Icon Genetics's MagICON® technology.³² Transgenic plants, on the other hand, require a longer time for the generation and characterization of production lines and the generation of a sufficient amount of seed material. But after this, an almost unlimited and sustainable production capacity can be reached using the established agricultural infrastructure. This strategy is already applied commercially using bulk crop plants like tobacco, corn, rice, or soybean^{15,18} or special plant species like soapworts (Saponin Inc., Saskatoon, Canada), safflower (SemBioSys Genetics Inc., Calgary, Alberta Canada), or alfalfa (Medicago Inc., Quebec Canada). An alternative to open-field plants is the cultivation of the transgenic plants in greenhouses, which enables more controlled production conditions, but increases the production costs. This decision depends on the quantity demand of the target protein and the regulatory issues of the specific country in terms of release of transgenic plants into the environment and GMP requirements of the production process.

AQUATIC PLANTS AS EXPRESSION SYSTEMS

Free floating water plants as production hosts for recombinant protein production are a quite new and innovative field of molecular farming, providing a further alternative to open-field plants. This includes higher, free floating water plants as well as algae. All these have the advantage of a fast vegetative growth rate and the cost-effective cultivation on water and inorganic nutrients. Depending on the safety and mass requirements, these organisms can be held in contained *in vitro* cultures, in controlled greenhouse containers, or in open ponds of any size for bulk biomass production. From eucaryotic algae, mostly

Chlamydomonas reinhardtii is used.³³ But other algae species eventually have been tested for that purpose, for example, *Chlorella ellipsoidea*.³⁴ The specific algae-related problem of a codon usage different from animal and human could be overcome by the use of completely synthetic genes with adopted codon usage.³⁵

Regarding higher plants, members of the *Lemnaceae* (duckweed) family successfully are already commercially exploited as a source for recombinant therapeutic proteins, namely *Lemna minor* by Biolex Inc. (Pittsboro, NC, USA) and *Spirodela oligorhiza* by LemnaGene (France). Also, species of the genus *Wolffia* are under development for that purpose.^{36,37} A specific advantage of duckweed is that these plants are rich in proteins and can serve as animal feed.³⁸ In addition, *Wolffia* plants were also cultivated as “water eggs” and used for human nutrition in east Asia in former times.³⁹

SPATIAL AND TEMPORAL REGULATION OF GENE EXPRESSION

In most cases, the target protein is expressed in the whole plant and the gene is driven by constitutive promoters, for example, CaMV 35S or *mas* promoter without any regulation. This is to reach the maximum protein yield. But the temporal and spatial regulation of transgene expression within the plant can be advantageous for specific applications. This regulation is reached by the use of plant promoters that are active only in a specific plant organ or time frame within the plant's life. If the transgene is expressed constitutively in the whole plant, the recombinant protein has to be extracted right after the plant harvest and the crude protein extract or the purified protein has to be distributed under elaborate conditions in a cooling chain. In contrast, targeting the expression to one of the plant storage organs leads to an accumulation of the resulting protein exclusively in these plant parts. Here, the protein remains stable and active at ambient temperature for an extended time span. After harvest, the organs can be stored and transported without any cooling or stabilization and can be extracted at a place distant from the harvesting location. The target storage organs mostly were seeds (cereals, corn) or tubers (potato). This strategy is applied successfully to several technical proteins and enzymes and is also considered for therapeutic protein production.⁴⁰ To make protein production in plants more safe and controllable, it can be useful to put the target gene under the control of an inducible promoter. This avoids transgenic plants containing recombinant protein on the field and the deliberate induction of gene expression enables protein production after the harvest right before extraction. This possibility successfully has been applied using a stress-inducible promoter (MeGATM promoter) in tobacco by CropTech Corp (Blacksburg, VA, USA).⁴¹ Here, the foreign gene expression is induced by chopping the freshly harvested tobacco plants under contained conditions. In another strategy, a

germination-specific promoter drives gene expression in barley seeds. After harvest and storage of the seed granules, the protein production can be induced at the desired time point and place by germinating (malting) the granules. This technique is proprietary of MALTagen GmbH (Andernach, Germany).

EFFECTS ON THE INTRACELLULAR TARGETING OF THE RECOMBINANT PROTEIN

The intracellular targeting of the resulting protein is of great influence to protein yield and its biological properties. At the current state of the art, the targeting of all intracellular compartments is feasible using known signal sequences that can be fused to the target gene. The intracellular locations that have been tested and compared for foreign protein production include cytosol, the endoplasmic reticulum (ER), the apoplastic space, the vacuole, and the chloroplast (see below). Several studies have been performed to compare the efficacy of protein production using cytoplasmic, ER, and apoplastic accumulation in tobacco⁴²⁻⁴⁴ or rice.⁴⁵ Conclusively, ER targeting gives rise to the highest yield of biologically active protein. In contrast, cytosolic expression mostly leads only to poor protein yield. The reason is that the ER contains many important posttranslational protein maturation elements like chaperones, protein-disulfide isomerase, and glycosylation enzymes in a reducing environment. These features are not at all or scarcely present in the cytosol. Vacuolar targeting and secretion into the apoplastic space is also coupled with ER/Golgi passage and the above-mentioned maturation and stabilization properties. However, the protein yield often is found to be diminished compared to ER accumulation, probably due to unfavorable protein stability properties of these compartments.

CHLOROPLAST TRANSFORMATION

The targeting of the foreign gene's expression to the chloroplasts is a completely different strategy that cannot be accomplished using the respective signal sequence for chloroplast protein import but by a specific transformation method of the chloroplast itself using particle bombardment.^{46,47} Successful chloroplast transformation, which is selected via chloroplast-specific selection markers, leads to "transplastomic" rather than transgenic plants. After the generation of fully homotransplastomic plants by repeated rounds of selection, the protein yield reaches up to 46.1% total leaf protein⁴⁶ and, thus, is superior to all other transgenic expression strategies with regard to protein yield. This results from the high copy number of the chloroplast genome and of the integrated transgene coupled with high transcription rates. Some more advantages render the chloroplastic expression of foreign genes to a

promising strategy to compete with other nonplant expression systems regarding protein yield: Due to the procaryotic nature of the chloroplasts, it is possible to transfer whole polycistrons with several genes under the same regulation and to get their coordinate expression. Moreover, homologous recombination is feasible to integrate genes into specific genome sites without gene silencing or position effects, which leads to reduced variability in expression levels of the introduced transgene. Finally, the plants lack transgenic pollen due to the maternal inheritance of the chloroplast genome. The only disadvantage that results from the procaryotic organelle character is the decreased ability for necessary posttranslational modifications present in the ER. Thus, the range of proteins that can be obtained in a biologically active manner from chloroplast transformation is limited similarly to the bacterial expression systems. The chloroplast transformation was first established in tobacco, but the range of transformable plant species is increasing in recent years, including cotton,⁴⁸ soybean,⁴⁹ oilseed rape,⁵⁰ tomato,⁵¹ and potato.⁵² A growing number of therapeutic relevant proteins have been expressed using this strategy, including interferon gamma⁵³ vaccines⁵⁴⁻⁵⁶ and other therapeutic relevant proteins.⁴⁶ This production strategy has already been commercialized, for example, by Chlorogen, Inc. (St. Louis, MO, USA) and Icon Genetics (Halle, Germany).

EDIBLE VACCINES

Besides living, attenuated, and inactivated strains of viruses and bacteria, it has been found that also isolated parts or even single proteins of these pathogens (so called subunit vaccines) can provoke an active, prophylactic immunization, if delivered by injection. These subunit vaccines are another class of therapeutic proteins that can actively be expressed using plants.⁵⁷ An alternative vaccine delivery to reach an active immunization efficiently is oral or nasal exposure of the vaccines to mucosal tissue of nose, throat, and gut. Here, the vaccines elicit the mucosal (sIgA) as well as systemic (IgG) immune response.⁵⁸ These findings paved the road to a very fascinating combination of medicine and plant science: The plants expressing oral vaccines do not only serve as the production system but at the same time as delivery vehicle for the vaccines. Oral immunization simply can be achieved by eating edible plant parts expressing the subunit vaccine gene. This enormously simplifies delivery and administration and reduces the production costs, since any kind of downstream processing and protein purification is unnecessary. Thus, the production of vaccines in edible plant parts like fruits is of unique advantage. Choosing an appropriate host species like banana, it opens up the possibility for low-cost immunization in underprivileged countries with poor medical supply.

Vaccines belonged to the first recombinant proteins that have been generated using transgenic plants.⁵⁹ The successful active immunization after oral administering of vaccine-expressing plant parts has been proven several times.⁶⁰

Since then, numerous vaccines have been produced in transgenic plants, including hepatitis B envelope surface protein, Norwalk virus capsid protein, heat-labile toxin B-subunit of enterotoxigenic *E. coli* for human immunization, and VP1 gene of foot-and-mouth disease virus or glycoprotein S of transmissible gastroenteritis coronavirus for veterinary applications.¹⁵ Relevant host plants for the expression were especially those generating edible plant parts that can be eaten without any processing or cooking. This includes tomato⁶¹ and carrot.⁶² Although no report about vaccine production in banana is available to date, this plant species is especially prone for vaccine production in developing countries, where it can be cultivated easily. Additionally, leaves can in some cases be eaten raw and, thus, used for oral vaccine production, as for example, alfalfa⁶³ or lettuce.⁶⁴ Potato tubers as an accumulation organ for vaccines often have successfully been used as model system⁶⁵ but lack practical relevance, since potatoes have to be cooked for human food, destroying much of the vaccine protein. In conclusion, if some regulatory issues like homogenous vaccine dose per gram fresh weight can be solved satisfactorily, plant-derived vaccines will hold many promises for safe and cost-effective mass immunization of mankind much more than any other production system.

COMMERCIALIZATION OF PLANT-BASED PROTEIN PRODUCTION

Because the perspective for an economically reasonable protein production in transgenic plants is that promising, several companies, especially in the United States and Canada, have been founded that focus on the production of therapeutic proteins in open-field-cultivated plants, including Large Scale Biology (www.lsb.com), Ventria Bioscience (www.ventria.com), Planet Biotechnology (www.planetbiotechnology.com), Dow Agroscience (www.dowagro.com), and Agracetus (www.agracetus.com) in the United States, Medicago (www.medicago.com), Plantigen (www.lhsc.on.ca/plantigen), and SemBioSys (www.sembiosys.ca) in Canada, Icon Genetics (www.icongenetics.com), Novoplant (www.novoplant.de), Sungene (www.sungene.de), and MALTagen (www.maltagen.de) in Germany, or Méristem Therapeutics (www.meristem-therapeutics.com) in France. Many of the numerous therapeutic proteins under development have already entered clinical phase trials. However, no plant-derived product has entered the market to date. The first product, a sIgA/IgG chimeric antibody against *Streptococcus mutans* for caries treatment, will be launched by Planet Biotechnology in 2006, according to the company.⁶⁶

PLANT *IN VITRO* CULTURES

In contrast, production systems using plant *in vitro* cultures are not used at a commercial level so far, but several systems are under development at the

academia.^{19,20,67} Plant cell cultures are more comparable to mammalian cell cultures from the view of biochemical engineering. Although no calculation of overall production costs using plant cell cultures can be found in literature, these systems promise to be more cost effective. This is due to the simple media using cheaper medium components (mostly inorganic salts and sucrose) and the lack of human pathogenic particles that have to be eliminated in the downstream processing. As a first estimation, plant cell cultures cannot compete with transgenic plants in open-field cultivation with regard to production costs, but still promise to be more cost-effective than mammalian cell cultures. This, however, is also strictly dependent on the level of gene expression and, thus, protein yield. First feasibility studies demonstrate the successful expression of IgG,⁶⁸ interleukin-12,⁶⁹ human granulocyte-macrophage colony stimulation factor,⁷⁰ or immunotoxins.⁷¹ However, protein yield remains low, averaging 1–5 mg/L cell suspension culture.¹⁹ Exceptional high protein yield has been reported rarely, for example, 200 mg/L of α -1-antitrypsin in rice cell cultures.⁷² This, however, highlights the potential of plant *in vitro* systems for therapeutic bioproduction purposes. To finally reach economical profitability, much effort has to be put on consequent strain and genotype selection, media component optimization, development of appropriate promoters and enhancer elements, codon optimization, et cetera. to enhance productivity by a factor of at least 500. This has been done in a similar way with animal cell culture strains over the last 20 years, which finally led to today's productivity in this area. It remains questionable, however, whether there is enough interest from the industry side to push the development of such profitable plant cell culture systems in the future in the face of available, optimized, and approved animal cell production lines. If so, the development will certainly focus on few well-characterized lines, for example, BY-2 tobacco cells,⁷³ rice cell culture,⁴⁵ or that of the moss *Physcomitrella patens*.⁷⁴

REFERENCES

1. WINK, M. 1988. Plant breeding – Importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* **75**: 225–233.
2. VERPOORTE, R., A. CONTIN & J. MEMELINK. 2002. Biotechnology for the production of plant secondary metabolites. *Phytochem. Rev.* **1**: 13–25.
3. ZONG, J.J. 2002. Plant cell culture for production of paclitaxel and other taxanes. *J. Biosci. Bioeng.* **94**: 591–599.
4. YAZAKI, K., H. MATSUOKA, T. UJIHARA & J. SCHELL. 1999. Shikonin biosynthesis in *Lithospermum erythrorhizon*: light-induced negative regulation of secondary metabolism. *Plant Biotechnol.* **16**: 335–342.
5. HORSCH, R.B., J.E. FRY, N.L. HOFFMANN, *et al.* 1985. A simple and general method for transferring genes into plants. *Science* **227**: 1229–1231.
6. EVERETT, N.P., K.E.P. ROBINSON & D. MASCARENHAS. 1987. Genetic engineering of sunflower (*Helianthus annuus* L.). *Bio/Technology* **5**: 1201–1294.

7. YUN, D., T. HASHIMOTO & Y. YAMADA. 1992. Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc. Natl. Acad. Sci. USA **89**: 11799–11803.
8. HAMILL, J.D., R.J. ROBINS, A.J. PARR, *et al.* 1990. Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Mol. Biol. **15**: 27–38.
9. HIATT, A., R. CAFFERKEY & K. BOWDIS. 1989. Production of antibodies in transgenic plants. Nature **342**: 76–77.
10. DÜRING, K., S. HIPPE, F. KREUZALER & J. SCHELL. 1990. Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. Plant Mol. Biol. **15**: 281–293.
11. STEINER, U. 2005. State of the biologic market. Oral presentation held at the Conference of plant-made pharmaceuticals, Montréal (<http://archives.cpmp2005.org>).
12. FISCHER, R. 2005. Qualitative and quantitative comparison of protein expression systems. Oral presentation held at the Conference of plant-made pharmaceuticals, Montréal (<http://archives.cpmp2005.org>).
13. MACAULEY-PATRICK, S., M.L. FAZENDA, B. MCNEIL, & L.M. HARVEY. 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast **22**: 249–270.
14. CHEN, C.-C., P.-H. WU, C.-T. HUANG & K.-J. CHENG. 2004. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of a *Escherichia coli* phytase. Enz. Microb. Technol. **35**: 315–320.
15. DANIELL, H., S.J. STREATFIELD & K. WYCOFF. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines on plants. Trends Plant Sci. **6**: 219–226.
16. DEPALMA, A. 2002. Capacity mismatch for production of monoclonals. Gen. Eng. News **22**: 62–66.
17. MA, J.K.-C., P.M.W. DRAKE & P. CHRISTOU. 2003. The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet. **4**: 794–805.
18. GIDDINGS, G., G. ALLISON, D. BROOKS & A. CARTER. 2000. Transgenic plants as factories for biopharmaceuticals. Nat. Biotech. **18**: 1151–1155.
19. HELLWIG, S., J. DROSSARD, R.M. TWYMAN & R.FISCHER. 2004. Plant cell cultures for the production of recombinant proteins. Nat. Biotech. **22**: 1415–1422.
20. DORAN, P.M. 2000. Foreign protein production in plant tissue culture. Curr. Opin. Biotech. **11**: 199–204.
21. STÖGER, E., M. SACK, R. FISCHER & P. CHRISTOU. 2002a. Plantibodies: application, advantages and bottlenecks. Curr. Opin. Biotechnol. **13**: 161–166.
22. MOR, T.S., M. STERNFELD, H. SOREQ, *et al.* 2001. Expression of recombinant human acetylcholinesterase in transgenic tomato plants. Biotechnol. Bioeng. **75**: 259–266.
23. SAWAHEL, W.A. 2002. The production of transgenic potato plants expressing human alpha-interferon using lipofectin-mediated transformation. Cell Mol. Biol. Lett. **7**: 19–29.
24. TREXLER, M.M., K.A. McDONALD & A.P. JACKMAN. 2005. A cyclical semicontinuous process for production of human alpha 1-antitrypsin using metabolically induced plant cell suspension cultures. Biotechnol. Prog. **21**: 321–328.
25. MA, J.K.-C, B.Y. HIKMAT, K. WYCOFF, *et al.* 1998. Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. Nat. Med. **4**: 601–606.

26. FISCHER, R., S. SCHILLBERG & N. EMANS. 2001. Molecular farming: a field of growing promise. *Outlook Agr.* **30**: 31–36.
27. EVANGELISTA, R.L., A.R. KUSNADI, J.A. HOWARD & Z. L. NIKOLOV. 1998. Process and economic evaluation of the extraction and purification of recombinant b-glucuronidase from transgenic corn. *Biotechnol. Prog.* **14**: 607–614.
28. FISCHER, R., C. VAQUERO-MARTIN, M. SACK, *et al.* 1999b. Towards molecular farming in the future: transient protein expression in plants. *Biotechnol. Appl. Biochem.* **30**: 113–114.
29. VAQUERO, C., M. SACK, J. CHANDLER, *et al.* 1999. Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc. Natl. Acad. Sci. USA* **96**: 11128–11133.
30. KAPILA, J., R. DE RYCKE, M. VAN MONTAGUM & G. ANGENON. 1997. An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci.* **122**: 101–108.
31. POGUE, G.P., J.A. LINDBO, W.O. DAWSON & T. H. TURPEN. 1998. Tobamovirus transient expression vectors: tools for plant biology and high-level expression of foreign proteins in plants. *In Plant Molecular Biology Manual*. S.B. Gelvin & R.A. Schilperoot, Eds.: L4, 1–27. Kluwer Academic Publishers. Dordrecht, the Netherlands.
32. MARILLONNET, S., C. THOERINGER, R. KANDZIA, *et al.* 2005. Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat. Biotech.* **23**: 718–723.
33. FRANKLIN, S.E. & S.P. MAYFIELD. 2005. Recent developments in the production of human therapeutic proteins in eucaryotic algae. *Expert Opin. Biol. Th.* **5**: 225–235.
34. KIM, D.H., Y.T. KIM, J.J. CHO, *et al.* 2002. Stable integration and functional expression of flounder growth hormone gene in transformed microalga, *Chlorella ellipsoidea*. *Mar. Biotechnol.* **4**: 63–73.
35. FUHRMANN, M., W. OERTEL & P. HEGEMANN. 1999. A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. *Plant J.* **19**: 353–361.
36. BOEHM, R., C. KRUSE, D. VOESTE, *et al.* 2001. A transient transformation system for duckweed (*Wolffia columbiana*) using *Agrobacterium*-mediated gene transfer. *J. Appl. Bot.* **75**: 107–111.
37. KRUSE, C., R. BOEHM, D. VOESTE, *et al.* 2001. Transient transformation of *Wolffia columbiana* by particle bombardment. *Aquatic Bot.* **72**: 175–181.
38. ISLAM, K.M.S. 2002. Feasibility of duckweed as poultry feed—a review. *Indian J. Anim. Sci.* **72**: 486–491.
39. APPENROTH, K.-J. & H. AUGSTEN. 1996. Wasserlinsen und ihre Nutzung. *Biologie in unserer Zeit* **26**: 187–194.
40. STÖGER, E., J.K.C. MA, R. FISCHER & P. CHRISTOU. 2005. Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotech.* **16**: 167–173.
41. CRAMER, C.L. & D.L. WEISSENBORN. 1997. HMG2 promoter expression system and post-harvest production of gene products in plants and plant cell cultures. US. patent 5,670,349.
42. SCHILLBERG, S., S. ZIMMERMANN, A. VOSS & R. FISCHER. 1999. Apoplastic and cytosolic expression of full-size antibodies and antibody fragments in *Nicotianum tabacum*. *Transgenic Res.* **8**: 255–263.

43. FISCHER, R., D. SCHUMANN, S. ZIMMERMANN, *et al.* 1999c. Expression and characterization of bispecific single-chain Fv fragments produced in transgenic tobacco. *Eur. J. Biochem.* **262**: 810–816.
44. STÖGER, E., M. SACK, Y. PERRIN, *et al.* 2002b. Practical consideration for pharmaceutical antibody production in different crop systems. *Mol. Breeding* **9**: 149–158.
45. TORRES, E., C. VAQUERO, L. NICHOLSON, *et al.* 1999. Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Res.* **8**: 441–449.
46. DANIELL, H., S. CHEBOLU, S. KUMAR, *et al.* 2005. Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine* **23**: 1779–1783.
47. BOCK, R.. 2001. Transgenic plastids in basic research and plant biotechnology. *J. Mol. Biol.* **312**: 425–438.
48. KUMAR, S., A. DHINGRA & H. DANIELL. 2004. Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Mol. Biol.* **56**: 203–216.
49. DUFOURMANTEL, N., B. PELISSIER, F. GARCON, *et al.* 2004. Generation of fertile transplastomic soybean. *Plant Mol. Biol.* **55**: 479–489.
50. HOU, B.K., Y.H. ZHOU, L.H. WAN, *et al.* 2003. Chloroplast transformation in oilseed rape. *Transgenic Res.* **12**: 111–114.
51. RUF, S., M. HERMANN, I.J. BERGER, *et al.* 2001. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotech.* **19**: 870–875.
52. SIDOROV, V.A., D. KASTEN, S.Z. PANG, *et al.* 1999. Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant J.* **19**: 209–216.
53. LEELAVATHI, S. & V.S. REDDY. 2003. Chloroplast expression of His-tagged GUS-fusions: a general strategy to overproduce and purify foreign proteins using transplastomic plants as bioreactors. *Mol. Breeding* **11**: 49–58.
54. KANG, T.J., S.C. HAN, M.Y. KIM, *et al.* 2004. Expression of non-toxic mutant of *Escherichia coli* heat-labile enterotoxin in tobacco chloroplasts. *Protein Exp. Purif.* **38**: 123–128.
55. TREGONING, J., P. MALIGA, G. DOUGAN & P. J. NIXON. 2004. New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC. *Phytochem.* **65**: 989–994.
56. SUN, M., K.X. QIAN, N. SU, *et al.* 2003. Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. *Biotechnol. Lett.* **25**: 1087–1092.
57. STREATFIELD, S.J. & J.A. HOWARD. 2003. Plant-based vaccines. *Int. J. Parasitol.* **33**: 479–493.
58. WARZECHA, H. 2000. Essbare Impfstoffe. *DAZ* **31**: 45–50.
59. MASON, H.S., D.M. LAM & C.J. ARNTZEN. 1992. Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* **96**: 11128–11133.
60. WALMSLEY, A.M. & C.J. ARNTZEN. 2003. Plant cell factories and mucosal vaccines. *Curr. Opin. Biotech.* **14**: 145–150.
61. HUANG, Z., G. ELKIN, B.J. MALONEY, *et al.* 2005. Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. *Vaccine* **23**: 1851–1858.
62. IMANI, J., A. BERTING, S. NITSCHKE, *et al.* 2002. The integration of a major hepatitis B virus gene into cell-cycle synchronized carrot cell suspension cultures and its expression in regenerated carrot plants. *Plant Cell Tiss. Org. Cult.* **71**: 157–164.

63. WIGDOROVITZ, A., D.M.P. FILGUEIRA, N. ROBERTSON, *et al.* 1999. Protection of mice against challenge with foot and mouth disease virus (FMDV) by immunization with foliar extracts from plants infected with recombinant tobacco mosaic virus expressing the FMDV structural protein VP1. *Virology* **264**: 85–91.
64. KAPUSTA, J., A. MODELSKA, M. FIGLEROWICZ, *et al.* 1999. A plant-derived edible vaccine against hepatitis B virus. *FASEB J.* **13**: 2339–2340.
65. KONG, Q., L. RICHTER, Y.Y. FANG, *et al.* 2001. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc. Natl. Acad. Sci. USA* **98**: 11539–11544.
66. FINEMAN, E. 2005. Host presentation of Planet Biotechnology. Oral presentation held at the Conference of plant-made pharmaceuticals, Montréal (<http://archives.cmpmp2005.org>).
67. FISCHER, R., N. EMANS, F. SCHUSTER, *et al.* 1999a. Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. *Biotechnol. Appl. Biochem.* **30**: 109–112.
68. SHARP, J.M. & P.M. DORAN. 2000. Characterization of monoclonal antibody fragments produced by plant cells. *Biotechnol. Bioeng.* **73**: 338–346.
69. KWON, T.H., J.E. SEO, J. KIM, *et al.* 2003a. Expression and secretion of the heterodimeric protein Interleukin-12 in plant cell suspension culture. *Biotechnol. Bioeng.* **81**: 870–875.
70. KWON, T.-H., Y.-S. KIM, J.-H. LEE & M.-S. YANG. 2003b. Production and secretion of biologically active human granulocyte-macrophage colony stimulation factor in transgenic tomato suspension cultures. *Biotechnol. Lett.* **25**: 1571–1574.
71. FRANCISCO, J.A., S.L. GAWLAK, M. MILLER, *et al.* 1997. Expression and characterization of Bryodin 1 and a Bryodin 1-based single-chain immunotoxin from tobacco cell culture. *Bioconjugate Chem.* **8**: 708–713.
72. HUANG, J.M., T.D. SUTLIFF, L.Y. WU, *et al.* 2001. Expression and purification of functional human alpha-1-antitrypsin from cultured plant cells. *Biotechnol. Prog.* **17**: 126–133.
73. NAGATA, T., Y. NEMOTO & S. HASEZAWA. 1992. Tobacco BY-2 cell line as the “HeLa” cell in the cell biology of higher plants. *Int. Rev. Cytol.* **132**: 1–30.
74. DECKER, E.L. & R. RESKI. 2004. The moss bioreactor. *Curr. Opin. Plant Biol.* **7**: 166–170.