nature biotechnology

Plant cell cultures for the production of recombinant proteins

Stephan Hellwig[1], Jürgen Drossard[1], Richard M Twyman[2] & Rainer Fischer[1,3]

The use of whole plants for the synthesis of recombinant proteins has received a great deal of attention recently because of advantages in economy, scalability and safety compared with traditional microbial and mammalian production systems. However, production systems that use whole plants lack several of the intrinsic benefits of cultured cells, including the precise control over growth conditions, batch-to-batch product consistency, a high level of containment and the ability to produce recombinant proteins in compliance with good manufacturing practice. Plant cell cultures combine the merits of whole-plant systems with those of microbial and animal cell cultures, and already have an established track record for the production of valuable therapeutic secondary metabolites. Although no recombinant proteins have yet been produced commercially using plant cell cultures, there have been many proof-of-principle studies and several companies are investigating the commercial feasibility of such production systems.

Techniques for the propagation of plant cells were developed in the 1950s when it was realized that plant cell cultures had the potential to synthesize a variety of useful, low molecular weight molecules[1]. Although the use of plant cells to produce such molecules has been studied extensively, only two secondary metabolites—shikonin and paclitaxel (Taxol)—have thus far been produced on a commercial scale. Part of the reason for this is the difficulty in achieving even moderate yields of most target compounds, a bottleneck that has stimulated a significant interest in the metabolic engineering of a wide variety of medicinal and nonmedicinal plants[2].

In contrast, the application of plant cell culture to recombinant protein production has focused only on a small number of well-characterized plant cell lines, the most popular of which are derived from the tobacco cultivars Bright Yellow 2 (BY-2) and *Nicotiana tabacum* 1 (NT-1). The first recombinant protein produced in plant cells was reported nearly 15 years ago[3]. Since this initial demonstration, over 20 different recombinant proteins have been produced in plant cell cultures, including antibodies, enzymes, hormones, growth factors and cytokines (**Table 1**).

In this review, we consider how plant cell cultures are now being developed as production systems for recombinant proteins, and highlight recent achievements that identify the unique benefits of this safe, flexible and efficient production platform.

Why plant cell culture systems?

Although there has been considerable interest in the development of whole plants for the production of recombinant proteins, the advantages of agricultural-scale production (low capital equipment costs and scalability) can be outweighed by the long development times, variations in product yield and quality, and the difficulty in applying good manufacturing practice (GMP) to the early stages of production[4–6]. In whole plants, the possibility of contamination with agrochemicals and fertilizers must be considered, as well as the impact of pests and diseases, and the variable cultivation conditions due to local differences in soil quality and microclimate.

Plant cell culture as an expression system for recombinant proteins avoids these problems while retaining the advantages. Like microbes, plant cells are inexpensive to grow and maintain, but because they are higher eukaryotes they can carry out many of the post-translational modifications that occur in human cells. Plant cells are also intrinsically safe, because they neither harbor human pathogens nor produce endotoxins.

Plant cells, like microbes, can be maintained in simple, synthetic media, but like animal cells they can synthesize complex multimeric proteins and glycoproteins, such as immunoglobulins[7,8] and interleukins[9]. Recombinant human glycoproteins synthesized in plants show much greater similarity to their native counterparts in terms of *N*-glycan structure compared to the same proteins produced in yeast, bacteria and filamentous fungi[10]. Unlike field-grown plants, the performance of cultured plant cells is independent of the climate, soil quality, season, day length and weather. There is no risk of contamination with mycotoxins, herbicides or pesticides[11] and there are fewer by-products (e.g., fibers, oils, waxes, phenolics and adventitious agents). Perhaps the most important advantage of plant cells over whole plants is the much simpler procedure for product isolation and purification[11–13] especially when the product is secreted into the culture medium. This means that GMP can be implemented throughout the production pipeline.

Principles of plant cell culture

Several approaches can be used for the *in vitro* cultivation of plant cells, including the derivation of hairy roots[14], shooty teratomas[15],

[1]Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Worringerweg 1, D-52074 Aachen, Germany. [2]Department of Biology, University of York, Heslington, York YO10 5DD, United Kingdom. [3]Institut für Biologie VII (Molekulare Biotechnologie), Aachen Technical University, D-52056 Aachen, Germany. Correspondence should be addressed to R.F. (fischer@molbiotech.rwth-aachen.de).

Published online 4 November 2004; doi:10.1038/nbt1027

immobilized cells[16] and suspension cell cultures[17]. Even so, researchers have focused their attention on suspension cells because these are the most amenable to GMP procedures and they can be cultivated relatively easily in large-scale bioreactors[18,19]. Suspension cell cultures have been prepared from several different plant species, including *Arabidopsis thaliana*[20], *Taxus cuspidata*[21], *Catharanthus roseus*[22] and important domestic crops such as tobacco, alfalfa, rice, tomato and soybean[23–27]. The popularity of these different systems for recombinant protein manufacture is discussed below.

Plant suspension cells are prepared by the agitation of friable callus tissue in shaker flasks or fermenters to form single cells and small aggregates. Callus is undifferentiated tissue obtained by cultivating explants on solid medium containing the appropriate mixture of plant hormones to maintain the undifferentiated state. The cells are grown in liquid culture medium containing the same hormones to promote rapid growth and prevent differentiation[28–30].

If transgenic plants expressing the recombinant protein of interest are used as the source of callus tissue, further genetic manipulation is

Table 1 Proteins of medical relevance produced in plant cell culture

Expressed protein	Expression host	Promoter	Localization, yield	Reference
Human serum albumin	*N. tabacum* suspension culture initiated from transgenic plants	Modified CaMV 35S	Secretion/apoplast targeting, 0.25 μg mg^{-1} protein in supernatant	3
scFv antibody fragment	*N. tabacum* suspension culture initiated from transgenic plants	CaMV 35S	Secretion, up to 0.5 μg l^{-1} up to 0.5% of TSP	79
Human erythropoietin	*N. tabacum* cv BY-2 suspension culture	CaMV 35S	Secreted, 1 pg g^{-1} FW	43, 80
Mouse monoclonal heavy-chain γ	*N. tabacum* cv NT-1 suspension culture	CaMV 35 S	Native heavy-chain secretion signal, ca. 10 μg l^{-1} extracellular without, 350 μg l^{-1} with PVP	61
Mouse $IgG_{2b/κ}$	*N. tabacum* cv Petite Havana SR-1	Enhanced CaMV 35S	15 μg g^{-1} FW, ~0.3% of TSP	8
Heavy chain mAb	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted up to 10 μg l^{-1}, with stabilization up to 350 μg l^{-1}	52
Bryodin 1	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted up to 30 mg l^{-1}	57
Human interleukin-2 and interleukin-4	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted (native signal peptides), 8–180 μg l^{-1} of culture broth	81
Recombinant ricin	*N. tabacum* suspension culture	CaMV 35S	25–37.5 μg l^{-1}	82
scFv antibody fragment	*Oryza sativa* cv Bengal (rice) callus culture	Maize ubiquitin	Apoplast targeting (optimized Ig leader peptides) and ER-retention, up to 3.8 μg g^{-1} callus FW	37
Full size IgG-2b/κ	*N. tabacum* cv Petite Havana SR-1	Enhanced CaMV 35S	0.3% of TSP or 15 μg/g wet weight	8
Human $α_1$-antitrypsin	*O. sativa* cv Taipei 309 suspension culture	*RAmy3D*	Secreted, 85 mg l^{-1} in shake flask, 25 mg l^{-1} in bioreactor	36
biscFv antibody fragment	*N. tabacum* cv BY-2 suspension culture	Enhanced CaMV 35S	Cytosolic (at detection limit), apoplast-targeted (up to 0.0064% of TSP), ER-retained (up to 0.064% of TSP)	54
Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	*N. tabacum* cv NT-1 suspension culture	CaMV 35S	Secreted/targeted to the apoplast ~250 μg l^{-1} extracellular, ~150 μg l^{-1} intracellular (based on culture volume)	62
scFv antibody fragment	*N. tabacum* suspension culture generated from transgenic plants	CaMV 35S	Apoplast targeting (sporamin secretion signal) 1 mg l^{-1} extracellular, 5 mg l^{-1} intracellular	83
Human $α_1$-antitrypsin	*O. sativa* suspension culture	*RAmy3D*	Up to 200 mg l^{-1} (calli suspended to 40% (vol/vol)) cell density in induction medium	39
Hepatitis B surface antigen (HBsAg)	*Glycine max* cv Williams 82 (soybean) and *N. tabacum* NT-1 suspension cultures	$(ocs)_3mas$	Intracellular up to 22 mg l^{-1} in soybean ~2 mg l^{-1} in tobacco	40
hGM-CSF	*N. tabacum*	CaMV 35S	1.6 to 6.6 μg ml^{-1} upon homogenizing the entire culture broth	84
Human lysozyme	*O. sativa* cv Taipei 309 suspension culture	*RAmy3D*	Intracellular (although *RAmy3D* signal peptide was used), up to 3%–4% of TSP	38
IL-12	*N. tabacum* cv Havana suspension culture	Enhanced CaMV 35 S	Secreted, up to 800 μg l^{-1} of supernatant	9
hGM-CSF	*Lycopersicum esculentum* cv Seokwang (tomato) suspension culture	Enhanced CaMV 35S	Secreted, up to 45 μg l^{-1} of supernatant	26
HBsAg	*N. tabacum* NT-1 suspension culture	*A. thaliana* ubq3	Secreted, up to 10 μg l^{-1} of particulate HBsAg	41
mAb against HBsAg	*N. tabacum* cv BY-2 suspension culture	CaMV 35S	Secreted, ~50/50 between supernatant and cells, total max ~15 mg l^{-1}	58

TSP, total soluble protein; ER, endoplasmic reticulum; PVP, polyvinylpyrrolidone; FW fresh weight.

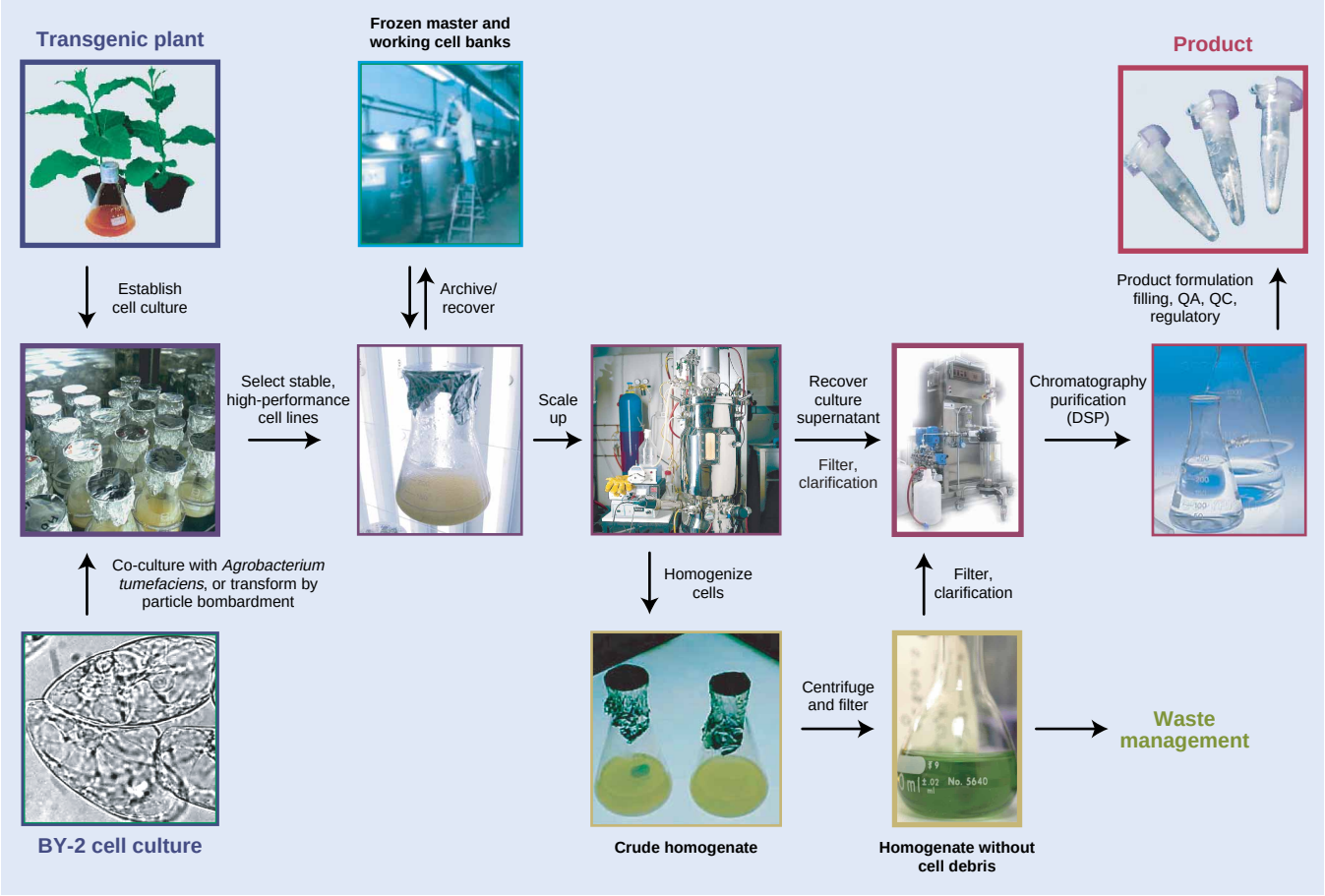

Figure 1 Overview of biopharmaceutical production in plant cells. For secreted proteins, the product is recovered directly from the culture supernatant, whereas for intracellular proteins, homogenization and filtration to remove coarse debris is necessary. DSP, downstream processing; QA, quality assurance; QC, quality control.

unnecessary (that is, the callus and/or suspension does not have to be selected for transformed cells). Alternatively, wild-type cell suspensions can be transformed with recombinant plasmids either by cocultivation with *Agrobacterium tumefaciens* or particle bombardment (**Fig. 1**)

The principles applied to the culture of microbial cells apply also to plant cells, although cell densities and growth rates are lower[31]. The growth rates of microbial cultures range from 0.1 h^{-1} to 1 h^{-1}, whereas the growth rate of *C. roseus* suspension cells ranges from 0.019 h^{-1} to 0.028 h^{-1}, with wild-type cells usually showing faster growth than transformed cells[32,33]. The popular tobacco cell line BY-2 has a particularly high growth rate for plant cells (up to 0.044 h^{-1}) and shows growth synchronicity and very low levels of nicotine compared with whole tobacco plants[27].

Oxygen uptake rates (and thus the oxygen transfer rates the bioreactor has to deliver) are also relatively low in plant cells. For example, Taticek *et al.*[31] reported an oxygen uptake rate (OUR) of 1–3.5 mmol l^{-1} h^{-1} in plant cell cultures, compared with ~5–90 mmol l^{-1} h^{-1} in bacterial cultures. Despite these differences, conventional fermenter equipment can be modified easily to work with plant cells, and many of the fermentation strategies applied to microbial cultures can also be applied to plants[34,35].

The cryocultivation of plant cell cultures is still in its infancy and is therefore not widely used except in commercial processes such as the production of paclitaxel. However, since this procedure is vital for reproducible processes, it will have to be developed before recombinant proteins can be produced on a commercial basis using cultured plant cells. In our laboratory, a protocol has been established for the cryocultivation of transformed BY-2 cells.

Recombinant protein production

Tobacco suspension cells, particularly those from the closely related cultivars BY-2 and NT-1, are frequently chosen as host cell lines because transformation and propagation are simple and well-established, and they have favorable growth characteristics. Although intact plants can be a useful source of suspension cells, freshly initiated plant suspension cultures may take a long time to acquire the favorable growth characteristics of BY-2 and NT-1. Other plant suspension cultures used for the production of recombinant proteins include rice[36–39], soybean[40] and tomato[26]. Such cell lines have been studied because of the possibility that they could be more favorable than tobacco in terms of by-product levels and, since they are derived from food crops, regulatory compliance. Other anticipated benefits of exploring diverse cell lines include faster growth, higher expression levels, more efficient secretion and other advantages concerning process compatibility. Some researchers focus on plants with a higher protein content, for example, soybean and lupin, assuming that these might more readily facilitate higher expression levels.

Table 2 Medium engineering approaches to improve the yield of recombinant proteins in plant cells

Medium additive	Expression host	Expressed protein	Effect	Reference
PVP	*N. tabacum* NT-1 suspension culture	Heavy-chain monoclonal antibody	Addition of 0.75% PVP 360.000 increased secreted product accumulation 35-fold	52, 61
BSA, NaCl	*N. tabacum* cv BY-2 suspension culture	Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Enhanced secreted product accumulation 100% (BSA), or 50% (NaCl)	62
BrefeldinA	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG_1	Inhibited the secretory pathway and thereby prevented degradation of secreted protein increased mAb accumulation 2.7-fold	15
Reducing manganese	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG_1	In manganese-reduced medium the stability and accumulation of secreted IgG_1 was increased –1.7-fold	44
Pluronic antifoam, PEG	*N. tabacum* cv Havana SR1 suspension culture	hGM-CSF	Pluronic antifoam addition increased the growth rate almost twofold, PEG-8000 increased hGM CSF accumulation fourfold	84
Gelatin, PVP, PEG	*N. tabacum* cv Havana SR1 suspension culture	hGM-CSF	2% gelatin increased accumulation 4.6-fold, PVP and PEG showed no effect	53
Gelatin, PVP, PEG	*N. tabacum* cv Havana	IL-12 heterodimer	2% gelatin increased IL-2 accumulation sevenfold	9

Media additives may act in different ways, for example, as enhancers of protein synthesis, enhancers or inhibitors of secretion, inhibitors of intracellular protein degradation, or as extracellular stabilizing agents. The effect of such additives must be determined empirically for each culture system and recombinant protein, and it should be noted that the addition of these compounds may, in some cases, interfere with downstream processing. PEG, polyethylene glycol; BSA, bovine serum albumin.

The design of the construct used to express the recombinant protein is a key factor in determining yield. Promoter choice affects the yield by determining the rate of transcription. The most commonly used promoter is the cauliflower mosaic virus (CaMV) 35S promoter or its enhanced version (**Table 1**) but a number of alternative constitutive promoters can be used, including the hybrid $(ocs)_3mas$ promoter (constructed from octopine synthase (*ocs*) and mannopine synthase (*mas*) promoter sequences) and the ubiquitin promoters from maize and *A. thaliana*[33,37,40,41]. In contrast to these constitutive promoters, the rice α-amylase *RAmy3D* promoter is induced by sugar deprivation[36].

Another important aspect of construct design is the presence or absence of a leader peptide, which directs the recombinant protein to the secretory pathway. Leader peptides from plant and nonplant proteins appear to function equivalently, and many human secreted proteins have been expressed using their endogenous leaders (**Table 1**). Proteins directed to the secretory pathway in cultured cells will eventually reach the apoplast, from where they will diffuse through the cell wall and into the culture medium unless they become trapped in the cell wall matrix. This depends on the size of the protein as well as other physicochemical properties[42–44]. Proteins of less than 30 kDa tend to be secreted into the medium whereas larger proteins are quantitatively retained, but large proteins including full-sized antibodies can be secreted efficiently whereas some small proteins remain trapped, suggesting that charge and/or hydrophobicity may also be important determinants.

Specific challenges

As well as the slow growth rates of plant cells compared with microbial cells, other challenges associated with suspension cells include the formation of aggregates, the tendency for cells to adhere to the walls of the fermenter vessel, gene drift (somaclonal variation), gene silencing and in some cases shear sensitivity[45–47]. Many of these issues have been addressed through improved fermenter design and agitation or aeration conditions[48,49], optimization of the nutrient supply[50,51] or by the careful selection of callus cell lines with respect to product formation, growth characteristics and genetic stability[38].

Another challenge is the relatively low protein yields from plant cells compared with plant storage organs. This partly reflects the undifferentiated state of the suspension cells and the lack of promoters that are very active under such conditions. Yields are also affected by protein stability, as there is significant degradation of proteins secreted into the culture medium because of proteolysis, aggregation and further unknown factors[26,52,53]. For example, up to 80% of a purified IgG_1 added to sterile, conditioned Murashige & Skoog and Gamborg's B5 media was degraded within 2 h[44].

Improving productivity

The productivity of plant cell cultures can vary considerably, with recombinant protein levels ranging from 0.0064% to 4% of total soluble protein (TSP) or from 0.5 $\mu g\ l^{-1}$ to 200 $mg\ l^{-1}$ based on the culture volume. However, different experiments are very difficult to compare because extraction procedures and the methods used to determine protein concentration are also variable and have not been standardized. In most cases, the expression and recovery levels obtained with plant cell cultures are approximately one or two orders of magnitude below the threshold where processes become economically feasible. One important factor in this respect is whether the product is a secreted protein that can be purified readily from the fermentation supernatant.

Separation of plant cells from the fermentation supernatant is simple compared with the same process in microbial fermentations and can often be accomplished by straightforward filtration steps. Moreover, plant cell culture media usually contain very few proteins compared with the supernatants of microbial cultures, which further facilitates recovery. Unfortunately, it has been shown that proteins such as antibodies can be very unstable in plant cell culture media[15,44], which is probably why proteins targeted to the endoplasmic reticulum using a retention signal accumulate to levels (based on cultivation

Table 3 Process engineering approaches to improve the yield of recombinant proteins in plant cells

Bioreactor type, scale and fermentation mode	Expression host	Expressed protein	Effect	Reference
Semicontinuous production of CAT in repeated batch and continuous fermentation at 0.25 d^{-1} in a bubble column	*N. tabacum* suspension culture	Chloramphenicol-acetyltransferase (CAT)	Constant product concentration in harvest	85
Shake-flask, semicontinuous perfusion/ continuous mode, replacing 33% of the medium every 12 h, equivalent to 0.66 d^{-1}	*N. tabacum* NT-1 suspension culture	Heavy-chain monoclonal antibody	+/– Constant levels of secreted heavy chain mAb in harvest	52
Stirred tank bioreactor, 10-liter continuous, 0.2 d^{-1}	*N. tabacum* cv BY-2 suspension culture	Carrot acidic invertase	Increased overall productivity fourfold	33
Encapsulation of suspension cultured cells in alginate, shake-flask	*N. tabacum* NT-1 suspension culture	Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)	Increased levels of secreted hGM-CSF 107 to 178 μg l^{-1}	86
Shake flask, periodic harvesting using hydroxyapatite-resin	*N. tabacum* suspension culture initiated from transgenic plants	Mouse IgG_1 monoclonal	Periodic harvesting increased overall yield	15

volume) 10- to 100-fold higher than identical proteins targeted for secretion[37,54–56]. However, the productivities achieved by some groups are very encouraging. The use of the inducible *RAmy3D* promoter in combination with rice cell lines[36,38,39] has provided impressive yields, even though the growth rates and characteristics of rice cell lines are inferior to those of BY-2 and NT-1 cell lines. Recombinant protein concentrations >10 mg l^{-1} based on culture supernatant volume constitute promising starting points for process development[36,39,57,58].

The recovery of recombinant proteins from plant cell cultures can be enhanced by modifying the culture conditions or adding various agents to the growth medium (**Table 2**). Substances that have been tested include simple inorganic compounds[59], amino acids to provide precursors for protein synthesis[8], stabilizing agents such as dimethylsulfoxide[60], polyethylene glycol[9,53], polyvinylpyrrolidone[9,15,52,53,59,61], gelatin[9,53,59] and bovine serum albumin[62] (these are thought to work by protecting the protein from degradation and preventing precipitation or adsorption to the vessel walls), gibberellic acid and haemin (for increasing the rate of protein synthesis)[44] and Brefeldin A, which inhibits secretion[15].

Product yields can be improved not only by medium optimization but also through process development (**Table 3**). All major fermentation strategies (batch, fed-batch, repeated batch or draw/fill, continuous culture and perfusion culture) can be applied to plant cell culture[35,63,64]. Although the growth of plant cells in batch culture in many cases does not follow the simple growth kinetics of microbes[12,27], plant cells are suitable for continuous fermentation as described for secondary metabolite production[35,64–66]. Problems associated with the continuous culture of plant cells include genetic instability and non-growth-coupled production[35], although the latter can be circumvented using two-stage bioreactors, as described, for example, by Sahai and Shuler[67]. Even so, it remains to be seen whether simple batch processes or long-term draw-fill, semicontinuous or continuous perfusion processes will emerge as the most suitable fermentation strategies for the production of proteins using plant cell cultures.

Downstream processing and regulatory considerations

Downstream processing is an integral part of every biomanufacturing process and is concerned with the isolation and purification of the product from the raw biomass. Regardless of the production system, downstream processing represents up to 80% of overall production costs, although this depends on the required level of purity, which is highest for clinical-grade materials. In many cases, it is necessary to develop specific processing steps for each product, although certain classes of product can be isolated using a standardized approach (e.g., the use of affinity chromatography to isolate recombinant antibodies or recombinant proteins expressed with integral epitope tags[68]). Several aspects of downstream processing have to be customized specifically for whole-plant systems, including adding steps for the removal of fibers, oils and other by-products, and process optimization for the treatment of different plant species and tissues.

Such issues do not apply so much to plant cell cultures, where the main choice is between the extraction of proteins from the wet biomass and purification of secreted proteins directly from the culture medium (**Fig. 1**). Secreted proteins are advantageous because they circumvent several unit operations during the purification process (that is, cell disruption and removal of debris) and provide a starting material, the culture supernatant, which has a much lower content of contaminating proteins and other metabolites than whole cell extracts. The downside of this approach is that the recombinant protein is highly diluted, so large volumes of liquid must be processed, and that the proteins can undergo significant degradation for the reasons discussed above.

If plant cell cultures are used to produce clinical-grade proteins, then downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regime of quality assurance and quality control to achieve approval of regulatory agencies[69]. Regulatory guidance for biopharmaceutical production in plants currently exists only as draft legislation, published in 2002 by both the Food and Drug Administration[70] and the European Agency for the Evaluation of Medicinal Products[71]. Although not explicitly excluding plant cell cultures as production systems, both documents focus on intact plants as production hosts, making the situation regarding cultured cells less than clear. In addition to the applicable parts of these documents, other nonplant-specific GMP guidelines for biotechnological production (e.g., Annex 18 of the EU Guide to Good Manufacturing Practice) will probably be relevant for pharmaceutical production in plant cell cultures. One of the most important requirements is a thorough description of the genetic background and genetic stability of the host cell as well as the precise documentation of all events associated with the introduction of the transgene into the plant cell. Many of the plant cell lines currently used for recombinant protein expression have a long history in

the public domain (including the BY-2 and NT-1 cell lines) and have not been characterized or banked sufficiently to fulfill GMP requirements. Cell banking is a prerequisite for the reliable supply of well-defined starting material, and a routine procedure for the cryopreservation of plant cells will have to be developed and validated.

The initial stages of processing display the greatest variability and have to be optimized in a system-specific manner. The secreted protein of interest, even though highly dilute, should form the major proteinaceous component of the harvest broth, and an important task is to find the appropriate harvesting window, taking into consideration the dynamics of protein expression and stability with respect to the production cycle. For example, in batch or fed-batch processes, peak production of the recombinant protein often occurs in the exponential phase, and begins to decline at the point where the cell mass and total protein content of the culture reach their maximum levels[15]. Once the appropriate window has been determined, clarification, concentration and product capture are the next processing steps. Large-scale clarification is generally carried out by dead-end or cross-flow filtration (or a combination thereof), sometimes preceded by bulk cell mass removal using a decanter, disk-stack separator or a semicontinuous or continuous centrifuge. Of these methods, cross-flow filtration provides the best clarified feed for packed-bed chromatography, but it is also the most demanding technique for method development and optimization. Experiments in our laboratory (unpublished data) have shown that considerable amounts of extracellular polysaccharides are produced by some transformed BY-2 cell lines and lead to the rapid formation of a gel layer on membrane surfaces, drastically reducing permeate flow and trapping the protein of interest within the layer. Hollow-fiber systems may therefore be a more suitable choice for these initial microfiltration steps.

Alternatively, advanced chromatographic technologies such as expanded bed adsorption (EBA) may help to capture the target protein from particulate feed material. This technique has been used to capture recombinant proteins from several industrial-scale microbial and animal cell cultures, and a recent publication describes its use with plant cells although there is need for further improvements in column hardware and adsorbent material design[72]. If these hurdles can be overcome, EBA may be an efficient method for simultaneous clarification, concentration and initial purification of proteins from plant cell fermentation broth or cell extracts[73]. Another alternative is the use of aqueous two-phase systems for cultivation and, eventually, *in-situ* extraction from plant cell cultures[74].

The initial purification of intracellular recombinant proteins expressed in plant cells requires downstream procedures similar to those used in whole transgenic plants. In both cases, disruption of cell walls and membranes is the first post-harvesting step, but the range of techniques available for plant suspension cells is wider that that used for whole plant tissue because there are no constraints reflecting different tissue types (leaves, seeds, fruits). Therefore, wet milling, sonication, pressure homogenization and enzymatic treatment have all been used to process plant cells, and the method used depends primarily on equipment availability (although it should be noted that chemical treatment adds another component to the feed material that has to be removed in subsequent steps). Wet milling and sonication are also the most difficult to scale up.

After cell disruption, clarification of the extract is carried out as described above, although cell debris and fines generated during homogenization may be harder to remove than intact cells. The advantages of intracellular expression for initial downstream processing lie in the smaller volume of the starting material and the generally higher concentration of the target protein, whereas the major disadvantage is the more complex composition of the feedstream and the liberation of proteolytic and oxidizing substances.

Several liquid chromatography steps are included in a full purification protocol, and again the initial chromatographic steps require the most development of the specific production system. In industrial processing, robust and inexpensive chromatography media are used in the initial steps, accepting that there will be some loss of selectivity and resolution[75]. However, important exceptions include the use of Protein A or Protein G affinity chromatography for antibody purification, and the use of affinity tags and their respective capture agents (e.g., His_6 and Ni-NTA resin), which are highly selective initial capturing methods.

Conclusions

Although the advantages of plant cell culture are widely recognized and appreciated, improvements are required in several areas before the platform becomes acceptable and commercially feasible. Most importantly, promoters need to be identified that provide yields comparable to other expression platforms, that is, 10%–20% of total mRNA. Further work must address the reasons for protein instability and identify strategies to enhance protein recovery.

There is also much interest in addressing the differences in post-translational modification between mammalian and plant cells. Whereas the glycosylation steps occurring in the endoplasmic reticulum are conserved, they diverge in the late Golgi apparatus so that core α(1,6)-linked fucose and terminal sialic acid residues are added in mammals, whereas bisecting β(1,2)-xylose and core α(1,3)-fucose residues are added in plants. Although these differences have not thus far been shown to affect the biological activity of recombinant proteins, some reports have identified pharmacokinetic differences between plant-derived recombinant proteins and their native counterparts, for example, the significantly reduced half-life of plant-derived α1-antitrypsin, which Huang, J. *et al.* speculated was due to the lack of sialic acid[39].

Nonmammalian glycans are furthermore regarded as potential immunogens or allergens particularly when the recombinant protein is intended for injection into human patients. Much emphasis has been placed on this issue by the regulatory authorities, which is understandable given conflicting reports on immunogenicity[76,77]. Efforts to avoid nonmammalian glycosylation patterns include the prevention of late glycosylation by directing proteins to the endoplasmic reticulum through addition of KDEL-tags (a strategy incompatible with protein secretion), the introduction of point mutations to eliminate glycosylation completely, or the humanization of the plant glycosylation machinery. As an example of the last strategy, Palacpac *et al.*[78] have expressed human β(1,4)galactosyltransferase in BY-2 cells, showing that plant glycan structures could be modified successfully.

The overall goal should be to produce in plant cells human proteins that are structurally and functionally equivalent to their native counterparts. If these challenges can be met, plant cells have the potential to compete with other expression platforms for the commercial production of recombinant proteins, as has already been achieved with small-molecule drugs.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturebiotechnology/

1. Gamborg, O.L. Plant tissue culture. Biotechnology milestones. *In Vitro Cell. Dev. Biol.* **38**, 84–92 (2002).
2. Verpoorte, R., van der Heijden, R. & Memelink, J. Engineering the plant cell factory for secondary metabolite production. *Transgenic Res.* **9**, 323–343; discussion 321 (2000).

3. Sijmons, P.C. *et al.* Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* **8**, 217–221 (1990).
4. Ma, J.K.-C., Drake, P.M.W. & Christou, P. The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* **4** (2003).
5. Fischer, R., Stoger, E., Schillberg, S., Christou, P. & Twyman, R.M. Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* **7**, 152–158 (2004).
6. Twyman, R.M., Stoger, E., Schillberg, S., Christou, P. & Fischer, R. Molecular farming in plants: host systems and expression technology. *Trends Biotechnol.* **21**, 570–578 (2003).
7. Drossard, J., Nähring, J.M., Hellwig, S. & Fischer, R. Production of engineered antibodies in tobacco plants and cell suspension cultures. in *Antibody Engineering, New Technology, Application and Commercialization*, vol. 2, IBC's 8th annual international conference on antibody engineering, 69–93 (IBC Library Services, Southborough, Massachusetts, 1997).
8. Fischer, R., Liao, Y.C. & Drossard, J. Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. *J. Immunol. Methods* **226**, 1–10 (1999).
9. Kwon, T.H. *et al.* Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture. *Biotechnol. Bioeng.* **81**, 870–875 (2003).
10. Gomord, V. & Faye, L. Posttranslational modification of therapeutic proteins in plants. *Curr. Opin. Plant Biol.* **7**, 171–181 (2004).
11. Doran, P.M. Foreign protein production in plant tissue cultures. *Curr. Opin. Biotechnol.* **11**, 199–204 (2000).
12. Fischer, R., Liao, Y.C., Hoffmann, K., Schillberg, S. & Emans, N. Molecular farming of recombinant antibodies in plants. *Biol. Chem.* **380**, 825–839 (1999).
13. Fischer, R., Emans, N., Schuster, F., Hellwig, S. & Drossard, J. Towards molecular farming in the future: using plant-cell-suspension cultures as bioreactors. *Biotechnol. Appl. Biochem.* **30**, 109–112 (1999).
14. Hilton, M.G. & Rhodes, M.J.C. Growth and hyoscyamine production of hairy root cultures of *Datura stramonium* in a modified stirred tank reactor. *Appl. Microbiol. Biotechnol.* **33**, 132–138 (1990).
15. Sharp, J.M. & Doran, P.M. Strategies for enhancing monoclonal antibody accumulation in plant cell and organ cultures. *Biotechnol. Prog.* **17**, 979–992 (2001).
16. Archambault, J. Large-scale (20-L) culture of surface-immobilized *Catharanthus roseus* cells. *Enzyme Microb. Technol.* **13**, 882–892 (1991).
17. Kieran, P.M., MacLoughlin, P.F. & Malone, D.M. Plant cell suspension cultures: some engineering considerations. *J. Biotechnol.* **59**, 39–52 (1997).
18. Schlatmann, J.E., ten Hoopen, H.J. & Heijnen, J.J. Large-scale production of secondary metabolites by plant cell cultures. in *Plant Cell Culture Secondary Metabolism: Toward Industrial Application.* (eds. DiCosmo, F. & Misawa, M.) 11–52 (CRC Press, Boca Raton, Florida, 1996).
19. Wen, W.S. Bioprocessing technology for plant cell suspension cultures. *Appl. Biochem. Biotechnol.* **50**, 189–216 (1995).
20. Desikan, R., Hancock, J.T., Neill, S.J., Coffey, M.J. & Jones, O.T. Elicitor-induced generation of active oxygen in suspension cultures of *Arabidopsis thaliana*. *Biochem. Soc. Trans.* **24**, 199S (1996).
21. Seki, M., Ohzora, C., Takeda, M. & Furusaki, S. Taxol (Paclitaxel) production using free and immobilized cells of *Taxus cuspidata*. *Biotechnol. Bioeng.* **53**, 214–219 (1997).
22. Van der Heijden, R., Verpoorte, R. & ten Hoopen, H.J. Cell and tissue cultures of *Catharanthus roseus* (L) Don G. A literature survey. *Plant Cell Tissue Organ Cult.* **18**, 231–280 (1989).
23. Hoehl, U., Upmaier, B. & Barz, W. Growth and nicotinate biotransformation in batch cultured and airlift fermenter grown soybean cell suspension cultures. *Appl. Microbiol. Biotechnol.* **28**, 319–323 (1988).
24. Daniell, H., Khan, M.S. & Allison, L. Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.* **7**, 84–91 (2002).
25. Chen, M.-H., Liu, L.-F., Chen, Y.-R., Wu, H.-K. & Yu, S.-M. Expression of α-amylases, carbohydrate metabolism, and autophagy in cultured rice cells is co-ordinately regulated by sugar nutrient. *Plant J.* **6**, 625–636 (1994).
26. Kwon, T.H., Kim, Y.S., Lee, J.H. & Yang, M.S. Production and secretion of biologically active human granulocyte-macrophage colony stimulating factor in transgenic tomato suspension cultures. *Biotechnol. Lett.* **25**, 1571–1574 (2003).
27. Nagata, T., Nemoto, Y. & Seiichiro, H. Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Int. Rev. Cytol.* **132**, 1–30 (1992).
28. Murashige, T. & Skoog, F. A revised medium for rapid growth and bioassay for tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497 (1962).
29. Gamborg, O.L., Murashige, T., Thorpe, T.A. & Vasil, I.K. Plant tissue culture media. *In Vitro* **12**, 473–478 (1976).
30. White, P.R. *The Cultivation of Animal and Plant Cells*, edn. 2 (Ronald Press, New York, 1963).
31. Taticek, R.A., Lee, C.W.T. & Shuler, M.L. Large-scale insect and plant cell culture. *Curr. Opin. Biotechnol.* **5**, 165–174 (1994).
32. Drapeau, D., Blanch, H.W. & Wilke, C.R. Growth kinetics of *Dioscorea deltoidea* and *Catharanthus roseus* in batch culture. *Biotechnol. Bioeng.* **28**, 1555–1563 (1986).
33. Des Molles, D., Gomord, V., Bastin, M., Faye, L. & Courtois, D. Expression of a carrot invertase gene in tobacco suspension cells cultivated in batch and continuous culture conditions. *J. Biosci. Bioeng.* **87**, 302–306 (1999).
34. Hooker, B.S., Lee, J.M. & An, G. Cultivation of plant cells in a stirred tank reactor. *Biotechnol. Bioeng.* **35**, 296–304 (1990).
35. Ten Hoopen, H.J.G., van Gulik, W.M. & Heijnen, J.J. Continuous culture of suspended plant cells. *In Vitro Cell. Dev. Biol.* **28**, 115–120 (1992).
36. Terashima, M. *et al.* Production of functional human α1-antitrypsin by plant cell culture. *Appl. Microbiol. Biotechnol.* **52**, 516–523 (1999).
37. Torres, E. *et al.* Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Res.* **8**, 441–449 (1999).
38. Huang, J. *et al.* Expression of functional recombinant human lysozmye in transgenic rice cell culture. *Transgenic Res.* **11**, 229–239 (2002).
39. Huang, J. *et al.* Expression and purification of functional human α1-antitrypsin from cultured plant cells. *Biotechnol. Prog.* **17**, 126–133 (2001).
40. Smith, M.L., Mason, H.S. & Shuler, M.L. Hepatitis B surface antigen (HBsAg) expression in plant cell culture: kinetics of antigen accumulation in batch culture and its intracellular form. *Biotechnol. Bioeng.* **80**, 812–822 (2002).
41. Sunil Kumar, G.B., Ganapathi, T.R., Revathi, C.-J., Prasad, K.S.N. & Bapat, V.A. Expression of hepatitis B surface antigen in tobacco cell suspension cultures. *Protein Expr. Purif.* **32**, 10–17 (2003).
42. Carpita, N., Sabularse, D., Montezinos, D. & Delmer, D.P. Determination of the pore size of living plant cells. *Science* **205**, 1144–1147 (1979).
43. Matsumoto, S., Ikura, K., Ueda, M. & Sasaki, R. Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. *Plant Mol. Biol.* **27**, 1163–1172 (1995).
44. Tsoi, B.M. & Doran, P.M. Effect of medium properties and additives on antibody stability and accumulation in suspended plant cell cultures. *Biotechnol. Appl. Biochem.* **35**, 171–180 (2002).
45. Meijer, J.J., ten Hoopen, H.J.G., van Gameren, Y.M., Luyben, K.C.A.M. & Libbenga, K.R. Effects of hydrodynamic stress on the growth of plant cells in batch and continuous culture. *Enzyme Microb. Technol.* **16**, 467–477 (1994).
46. Yu, S.X., Kwok, K.H. & Doran, P.M. Effect of sucrose, exogenous product concentration, and other culture conditions on growth and steroidal alkaloid production by *Solanum aviculare* hairy roots. *Enzyme Microb. Technol.* **18**, 238–243 (1996).
47. Offringa, R. *et al.* Extrachromosomal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. *EMBO J.* **9**, 3077–3084 (1990).
48. Böhme, C., Schröder, M.B. Jung-Heiliger, H. & Lehmann, J. Plant cell suspension culture in a bench-scale fermenter with a newly designed membrane-stirrer for bubble-free aeration. *Appl. Microbiol. Biotechnol.* **48**, 149154 (1997).
49. Doran, P. Design of reactors for plant cells and organs. in *Bioprocess Design and Control, Vol. 48.* (ed. Fiechter, A.) 115–168 (Springer, New York, 1993).
50. Sato, K., Nakayama, M. & Shigeta, J. Culturing conditions affecting the production of anthocyanin in suspended cell cultures of strawberry. *Plant Sci.* **113**, 91–98 (1996).
51. Sakamoto, K. *et al.* Effects of nutrients on anthocyanin production in cultured cells of *Aralia cordata*. *Phytochemistry* **33**, 357–360 (1993).
52. LaCount, W., An, G. & Lee, J.M. The effect of polyvinylpyrrolidone (PVP) on the heavy chain monoclonal antibody production from plant suspension cultures. *Biotechnol. Lett.* **19**, 93–96 (1997).
53. Lee, J.H., Kim, N.S., Kwon, T.H., Jang, Y.S. & Yang, M.S. Increased production of human granulocyte-macrophage colony stimulating factor (hGM-CSF) by the addition of stabilizing polymer in plant suspension cultures. *J. Biotechnol.* **96**, 205–211 (2002).
54. Fischer, R. *et al.* Expression and characterization of bispecific single-chain Fv fragments produced in transgenic plants. *Eur. J. Biochem.* **262**, 810–816 (1999).
55. Schouten, A. *et al.* The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway. *Plant Mol. Biol.* **30**, 781–793 (1996).
56. Xu, H. *et al.* Combined use of regulatory elements within the cDNA to increase the production of a soluble mouse single-chain antibody, scFv, from tobacco cell suspension cultures. *Protein Expr. Purif.* **24**, 384–394 (2002).
57. Francisco, J.A. *et al.* Expression and characterization of bryodin 1 and a bryodin 1-based single-chain immunotoxin from tobacco cell culture. *Bioconjug. Chem.* **8**, 708–713 (1997).
58. Yano, A., Maeda, F. & Takekoshi, M. Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. *J. Med. Virol.* **73**, 208–215 (2004).
59. Wongsamuth, R. & Doran, P.M. Production of monoclonal antibodies from tobacco hairy roots. *Biotechnol. Bioeng.* **54**, 401–415 (1997).
60. Wahl, M.F., An, G.H. & Lee, J.M. Effects of dimethylsulfoxide on heavy-chain monoclonal antibody production from plant cell culture. *Biotechnol. Lett.* **17**, 463–468 (1995).
61. Magnuson, N.S. *et al.* Enhanced recovery of a secreted mammalian protein from suspension culture of genetically modified tobacco cells. *Protein Expr. Purif.* **7**, 220–228 (1996).
62. James, E.A. *et al.* Production and characterization of biologically active human GM-CSF secreted by genetically modified plant cells. *Protein Expr. Purif.* **19**, 131–138 (2000).
63. Chang, H.N. & Sim, S.J. Extractive plant cell culture. *Curr. Opin. Biotechnol.* **6** (1995).
64. Van Gulik, W.M., ten Hoopen, H.J. & Heijnen, J.J. The application of continuous culture for plant cell suspensions. *Enzyme Microb. Technol.* **28**, 796–805 (2001).
65. Tulecke, W. Continuous cultures of higher plant cells in liquid media: the advantages and potential use of a phytostat. *Ann. NY Acad. Sci.* **139**, 162–175 (1966).
66. Miller, R.A., Shyluk, J.P., Gamborg, O.L. & Kirkpatrick, J.W. Phytostat for continuous culture and automatic sampling of plant-cell suspensions. *Science* **159**, 540–542 (1968).
67. Sahai, O.P. & Shuler, M.L. Multistage continuous culture to examine secondary metabolite formation in plant cells: phenolics from *Nicotiana tabacum*. *Biotechnol. Bioeng.* **26**, 27–36 (1984).
68. Stoger, E., Sack, M., Nicholson, L., Fischer, R. & Christou, P. Recent progress in plantibody technology. *Current Pharmaceutical Design* (in the press).

69. Fahrner, R.L. *et al.* Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes. *Biotechnol. Genet. Eng. Rev.* **18**, 301–327 (2001).
70. FDA. Guidance for industry. Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. (Food and Drug Administration, Rockville, Maryland, 2002).
71. CPMP. Point to consider on quality aspects of medicinal products containing active substances produced by stable transgene expression in higher plants (CPMP/BWP/764/02). (The European Agency for the Evaluation of Medicinal Products, London, 2002).
72. Valdes, R. *et al.* Large-scale purification of an antibody directed against hepatitis B surface antigen from transgenic tobacco plants. *Biochem. Biophys. Res. Commun.* **308**, 94–100 (2003).
73. Bai, Y. & Glatz, C.E. Capture of a recombinant protein from unclarified canola extract using streamline expanded bed anion exchange. *Biotechnol. Bioeng.* **81**, 855–864 (2003).
74. Choi, J.W., Cho, G.H., Byun, S.Y. & Kim, D.I. Integrated bioprocessing for plant cell cultures. *Adv. Biochem. Eng. Biotechnol.* **72**, 63–102 (2001).
75. Bai, Y. & Glatz, C.E. Bioprocess considerations for expanded-bed chromatography of crude canola extract: sample preparation and adsorbent reuse. *Biotechnol. Bioeng.* **81**, 775–782 (2003).
76. Chargelegue, D., Vine, N.D., van Dolleweerd, C.J., Drake, P.M. & Ma, J.K. A murine monoclonal antibody produced in transgenic plants with plant-specific glycans is not immunogenic in mice. *Transgenic Res.* **9**, 187–194 (2000).
77. Cabanes-Macheteau, M. *et al. N*-glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology* **9**, 365–372 (1999).
78. Palacpac, N.Q. *et al.* Stable expression of human beta1,4-galactosyltransferase in plant cells modifies *N*-linked glycosylation patterns. *Proc. Natl. Acad. Sci. USA* **96**, 4692–4697 (1999).
79. Firek, S. *et al.* Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. *Plant Mol. Biol.* **23**, 861–870 (1993).
80. Matsumoto, S., Ishii, A., Ikura, K., Ueda, M. & Sasaki, R. Expression of human erythropoietin in cultured tobacco cells. *Biosci. Biotechnol. Biochem.* **57**, 1249–1252 (1993).
81. Magnuson, N.S. *et al.* Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture. *Protein Expr. Purif.* **13**, 45–52 (1998).
82. Sehnke, P.C. & Ferl, R.J. Processing of preproricin in transgenic tobacco. *Protein Expr. Purif.* **15**, 188–195 (1999).
83. Ramirez, N. *et al.* Single-chain antibody fragments specific to the hepatitis B surface antigen, produced in recombinant tobacco cell cultures. *Biotechnol. Lett.* **22**, 1233–1236 (2000).
84. Lee, S.Y. & Kim, D.I. Stimulation of murine granulocyte macrophage-colony stimulating factor production by pluronic F-68 and polyethylene glycol in transgenic *Nicotiana tabacum* cell culture. *Biotechnol. Lett.* **24**, 1779–1783 (2002).
85. Hogue, R.S., Lee, J.M. & An, G. Production of a foreign protein product with genetically modified plant cells. *Enzyme Microb. Technol.* **12**, 533–538 (1990).
86. Bodeutsch, T., James, E.A. & Lee, J.M. The effect of immobilization on recombinant protein production in plant cell culture. *Plant Cell Rep.* **20**, 562–566 (2001).