Plant cell cultures for the production of recombinant proteins

Stephan Hellwig\textsuperscript{1}, Jürgen Drossard\textsuperscript{1}, Richard M Twyman\textsuperscript{2} & Rainer Fischer\textsuperscript{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\textsuperscript{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\textsuperscript{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 \textsuperscript{1} (NT-1). The first recombinant protein produced in plant cells was reported nearly 15 years ago\textsuperscript{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\textsuperscript{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 multimolecular proteins and glycoproteins, such as immunoglobulins\textsuperscript{7,8} and interleukins\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{12–13} 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 \textit{in vitro} cultivation of plant cells, including the derivation of hairy roots\textsuperscript{14}, shooty teratomas\textsuperscript{15},...
immobilized cells and suspension cell cultures. Even so, researchers have focused their attention on suspension cells because these are the most amenable to GMP procedures and can be cultivated relatively easily in large-scale bioreactors. Suspension cell cultures have been prepared from several different plant species, including Arabidopsis thaliana, Taxus cuspidata, Catharanthus roseus and important domestic crops such as tobacco, alfalfa, rice, tomato and soybean. 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.

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

<table>
<thead>
<tr>
<th>Expressed protein</th>
<th>Expression host</th>
<th>Promoter</th>
<th>Localization, yield</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human serum albumin</td>
<td><em>N. tabacum</em> suspension culture initiated from transgenic plants</td>
<td>Modified CaMV 35S</td>
<td>Secretion/apoplast targeting, 0.25 µg mg⁻¹ protein in supernatant</td>
<td>3</td>
</tr>
<tr>
<td>scFv antibody fragment</td>
<td><em>N. tabacum</em> suspension culture initiated from transgenic plants</td>
<td>CaMV 35S</td>
<td>Secretion, up to 0.5 µg l⁻¹ up to 0.5% of TSP</td>
<td>79</td>
</tr>
<tr>
<td>Human erythropoietin</td>
<td><em>N. tabacum</em> cv BY-2 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreced, 1 µg g⁻¹ FW</td>
<td>43, 80</td>
</tr>
<tr>
<td>Mouse monoclonal heavy-chain γ</td>
<td><em>N. tabacum</em> cv NT-1 suspension culture</td>
<td>CaMV 35S</td>
<td>Native heavy-chain secretion signal, ca. 10 µg l⁻¹ extracellular without, 350 µg l⁻¹ with PVP</td>
<td>61</td>
</tr>
<tr>
<td>Mouse IgG2β</td>
<td><em>N. tabacum</em> cv Petite Havana SR-1</td>
<td>Enhanced CaMV 35S</td>
<td>15 µg l⁻¹ FW, −0.3% of TSP</td>
<td>8</td>
</tr>
<tr>
<td>Heavy chain mAb</td>
<td><em>N. tabacum</em> cv NT-1 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreted up to 10 µg l⁻¹, with stabilization up to 350 µg l⁻¹</td>
<td>52</td>
</tr>
<tr>
<td>Bryodin 1</td>
<td><em>N. tabacum</em> cv NT-1 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreced up to 30 mg l⁻¹</td>
<td>57</td>
</tr>
<tr>
<td>Human interleukin-2 and interleukin-4</td>
<td><em>N. tabacum</em> cv NT-1 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreted (native signal peptides), 8–180 µg l⁻¹ of culture broth</td>
<td>81</td>
</tr>
<tr>
<td>Recombinant ricin</td>
<td><em>N. tabacum</em> suspension culture</td>
<td>CaMV 35S</td>
<td>25–37.5 µg l⁻¹</td>
<td>82</td>
</tr>
<tr>
<td>scFv antibody fragment</td>
<td><em>Oryza sativa</em> cv Bengal (rice) callus culture</td>
<td>Maize ubiquitin</td>
<td>Apollast targeting (optimized lg leader peptides) and ER-retention, up to 3.8 µg g⁻¹ callus FW</td>
<td>37</td>
</tr>
<tr>
<td>Full size IgG-2bκ</td>
<td><em>N. tabacum</em> cv Petite Havana SR-1</td>
<td>Enhanced CaMV 35S</td>
<td>0.3% of TSP or 15 µg/g wet weight</td>
<td>8</td>
</tr>
<tr>
<td>Human α1-antitrypsin</td>
<td><em>O. sativa</em> cv Taipei 309 suspension culture</td>
<td>RAmy3D</td>
<td>Secreted, 85 mg l⁻¹ in shake flask, 25 mg l⁻¹ in bioreactor</td>
<td>36</td>
</tr>
<tr>
<td>biscFv antibody fragment</td>
<td><em>N. tabacum</em> cv BY-2 suspension culture</td>
<td>Enhanced CaMV 35S</td>
<td>Cytosolic (at detection limit), apollast-targeted (up to 0.0064% of TSP), ER-retained (up to 0.064% of TSP)</td>
<td>54</td>
</tr>
<tr>
<td>Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)</td>
<td><em>N. tabacum</em> cv NT-1 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreted/targeted to the apoplast −250 µg l⁻¹ extracellular, −150 µg l⁻¹ intracellular (based on culture volume)</td>
<td>62</td>
</tr>
<tr>
<td>scFv antibody fragment</td>
<td><em>N. tabacum</em> suspension culture generated from transgenic plants</td>
<td>CaMV 35S</td>
<td>Apollast targeting (sporamin secretion signal) 1 mg l⁻¹ extracellular, 5 mg l⁻¹ intracellular</td>
<td>83</td>
</tr>
<tr>
<td>Human α1-antitrypsin</td>
<td><em>O. sativa</em> suspension culture</td>
<td>RAmy3D</td>
<td>Up to 200 mg l⁻¹ (calli suspended to 40% (vol/vol)) cell density in induction medium</td>
<td>39</td>
</tr>
<tr>
<td>Hepatitis B surface antigen (HBsAg)</td>
<td><em>Glycine max</em> cv Williams 82 (soybean) and <em>N. tabacum</em> NT-1 suspension cultures</td>
<td>(ocs)3mas</td>
<td>Intracellular up to 22 mg l⁻¹ in soybean −2 mg l⁻¹ in tobacco</td>
<td>40</td>
</tr>
<tr>
<td>hGM-CSF</td>
<td><em>N. tabacum</em></td>
<td>CaMV 35S</td>
<td>1.6 to 6.6 µg ml⁻¹ upon homogenizing the entire culture broth</td>
<td>84</td>
</tr>
<tr>
<td>Human lysozyme</td>
<td><em>O. sativa</em> cv Taipei 309 suspension culture</td>
<td>RAmy3D</td>
<td>Intracellular (although RAmy3D signal peptide was used), up to 3%–4% of TSP</td>
<td>38</td>
</tr>
<tr>
<td>IL-12</td>
<td><em>N. tabacum</em> cv Havana suspension culture</td>
<td>Enhanced CaMV 35S</td>
<td>Secreted, up to 800 µg l⁻¹ of supernatant</td>
<td>9</td>
</tr>
<tr>
<td>hGM-CSF</td>
<td><em>Lycopersicum esculentum</em> cv Seokwang (tomato) suspension culture</td>
<td>Enhanced CaMV 35S</td>
<td>Secreted, up to 45 µg l⁻¹ of supernatant</td>
<td>26</td>
</tr>
<tr>
<td>HBsAg</td>
<td><em>N. tabacum</em> NT-1 suspension culture</td>
<td><em>A. thaliana</em> ubq3</td>
<td>Secreted, up to 10 µg l⁻¹ of particulate HBsAg</td>
<td>41</td>
</tr>
<tr>
<td>mAb against HBsAg</td>
<td><em>N. tabacum</em> cv BY-2 suspension culture</td>
<td>CaMV 35S</td>
<td>Secreted, ~50/50 between supernatant and cells, total max ~15 mg l⁻¹</td>
<td>58</td>
</tr>
</tbody>
</table>

TSP, total soluble protein; ER, endoplasmic reticulum; PVP, polyvinylpyrrolidone; FW, fresh weight.
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\(^{2,33}\). 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.

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**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.
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. thaliana33,37,40,41. In contrast to these constitutive promoters, the rice α-amylase Ramy3D promoter is induced by sugar deprivation38.

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 properties42–44. 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.

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

<table>
<thead>
<tr>
<th>Medium additive</th>
<th>Expression host</th>
<th>Expressed protein</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVP</td>
<td>N. tabacum NT-1 suspension culture</td>
<td>Heavy-chain monoclonal antibody</td>
<td>Addition of 0.75% PVP 360.000 increased secreted product accumulation 35-fold</td>
<td>52, 61</td>
</tr>
<tr>
<td>BSA, NaCl</td>
<td>N. tabacum cv BY-2 suspension culture</td>
<td>Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)</td>
<td>Enhanced secreted product accumulation 100% (BSA), or 50% (NaCl)</td>
<td>62</td>
</tr>
<tr>
<td>BrefeldinA</td>
<td>N. tabacum suspension culture initiated from transgenic plants</td>
<td>Mouse IgG1</td>
<td>Inhibited the secretory pathway and thereby prevented degradation of secreted protein increased mAb accumulation 2.7-fold</td>
<td>15</td>
</tr>
<tr>
<td>Reducing manganese</td>
<td>N. tabacum suspension culture initiated from transgenic plants</td>
<td>Mouse IgG1</td>
<td>In manganese-reduced medium the stability and accumulation of secreted IgG1 was increased –1.7-fold</td>
<td>44</td>
</tr>
<tr>
<td>Pluronic antifoam, PEG</td>
<td>N. tabacum cv Havana SR1 suspension culture</td>
<td>hGM-CSF</td>
<td>Pluronic antifoam addition increased the growth rate almost twofold, PEG-8000 increased hGM CSF accumulation fourfold</td>
<td>84</td>
</tr>
<tr>
<td>Gelatin, PVP, PEG</td>
<td>N. tabacum cv Havana SR1 suspension culture</td>
<td>hGM-CSF</td>
<td>2% gelatin increased accumulation 4.6-fold, PVP and PEG showed no effect</td>
<td>53</td>
</tr>
<tr>
<td>Gelatin, PVP, PEG</td>
<td>N. tabacum cv Havana SR1 suspension culture</td>
<td>IL-12 heterodimer</td>
<td>2% gelatin increased IL-2 accumulation sevenfold</td>
<td>9</td>
</tr>
</tbody>
</table>

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.

### 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 µg I⁻¹ to 200 mg I⁻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 media45,44, which is probably why proteins targeted to the endoplasmic reticulum using a retention signal accumulate to levels (based on cultivation
and Shuler 67.E ven so, it remains to be seen whether simple batch vented using two-stage bioreactors, as described, for example, by Sahai
costs, although this depends on the required level of purity, which is
downstream processing represents up to 80% of overall production
product from the raw biomass. Regardless of the production system,
regulatory considerations
gies for the production of proteins using plant cell cultures.
fusion processes will emerge as the most suitable fermentation strate-
processes or long-term draw-fill, semicontinuous or continuous per-

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 compounds59, amino acids to provide
precursors for protein synthesis8, stabilizing agents such as dimethyl-
sulfoxide60, polyethylene glycol9,53, polyvinylpyrrolidone9,15,52,53,59,61,
gelatin9,53,59 and bovine serum albumin62 (these are thought to work
mass and purification of secreted proteins directly from the culture
context promising starting points for process development36,39,57,58.

The downside of this approach is that the recombinant protein is
contaminating proteins and other metabolites than whole cell extracts.
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, includ-
ing a strict regime of quality assurance and quality control to achieve
approval of regulatory agencies69. Regulatory guidance for biophar-
maceutical production in plants currently exists only as draft legisla-
tion to the applicable parts of these documents, other nonplant-
specific GMP guidelines for biotechnological production (e.g., Annex
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
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 cur-
cently used for recombinant protein expression have a long history in

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

<table>
<thead>
<tr>
<th>Bioreactor type, scale and fermentation mode</th>
<th>Expression host</th>
<th>Expressed protein</th>
<th>Effect</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Semi-continuous production of CAT in repeated batch and continuous fermentation at 0.25 d⁻¹ in a bubble column</td>
<td><em>N. tabacum</em> suspension culture</td>
<td>Chloramphenicol-acetyltransferase (CAT)</td>
<td>Constant product concentration in harvest</td>
<td>85</td>
</tr>
<tr>
<td>Shake-flask, semi-continuous perfusion/continuous mode, replacing 33% of the medium every 12 h, equivalent to 0.66 d⁻¹</td>
<td><em>N. tabacum</em> NT-1 suspension culture</td>
<td>Heavy-chain monoclonal antibody</td>
<td>+/- Constant levels of secreted heavy chain mAb in harvest</td>
<td>52</td>
</tr>
<tr>
<td>Stirred tank bioreactor, 10-liter continuous, 0.2 d⁻¹</td>
<td><em>N. tabacum</em> cv BY-2 suspension culture</td>
<td>Carrot acidic invertase</td>
<td>Increased overall productivity fourfold</td>
<td>33</td>
</tr>
<tr>
<td>Encapsulation of suspension cultured cells in alginate, shake-flask</td>
<td><em>N. tabacum</em> NT-1 suspension culture</td>
<td>Human granulocyte-macrophage colony-stimulating factor (hGM-CSF)</td>
<td>Increased levels of secreted hGM-CSF 107 to 178 µg l⁻¹</td>
<td>86</td>
</tr>
<tr>
<td>Shake flask, periodic harvesting using hydroxypatite-resin</td>
<td><em>N. tabacum</em> suspension culture initiated from transgenic plants</td>
<td>Mouse IgG₁ monoclonal</td>
<td>Periodic harvesting increased overall yield</td>
<td>15</td>
</tr>
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</table>

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

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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. 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. 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. Another alternative is the use of aqueous two-phase systems for cultivation and, eventually, in-situ extraction from plant cell cultures.

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. 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., His₆ 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.

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. 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. 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.

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