Molecular farming in plants: host systems and expression technology

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Plants provide an inexpensive and convenient system for the large-scale production of valuable recombinant proteins. This principle has been demonstrated by the commercial success of several first-generation products, and many others are currently under development. Over the past ten years, several efficient plant-based expression systems have emerged, and >100 recombinant proteins have now been produced in a range of different species. Plants have many advantages over other production systems, particularly in terms of practicality, economy and safety. However, several constraints that hinder the widespread use of plants as bioreactors remain to be addressed. Important factors include quality and homogeneity of the final product, the challenge of processing plant-derived pharmaceutical macromolecules under good manufacturing practice conditions and concerns about biosafety. Molecular farming in plants will only realize its huge potential if these constraints are removed through rigorous and detailed science-based studies.

In the past decade, plant-based expression systems have emerged as a serious competitive force in the large-scale production of recombinant proteins. The first plant-derived technical proteins have already reached the market [1,2], and detailed economic evaluations have demonstrated their competitiveness against established market sectors [3,4]. Now, several plant-derived biopharmaceutical proteins are reaching the late stages of commercial development. These products include antibodies, vaccines, human blood products, hormones and growth regulators [5,6]. For such products, plants offer practical and safety advantages as well as lower production costs compared with traditional systems based on microbial or animal cells, or transgenic animals. With an increasing number of products in development, molecular farming in plants is finally coming of age. In this, the first of two reviews on the subject, we consider recent technological developments in molecular farming, focusing on plant host systems and expression technology. In a future issue of *Trends in Biotechnology*, we discuss the types of recombinant proteins that are expressed in plants and consider production, biosafety and regulatory matters, including timelines, economics and progress towards clinical trials. These issues govern the creation and release of transgenic plants for molecular farming, and help to shape the emerging regulatory framework covering the use of plant-derived pharmaceuticals.

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Transgenic plants: benefits and drawbacks for the production of recombinant proteins

Production costs

A major advantage of transgenic plants for molecular farming is the comparatively low cost of large-scale production (Figure 1). Both capital and running costs are significantly lower than those of cell-based production systems because there is no need for fermenters or the skilled personnel to run them. It is estimated that recombinant proteins can be produced in plants at 2–10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell cultures, although this depends on the product yield [6]. For proteins that can be produced at high yields, the economic advantages of plant production systems are clear. One bushel of maize producing recombinant avidin at 20% total soluble seed protein has the same total yield as one tonne of chicken eggs – the natural source of avidin – but at 0.5% of the cost [7]. Not many proteins can be produced at this level, but yields of 0.1–1.0% total soluble protein (TSP), the typical levels observed for the production of pharmaceutical proteins, such as recombinant antibodies, are sufficiently competitive with other expression systems to make plants economically viable [8]. Where such yields cannot be achieved, molecular farming in plants might not be economical, particularly if the product has a low market value (Box 1).

For any given expression system, scalability is an important commercial advantage. Fermentation systems and transgenic animals have limited potential in this respect, whereas the scale of plant-based production can be modulated rapidly in response to market demand simply by using more or less land as required. The speed of scale-up is also important. It can take several years to achieve tenfold scale-up in a herd of transgenic sheep using natural breeding cycles, but a field of transgenic plants can be scaled-up more than 1000-fold in a single generation owing to the prolific seed output [9]. Where scale-down is

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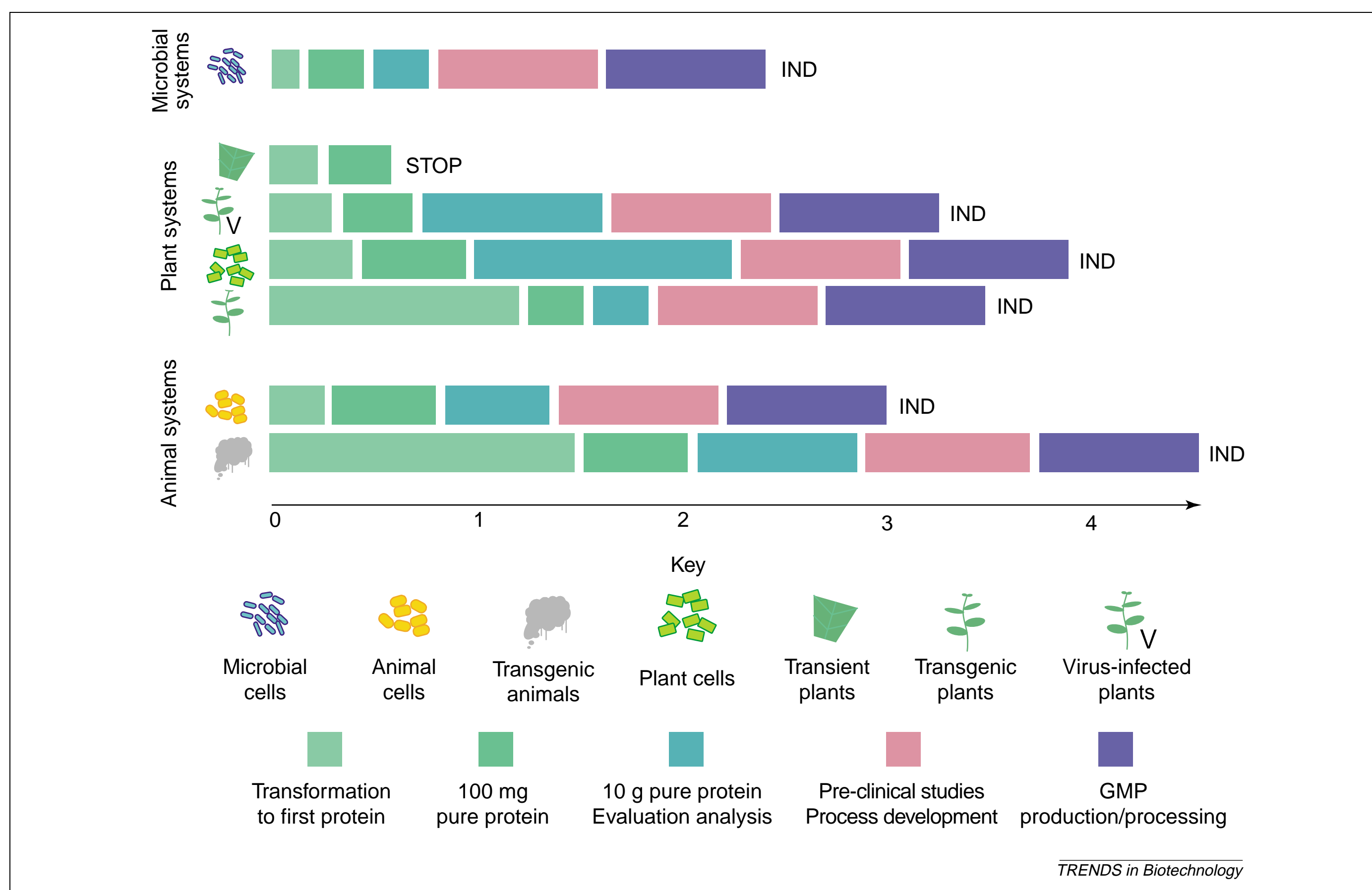

Figure 1. Performance of plant-based production systems in comparison to other commercial platforms for the production of recombinant proteins. Abbreviations: IND, investigational new drug.

required, surplus transgenic animals have to be sacrificed or maintained at a loss, whereas the amount of land planted to a specific transgenic crop can be adjusted as required, and transgenic plant lines can be stored indefinitely and inexpensively as seed [9].

The production costs of a recombinant protein also depend significantly on the required purity because $>85\%$ of expenditure reflects downstream processing rather than production *per se*. Because similar methods are used for protein purification regardless of the expression system, the costs of processing are similar across the board when high purity is required. However, plants are advantageous because several types of recombinant protein can be used in unprocessed or partially processed material, therefore removing many of the downstream costs. For example, recombinant subunit vaccines produced in plants can be administered by the consumption of raw or part-processed fruits and vegetables [10], and antibodies for passive immunization can be administered as topically applied pastes following minimal purification. Similarly, industrial enzymes such as glucanase (which is used to break down cellulose in animal feed) and phytase (which breaks

Box 1. Portrait of an industry

The commercial success of molecular farming in plants depends on technology, economic considerations and public acceptance. This review deals with the first of those issues and makes the simple assumption that, in most cases, the technology will succeed. The most significant driver of the plant biopharming industry is the expected cost reductions and the knock-on effects this will have on the biopharmaceutical markets overall. At present, the politicians and public of Europe are unwilling to accept biotechnology-derived foods, but biotechnology-derived drugs are more likely to meet with approval, particularly with the enforcement of strict regulatory measures.

Although in its infancy, the plant biopharming industry has demonstrated considerable growth and has attracted a large amount of investment. At present, it comprises ~100 small companies, each focusing on the development of a few products. The market leaders are companies such as Prodigene (http://www.prodigene.com/), Epicyte (http://epicyte.com), Large Scale Biology (http://www.lsbc.com/), SemBioSys Genetics (http://www.sembiosys.com/) and the European leader Meristem Therapeutics (http://www.meristem-therapeutics.com), each of which has tens of products in the pipeline. In some cases, there are already productive collaborations with pharmaceutical companies, such as the collaboration of Meristem Therapeutics with Solvay Pharmaceuticals (http://www.solvay.com) for the development of plant-derived human gastric lipase. This is one of two plant-derived products currently in Phase II clinical trials, the other being CaroRx™, a recombinant antibody used for the prevention of dental caries (Planet Biotechnology; http://www.planetbiotechnology.com/products.html#carorx). An industry high-point this year was the founding of Chlorogen (http://www.chlorogen.com/) to exploit the chloroplast transgenic system for the commercial production of human pharmaceuticals. However, the industry has also had its low points, a prominent example being the collapse of CropTech, owing to its inability to achieve the expression levels required for commercial feasibility.

down phytic acid and releases bioavailable phosphorus) can be introduced into the industrial process either as part-processed plant material or expressed directly in the plant that needs to be processed [11–14].

For pharmaceutical proteins that need to be purified before use, several strategies have been developed to reduce downstream processing costs. As the cost of processing is inversely proportional to the concentration of product in the starting material, the yield of protein per unit of plant biomass is crucial. As discussed later, this depends on both the expression system and the product itself, and needs to be evaluated on an empirical basis. One of the advantages of recombinant protein expression in the seeds of transgenic cereal plants is that high levels of the product can accumulate in a small volume, which minimizes the costs associated with processing [15,16]. Where conventional extraction from seeds is too expensive, further strategies to assist purification can be used. An example is the oleosin-fusion platform developed by SemBioSys Genetics (http://www.sembiosys.com/), in which the target recombinant protein is expressed in oilseed crops as a fusion with oleosin. The fusion protein can be recovered from oil bodies using a simple extraction procedure and the recombinant protein is separated from its fusion partner by endoprotease digestion [17]. Similarly, we have devised a strategy in which recombinant proteins are expressed as fusion constructs containing an integral membrane-spanning domain derived from the human T-cell receptor [18]. The recombinant protein accumulates at the plasma membrane and can be extracted in a small volume using appropriate buffers and detergents.

Another approach is to exploit the ability of vegetative tissues (e.g. leaves or roots) to secrete recombinant proteins in their exudates, enabling proteins to be collected continuously. Such a system for the production of human secreted alkaline phosphatase is being developed by Phytomedics (http://www.phytomedics.com/) [19,20] and has also been used for the synthesis of recombinant antibodies [21]. However, this type of strategy is suitable only for small-scale production in high-containment facilities because field deployment would result in large amounts of recombinant protein leaching into the soil and groundwater.

Development timescale

The gene-to-protein time for transgenic plants encompasses the preparation of expression constructs, transformation, regeneration and the production and testing of several generations of plants. The testing phase is necessary to ensure transgene and expression stability and the biochemical activity of the product, as well as the absence of adverse phenotypic changes in the host plant. These processes take up to two years depending on the plant species, although milligram amounts of protein might be available after several months for initial testing [8]. Microbial expression systems and animal cells are more advantageous here because they can produce the first batches of recombinant protein more quickly than plants, but this might be outweighed by their limitations in terms of overall scalability and cost. Furthermore, several plant-based transient expression systems are available for the rapid production and testing of recombinant proteins on a small scale, and these can be used to evaluate expression constructs and product quality before committing to the expense of transgenic plants [22]. Plant-cell-suspension cultures also produce recombinant proteins more rapidly than transgenic plants because the development and testing schedule is much shorter. Suspension cells have therefore been used for molecular farming, especially where high containment is an advantage [23]; this strategy is discussed in more detail later.

Product authenticity

As a production system for pharmaceutical proteins, plants are considered to be much safer than both microbes and animals because they generally lack human pathogens, oncogenic DNA sequences, and endotoxins [24]. However, it is necessary to consider the intrinsic safety of plant-derived human proteins because the structural authenticity of such proteins influences their behaviour *in vivo*. The protein synthesis pathway is conserved between plants and animals so plants appear to fold and assemble recombinant human proteins efficiently. This is a great advantage over bacterial expression systems, in which many proteins fail to fold properly and are either degraded (resulting in low yields) or accumulate as insoluble inclusion bodies. The ability of plants to fold and assemble complex proteins correctly is demonstrated by their capacity to produce functional serum antibodies, which comprise four polypeptide chains covalently linked by disulphide bonds [9,25]. More remarkably, plants produce functional secretory antibodies, which are dimers of the typical serum immunoglobulins and have two additional polypeptide components, making ten separate polypeptides in total [8,26,27]. Two different cell types are required to assemble such antibodies in mammals.

Although the protein synthesis pathway is the same in plants and animals, there are some differences in post-translational modifications, particularly with respect to glycan-chain structure. Thus, plant-derived recombinant human proteins tend to have the carbohydrate groups $\beta(1 \rightarrow 2)$-xylose and $\alpha(1 \rightarrow 3)$-fucose, which are absent in mammals, but lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins (Box 2). Experience with other production systems has shown that even minor differences in glycan structures can change the distribution, activity or longevity of recombinant proteins compared with their native counterparts, and could render such proteins immunogenic when administered to humans. Several changes in the glycosylation pathway are required to produce proteins with typical human glycan structures in plants [28]. Strategies that have already been used in an attempt to ‘humanize’ the glycan structures of recombinant human glycoproteins include: (i) the use of purified human β(1,4)-galactosyltransferase and sialyltransferase enzymes for the *in vitro* modification of plant-derived recombinant proteins [29]; and (ii) expression of human β(1,4)-galactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans [30]. In the case of (ii), 30% of the antibody was

Box 2. Glycosylation of proteins in plants

The first part of the *N*-linked glycosylation pathway is similar in all eukaryotes, but diversity is generated at later stages by the predominance of different modification enzymes in plants, mammals, insects and yeast. In plants, co-translational transfer of the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ is followed by trimming to $Man_5GlcNAc_2$ as the protein enters the medial-Golgi. The trimannosyl core is substituted by several different carbohydrate groups of which $\beta(1 \rightarrow 2)$-xylose and $\alpha(1 \rightarrow 3)$-fucose residues are not found in mammals [85] (Figure I).

Although sometimes described as plant-specific, the paucimannosidic-type glycans are similar to those found in insects [86]. They have $\alpha(1,3)$-fucose and/or $\beta(1,2)$-xylose linked to the proximal GlcNAc and the β-mannose residue of the core glycan, respectively. These structures probably occur by the elimination of terminal residues from complex glycans using exoglycosidase or *N*-acetyl glucosaminidase. Most vacuolar and seed storage proteins have this type of glycan structure [86].

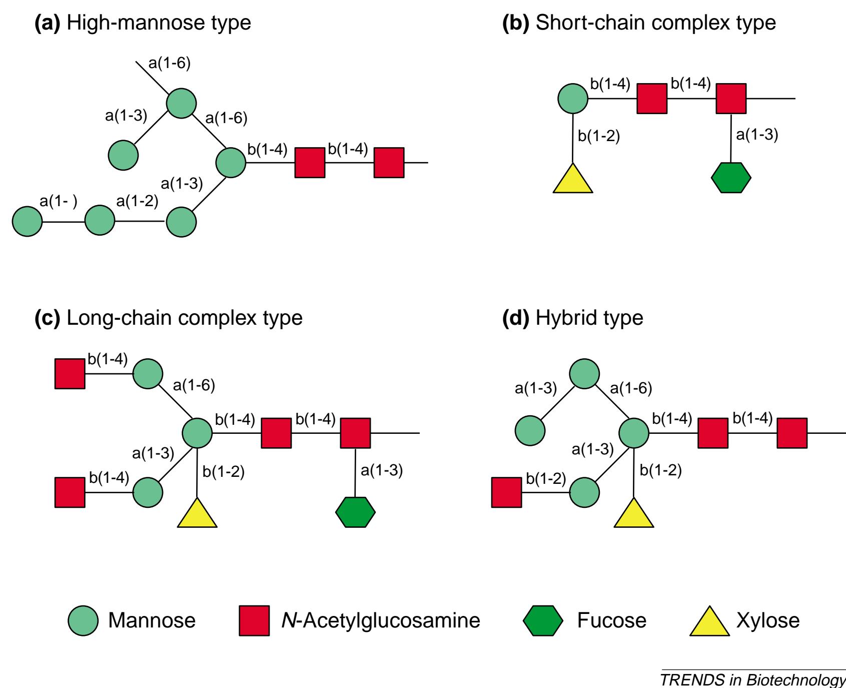

Figure I. Examples of *N*-linked oligosaccharide groups found in plant glycoproteins. **(a)** High-mannose type (dots indicate uncertain linkage architecture). **(b)** Short-chain complex type. **(c)** Long-chain complex type. **(d)** Hybrid type.

galactosylated, which is similar to the proportion found in hybridoma cells. *In vivo* sialylation will be more difficult to achieve because plants lack the precursors and metabolic capability to produce this carbohydrate group. Although these approaches involve the addition of carbohydrate groups missing in plants, there is also the problem of removing plant-specific carbohydrates. Inhibition of the enzymes fucosyltransferase and xylyltransferase using antibodies, ribozymes or RNA interference could be used to achieve this, and gene targeting by homologous recombination has been used to produce recombinant proteins lacking plant-specific glycans in the moss *Physcomitrella patens* (G. Gorr, pers. commun.). Although achieving authenticity should be a major goal in molecular farming, it is notable that studies in which mice were administered a recombinant antibody containing plant-specific glycans showed no evidence of an immune reaction [31].

Maximizing yields in transgenic plants

One of the most important factors determining the commercial viability of molecular farming is the achievement of adequate recombinant protein yields. Absolute yields depend on the species used for production but the choice of production crop depends on a variety of factors in addition to yield potential, which we discuss in more detail later. Here, we describe recent progress in expression technology that can help to achieve maximum yields of recombinant protein in any given species.

High-level transgene expression

Expression-construct design can help to achieve high yields in transgenic plants by maximizing the rates of transcription and translation. For dicotyledonous species, the strong and constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter is often chosen to drive transgene expression [32]. In cereals, the CaMV 35S promoter has a lower activity, therefore the maize ubiquitin-1 (*ubi*-1) promoter is preferred [33]. The rate of transcription in cereals is also increased by the inclusion of an intron; this phenomenon is known as intron-mediated enhancement [34].

Regulated promoters can be used in preference to constitutive promoters because these often have both practical and biosafety advantages. For example, although constitutive promoters enable high-level accumulation of

recombinant proteins in seeds, the proteins are also expressed in leaves, pollen and roots. This might adversely affect the growth and development of vegetative parts of the plant, and could expose herbivores, pollinating insects and microbes in the rhizosphere to the effects of the recombinant protein. Restriction of protein accumulation to seeds helps to reduce these risks [16,24]. Inducible promoters [35] can be used to limit recombinant protein expression to just before or following harvest, as has been demonstrated with recombinant glucocerebrosidase expressed in tobacco [36].

The rate of translation can be optimized by ensuring that any mRNA instability sequences are eliminated from the transgene construct, and that the translational start-site matches the Kozak consensus for plants [37,38]. It might be necessary to modify codon usage in some transgenes, not only to maximize the rate of protein synthesis, but also to eliminate cryptic introns and instability sequences [39].

Both transcription and translation can be maximized by taking precautions against transgene silencing, a group of epigenetic phenomena that can inhibit the expression of even structurally intact transgenes at either the transcriptional or post-transcriptional level. Strong triggers for silencing include prokaryotic DNA sequences (such as those found on the vector backbone) and sequences that have the potential to form hairpin secondary structures at the DNA level, or double-stranded RNA when expressed [40–43]. Useful strategies to minimize the frequency of silencing include the use of clean DNA procedures to avoid vector backbone integration [44].

Protein targeting

We have already discussed the targeting of recombinant proteins to oil bodies or the plasma membrane as a strategy to facilitate isolation and purification. However, subcellular targeting can also be used as a general method to increase the yield of recombinant proteins because the compartment in which a recombinant protein accumulates strongly influences the interrelated processes of folding, assembly and post-translational modification. All of these contribute to protein stability and, hence, help to determine the final yield [9].

Comparative targeting experiments with full-size immunoglobulins and single-chain Fv fragments have shown that the secretory pathway is a more suitable compartment for folding and assembly than the cytosol, and is therefore advantageous for high-level protein accumulation [45,46]. Because many plant-derived recombinant proteins under development are human proteins that normally pass through the endomembrane system, this principle can be applied not only to antibodies but also more generally. Antibodies targeted to the secretory pathway using either plant or animal N-terminal signal peptides usually accumulate to levels that are several orders-of-magnitude greater than those of antibodies expressed in the cytosol. Occasional exceptions to this general observation suggest that intrinsic features of each antibody might also contribute to overall stability [47,48]. The endoplasmic reticulum (ER) provides an oxidizing environment and an abundance of molecular chaperones, while there are few proteases. These are likely to be the most important factors affecting protein folding and assembly. It has been shown recently that antibodies targeted to the secretory pathway in transgenic plants interact specifically with the molecular chaperone BiP (binding protein) [49].

In the absence of further targeting information, the expressed protein is secreted to the apoplast. Depending on its size, the protein can be retained therein or might leach from the cell, with important implications for production systems based on cell-suspension cultures. The stability of antibodies in the apoplast is lower than in the lumen of the ER. Therefore, antibody expression levels can be increased even further if the protein is retrieved to the ER lumen using an H/KDEL C-terminal tetrapeptide tag [50]. Accumulation levels are generally 2–10-fold greater compared with an identical protein lacking the H/KDEL signal [9]. As an added benefit, antibodies retrieved in this way are not modified in the Golgi apparatus, which means they have high-mannose glycans but not plant-specific xylose and fucose residues [51]. Again, although the principles of ER-retention in molecular farming have been established using antibodies, it is probable that they will also apply to many other proteins.

High levels of protein expression have also been demonstrated when transgenes are introduced into the chloroplast rather than the nuclear genome [52]. Several examples of chloroplast-based molecular farming have been reported in tobacco, where the technology for chloroplast gene transfer is most advanced. These include: (i) production of human growth hormone at levels approaching 8% TSP [53]; (ii) production of human serum albumin at levels exceeding 11% TSP [54]; (iii) production of cholera and tetanus toxin fragments at up to 25% TSP [55,56]; and (iv) production of a thermostable xylanase at 6% TSP, with 85% recovery [57]. The high yields possible with the chloroplast transgenic system are attractive, and chloroplasts also offer biosafety advantages in terms of transgene containment, owing to maternal inheritance. However, the use of this system as a general approach in molecular farming is limited by the inability of chloroplasts to carry out many post-translational modifications, including glycosylation [52], and biosafety concerns have been raised by the recent demonstration of horizontal gene transfer from the chloroplasts of transplastomic plants to bacteria, under laboratory conditions [58]. An alternative approach is to express the protein from the nuclear genome but introduce a chloroplast targeting sequence. In an interesting recent report, Jobling *et al.* [59] expressed a camelid heavy-chain antibody in potatoes, and succeeded in targeting the protein to the chloroplast where it inhibited the starch-branching enzyme. Although the intention of the study was metabolic engineering rather than molecular farming, that camelid heavy-chain antibodies are extremely stable makes these molecules useful candidates for both medical and industrial applications.

Choice of production species

Leafy crops

Tobacco has a long history as a successful crop system for molecular farming and is therefore one of the strongest

candidates for the commercial production of recombinant proteins [9,25,60]. The major advantages of tobacco include the well-established technology for gene transfer and expression, high biomass yield, prolific seed production and the existence of a large-scale processing infrastructure. Because tobacco is neither a food nor a feed crop, there is little risk that tobacco material will contaminate either the food or feed chains. Although many tobacco cultivars produce high levels of toxic alkaloids, there are low-alkaloid varieties that can be used for the production of pharmaceutical proteins, and these metabolites are absent from tobacco cell suspensions, which can also be used to produce recombinant proteins.

Alternative leafy crops that are being investigated for molecular farming include alfalfa, soybean and lettuce. Alfalfa and soybean have the major advantage of using atmospheric nitrogen through nitrogen fixation, therefore reducing the need for chemical fertilizers [61]. Alfalfa is particularly useful because it has a large dry biomass yield per hectare and can be harvested up to nine times a year. Both of these legumes have been used to produce recombinant antibodies [62,63]. Soybean has been used to produce *Aspergillus* phytase [11], whereas alfalfa is being used to produce an increasing number of products, mainly through the efforts of the Canadian biotechnology company Medicago (http://www.medicago.com/). Lettuce is also being investigated as a production host for edible recombinant vaccines and has been used in one series of clinical trials for a vaccine against the hepatitis B virus [64]. One of the greatest disadvantages of leafy crops is that recombinant proteins are synthesized in an aqueous environment and are often unstable, resulting in low yields. The leaves must be frozen or dried for transport, or processed soon after harvest to extract useful amounts of the product. Tobacco leaves contain phenolic substances that are released during grinding and protein extraction, which can interfere with downstream processing. The expression of recombinant proteins in vegetative organs such as leaves could potentially interfere with plant growth and development. Biosafety concerns include the potential exposure of herbivores to pharmaceutical products expressed in leaves, and the leaching of recombinant proteins into the environment.

Cereal and legume seeds

In contrast to leafy crops, the expression of proteins in seeds enables long-term storage, even at room temperature, because seeds have the appropriate biochemical environment to promote stable protein accumulation. It has been demonstrated that antibodies expressed in seeds remain stable for at least three years at ambient temperatures with no detectable loss of activity [16]. Cereal seeds also lack the phenolic compounds present in tobacco leaves, thus improving the efficiency of downstream processing. However, the overall yields of recombinant proteins in seed crops are much lower than in tobacco, and the most appropriate expression system must be determined on a case-by-case basis. The specific expression of recombinant proteins in seeds has biosafety advantages in that it reduces exposure to herbivores and other non-target organisms. However, it is necessary for the transgenic plants to go through a flowering cycle to produce seeds, whereas proteins produced in vegetative organs can be harvested before flowering, therefore preventing the release of pollen and eliminating gene flow by pollen transfer.

Several different crops have been investigated for seed-based production, including cereals (rice, wheat and maize) and legumes (pea and soybean) [60,65,66]. Important factors that must be considered when choosing a production crop include biomass yield per hectare, yield of recombinant protein per unit biomass, ease of transformation, and scalability. Rice, wheat, pea and tobacco have been used to express the same single-chain Fv antibody to compare the merits of each production system [60]. With the optimal promoter (the enhanced CaMV 35S promoter for tobacco and the *ubi*-1 promoter for rice), rice plants showed the highest yields per unit biomass, even exceeding tobacco leaves, although tobacco has a higher overall yield owing to the greater amount of biomass produced per hectare. Levels were lower in wheat and pea seeds although these systems are still at early stages of development [60].

Maize was chosen by Prodigene (http://www.prodigene.com/) as the first plant species for commercial molecular farming. The major factors in this decision were high biomass yield, ease of transformation and *in vitro* manipulation, and convenience of scale-up. Maize has been used for the commercial production of recombinant avidin and β-glucuronidase [1,2] and its use for the production of recombinant antibodies [8] and further technical enzymes, such as laccase, trypsin and aprotinin, is being explored [65].

Fruit and vegetable crops

A major advantage of protein expression in fruit and vegetable crops is that the edible organs can be consumed as uncooked, unprocessed or partially-processed material, making them ideal for the production of recombinant subunit vaccines, nutriceuticals and antibodies designed for topical application. Potatoes are the major system for vaccine production [67–69] and transgenic potato tubers have been administered to humans in at least three clinical trials carried out to date. A recent report describes the production of rotavirus VP6 capsid protein in transgenic potatoes for vaccination against acute viral gastroenteritis [70]. Artsaenko and colleagues showed how potatoes could be used for antibody production [71] and this system has been investigated as a possible bulk-antibody-production platform [72]. Potatoes have also been used for the production of glucanases [12], diagnostic antibody-fusion proteins [73] and proteins from human milk [74,75]. Tomatoes are more palatable than potatoes and have other advantages, including high biomass yield and use of greenhouses for increased containment. Tomatoes were first used for the production of a rabies vaccine candidate, and have also been used to produce antibodies, although yields thus far are lower than 3 μg per gram fresh weight [60,76]. Bananas are attractive vehicles for edible vaccine distribution because they are widely grown and consumed by both children and adults

in Africa where vaccination programmes are badly needed [10,77].

Fibre and oil crops

The advantage of fibre and oil crops, such as flax, cotton and oilseed rape, is the potential for the costs of recombinant protein production to be offset to a certain degree by secondary revenues from alternative products. However, both fibre and oil can interfere with downstream processing so this applies mainly to proteins that do not need to be extensively purified. As discussed earlier, an exception is the oleosin-fusion technology developed by SemBioSys Genetics, in which the targeting of recombinant proteins to oil bodies can be used to facilitate purification [17].

Alternative plant-based production systems

Transient expression can be used to verify expression constructs and produce small amounts of product for functional analysis before proceeding to transgenic plants. However, transient expression by agro-infiltration in tobacco leaves can also be used to produce larger quantities of protein [78] (e.g. milligram amounts of recombinant antibodies in a matter of days or weeks [79]), and could therefore find use as a mainstream production platform. Virus-infected plants have also been used to produce pharmaceutical proteins, including antibodies [80,81] and subunit vaccines (reviewed in [82]). Again, the advantage here is the rapid gene-to-protein cycle, but there are also drawbacks. For example, agro-infiltration has a low capacity for scale-up, and viral vectors provide additional concerns in terms of containment.

As an alternative to transgenic plants or transient expression systems, plant-cell-suspension cultures can also be used to produce recombinant proteins [23]. Suspension cells are particularly advantageous when defined and sterile production conditions in addition to straightforward purification protocols are required, as these conditions are particularly applicable to the production of therapeutic proteins [83]. Indeed, we have expressed a wide panel of recombinant antibodies in BY-2 tobacco and rice-cell-suspension cultures, including full-size immunoglobulins, Fab fragments, single chains, bispecific antibody fragments and fusion proteins [23,84].

Recombinant proteins expressed in plant-cell-suspension cultures can be secreted into the culture supernatant or retained within the cells. Localization depends on protein targeting, as discussed earlier, and the permeability of the plant cell wall to the protein in question. The fate of proteins targeted for secretion depends to a large extent on their size: molecules of 20–30 kDa (the size-range of single-chain Fvs) will generally pass through the plant cell wall and be secreted into the culture medium, whereas larger proteins (such as IgGs) will be retarded in a size-dependent manner. For the reasons discussed earlier, the inclusion of a C-terminal H/KDEL sequence results in higher levels of antibody accumulation in cultured cells. However, this requires disruption of the cells to isolate the protein, which requires additional processing time and causes the release of phenolic molecules that interfere with purification and reduce the overall yield. Therefore, our preferred approach is to secrete the target proteins and capture them from the culture supernatant or release them from the cells by disruption or mild enzymatic digestion.

Concluding remarks

Research over the past 10 years has significantly increased our knowledge of gene regulation and protein synthesis in different plants. This has enabled a move from proof-of-principle studies in model species to exploration of a variety of different plants for the production of recombinant proteins. A diverse repertoire of technical, pharmaceutical and industrial proteins has been produced in plants, some on a commercial basis. Much effort is being devoted to overcoming the technical limitations of molecular farming, particularly by increasing the low yields in certain expression systems and producing human proteins with authentic glycan chains. However, there are several further challenges concerning issues of environmental impact, biosafety and risk assessment, which reflect the release and agricultural-scale cultivation of transgenic plants, as well as the safety of the plant-derived products themselves. The progress of plant-derived pharmaceutical proteins through preclinical development and clinical trials is a significant bottleneck. These crucial issues, which will probably have more of an impact on the commercial success of molecular farming than the technological hurdles, will be discussed in our forthcoming review article in this journal.

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