

Expert Opinion

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Transgenic plants in the biopharmaceutical market

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Many of our 'small-molecule-drugs' are natural products from plants, or are synthetic compounds based on molecules found naturally in plants. However, the vast majority of the protein therapeutics (or biopharmaceuticals) we use are from animal or human sources, and are produced commercially in microbial or mammalian bioreactor systems. Over the last few years, it has become clear that plants have great potential for the production of human proteins and other protein-based therapeutic entities. Plants offer the prospect of inexpensive biopharmaceutical production without sacrificing product quality or safety, and following the success of several plant-derived technical proteins, the first therapeutic products are now approaching the market. In this review, the different plant-based production systems are discussed and the merits of transgenic plants are evaluated compared with other platforms. A detailed discussion is provided of the development issues that remain to be addressed before plants become an acceptable mainstream production technology. The many different proteins that have already been produced using plants are described, and a sketch of the current market and the activities of the key players is provided. Despite the currently unclear regulatory framework and general industry inertia, the benefits of plant-derived pharmaceuticals are now bringing the prospect of inexpensive veterinary and human medicines closer than ever before.

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1. Background

Plants have been used for thousands of years to prevent and cure disease, but only in the last 15 years has the plant genome been manipulated for the specific purpose of producing therapeutic molecules [1]. About 25% of the small-molecule drugs used in the developed world are either plant-derived or contain components from plants, and this proportion is even higher in developing countries, which have limited access to modern drugs and continue to rely primarily on ethnobotanical medicines [2]. In most cases, botanical drugs are derived from wild or cultivated plants that have not been genetically altered. The majority of therapeutic molecules that researchers have attempted to produce in genetically modified plants are in fact animal proteins, which were originally obtained directly from animal sources or human cadavers and are now produced commercially either in microbial fermenters or cultured mammalian cells. The ability of plants to express human genes was established in 1986, when Barta and colleagues [3] showed that tobacco and sunflower callus tissue (undifferentiated plant tissue) could produce transcripts of a fusion gene comprising the human growth hormone gene and the *Agrobacterium tumefaciens nos* gene. *A. tumefaciens* is a bacterium that can transfer DNA into the plant genome and is widely used as a transformation vehicle. The *nos* gene encodes an enzyme, nopaline synthase, which the bacterium uses to synthesise opines, which are amino acid derivatives that the bacterium uses as a nutrient source. The first potentially therapeutic protein expressed in plants was human serum albumin, which was produced in tobacco and potato leaves and suspension cells in 1990 [4]. Since then, > 100 therapeutic and diagnostic recombinant proteins and vaccines have been

produced in a variety of plants, including tobacco, cereals, legumes, fruit and vegetable crops, fodder crops, edible foliage such as lettuce and spinach, oilseeds, and aquatic or unicellular plant species grown in bioreactors [5,6]. Plants have many advantages over traditional production systems based on microbial or mammalian cells, particularly in terms of economy, production scale, safety and practicality [5,7-9]. A large number of companies have now been set up to investigate and exploit the potential of plant-derived recombinant vaccines, antibodies and other therapeutic entities. Although no such products are yet available as prescription drugs or over the counter medicines, a number of plant-derived recombinant proteins such as avidin, trypsin and β -glucuronidase have reached the market as technical reagents [10,11], and several diagnostic/therapeutic recombinant proteins from plants have progressed through clinical trials and are approaching regulatory approval.

2. Medical need

A wide range of medically relevant proteins has been produced in plants, and these products do not address any one specific medical need [1,7,9]. The advantage of plant-derived pharmaceuticals is that they address the rapidly increasing demand for diagnostic and therapeutic proteins and vaccines, and the insufficient capacity of the current supply chain to provide them. It has been estimated that fermenter production capacity must increase at least 10-fold over the next decade to meet the projected increase in demand, given that > 300 novel protein-based drugs and diagnostics are currently going through clinical trials and many more are in the development pipeline [12].

The reliance on microbial and mammalian cell cultures for recombinant protein production not only places a heavy demand on worldwide production capacities, but also drives up the price of the resulting drugs. The expense of building and running fermenter systems makes the production of all but the most valuable recombinant proteins economically unfeasible. This reflects the need for equipment, skilled workers, and in the case of mammalian cell cultures, expensive culture media. Transgenic plants on the other hand can be grown and harvested using traditional agricultural practices and low-skilled labour, and require nothing more than sunlight, water and fertiliser for growth [5,7]. The cost of scaling fermenter-based production up or down in response to demand can be high, whereas with plants the same can be achieved simply by cultivating more or less land. Another advantage of plants is that no plant pathogens are known to infect humans – after all, most people eat fresh or cooked plant material every day with no ill effects. In contrast, the potential presence of viruses, prions and other undesirable components in mammalian cell cultures and transgenic animals is an ever-present worry, which increases the cost of processing and purification. The presence of endotoxins in bacterial cultures can place similar limitations on the use of microbial production

systems. A disadvantage of field-grown plants is that it will be much more difficult to apply the principles of good manufacturing practice (GMP) given the variable environment in terms of climate, soil composition and weather. It remains to be seen how the rigid regulatory framework that currently governs fermenter-based production systems can be adapted for field-grown plants.

Another issue addressed by the use of plant production systems is the delivery and storage of medicines. The developed world is accustomed to readily available injections, pills and liquid preparations, and most of us take for granted the ability to store and distribute such formulations in the appropriate way to maintain their viability (e.g., chilled or frozen). In the developing world, without reliable storage or distribution networks, getting medicines to those most in need can be difficult. However, plants provide the ideal solution to this problem because proteins expressed in certain plant tissues (e.g., cereal seeds) remain stable for years at ambient temperatures without loss of activity, and certain classes of recombinant proteins can be consumed in raw fruits or leaves, or applied in the form of pastes derived from the plant material with minimal processing [1,7,9].

3. Existing technologies

As this article focuses on a production technology rather than a specific disease or drug class, this section describes existing platforms for recombinant proteins, and their advantages and disadvantages compared with transgenic plants. Many of the production systems described below are already used for the commercial manufacture of protein therapeutics, whereas some, like plants, are emerging technologies that have yet to see a product reach the market.

3.1 Bacterial production systems

Although many different bacteria have been investigated as potential expression hosts for pharmaceutical production, the bacterial field is still dominated by the laboratory workhorse *Escherichia coli* [13,14]. This was the first bacterium used to produce a recombinant human protein in the laboratory (somatostatin, in 1977), and was the first to be used for the production of a commercial therapeutic protein (recombinant human insulin, approved in 1982 and marketed by Eli Lilly & Co. under licence from Genentech). Another early success was human growth hormone, which was launched in 1985 and marketed by Genentech as Protropin®. These products are typical of protein therapeutics produced in bacterial systems in that they are relatively simple glycosylated proteins that accumulate as inclusion bodies within the bacterial cell and must be subjected to *in vitro* denaturation and refolding. The *E. coli* system has been superseded in many cases by mammalian cells because the latter can produce more complex proteins and achieve correct folding and carry out post-translational modification *in vivo*. However, *E. coli* is much cheaper to cultivate than mammalian cells and more recent

advances such as low gene dosage systems that facilitate the expression of soluble proteins [15], and targeting systems that direct recombinant proteins to the periplasm [16], have been very successful. Mammalian proteins targeted to the bacterial periplasm are more likely to fold correctly because this compartment has the ability to form and isomerise disulfide bonds. An alternative strategy, which has also been successful, is the expression of protein disulfide isomerases in the bacterial cytosol along with the recombinant protein of interest [17].

3.2 Yeast and filamentous fungi as production systems

Yeast cells grow in a similar manner to bacterial cells and, like bacteria, require simple and relatively inexpensive media for growth. However, they are eukaryotes and are, therefore, able to fold and assemble complex recombinant proteins much more efficiently than bacteria. The secretion of recombinant proteins from cultured yeast cells allows the formation of disulfide bonds, proteolytic maturation, N- and O-linked glycosylation and other post-translational modifications that occur either not at all or very inefficiently in bacteria. *Saccharomyces cerevisiae* was the first yeast used for recombinant protein production, a choice based on the widespread use of this organism as a laboratory model [18]. It is used commercially for the production of the hepatitis B virus (HBV) vaccine, which is a subunit vaccine based on the HBV surface antigen [19].

Unfortunately, as a general production system for recombinant pharmaceuticals, *S. cerevisiae* suffers from a number of limitations including low product yield, poor plasmid stability, difficulties in scaling up production, the hyperglycosylation of recombinant human glycoproteins, and inefficient secretion [20]. Although improved *S. cerevisiae* strains have been described, some of which are capable of human-compatible high-mannose type glycosylation [21], other yeast species have been developed as alternative production hosts. These include another popular model organism – *Schizosaccharomyces pombe* – as well as the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha*, the dairy yeast *Kluyveromyces lactis*, and others such as *Schwanniomyces occidentalis* and *Yarrowia lipolytica*. These organisms often out-perform *S. cerevisiae* in terms of yield, reduced hyperglycosylation and secretion efficiency [20]. The methylotrophic yeasts are now emerging as competitive production systems [22]. A recent report has shown how proteins with humanised glycan structures can be synthesised in *P. pastoris* [23].

Filamentous fungi have also been explored as production systems for recombinant therapeutic proteins, reflecting their high capacity for protein secretion. Furthermore, many filamentous fungi are already used in the food industry and, therefore, have 'generally regarded as safe' status [24,25]. Heterologous gene expression was first achieved in filamentous fungi using the laboratory organism *Aspergillus nidulans*, but can now also be carried out in a variety of industrially important species, such as *A. niger*, *A. oryzae*, *Trichoderma reesei*, *Acremonium chrysogenum* and *Penicillium chrysogenum* [25]. The use of yeast and filamentous fungi for the production of human therapeutic proteins has recently been reviewed [26].

3.3 Mammalian cells as production systems

Mammalian cells have dominated the biopharmaceutical industry since the mid 1990s for one simple reason: they can produce authentic complex recombinant glycoproteins that fold *in vivo* and are functionally and, in many cases, structurally identical to their native counterparts [12]. Despite the economy and efficiency of the yeast and fungal systems mentioned above, there are major differences in the glycan structures of native human proteins and recombinant proteins produced by fungal cells. Therefore, although they are much more expensive to maintain, mammalian cells are used to produce the majority of today's pharmaceutical proteins, including all antibodies [27]. Indeed, more than half of all biological products – therapeutics, diagnostics and vaccines – approved by the Federal Drug Administration (FDA) are produced in mammalian cells. There are several principal cell lines of choice: Chinese hamster ovary (CHO) cells, the murine myeloma cells lines NS0 and SP2/0, BHK and HEK-293 cells, and the human retinal line PER-C6. These have been used to produce nearly all the mammal-derived recombinant therapeutic products licensed by the FDA up to June 2004 [27,28]. The yield of recombinant protein from mammalian cells is in the g/l range in the very best cases [28]. This situation is continuously improving thanks to the development of better expression vectors, gene amplification systems (e.g., the dihydrofolate reductase marker with methotrexate selection in CHO cells) and the identification of genomic integration sites that are permissive for transgene expression. Despite progress towards the use of serum-free medium, the major disadvantage of mammalian cells remains the cost of production, particularly reflecting downstream processing costs. Further disadvantages include the limited capacity for scale-up and the risk of contamination with human pathogens. Finally, it should be noted that although mammalian cells can synthesise typically mammalian glycan structures, these are often variable and dependent on culture conditions as well as the recombinant protein itself. There has been a considerable effort to optimise the production of high-quality and homogeneous recombinant glycoproteins by manipulating the glycosylation process through the overexpression of glycosyltransferases and other enzymes [29].

3.4 Transgenic mammals as production systems

Transgenic mammals can produce recombinant therapeutic proteins in their body fluids, providing a renewable source of the product so that periodic harvesting is possible without killing the animals [30,31]. Although most proteins have been expressed using mammary-gland-specific promoters and targeting signals that allow secretion in the milk, various other body fluids have been investigated as expression systems, including blood serum, urine and seminal fluid [32]. Milk is the most popular expression system because it is produced in large amounts and can be drawn regularly without affecting the animal's health, providing the potential for very high protein yields [33]. Many farm species and other domestic

mammals have been considered as expression hosts [32,33], but mice are often used as the test species because the technology for mouse transgenesis is advanced and transgenic mice can be produced inexpensively in a matter of weeks. However, the amount of milk generated by mice is low, as is the total yield of recombinant protein. For commercial production, attention has focused on rabbits, cows, pigs, sheep and goats, which produce much larger volumes of milk. For example, a humanised version of the BR96 anti-Lewis Y monoclonal antibody developed by Bristol-Myers Squibb for cancer therapy has been produced in goats. Expression levels were in the range of 0.1 – 14 g/l, the antibody was functional and it could be isolated and prepared to a purity of > 99% [34].

Although the productivity of animal bioreactors is high, the breeding and maintenance of transgenic animals is time-consuming and expensive, and the regulatory framework is restrictive. Most transgenic embryos are lost during the procedure and many of the transgenic animals that survive to term do not express the transgene at sufficient levels and have to be sacrificed. A further disadvantage is the complexity of the body fluids in terms of protein composition, which makes extraction and purification more difficult. Moreover, as discussed above for mammalian cells, the purification of pharmaceutical proteins from animal body fluids carries the risk of pathogen contamination. Finally, pharmaceutical proteins have the potential in some cases to affect the health and physiology of the production host.

3.5 Recombinant protein production in hens' eggs

Domesticated chickens lay as many as 300 eggs each year, each containing about 6 g of protein [35]. Although there have been few studies thus far, it has been estimated that a flock of 5000 chickens could produce 150 kg of recombinant protein per year, assuming 100 mg of recombinant protein per egg (0.1 – 0.2% total egg protein) [36]. This yield is a realistic goal given that 2 g of each egg is made up of the protein ovalbumin, which is the product of a single gene. Chickens have a shorter generation time than most domestic mammals and the production and housing of transgenic chickens is less expensive than herds of cows, goats, pigs or sheep. Furthermore, the glycan structures of chicken glycoproteins are closer in some respects to human glycoproteins than those of goats, sheep and cows [37]. However, further evaluation of this production system will be required before it can be judged against the more-established transgenic mammal systems.

3.6 Insect production systems

Recombinant proteins can be produced in insect cells either by stable transformation (analogous to the mammalian cell systems discussed above) or by infection with recombinant baculovirus vectors [38]. The baculovirus system has been used for many years for high-yield transient expression of recombinant proteins in insect cells, routinely achieving yields of > 200 mg/l of culture [39,40]. However, virus infection interferes with the secretory pathway so that the yields of secreted

proteins are much lower than those of intracellular proteins. In contrast, the stable transfection of insect cells with plasmid vectors leads to lower short-term yields, but higher yields of secreted proteins over a longer timescale [41]. The major advantages of insect cells over mammalian cells are the reduced costs of the culture medium and the relative ease of scale-up, as has been shown for the production of human tissue plasminogen activator [42]. However, as is the case with yeast cells, there are significant differences in the glycan structures of recombinant human glycoproteins produced in insect cells, and attempts have been made to address this issue through the co-expression of galactosyltransferases and sialyltransferases [43,44]. More recently, the potential of silkworm larvae as production hosts for human collagen has also been explored [45].

4. Current research goals

Notwithstanding the benefits of transgenic plants as a production system, several issues need to be addressed in order for this technology platform to become widely accepted as a mainstream alternative to microbial and mammalian cell fermentation. First and foremost, it will be necessary to increase the yields of recombinant proteins that can be achieved using transgenic plants, as in most cases these fall below the threshold required for commercial viability. A standardised system for reporting yields also needs to be agreed by the community. At the current time, researchers in different laboratories present their yields in various ways that are difficult to compare directly (e.g., as percentage total soluble protein, or as yield/g fresh weight or dry weight, or as units of enzyme activity/g fresh or dry weight, or as a concentration in exudates or culture supernatant). Researchers are also breaking away from the idea that achieving high expression levels is the be all and end all of molecular farming. Attention is turning to concepts such as improving protein stability, and improving the downstream processing stages of production to increase protein quality and batch-to-batch consistency. In this context, the importance of protein glycosylation cannot be understated. Although plants and mammals add glycan chains to the same positions on recombinant proteins, the precise structure of the glycans is distinct. This, together with the problem of heterogeneous glycosylation, needs to be addressed before plants can be considered as suitable for production under GMP conditions. The products themselves also need to be subjected to a battery of tests for toxicity, stability and allergenicity to ensure they are 'biosimilar' to equivalent recombinant products synthesised in fermenter-based systems. Another widespread public concern about the use of transgenic plants as pharmaceutical factories is that of biosafety, which is based on fears of potential transgene spread in the environment, pollution of the human food chain with recombinant plant material and the unknown effects of transgenic plants expressing pharmacologically active molecules on non-target organisms in the environment. These issues can be resolved through appropriate advances in

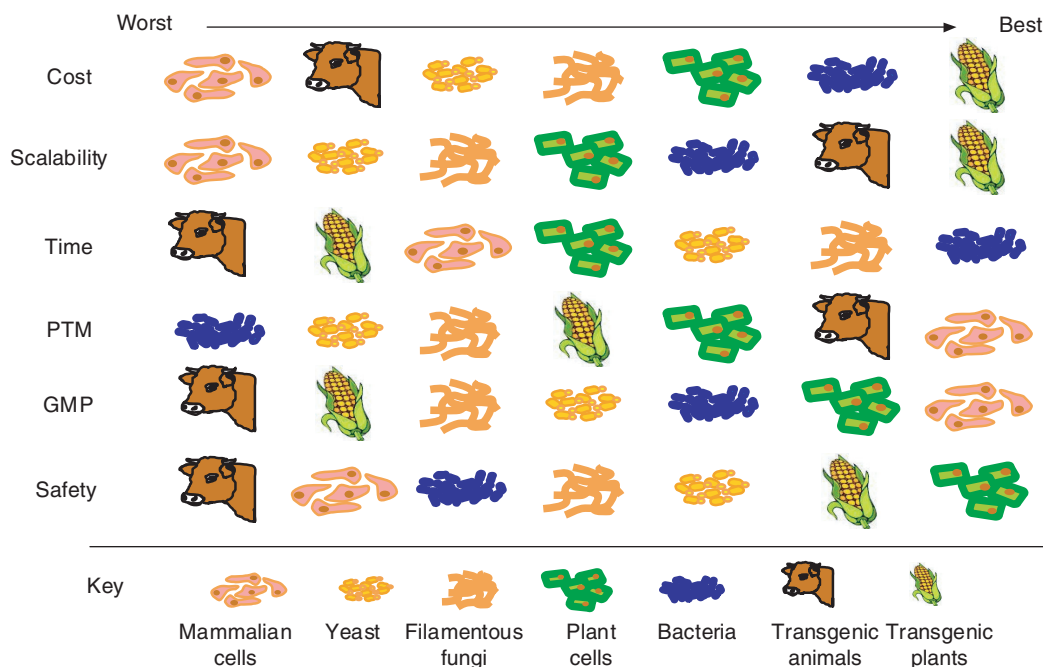


Figure 1. Benefits and limitations of some of the different production systems for recombinant proteins. Most recombinant human proteins are produced either in mammalian cells or in microbial fermenter systems (bacteria, yeast, or filamentous fungi). Undoubtedly, mammalian cells and transgenic mammals are more likely to produce recombinant human proteins that closely resemble the structure and glycosylation pattern of native human proteins, as the host cells are equipped with the correct apparatus for authentic post-translational modification. However, mammalian systems are among the most expensive to establish and maintain, and there is the ever-present risk of contamination with viruses and other pathogens. Microbial cultures are less expensive to set up and maintain, but they may not carry out authentic post-translational modifications and so cannot be used to produce complex proteins and glycoproteins. Bacteria also synthesize endotoxins that must be removed during processing, adding significantly to production costs. The use of terrestrial plants addresses many of these economical, quality and safety issues, but it is more difficult to conform to GMP requirements using field plants, which is why cultured plant cells have the greatest combined benefits for pharmaceutical product safety. GMP: Good manufacturing practice; PTM: Posttranslational modification.

formulation, quality assurance and control, regulatory approval and stewardship with consumers and non-governmental organisations (NGOs) and waste disposal.

5. Scientific rationale

5.1 Economic considerations

As stated above, it is predicted that the major advantage of transgenic plants for pharmaceutical production will be the comparatively low cost of large-scale production. Figure 1 compares the relative merits of the major existing production systems, including the costs of set up and maintenance. Both the capital investments and running costs required for transgenic plants are significantly lower than those of cell-based production systems and it has been 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 lines, depending on the yield [9]. The actual cost saving depends on the required purity, as the majority of production costs lie not in the production system itself, but in the downstream processing steps required to extract and purify the pharmacologically active ingredient.

The fewer processing steps required, the lower the cost. Therefore, plants are probably the best system in economic terms for the production of oral vaccines, as only minimal processing is required – the product can be administered as unprocessed or partially processed plant material [46]. For such products, the yield of protein becomes critical, as this determines the amount of plant material that constitutes a dose of the vaccine, and the number of doses required. For the production of other clinical grade proteins, such as blood products, cytokines, enzymes and antibodies, the processing steps are similar to those of any other production system, and the savings must be made upstream in the generation, growth and harvesting of the transgenic material.

Downstream processing costs in plants can be minimised through the exploitation of particular tissues, or the use of specific expression technologies. The cost of processing is related to the concentration of the product in the starting material, so the yield of protein as a proportion of plant biomass is very important. Recombinant protein expression in cereal seeds is advantageous in this context because the product is concentrated in a small tissue volume that has a fairly simple composition compared with vegetative tissues

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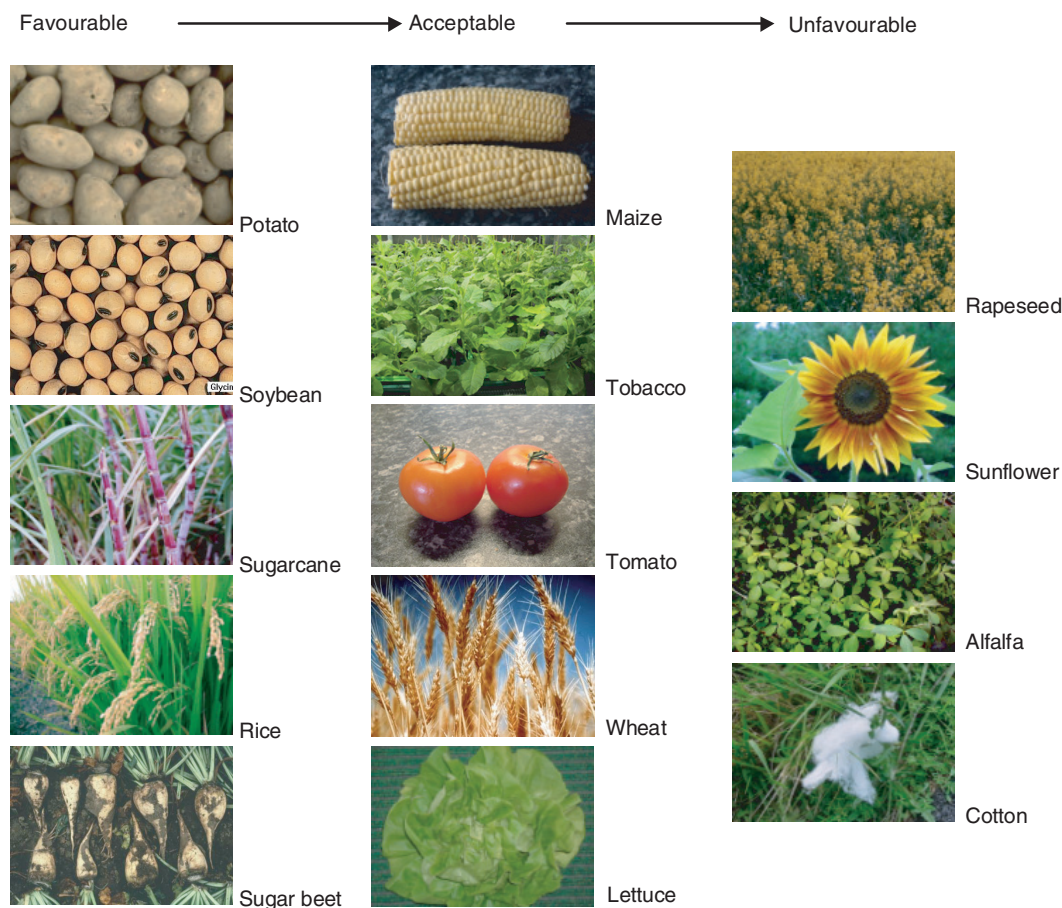


Figure 2. Suitability of different crops for the production recombinant proteins, from a regulatory perspective. The most favourable crops have beneficial features for environmental safety and regulatory compliance (e.g., male-sterility in potato, self-pollination in rice). Sugar beet is only favourable in the first year, when it undergoes vegetative growth. The least favourable plants show lower yields and have undesirable features such as open pollination, sexually-compatible wild relatives and so on. Note that the most suitable species in terms of biosafety are not necessarily those with the best features for production (e.g., alfalfa, which is advantageous due to its homogenous glycans, is bee-pollinated and is sexually compatible with local wild relatives, whereas soybeans, which are self-pollinating and isolated, suffer from excessive protein degradation).

[47,48]. Different aspects of plant physiology can also be exploited to simply downstream processing. For example, SemBioSys Genetics, Inc. has developed a proprietary technology used with oilseed crops in which the target protein is expressed as a fusion with oleosin, an endogenous protein that is restricted to oil bodies. The fusion protein can be recovered from oil bodies using a simple extraction procedure and the recombinant protein separated from its fusion partner by endoprotease digestion [49]. In a similar approach developed in the authors' laboratory, recombinant proteins are expressed as fusion constructs containing an integral membrane-spanning domain derived from the human T cell receptor [50]. The recombinant protein accumulates at the plasma membrane and can be extracted in a small volume using appropriate buffers and detergents. A rather different approach is the excretion of recombinant proteins into the root exudates or leaf guttation fluid of transgenic tobacco plants. This system

is being developed by Phytomedics, Inc., initially for the production of human secreted alkaline phosphatase [51,52], but now also for the production of recombinant antibodies and other proteins [53]. The requirement for hydroponic facilities limits the scale of this system, but it may prove useful for the production of diagnostic antibodies and pharmaceuticals with small, specialised markets.

5.2 Product yields – choice of plant species and platform

The species used for production and the type of production platform each play a significant role in the final yield of recombinant protein. A decision must be made to use terrestrial plants, aquatic or unicellular plants, or plant cell suspensions. Terrestrial plants have the advantage of scalability, whereas the others offer containment and the ability to use defined growth conditions that will be more likely to comply

with GMP. Among the terrestrial plants, a decision must be made to use either food or non-food crops, and then to use leafy crops, cereals, fruit/vegetable crops, fibre crops or oilseeds.

Tobacco is the most widely used leafy crop because it has a massive biomass yield and well-established procedures for gene transfer and expression. As a non-food crop, pharmaceutical tobacco material is unlikely to contaminate the human food chain, even if the existing infrastructure for large-scale tobacco cultivation, harvesting and processing is exploited. One disadvantage of tobacco, however, is the presence of toxic alkaloids in vegetative tissues (although there are low-alkaloid varieties that can be used for the production of pharmaceutical proteins). Another is the heterogeneous glycan structures that are produced by tobacco cells.

Alternative leafy crops for pharmaceutical production include alfalfa, lettuce and spinach. Alfalfa is a legume, and its ability to fix nitrogen is beneficial in terms of reduced input costs [54]. Soybean is also a legume, and although typically classed as a seed-based production vehicle, the only pharmaceutical to be expressed in soybean thus far has been expressed in the leaves. Both alfalfa and soybean have been used to produce a number of pilot products including recombinant antibodies [55,56]. Like tobacco, these crops have a high biomass yield. Alfalfa has been shown to produce homogeneous glycans, which may make it more compatible with GMP conditions than tobacco [57]. However, alfalfa is low on the list of desirable crops for biosafety reasons – it is mostly cross-pollinated and has wild relatives in areas where it is cultivated – whereas soybean is more desirable because it is self-pollinating and has no wild relatives, at least in the US (Figure 2). Even so, alfalfa is being developed as a proprietary production system by the Canadian biotechnology company Medicigo, whereas soybean is no longer being pursued as a production system by most companies. Lettuce, as an edible salad crop, is suitable for the production of oral vaccines and has been used in one series of clinical trials for a vaccine against hepatitis B virus [58]. It has a higher biomass yield than alfalfa and, like soybean, is favourable in biosafety terms because it is usually self-pollinating. Spinach has not been used as a transgenic production platform, but as a host for recombinant plant viruses expressing vaccine antigens [59]. These have been used in clinical trials as discussed later.

Although favourable in terms of biomass yield, perhaps the greatest common disadvantage of leafy crops is that recombinant proteins are synthesised in an aqueous environment and are subject to rapid proteolytic degradation after harvesting [5]. To maintain high yields, foliage must be processed on-site, or transported as dried or frozen material adding considerably to production costs. Furthermore, oxidising substances released from tobacco and alfalfa leaves during initial grinding and extraction can interfere with downstream processing. Finally, it should be noted that the expression of active recombinant proteins in vegetative tissues, such as leaves, has the potential to interfere with plant growth and development,

although this has not been demonstrated thus far for any pharmaceutical protein. This is not the case with the seed crops discussed below.

In contrast to leafy crops, the expression of proteins in cereal or grain legume seeds allows long-term storage, even at room temperature, because mature seeds are desiccated and have evolved to accumulate storage proteins in a stable environment. It has been demonstrated that antibodies expressed in seeds remain stable for at least 3 years with no detectable loss of activity [60]. Cereal seeds also lack the oxidising compounds present in tobacco and alfalfa leaves, thus improving the efficiency of downstream processing. Furthermore, because seeds are not vegetative organs, potentially toxic proteins accumulating in seeds will not affect vegetative plant growth. Despite these advantages, seeds can be regarded as viable genetically-modified organisms in their own right, which could have regulatory implications. Four cereal species have been evaluated as production systems for pharmaceutical proteins: maize, rice, wheat and barley. Maize is the most widely adopted and has been used as the major production crop by a number of molecular farming companies (including Prodigene, Inc., which used maize to produce the first commercial recombinant proteins from plants – the technical reagents avidin, trypsin and β -glucuronidase). This largely reflects the fact that maize is the most widely grown crop in North America, is easy to transform and manipulate in the laboratory, has the largest biomass yield of the four species and is the easiest to scale up in the field. However, maize is also a cross-pollinating species and a food crop. For use in molecular farming, research is ongoing into the use of visibly distinct varieties that are unlikely to be mixed accidentally with food. Rice and barley have been developed as production hosts by other companies (e.g., Ventria Bioscience for rice and Maltagen and ORF Genetics for barley). High product yields have been demonstrated for various pharmaceutical products in all three cereals, whereas lower yields have been achieved in wheat and pea. Rice, wheat, pea and tobacco have been compared in a head-to-head study with the same recombinant antibody expressed using the strongest promoter available for each host plant [61]. Rice plants showed the highest overall yields per unit biomass, but tobacco had a higher overall yield due to the greater biomass output per hectare.

Fruit and vegetable crops have also been investigated as production systems, mainly for oral vaccines because fruits and many vegetables can be eaten raw or as partially processed tissue. Potato has been developed as the major model system for vaccine production [62-65] and has been used in three of the six clinical trials involving vaccines from transgenic plants [62,63]. Potatoes have also been used as a general production host for antibodies [66,67], other biopharmaceuticals based on antibodies [68] and human milk proteins [69,70]. Tomato has been used for the production of several candidate vaccines (e.g., [71-73]) as well as recombinant antibodies [61]. This fruit is potentially advantageous because it has a high biomass yield per hectare and is grown in glasshouses, offering better

containment than other fruits and vegetables. Carrot may also be useful as a vaccine-producing crop because the taproot is a natural storage organ and is the edible portion of the plant, which can be consumed raw. Several antigens have already been expressed in carrot, including the *Mycobacterium tuberculosis* MPT64 protein [74], glutamic acid decarboxylase [75] and derivatives of measles haemagglutinin [76,77]. Finally, bananas are attractive vehicles for oral vaccine distribution because this fruit is used in Africa to prepare baby food and is also eaten by adults [78].

Various other crops have been considered as hosts for recombinant protein expression, including oilseeds, cotton, sugarcane and sugar beet. One advantage is that by-products can be used to offset the price of production (e.g., specialty sugars from cane juice from which the recombinant protein has already been extracted). One downside is that some of these by-products, especially oils and fibres, can interfere with downstream processing, although this is turned to an advantage in the SemBioSys oleosin-fusion technology used in canola and safflower as described above. Another potential problem is that regulatory hurdles may prevent the use of by-products from transgenic plants producing pharmaceuticals.

5.3 Product yields – strategies to enhance transgene expression and protein accumulation

The intrinsic production capacity of the chosen expression platform is a property that cannot be modified easily because it is dependent on the overall biomass yield of the crop. However, the specific yield of recombinant protein per unit of plant biomass can be influenced by the optimisation of transgene expression, which is achieved through expression construct design. Perhaps the most important component of the expression construct is the promoter used to control the transcription of the transgene. For dicotyledonous species such as tobacco, potato and tomato, the strong and constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) is often chosen to drive transgene expression [79]. In cereals, the CaMV 35S promoter has a lower activity and the maize ubiquitin-1 (*ubi-1*) promoter is preferred [80]. Regulated promoters can be used in preference to constitutive promoters to improve practicality and biosafety. For example, although constitutive promoters allow high-level accumulation of recombinant proteins in seeds, the proteins are also expressed in leaves, pollen and roots. The use of seed-specific promoters largely restricts recombinant protein accumulation to the seeds, so the vegetative organs do not accumulate detectable levels of the recombinant protein. This increases the biosafety of the plants, as adventitious contact with non target organisms is unlikely [81]. Indeed, the use of inducible promoters [82] means that recombinant protein synthesis can be delayed until just before harvest, or even after harvest, as is the case for the CropTech MeGA promoter system (mechanical gene activation), which employs a wound-inducible promoter to activate gene expression when the harvested tobacco leaves are shredded prior to protein extraction [83]. However, many of

the regulated promoters that have been described show some leakiness; for example, many nominally seed-specific promoters are also active in pollen.

After promoter choice, the next most important aspect of construct design is the inclusion of sequences that control sub-cellular targeting of the protein. This is a general method to increase the yield of recombinant proteins because the compartment in which a recombinant protein accumulates influences its folding, assembly and post-translational modification [5,7]. 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 [84,85]. The endoplasmic reticulum (ER) provides an oxidising environment and an abundance of molecular chaperones, whereas there are few proteases. Proteins are directed to the secretory pathway using either a heterologous or endogenous signal peptide, located at the N-terminus of the native protein. Such proteins are cotranslationally imported into the ER and are eventually secreted to the apoplast, a supracellular network of interlinked compartments underlying the cell wall. Depending on its size, a protein can be retained in the cell wall matrix or it can leach from the cell. Although the majority of recombinant proteins are generally more stable in the apoplast than the cytosol, they are even more stable in the ER lumen. Therefore, antibody expression levels can be increased even further if the protein is retrieved to the ER using an H/KDEL C-terminal tetrapeptide tag in addition to the signal peptide [86]. Accumulation levels are generally 2- to 10-fold greater compared with an identical protein lacking the KDEL signal [87]. As an added benefit, antibodies retrieved in this manner are not modified in the Golgi apparatus, which means they possess high-mannose glycans, but not plant-specific xylose and fucose residues [88].

High-level recombinant protein expression has also been demonstrated following transformation of the chloroplast genome in higher plants [89]. Early work focussed on the tobacco plastid genome and several examples of pharmaceutical protein production in tobacco chloroplasts have already been reported. Expression of human growth hormone in tobacco chloroplast has been reported at levels approaching 8% total soluble protein (TSP) [90]. Similarly, human serum albumin has been produced at levels of > 11% TSP [91], and production of the cholera and tetanus toxin fragments at levels up to 25% TSP has been reported [92,93]. Further advantages of the chloroplast system are the natural containment both in terms of the transgene (functional chloroplast DNA is not found in the pollen of most crops, preventing transgene spread by outcrossing) and the product itself, which accumulates within the chloroplast, thus limiting its effects on the host cell. Limitations include the absence of reliable chloroplast transformation systems other than for algae and solanaceous plants, and the absence of glycosylation and other forms of post-translational modification in the chloroplast. Therefore, the chloroplast system is currently limited to the production of relatively simple aglycosylated proteins [89].

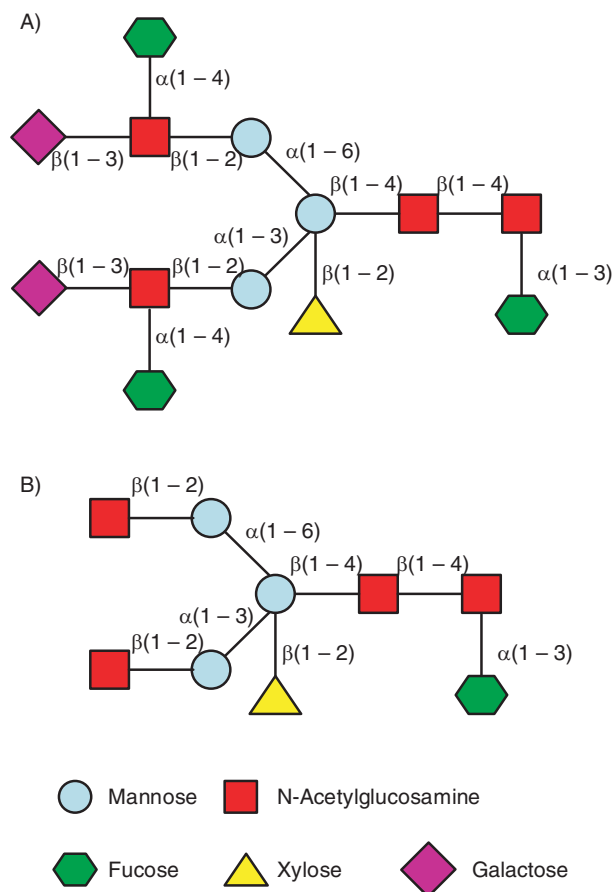


Figure 3. Two examples of complex glycans produced in plants. A) Galactose-extended complex glycan. **B)** Long-chain complex glycan. Note the presence of xylose residues and $\alpha(1,3)$ -linked fucose residues, which are not found in mammals.

Product yields can be increased not only through construct design, but also through the implementation of a selection and backcrossing programme once transgenic plants are available. In the case of maize, it has proven possible to increase the yield of a recombinant enzyme > 70-fold in only six generations, and to increase the yield of recombinant avidin > 150-fold in eight generations [94]. There is no reason why the same principles should not apply to pharmaceutical proteins.

5.4 Protein folding, assembly and glycosylation

The ability of plants to fold and assemble complex human proteins has been demonstrated by the successful production of functional serum antibodies using plants carrying two transgenes (encoding the heavy and light chain components, respectively). Such antibodies comprise four polypeptides – two heavy and two light chains – covalently joined by disulfide bonds [87,95]. More remarkably, plants also assemble functional secretory antibodies, which have 10 polypeptides representing 4 different polypeptide chains (the heavy and light chains, and additional joining chain and secretory component subunits)

[96,97]. Two different cell types are required to produce and assemble such antibodies in mammals.

Although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for post-translational modification. Plants do not, for example, hydroxylate proline residues in recombinant collagen. There are also various differences in glycan structure: plant-derived recombinant human glycoproteins tend to contain the carbohydrate groups $\beta(1\rightarrow2)$ xylose and $\alpha(1\rightarrow3)$ fucose, which are absent in mammals, but generally lack the terminal sialic acid residues that are found on many native human glycoproteins (Figure 3). Because glycan structures can impact on the solubility, stability, immunogenicity and biological activity of recombinant proteins, the ‘humanisation’ of glycan structures produced in plants has been an important topic of research and debate in the scientific community. Although no plant-derived recombinant proteins have thus far demonstrated unintended immunogenicity in either the human or murine immune systems [98], there has been considerable interest in modifying the plant glycosylation pathway to humanise the glycan profile of recombinant proteins. Several changes in the pathway are required to produce proteins with typical human glycan structures [99]. Strategies used include the *in vitro* modification of plant-derived recombinant proteins by purified human $\beta(1,4)$ -galactosyltransferase and sialyltransferase enzymes [100] and the expression of human $\beta(1,4)$ -galactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans [101]. In the latter case, 30% of the antibody was galactosylated, 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. A recent report documenting sialylation in *Arabidopsis thaliana* suspension cells has been challenged, although the subject remains a matter of controversy [102-104]. To remove the non-mammalian $\beta(1\rightarrow2)$ xylose and $\alpha(1\rightarrow3)$ fucose residues, some researchers have explored the possibility of inhibiting the enzymes responsible for synthesising these groups; in one case this goal has been achieved in whole *A. thaliana* plants by gene knock-out techniques [105]. The moss *Physcomitrella patens* can also be modified by gene targeting to eliminate these enzymes [106].

5.5 Biosafety concerns

The biosafety of transgenic plants producing recombinant pharmaceuticals has a major impact on public opinion and, in turn, influences the political and regulatory framework governing pharmaceutical production and biotechnology in general. Specific biosafety risks fall into two categories, which can be described as the risk of transgene escape and the risk of unintended exposure to the recombinant protein [81]. The risk of transgene spread reflects the potential for transgene DNA sequences to spread outside the intended host plants and production site. This can result in the growth of transgenic crops in other cultivated fields or in non-cultivated areas, the spread

of foreign DNA to other plants (and possibly to microbes and animals), and the uncontrolled production of recombinant proteins in natural settings. Mechanisms of transgene spread include the dispersal of transgenic plants or seeds by human and animal activities or the weather, and outcrossing via transgenic pollen. The risk of unintended exposure concerns the potential for any organism (including humans) to come into contact with the recombinant protein produced by a transgenic plant. Many different mechanisms can be involved, including herbivory and parasitism, the exposure of pollinating insects to transgenic pollen, the exposure of microbes in the rhizosphere to root exudates, the exposure of non-target microbes and animals to proteins secreted in the leaf guttation fluid, the release of recombinant proteins by dead and decaying transgenic plant material, and the contamination of food or feed crops during harvesting, transport, processing and/or waste disposal.

An appropriate choice of production species can go a long way to prevent or minimise transgene spread by dispersal or vertical gene transfer. Certain plants have been singled out as inappropriate hosts by regulatory organisations [107,108]. For example, alfalfa and rapeseed have been highlighted as unsuitable for field cultivation because they are bee-pollinated, sexually compatible with abundant and local weed species and the seeds can lie dormant for several years, making volunteer plants difficult to isolate and destroy (Figure 2). Note that this does not compromise their merits as production hosts if they are grown under containment. Other species have been more readily accepted because of the lower risks of transgene spread; for example, potato (male sterile), sugar beet and rice (self-pollinating). The major current production crops – maize and tobacco – have intermediate status.

Given an appropriate choice of host species, the only way to fully avoid transgene spread from field plants to compatible crops and wild species is by containment. The aim of containment is to prevent seed and pollen dispersal, prevent the survival of dispersed seeds and pollen, or prevent gene flow from viable pollen. The containment may be physical and based on habitat barriers. For example, transgenic plants can be maintained in greenhouses, in artificially-irrigated desert plots miles from any other plants, or in underground caverns and caves [109]. Alternatively, the physical containment may be focused on individual plants. For example, flowers can be emasculated before viable pollen has developed (not an option for seed- or fruit-based production, but suitable for leafy or vegetable crops), or the flowers/fruits may be concealed in plastic bags. Isolation zones are often placed around transgenic crops. These can be barren, but a more suitable alternative for insect-pollinated crops is to provide a zone of non-insect-pollinated plants, which would discourage the insects from leaving the transgenic zone. Barrier crops (i.e., a border of non-GM plants of the same species as the transgenic crop) are also useful as these can absorb much of the pollen released by transgenic plants and can then be destroyed after flowering.

Biological containment measures provide additional barriers to gene flow and many different strategies have been tested. In some cases, natural genetic barriers have been exploited. For example, pharmaceutical production in self-pollinating species (e.g., rice, wheat, pea) or crops with no sexually compatible wild relatives near the site of production provide a first level of defence against gene flow. Similarly, crops with asynchronous flowering times or atypical growing seasons are useful. Cleistogamy (self-fertilisation before flower opening) is an extension of the above, and could be engineered into crops used for molecular farming by modifying the architecture of flower development. In practice, however, there is always a residual risk of outcrossing. Another potential strategy, yet to be fully explored, is the exploitation of apomixis (embryo development in the absence of fertilisation). Transformation strategies can also be adapted to take advantage of natural barriers; for example, chloroplast transformation (which prevents gene flow by outcrossing, see above) and genomic incompatibility, which is suitable for polyploid species such as wheat that hybridise with wild relatives (in this approach, the transgene is placed on a wheat chromosome that does not contribute to the genome of the hybrid offspring). These natural mechanisms may be augmented with artificial genetic strategies including male sterility, transgene mitigation (tight linkage between the transgene encoding the recombinant protein and another gene that confers a selective disadvantage on wild plants carrying the gene, but not those cultivated under defined conditions) and genetically controlled seed sterility. As well as the transgenes encoding the pharmaceutical products of interest, marker genes used to facilitate plant transformation may also spread by the mechanisms discussed above. However, unlike the pharmaceutical transgenes, such markers are no longer necessary once the transgenic line has been established. For this reason, a suitable strategy to prevent the spread of marker genes is to excise these genes from the transgenic lines. The removal of marker genes from nuclear transgenics has been reviewed in detail [110] and has also been achieved in plastid transformants [111].

There are numerous genetic strategies to avoid unintended exposure both by 'natural' processes such as adventitious herbivory, and by human activities such as the unintentional mixing of transgenic and non-transgenic crops during harvesting, transport, refining and processing. Unintended exposure during cultivation can be prevented using restricted promoters, especially systems such as the CropTech postharvest expression technology discussed above. The protein can also be expressed as an inactive precursor which is safe if consumed accidentally. To avoid contamination by human error, there should be a clear distinction between pharmaceutical and food plant material. The best way to ensure segregation is through the use of non-food crops such as tobacco for production, because these never come into contact with food. Where food crops are used, a rigorous series of regulatory practices should be in place from the farm to the factory, ensuring complete isolation of

transgenic material during growth, harvesting, transport, storage, processing, extraction and waste disposal, and this should be supported by validated procedures for cleaning shared equipment. An important step towards segregation is identity preservation through the use of visually distinct non-food varieties for pharmaceutical production, such as purple maize [81].

6. Competitive environment

Because the use of transgenic plants for the production of therapeutic proteins is an emerging technology, there are relatively few plant-derived therapeutic proteins in clinical trials, but there are many such proteins in development and many others at the experimental stage in the laboratory. Medically-relevant proteins made in plants are often subdivided into three convenient categories – vaccines, recombinant antibodies and ‘all others’, the latter group including blood products, growth factors, cytokines, enzymes and structural proteins. Tables 1, 2 and 3 constitute a therapeutic class review for plant-derived recombinant proteins and list the recombinant protein products made in plants as reported in the scientific literature and announcements from industry and academia up to the end of September 2004. Table 4 is an overview of some of the companies (past and present) with major interests in the use of plants for the production of recombinant proteins (the information in the table is incomplete because only information generally available to the public, e.g., through press releases and websites, is reported). The rest of this section briefly summarises the features of some of the front runners, (i.e., those that are currently undergoing clinical trials and are the closest to commercialisation and regulatory acceptance).

6.1 Plant-derived vaccines – veterinary and clinical trials

Plant-derived vaccines can be divided into two categories – those designed for veterinary use and those designed for medical use. Veterinary vaccines are likely to become the first plant-derived recombinant vaccines to reach the market, given the recent press release by Dow AgroSciences concerning their proposal to commercialise a poultry vaccine by 2006. There is now a large body of both immunogenicity and challenge data to support the efficacy of such vaccines. In a number of reports, plant-derived recombinant subunit vaccines have protected animals against (in some cases lethal) challenges with the pathogen; for example, canine parvovirus [112], foot and mouth disease virus [113,114], chicken infectious bronchitis virus [115,116], mink enteritis virus [117], murine hepatitis virus [118] and rabbit haemorrhagic disease virus [119,120]. Clinical trials conducted by ProdiGene, Inc., a biopharmaceutical company based in College Station, TX, have demonstrated for the first time that an oral vaccine expressed in plants gives protection against a virulent viral pathogen in livestock. The trials were conducted on swine using an edible form of a vaccine for transmissible gastroenteritis virus [121].

There have been six human clinical trials involving plant-derived, recombinant oral vaccines, all of which have been successful in that they produced serum and/or secretory antibody responses against the antigen in the test subjects [58,59,62,63,122]. Tacket *et al.* [62] performed the first such trial with transgenic potatoes expressing the enterotoxigenic *E. coli* labile toxin B-subunit (LTB), which is one of the most potent known oral immunogens. The LTB content of the tubers varied in the range of 3.7 – 15.7 µg/g fresh weight. Fourteen volunteers were given either transgenic or non-transgenic potato on days 0, 7 and 21 of the trial. Almost all of those consuming the transgenic potatoes showed at least fourfold increases in serum IgG against LTB, whereas no such increase was seen in those consuming the non-transformed potatoes. Five of these individuals also demonstrated at least a fourfold increase in anti-LTB IgA, detected in stool samples. There were few side effects, such as nausea and diarrhoea. A more recent trial using LTB expressed in processed corn seed produced similar results to the potato study [122].

Tacket *et al.* also described the results of a clinical trial performed using transgenic potato tubers expressing the Norwalk virus capsid protein (NVCP) [63]. Twenty adult volunteers were given two or three 150-g doses each of raw transgenic potato tuber containing 215 – 750 µg NVCP. Despite the fact that only 50% of the NVCP subunits assembled into virus-like particles in the potato cells, thus reducing the effective dose of the vaccine, nearly all of the volunteers showed significant increases in the numbers of IgA-antibody forming cells (AFCs), and six of these individuals also showed increases in IgG AFCs. There were also noticeable increases in serum antibodies against NVCP and stool IgA antibodies in a few of the participants [63].

A clinical trial has also been carried out using orally delivered HBV surface antigen produced in lettuce [58]. Two of three volunteers who were given two 150-g doses of transgenic lettuce containing about 2 µg of the antigen per dose, produced protective serum antibodies after the second dose, although the titres declined in a few weeks. Even so, the study confirmed that naive subjects could be seroconverted by the oral delivery of a plant-derived viral antigen. A similar trial in the US involved the HBV surface antigen expressed in transgenic potatoes, although participants in this trial had already been seroconverted with the standard, yeast-derived vaccine [64]. Of 33 participants given either two or three 1-mg doses of the antigen, about half showed increased serum IgG titres against the virus.

Finally, Yusibov *et al.* [59] have carried out a trial involving 14 volunteers given spinach infected with alfalfa mosaic virus vectors expressing the rabies virus glycoprotein and nucleoprotein. Five of these individuals had previously received a conventional rabies vaccine. Three of those five and all nine of the initially naive subjects produced antibodies against rabies virus, whereas no such response was seen in those given normal spinach. It should be noted that the

spinach used in this trial was not strictly transgenic, as the recombinant protein was produced by an engineered plant virus.

6.2 Plant-derived antibodies in clinical trials

Three plant-derived antibodies have been tested in Phase II clinical trials. One of these is a full-length IgG specific for EpCAM (a marker of colorectal cancer), which is produced in maize and developed as the drug Avicidin® by NeoRx and Monsanto. Although Avicidin demonstrated some anticancer activity in patients with advanced colon and prostate cancers, it was withdrawn from Phase II trials in 1998 because it also resulted in a high incidence of diarrhoea [123]. This was not due to the use of plants as the expression host. Indeed, the same problems occurred with antibodies produced in mammalian cells, and the conclusion of the trial was that the two antibodies were comparable in terms of physicochemical properties, serum clearance, urine clearance and dosimetry. The side effects were possibly due to crossreaction with related epitopes on the cells lining the intestine.

The most advanced plant-derived antibody is CaroRx™, a chimeric secretory IgA/G produced in transgenic tobacco plants, which has completed Phase II trials [124]. As stated earlier, secretory antibody production requires the expression of four separate components, which in this case were initially expressed in four different plant lines that were crossed over two generations to stack all the transgenes in one line. The antibody is specific for the major adhesin of *Streptococcus mutans*, the organism responsible for tooth decay in humans. Topical application following elimination of bacteria from the mouth helped to prevent recolonisation by *S. mutans* and led to the replacement of this pathogenic organism with harmless endogenous flora.

The production of anti-idiotypic antibodies recognising malignant B cells is a useful approach for the treatment of diseases such as non-Hodgkin's lymphoma. McCormick and colleagues [125] produced a plant-derived scFv based on the well-characterised mouse lymphoma cell line 38C13. When administered to mice, the scFv stimulated the production of anti-idiotypic antibodies capable of recognising 38C13 cells. This provided immunity against lethal challenge with the lymphoma. It is envisaged that this strategy could be used as a rapid production system for tumour-specific vaccines customised for each patient and capable of recognising unique markers on the surface of any malignant B cells. The therapeutic product has now completed Phase I trials and is undergoing Phase II. The rapid derivation of such prophylactic antibodies is ensured by the use of virus-infected plants rather than stably-transformed transgenic plants.

6.3 Other plant-derived biopharmaceuticals in clinical trials

The only other reported clinical trial involving a plant-derived recombinant protein is the Phase I trial of canine gastric lipase, produced in maize by Meristem Therapeutics. This

safety study involved eight healthy volunteers (four males, four females) and was designed to assess safety and tolerance of the protein after single and repeated oral administration. Safety results of this Phase I study showed that the oral administration of gastric lipase, either as single doses or as three doses per day for 10 days to healthy male and female subjects was well tolerated. Gastric lipase is currently undergoing Phase IIa clinical trials in Germany and France, and is expected to be launched on the market in Europe in 2005. It will be used to treat exocrine pancreatic insufficiency, which occurs in pancreatic disorders, after surgery and as one of the symptoms of cystic fibrosis.

7. Potential development issues

7.1 Downstream processing

Downstream processing, which involves the isolation and purification of the recombinant product, is an integral part of every biomanufacturing process. Whichever production system is used, downstream processing represents up to 80% of overall production costs, although this depends on the required level of purity and 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 standardised approach (e.g., affinity chromatography to isolate recombinant antibodies [126]). Several aspects of downstream processing have to be customised specifically for plant systems, including the removal of fibres, oils and other by-products from certain crops, and process optimisation for the treatment of different plant species and tissues.

For the production of clinical grade proteins, downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regimen of quality assurance and quality control to achieve the approval of regulatory agencies [127]. Regulatory guidance for biopharmaceutical production in plants currently exists only as draft legislation, published in 2002 by both the FDA [107] and European Agency for the Evaluation of Medicinal Products [108]. The initial stages of processing display the greatest variability and have to be optimised in a system-specific manner. Disruption of cell walls and membranes is the first postharvesting step, but different tissue types (leaves, seeds, fruits etc.) require different forms of treatment (grinding, milling etc.). After cell disruption, clarification of the extract is often carried out by dead-end or cross-flow filtration, which is sometimes preceded by bulk cell mass removal using a decanter, plate separator or centrifuge.

Several liquid chromatography steps are required in a full purification protocol, and the initial chromatographic steps require the most specialisation for plant-based production. 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 [128]. 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.

7.2 Regulatory landscape

One of the greatest uncertainties surrounding the use of plants for the production of pharmaceuticals is the regulatory landscape. For plants grown in glasshouses and in enclosed bioreactors, the production of pharmaceuticals is regulated in the same way for other production systems, and comes under the authority of the FDA and equivalent agencies in other parts of the world. The switch to open-field conditions adds another layer of regulatory complexity because the transgenic plants then come under the authority of the Animal and Plant Health Inspection Service (part of the US Department Of Agriculture) or their counterparts in Europe and other regions. The involvement of multiple regulatory agencies makes the production process more complex because the extent of each authority's jurisdiction is not always clear, and at the current time only draft guidelines are available [107,108]. The impact of this is to suppress the market. It is likely that more companies will become interested in plant-derived pharmaceuticals when full guidelines become available, hopefully by 2005.

All recombinant pharmaceuticals, including those derived from plants, need to comply with the national and international GMP standards for product safety, quality, potency and efficacy. However, it is not clear at what stage GMP requirements should come into effect when plants are used as the production system, as the strict rules governing defined growth conditions are difficult to implement in the field, where variables such as the weather, differences in soil quality and the presence of other organisms need to be considered. This is increasingly important now that European regulatory requirements regarding GMP-compliance for the manufacture of medicinal products have extended to the production of clinical trial material (Directive 2001/20/EC).

7.3 Alternative plant-based production technologies

One way in which developmental issues surrounding the use of transgenic plants is being addressed is the use of transient production systems that avoid stable gene transfer and can be used for moderate-scale production in containment, particularly in the early stages of process development. One example of this is the transient expression of recombinant proteins in tobacco and alfalfa leaves through agroinfiltration, a process in which *A. tumefaciens* is introduced into the gaps between leaf cells by vacuum infiltration, resulting in the short-term production of recombinant proteins [129]. This has been used for several years as an initial test system to verify vector performance and allow the analysis of small amounts of recombinant proteins before committing to the expense and timescale required for transgenics [130], but now it is emerging as a useful production system in its own right. Baulcombe and colleagues have shown that the prevention of gene silencing in agroinfiltrated leaves can increase protein yields by up to 50-fold [131]. Furthermore,

researchers at Medicago, Inc. have described how the agroinfiltration of alfalfa leaves can be scaled up to 7500 leaves per week, for the production of micrograms of recombinant protein [132]. Similarly, it has been shown that 25 – 50 kg of wild-type tobacco leaves can be batch-processed by agroinfiltration resulting in the production of several hundred milligrams of protein (authors' unpublished data).

Virus-infected plants have also been used to produce pharmaceutical proteins including antibodies [125,133] and subunit vaccines (reviewed in [134]). Although these can theoretically at least be grown on the same scale as transgenic plants, the development of producer lines is much quicker because virus infections take days or weeks to establish compared with the months or years required to produce transgenic plants. High level expression is possible because viral replication is prodigious and infections are systemic. Virus-infected plants have been developed as a platform technology by several companies, including Large Scale Biology Corp., who have one scFv antibody in Phase II clinical trials and have just released their first commercial products, recombinant interferons 2 α and 2 β , under a biomanufacturing and commercial distribution agreement with Sigma-Aldrich. As well as viruses expressing full-length transgenes yielding recombinant proteins that are extracted from infected plants using the same procedures developed for transgenic plants, a quite separate technology platform involves the use of plant virus particles to display the epitopes of animal viruses and other pathogens as coat fusion proteins (reviewed in [135,136]). Plant-based vaccines developed using this technology are listed in Table 1.

Suspension cell cultures derived from whole plants (e.g., tobacco and rice) possess many of the advantages of whole plants in terms of safety and capacity for the folding and modification of human proteins [137-139]. They are also cheaper to maintain than mammalian cells because they require a relatively simple, synthetic growth medium and they allow the product to be secreted into the medium for purification. Current challenges that need to be addressed include the relatively low yields (these need to be increased at least 10-fold before the process becomes commercially feasible) and the genetic instability of many plant cultures [139]. As an alternative, bioreactors of aquatic plants such as *Lemna minor* [140], or cultures of algae [141] or moss [106] can be used as production systems. The moss *Physcomitrella patens* is a particularly promising system because it is one of the few plant species known to be amenable to gene targeting by homologous recombination. This provides the potential to knockout genes encoding the enzymes responsible for adding non-mammalian glycan structures to recombinant glycoproteins [106].

8. Expert opinion and conclusion

The production of recombinant pharmaceuticals in plants has emerged in the last few years as a potential competitor to the established fermenter-based production technologies, theoretically offering unlimited production scales at unprecedented low

Transgenic plants in the biopharmaceutical market

manufacturing costs. Technical limitations such as low yields, instability and non-authentic glycan structures are beginning to break down under the relentless weight of research, providing the field with novel promoters, a range of proprietary expression systems and strategies to humanise the glycan profiles of plant-derived glycoproteins. Despite the further limitations of a formative and, in some cases, restrictive regulatory framework, the potential of molecular farming can be seen in the rich intellectual property landscape and the multiple cross-licensing and collaborative ventures that are possible between companies developing production platforms, extraction and separation

technologies and those with experience in the latter stages of drug development and marketing. The expert opinion of the authors is that within 10 years, we shall see plants come to the forefront of pharmaceutical production and become a primary source of safe, inexpensive and high quality therapeutics, diagnostics and vaccines, including antibodies for passive and active immunisation, blood products, therapeutic enzymes, animal products, antibacterial and structural proteins. Cooperation between the researchers, breeders, producers and regulatory agencies will be necessary to find the correct path towards the goal of inexpensive medicines available to all.

Author Proof

Table 1. Vaccine antigens against infectious diseases produced by molecular farming in plants. Only vaccines with potential applications in infectious diseases are shown – auto-antigens and allergens are omitted.

Antigen	Production system	Comments	References
Vaccines for veterinary use			
Bovine herpes virus (type 1) glycoprotein D	Tobacco leaves	Humoral and cellular immune response in mice and cows following parenteral administration. Alleviation of symptoms in cows challenged with the virus	[142]
Bovine rotavirus	Tobacco leaves, transgenic and virus infected	Immunogenic and protective	[143,144]
Canine parvovirus VP2 epitope	<i>Arabidopsis thaliana</i> leaves	Expressed as fusion with bacterial enzyme β -glucuronidase. Specific antibodies detected and immunogenic in mice following parenteral administration	[145]
	Cowpea mosaic virus epitope display in cowpea	Immunogenic in mice following parenteral or nasal administration. Protective against lethal challenge in parenterally immunised dogs	[112,146]
	Plum pox virus epitope display in tobacco	Neutralising antibodies produced in mice. Immunogenic in mice and rabbits following parenteral administration	[147]
	Tobacco leaves (chloroplast expression)	Expressed as fusion of VP2 2L21 peptide with cholera toxin B subunit, or with green fluorescent protein. Induced neutralising antibodies in mice	[148]
Enterotoxigenic <i>E. coli</i> fimbrial subunit FaeG	Tobacco leaves	Prevented bacterial colonisation of piglet villi in an <i>in vitro</i> assay	[149]
Foot and mouth disease virus VP1 epitope	Alfalfa leaf	Specific antibody response in mice. Immunogenic in mice following parenteral or oral administration. Mice protected against viral challenge	[150,151]
	<i>Arabidopsis thaliana</i> leaves	Immunogenic in mice following parenteral administration. Mice protected against viral challenge	[113]
	Cowpea mosaic virus epitope display in cowpea		[152]
	Potato tuber	Immunogenic in mice. Mice protected against viral challenge	[114]
Infectious bronchitis virus S1 glycoprotein	Potato tuber	Neutralising antibodies produced. Immunogenic in chickens following parenteral, oral or nasal administration. Mice and chickens protected against viral challenge	[115,116]
<i>Mannheimia haemolytica</i> A1 leukotoxin 50	White clover leaves	Expressed as green fluorescent protein fusion. Rabbits produced neutralising antibodies. Immunogenic in rabbits following parenteral administration	[153]
Mink enteritis virus VP2 epitope	Cowpea mosaic virus epitope display in cowpea	Immunogenic in mink following parenteral administration. Protective against viral challenge	[117]
Murine hepatitis virus glycoprotein S 5B19 epitope	Tobacco mosaic virus vectors in tobacco	Immunogenic in mice following parenteral or nasal administration. Protected mice against virulent strain of the virus	[118]
Porcine epidemic diarrhoea virus spike protein	Tobacco leaves	Systemic and mucosal immune response following oral administration	[154]
Rabbit hemorrhagic disease virus VP60 epitope	Plum pox virus epitope display in tobacco	Neutralising antibodies produced. Immunogenic in mice and rabbits following parenteral administration. Rabbits survived lethal challenge following parenteral administration	[119,147]
	Potato leaves	Neutralising antibodies produced. Immunogenic in rabbits following parenteral administration. Rabbits survived lethal challenge following parenteral administration	[120]

Table 1. Vaccine antigens against infectious diseases produced by molecular farming in plants. Only vaccines with potential applications in infectious diseases are shown – auto-antigens and allergens are omitted.

Antigen	Production system	Comments	References
Rabies virus – see medical vaccines below			
Rinderpest virus haemagglutinin protein	Pigeon pea leaves, tobacco leaves	Neutralising antibodies detected in mice immunised with tobacco-derived vaccine	[155]
Transmissible gastroenteritis coronavirus N-terminal domain of the spike glycoprotein	<i>Arabidopsis thaliana</i> leaves	Neutralising antibodies detected. Immunogenic following parenteral administration	[156]
	Maize seeds	Neutralising antibodies produced in piglets. Reduced symptoms in piglets when challenged with virus following oral delivery	[121]
	Potato tubers	Specific antibodies produced following parenteral or oral administration	[157,158]
	Tobacco leaves	Specific antibodies produced in pigs following parenteral administration. Immunogenic in pigs following parenteral administration	[157]
Vaccines for medical use			
<i>Bacillus anthracis</i> protective antigen	Tobacco leaves		[159]
Enterotoxigenic <i>E. coli</i> conjugal pilus, A subunit	Tobacco leaves	Immunogenic in mice after oral administration	[160]
Enterotoxigenic <i>E. coli</i> heat labile toxin, B subunit	Maize seeds	Immunogenic and protective in mice after oral administration. Serum and secretory antibodies produced in humans	[121,161,162]
	Potato tubers	Immunogenic and protective in mice after oral administration. Immunogenic in human after oral administration	[62,163-165]
	Tomato fruit and leaves	Expressed as fusion with cholera toxin B subunit and rotavirus VP6 protein. Mice developed neutralising antibodies. Immunogenic in mice against enterotoxigenic <i>E. coli</i> , rotavirus, and <i>V. cholerae</i> following oral administration	[166]
<i>E. coli</i> O157:H7 intimin	Tobacco leaves	Expressed as native protein and as fusion to an immunocontraceptive antigen	[72]
Hepatitis B virus surface antigen	Tobacco leaves	Immunogenic and protective in primed mice after oral administration	[167]
	Cherry tomatillo leaves and fruit	Immunogenic in primed mice following oral administration	[168]
	Lettuce leaves	Immunogenic in humans following oral administration	[58]
	Lupin callus tissue	Immunogenic in mice when administered orally	[58]
	Potato tubers	Immunogenic in mice when administered orally	[64,169,170]
	Soybean cell suspension cultures		[171]
Hepatitis C virus HVR1 epitope of E2 envelope protein	Tobacco leaves	Immunogenic in parenterally vaccinated mice	[172,173]
	Tobacco leaves	Expressed as fusion with soybean vegetative protein (VSP α S). The fusion protein generated higher levels of serum IgG than native HBsAg in mice	[174]
Hepatitis C virus HVR1 epitope of E2 envelope protein	Cucumber mosaic virus epitope display in tobacco	Crossreactive with a wide range of human anti-HVR1 antibodies	[175]
	Tobacco leaves	Expressed as fusion with <i>V. cholerae</i> cholera toxin, B subunit. HVR1-CTB was immunogenic in mice following nasal administration	[176]

Table 1. Vaccine antigens against infectious diseases produced by molecular farming in plants. Only vaccines with potential applications in infectious diseases are shown – auto-antigens and allergens are omitted.

Antigen	Production system	Comments	References
Hepatitis E virus ORF2 protein	Tomato leaves and fruit		[177]
Human cytomegalovirus glycoprotein B	Tobacco seeds	Immunogenic in mice	[109,178]
Human immunodeficiency virus (type 1) p24 protein	Virus vectors in tobacco		[179, 180]
Human immunodeficiency virus (type 1) p120 protein	Virus vectors in tobacco	Neutralising antibodies produced. Immunogenic in mice following parenteral administration	[181, 182]
Human immunodeficiency virus (type 1) ELDKWA epitope	Potato virus X epitope display in tobacco leaf	Neutralising antibodies produced. Immunogenic in mice following parenteral or nasal administration	[183]
Human papillomavirus (type 11) L1 protein	Tobacco leaves, potato tuber	Immunogenic in parenterally vaccinated mice followed by oral boost	[184]
Human papillomavirus (type 16) E7 protein	Virus vectors in tobacco	Immunogenic and protective in parenterally vaccinated mice	[185]
Human papillomavirus (type 16) L1 protein	Tobacco leaves, potato tubers	Immunogenic when administered orally following a parenteral boost	[186,187]
Human rhinovirus (type 14) VP1 epitope	Cowpea mosaic virus epitope display in cowpea	Immunogenic in rabbits when delivered parenterally	[188]
Measles virus haemagglutinin	Carrot leaves and taproots	Immunogenic in parenterally vaccinated mice	[76,77]
	Tobacco leaves	Immunogenic after parenteral or oral delivery in mice	[189,190]
Norwalk virus capsid protein	Tobacco leaves, potato tubers	Immunogenic in mice and humans following oral administration	[63,191]
<i>Plasmodium falciparum</i> (PfMSP1)	Tobacco leaf	Immunogenic in mice	[192,193]
<i>Pseudomonas aeruginosa</i> membrane protein F epitope	Cowpea mosaic virus epitope display in cowpea	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with <i>P. aeruginosa</i>	[194,195]
	Tobacco mosaic virus epitope display in tobacco	Specific antibodies produced. Immunogenic in mice following parenteral administration. Protected mice against challenge with <i>P. aeruginosa</i>	[196]
Rabies virus glycoprotein	Tomato leaves and fruit		[71]
Rabies virus glycoprotein and nucleoprotein	Viral vectors in tobacco and spinach leaves	Immunogenic and protective in mice when delivered orally and parenterally. Immunogenic in humans following oral administration	[59,197,198]
Respiratory syncytial virus fusion protein	Tomato fruit	Immunogenic in mice following oral administration.	[73]
Respiratory syncytial virus G and F proteins	Alfalfa mosaic virus epitope display in tobacco	Neutralising antibodies produced. Immunogenic in mice following parenteral administration. Mice protected from viral challenge	[199]
Rotavirus NSP4 protein	Potato tubers	Expressed as fusion with cholera toxin B subunit. Specific serum and mucosal antibodies produced after oral vaccination. Th1 immune response observed	[200]
Rotavirus VP6 protein	Potato tubers	Immunogenic in mice when administered parenterally	[201]
		Expressed as fusion with cholera toxin B subunit and enterotoxigenic <i>E. coli</i> heat labile toxin, B subunit. Specific serum and intestinal antibodies produced. Immunogenic in mice against enterotoxigenic <i>E. coli</i> , rotavirus, and <i>V. cholerae</i> following oral delivery. Symptoms reduced following rotavirus challenge in pups	[166]
	Tomato suspension cells		[202,203]

Table 1. Vaccine antigens against infectious diseases produced by molecular farming in plants. Only vaccines with potential applications in infectious diseases are shown – auto-antigens and allergens are omitted.

Antigen	Production system	Comments	References
	Virus vectors in tobacco leaves		[204]
Rotavirus VP7 protein	Potato tubers	Immunogenic in mice following oral administration	[205]
Simian/human immunodeficiency virus 89.6p Tat	Potato tubers	Expressed as fusion with <i>V. cholerae</i> cholera toxin, B subunit. Assembled into pentamers	[206]
<i>Staphylococcus aureus</i> D2 epitope of fibronectin-binding protein	Cowpea mosaic virus epitope display in cowpea, and potato virus X epitope display in tobacco	Specific antibodies produced. Immunogenic in mice and rats following parenteral, oral or nasal delivery	[207]
Tetanus toxin, fragment C	Tobacco leaves (chloroplast expression)	Immunogenic in mice following nasal administration	[93]
<i>V. cholerae</i> cholera toxin, B subunit (as single antigen)	Potato tubers	Immunogenic and protective in mice when delivered orally	[208,209]
	Tobacco leaves	Codon optimised for enhanced expression	[210]
	Tobacco leaves (chloroplast expression)		[92]
	Tomato leaves and fruit		[211]

Table 2. Recombinant therapeutic or diagnostic recombinant antibodies produced by molecular farming in plants and reported in the scientific literature (many antibodies in commercial development remain undisclosed until intellectual property rights have been secured). Antibodies with alternative applications, such as phytomodulation or the prevention of plant disease, are not listed.

Antigen	Antibody format	Production system	Comments	References
B cell lymphoma, murine 38C13	scFv	Virus vectors in tobacco leaves	Maximum yield 30.2µg/g leaves	[125]
Carcinoembryonic antigen	scFv, IgG1	Tobacco agroinfiltration	Directed to apoplast or endoplasmic reticulum. Maximum yields 5µg scFv/g leaves, 1µg IgG/g leaves	[212]
	dAb	Tobacco, agroinfiltration and transgenic		[130]
	scFv	Rice, rice cell cultures	Directed to apoplast or ER. Maximum yields 3.8µg/g callus, 29µg/g leaves, 32µg/g seed	[48,213]
		Wheat	Directed to apoplast or ER. Maximum yields 900ng/g leaves, 1.5µg/g seed	[48]
		Pea	Directed to endoplasmic reticulum. Maximum yield 9 µg/g seed	[47,48]
CD-40	scFv-fusion	Tobacco suspension cells	Secreted to apoplast. Yield not reported	[152]
Colon cancer antigen	IgG	Virus vectors in tobacco leaves	Yield not reported	[97]
Creatine kinase	IgG1, Fab	Tobacco leaves, <i>Arabidopsis</i> leaves	Accumulated in nucleolus [214] or apoplast [215]. Maximum yield 1.3% TSP [215]	[214,215]
	scFv	Tobacco leaves	Direct to cytosol or apoplast. Maximum yield 0.01% TSP	[216]
Rhesus D antigen	IgG1	<i>Arabidopsis</i> leaves		[217]
Ferritin	scFv	Tobacco leaves		[218]
Hepatitis B virus surface antigen	IgG	Tobacco leaves	Up to 25 mg antibody/kg biomass	[219,220]
Herpes simplex virus 2	IgG1	Soybean	Secreted to apoplast. Yield not reported	[58]
HIV antibodies in blood	scFv-fusion	Tobacco leaves, barley grains, potato tubers	Yield 150 µg/g	[68]
Human choriogonadotropin	scFv, dAb, IgG	Tobacco leaves	Secreted to apoplast. Maximum yield 40 mg/kg FW	[221]
Human IgG	IgG1	Alfalfa	Secreted to apoplast. Maximum yield 1% TSP	[56]
Interleukin-4	scFv	Tobacco roots		[222]
Interleukin-6	scFv	Tobacco roots	Up to 0.18% TSP	[222]
Streptococcal surface antigen (I/II)	slgA	Tobacco leaves	Secreted to apoplast. Maximum yield 500 µg/g FW	[96]
	IgG1	Tobacco leaves	Directed to plasma membrane. Maximum yield 1.1% TSP leaves	[223]
	IgG1	Secretion from tobacco roots	Up to 11.7 µg/g dry root weight/day	[53]
Substance P	VH	Tobacco leaves	Secreted to apoplast. Maximum yield 1% TSP	[224]

dAB:Diabody; FW:Fresh weight; IgG:Immunoglobulin G; ScFv:Single chain fragment variable; SlgA:Secretory immunoglobulin A; TSP:Total soluble protein; VH: Heavy chain variable region.

Table 3. Plant-derived recombinant human proteins and animal proteins that can be used therapeutically in humans as reported in the scientific literature (many proteins in commercial development remain undisclosed until intellectual property rights have been secured).

Protein	Host plant system	Potential application	Comments	References
Adenosine deaminase	Maize	Severe combined immunodeficiency	Yield not reported	[225]
Angiotensin-1 converting enzyme	Tobacco and tomato (virus infected)	Hypertension	Yield not reported	[226]
Aprotinin	Maize	Bleeding, pancreatitis	Up to 0.07% TSP in T2 seeds. Improved yields reported by Prodigene, Inc. since this publication	[227]
Collagen, human	Tobacco	Tissue repair	Yields < 0.01% TSP fresh weight. In [163], the co-expression of a modification enzyme produced hydroxylated collagen	[228,229]
Enkephalins, human	<i>Arabidopsis</i> , canola seeds	Antihyperanalgesic	Maximum yield 0.1% seed protein	[230]
Epidermal growth factor, human	Tobacco	Wound repair, cell proliferation	Yields < 0.01% TSP	[231]
	Tobacco, transgenic and transient expression		Yields < 0.11% TSP	[232]
Erythropoietin, human	Tobacco suspension cells	Anaemia	Yields < 0.01% TSP	[233]
Factor XIII (A-domain)	Tobacco	Bleeding		[234]
Glutamic acid decarboxylase (GAD65)	Tobacco	Diabetes	Yields up to 0.04% TSP	[235]
Glucocerebrosidase	Tobacco	Gaucher's disease	1 – 10% TSP	[236]
Growth hormone, human	Tobacco, sunflower callus [3]	Pituitary dwarfism	Maximum yields 7% TSP in tobacco leaf chloroplasts [90]	[3,90,237]
	Tobacco leaves (chloroplast expression) [90]			
	Tobacco seeds [237]			
Haemoglobin (α - and β -globin), human	Tobacco seeds	Blood substitute	Yields 0.05% seed proteins	[238]
Hirudin, leech	Canola	Anticoagulant	Yields of 30% seed protein	[239]
Insulin-like growth factor-1	Tobacco, rice	Diabetes		[240]
Interleukin-2	Potato	Antiviral, anticancer	Activity of 115 units/g of tuber	[241]
Interleukin-4	Tobacco			[242]
Interleukin-10	Tobacco	Antiviral, anticancer	Yield not reported	[243]
Interleukin-12	Tobacco	Antiviral, anticancer		[244]
Interleukin-18	Tobacco	Antiviral, anticancer	0.01% TSP	[245]
Intrinsic factor	<i>Arabidopsis</i> leaves	Diagnostic, supplement	Maximum yield 70 mg/kg wet weight	[246]
Lactoferrin	Potato tubers	Antimicrobial	0.1% TSP	[69]
	Rice	Antimicrobial		[247,248]
	Tobacco	Antimicrobial		[69]
	Viral vectors in tobacco	Antimicrobial	N-lobe of protein only was expressed, yield up to 0.6% TSP	[249]

Table 3. Plant-derived recombinant human proteins and animal proteins that can be used therapeutically in humans as reported in the scientific literature (many proteins in commercial development remain undisclosed until intellectual property rights have been secured).

Protein	Host plant system	Potential application	Comments	References
Lysozyme	Rice grains, rice cell culture	Antimicrobial		[248,250,251]
Pancreatic lipase	Tobacco, maize	Exocrine pancreatic deficiency	Up to 7% TSP	[252]
Protein C, human	Tobacco	Anticoagulant	Yields < 0.01% TSP	[236]
Secreted alkaline phosphatase, human	Tobacco (secretion)	Ovarian and testicular cancer	Up to 3% of root exudates/guttation fluid	[51,52]
Serum albumin, human	Tobacco and potato leaves [4], tobacco chloroplasts [90], potato tubers [253]	Burns, liver cirrhosis	Maximum yields 11% TSP in tobacco leaf chloroplasts [91]	[4,91,253]
SMAP-29	Tobacco	Antimicrobial		[254]
Synthetic elastin	Tobacco, Tobacco chloroplasts	Tissue repair		[255]
α -Interferon, human	Rice, turnip	Hepatitis	Yield not reported	[256]
α -Trichosanthin, viral	Tobacco (virus infected)	HIV	Maximum yield 2% TSP	[257]

TSP: Total soluble protein.

Table 4. Selected companies, past and present, with a major or sole focus on plant-derived pharmaceuticals. Companies that provide support services, but that are not directly involved in recombinant protein production are not listed. The table is based on information available in press releases and company websites, and is not intended to be a comprehensive summary of the industry.

Company	History, major interests (relevant to plant-derived pharmaceuticals) and leading products	Website reference
Agrenvec S.L., Madrid, Spain	Founded in 2001 as a spin-off from INIA (Agriculture and Food Research National Institute). Focuses on the production of recombinant proteins and peptides in a variety of host plants using viral vectors based on turnip mosaic virus	[301]
AltaGen Bioscience Inc, Morgan Hill, CA	Formed in 2002 as a merger between Phytogenics and Sierra Biosource, acquired in June 2004 by Serologicals Corp. Focused on the production of pharmaceutical proteins from transgenic plants and plant cultures, greenhouse containment, and hydroponics	[302]
Axis Genetics PLC, Cambridge, UK	Formed in the mid 1980s after a management buyout. Focused on the production of vaccines in transgenic potatoes and in plants infected with recombinant cowpea mosaic virus. Liquidated in 1999 after failing to secure adequate funding	
Biocem SA	Part of the Limagrain Group. Played an early role in the development of canine gastric lipase in tobacco and maize, now under development by Meristem Therapeutics and progressing through Phase II clinical trials. Also involved in the production of other enzymes and rabies virus G glycoprotein, now being developed by Meristem Therapeutics	
Biolex Inc, Pitsboro, NC	Formed in 1999. Acquired Epicyte Pharmaceutical in May 2004. Focuses on the production of pharmaceutical proteins in <i>Lemna minor</i> (the LEX system). Many products in pipeline, with α -interferon likely to be the first investigational new drug. Acquisition of Epicyte will provide access to antibodies in the late stages of development. Biolex has formed corporate collaborations with Bayer Corporation (two proteins), Centocor, Inc. (three proteins), Debiopharm SA (one protein) and a further undisclosed pharmaceutical company	[303]
Calgene, Inc.	Founded in 1980. Played a major role in the development of plant biotechnology and the control of transgene expression in plants. Now part of the Monsanto group	
Ceres, Inc., Malibu, CA	Major developer in all areas of plant biotechnology, including pharmaceutical production	[304]
Chlorogen, St Louis, MO	Formed in 2002. Focuses on the production of pharmaceuticals, antibodies and vaccines in plant chloroplasts	[305]
Crop Design, Ghent, Belgium	Founded in 1998 as a spin-off from the Flanders Interuniversity Institute for Biotechnology. Focuses on protein expression technology in rice grains.	[306]
CropTech Development Corp., Blacksburg, VA	Formed in 1992, focussing on tobacco-based production, including > 20 products in a facility opened in 2001. MeGA PharM (mechanical gene activation) system allows postharvest expression. Liquidated in 2003 after failing to secure adequate funding. More than 20 of the former investors formed a new organisation, Tobacco Ventures, to secure former CropTech IP assets	
Dow AgroSciences LLC	Wholly owned subsidiary of Dow Chemical Co., formerly the joint venture Dow Elanco. Has a portfolio of plant-derived antibodies and vaccines, in partnership with Epicyte and Centocor	[307]
Epicyte Pharmaceutical Co	Founded in 1996, acquired in May 2004 by Biolex, Inc. Focused on plant-derived antibodies. Leading products (now under development by Biolex, Inc.) include HX8 against herpes simplex virus, R19 against respiratory syncytial virus, antibodies for contraception and antibodies against HIV and <i>Clostridium difficile</i>	
ERA Plantech, Barcelona, Spain	Founded in 2001. Focuses on plant-derived proteins accumulating in the endoplasmic reticulum	[308]

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Company	History, major interests (relevant to plant-derived pharmaceuticals) and leading products	Website reference
Evogene Ltd, Israel	Founded in 2002 as spin-off of Compugen. Has various technologies on offer including a molecular farming platform based on tomato trichomes for the secretion of recombinant proteins	[309]
Farmacule BioIndustries Ltd, Brisbane, Australia	Founded in 2001 as a spin-off from Queensland University of Technology. Focuses on precision gene expression in transgenic plants using the INPACT regulated expression technology	[310]
Fibrogen Inc, San Francisco, CA	Founded in 1995. Concentrates principally on the development of novel therapeutics for the treatment of fibrosis. Actively exploring the use of plants for the production of recombinant human collagen and related proteins	[311]
GenoMine, Inc., Pohang, Republic of Korea	Founded in 2001. Focus is primarily genomic and proteomic technologies in plants, but also plant-derived vaccines. Currently developing a human papillomavirus vaccine expressed in plants	[312]
Greenovation Biotech GmbH, Freiburg, Germany	Founded in 1999. Focuses on the production of pharmaceutical proteins in the moss bioreactor system <i>Physcomitrella patens</i>	[313]
Hayashibara Co. Ltd, Japan	At the forefront of Japanese biotechnology and marketing recombinant interferon since 1988. Now diversifying into plant-derived edible vaccines	
Icon Genetics, Freising-Weihestephan, Germany	Provides production systems based on transgenic foliage and virus infected plants	[314]
Integrated BioPharma, Hillside, NJ	Owns many subsidiaries, including NuCycle Therapy, Inc., which is currently collaborating with the US Navy in the development of a plant-based anthrax vaccine	[315]
Large Scale Biology Corp., Vacaville, CA	Formed in 1987, formerly Biosource Technologies, Inc., and built the world's first bioprocessing facility for plant-derived proteins. Focuses on the production of proteins in transgenic plants and plants infected with plant viruses (GENEWARE® platform). Most developed products are personalised vaccine scFv antibodies for the treatment of non-Hodgkins lymphoma, which are in Phase I clinical trials, and α -galactosidase for the treatment of Fabry disease. Further antibodies are being developed in collaboration with Prodigene, Inc. In July 2004 announced commercial scale production of interferons- α_{2a} and - α_{2b} in cooperation with Sigma Aldrich	[316]
LemnaGene LLC, Lyons, France	Formed in 2002. Forged a licensing agreement with Bayer CropScience and Yeda Research in 2003, enabling the completion of first round of financing. Focuses on the large-scale production of recombinant proteins in <i>Lemna</i>	[317]
Maltagen Forschung GmbH, Andernach, Germany	Formed in 1994. Focuses on the production of recombinant proteins in barley grains. Example products include human serum albumin, lactoferrin and lysozyme	[318]
Maxygen, Inc., Redwood City, CA	Formed in 1997. Focuses on the production of recombinant proteins in maize. Example products include interferons- β and - γ , and a vaccine for colorectal cancer. One of these is projected to achieve IND status in the next year	[319]
MPB-Cologne GmbH, Cologne, Germany	Formed in the 1999 as a spin-out from the Max Planck Institute, with a focus on the production of recombinant protein in potato tubers and rapeseed. Liquidated in 2002 due to lack of funding	
Medicago, Inc., Quebec, Canada	Formed in 1999. Focuses on the production of recombinant proteins in alfalfa (cell culture, transiently transformed plants and transgenics). Various antibodies, enzymes and blood products in the pipeline	[320]

Transgenic plants in the biopharmaceutical market

Table 4. Selected companies, past and present, with a major or sole focus on plant-derived pharmaceuticals. Companies that provide support services, but that are not directly involved in recombinant protein production are not listed. The table is based on information available in press releases and company websites, and is not intended to be a comprehensive summary of the industry.

Company	History, major interests (relevant to plant-derived pharmaceuticals) and leading products	Website reference
Meristem Therapeutics, Inc., Clermont-Ferrand, France	Formed in 1997 to implement the plant genetics programme 'molecular pharming' initiated by the Limagrain group. Focuses on the development of large-scale processes for the production of therapeutic proteins in tobacco, maize, rapeseed, potato and other crops. Example products include antitrypsin, pancreatic lipase, collagen, lactoferrin and canine gastric lipase (currently in Phase IIa trials)	[321]
Monsanto Protein Technology, St Louis, MO	Division of Monsanto concerned specifically with biopharmaceuticals with a focus on the development of large-scale processes for the production of therapeutic proteins in maize. Collaborated with NeoRX Corp. to produce the Avicidin anti-EpCAM antibody for the treatment of colorectal cancer (withdrawn during Phase II trials). Monsanto decided to withdraw from the plant-derived pharmaceuticals market in 2004	[322]
NeoRX Corp, Seattle, WA	Collaborator in the development of Avicidin® (see above)	[323]
Nexgen Biotechnologies, Inc., Daejeon City, Korea	Formed in 1999. Has Canadian subsidiary Guardian Biotechnologies, Inc. Focuses on the development of edible vaccines	[324]
Novoplant GmbH, Gatersleben, Germany	Formed in 1998. Focuses on plant-derived antibodies for the veterinary medicine industry	[325]
ORF Genetics, Reykjavik, Iceland	Formed in 2002. Focuses on the production of pharmaceutical proteins in barley, which does not grow naturally in Iceland offering a natural containment system, and lettuce. Example products: granulocyte colony stimulating factor, interleukin-3, stem cell factor, erythropoietin	[326]
Phycotransgenics LLC, Bloomington, IL	Formed in 1999. Focuses on the production of recombinant proteins in the alga <i>Chlamydomonas reinhardtii</i>	[327]
Phytomedics Inc, Dayton, NJ	Formed in 1996. Focuses on the production of recombinant proteins from tobacco roots by secretion. Example products include alkaline phosphatase	[328]
Phytoprotein Biotech Pte Ltd, Singapore	Formed in 2000, focuses on production of vaccines and antibodies in plant cells	
Plant Biotechnology, Inc., Hayward CA	Formed in 1998. Focuses on the production of recombinant secretory antibodies in tobacco. Several products are in advanced stages of clinical development. CaroRX™ (anti- <i>Streptococcus mutans</i>) has completed Phase II trials, whereas RhinoRX™ (against common cold virus) and DoxoRX™ (protects against chemotherapy side effects) are likely to enter trials in the next year. Agreement with Large Scale Biology Corp. to extract CaroRX made in July 2004	[329]
Plantechno SRL, Lombardia, Italy	Formed in 1995 as a spinout from <i>Università Cattolica S. Cuore di Agraria Istituto di Botanica e Genetica</i> . Focuses on the expression of pharmaceuticals in tobacco plants. Field trials have been carried out with tobacco expressing glucocerebrosidase	[330]
PlantGenix, Inc. Malvern, PA	Formed in 1999. Works on membrane transport technology to improve storage and accumulation of recombinant proteins	[331]
Plantigen, Inc., Ontario, Canada	Formed in 1999 by the London Health Sciences Centre in Canada. Focuses on the expression of pharmaceuticals in tobacco plants. Example products include glutamic acid decarboxylase and interleukins	[332]
Planton GmbH, Kiel, Germany	Formed in 2001. Focuses on the expression of pharmaceuticals in potato tubers. Example products include antimicrobial peptides	[333]

Table 4. Selected companies, past and present, with a major or sole focus on plant-derived pharmaceuticals. Companies that provide support services, but that are not directly involved in recombinant protein production are not listed. The table is based on information available in press releases and company websites, and is not intended to be a comprehensive summary of the industry.

Company	History, major interests (relevant to plant-derived pharmaceuticals) and leading products	Website reference
Prodigene, Inc., College Station, TX	Formed in 1996, and was the first company to commercialise proteins from transgenic plants (1998). Focuses on the expression of pharmaceuticals and technical proteins in maize. Example products: avidin, β -glucuronidase, trypsin, antibodies and vaccines	[334]
Quantum Tubers Corp, Delavan, WI	Specialises in potato technology. Currently producing the first crop of potato-based oral vaccines	[335]
SemBioSys Genetics, Inc., Calgary, Canada	Founded in 1994 as a spinout from the University of Calgary. Focuses on the expression of pharmaceuticals and technical proteins in oilseeds (safflower) using proprietary oleosin fusion technology. Recent deal with Syngenta to produce Syngenta's front-line biological products	[336]
Syngenta International AG, Switzerland	Formed in 2000 through the merger of Novartis agribusiness and Zeneca agrochemicals. Among many plant biotechnology products, Syngenta is involved in the development of at least six biopharma products with partners including SemBioSys Genetics, Inc.	[337]
Toxin Alert, Inc., Ontario, Canada	Founded in 1998. Focuses on food diagnostic products and the use of antibodies as diagnostic reagents. Now developing production capabilities in plants	[338]
UniCrop Ltd, Helsinki, Finland	Founded in 1998. Focuses on recombinant pharmaceuticals produced in sprouting plants in bioreactors	[339]
Ventria Bioscience, Sacramento, CA	Founded in 1993 as Applied Phytologics, Inc. Focuses on pharmaceutical production in transgenic rice grains. Example products include lactoferrin and lysozyme. Relocating to Maryville, MO in 2005	[340]

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