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Review

Molecular farming of recombinant antibodies in plants

S. Schillberg[a], R. Fischer[a,b,*] and N. Emans[b]

[a] Fraunhofer Institute for Molecular Biology and Applied Ecology, IME, Grafschaft, Auf dem Aberg 1, 57392 Schmallenberg (Germany)
[b] Institute for Molecular Biotechnology, Biology VII, RWTH Aachen, Worringerweg 1, 52074 Aachen (Germany), Fax: + 49 241 871062, e-mail: fischer@molbiotech.rwth-aachen.de

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Abstract. Antibodies represent a large proportion of therapeutic drugs currently in development. In most cases, they are produced in mammalian cell lines or transgenic animals because these have been shown to fold and assemble the proteins correctly and generate authentic glycosylation patterns. However, such expression systems are expensive, difficult to scale up and there are safety concerns due to potential contamination with pathogenic organisms or oncogenic DNA sequences. Plants represent an inexpensive, efficient and safe alternative for the production of recombinant antibodies. Research over the last 10 years has shown that plants can produce a variety of functional antibodies and there is now intense interest in scaling up production to commercial levels. In this review, we discuss the advantages of plants over traditional expression systems, describe how antibody expression in plants is achieved and optimized and then consider the practical issues concerning large-scale molecular farming in plants. The first plant-produced therapeutic antibodies are already in clinical trials, and, given the economic benefits of this production system, we are likely to see many more recombinant antibodies produced in this manner in the future.

Key words. Recombinant protein; transgenic plant; antibody production; purification; biotechnology.

Introduction

For thousands of years, plants have provided humans with food and other natural products such as medicines, dyes, poisons and materials like cotton and rubber. The 1980s saw the birth of a new era in biotechnology brought about by the development of techniques to introduce foreign DNA into plants. For the first time, this allowed plants to be used for the production of specific heterologous proteins. Extensive research over the past two decades has shown that a wide range of valuable proteins can be efficiently expressed in plants. Examples include human serum proteins and growth regulators, antibodies, vaccines, industrial enzymes, biopolymers and molecular biology reagents [1–3].

The success of these experiments suggested that plant systems could be used to produce recombinant proteins on a commercial scale. This is known as *molecular farming*, and traditionally has been carried out using microbial cultures, cultured animal cells and, more recently, transgenic animals [4, 5]. Despite initial scepticism, the first generation of recombinant proteins produced in transgenic plants is now reaching commercial status. Many of these are therapeutic proteins, including antibodies, vaccines and human hormones or growth regulators. This review discusses the advantages of plants for the molecular farming of therapeutic proteins, focussing on the production of antibodies.

* Corresponding author.

Antibodies

The importance of antibodies

Antibodies and their derivatives account for more than 20% of all the biopharmaceuticals under current development. Part of the reason for the prevalence of antibodies are their manifold uses. Their specificity for particular antigens allows them to be used as diagnostic agents, therapeutic drugs and even as novel industrial enzymes (abzymes). An estimated 1000 therapeutic antibodies are being developed by biopharmaceutical companies around the world, over 200 of which are already in clinical trials.

Types of antibody

An antibody molecule comprises two heavy chains and two light chains joined by disulphide bonds (fig. 1). The C-terminal region of the heavy chain forms the Fc portion of the antibody, performing particular effector functions. The N-terminal regions are variable and, along with the light chains, are involved in antigen binding. The Fc region contains a conserved asparagine residue at position 297 to which glycan chains are added during the processing of immunoglobulin chains [6].

As well as full-size antibodies, derivatives with alternative structures can also be produced [7]. These include Fab and $F(ab')_2$ fragments (which contain only the sequences distal to the hinge region of a full-length antibody), single-chain Fv fragments (scFvs, which contain the variable regions of the heavy and light chains joined by a flexible peptide linker), chimeric antibodies containing components of different classes (e.g. chimeric IgG/A), fusion proteins with additional functionality (e.g. interleukin-scFv fusions) and bispecific scFvs. These derivatized structures are also shown in fig. 1.

Molecular farming of antibodies

A number of different host expression systems have been used to produce recombinant proteins. These include bacteria, yeast, insect cells, mammalian cells, transgenic animals, plant cell cultures and transformed plants. Each system has its advantages and disadvantages as summarized in table 1 and discussed below in more detail. Special requirements need to be met for the production of antibodies, including the ability of the chosen expression system to correctly fold and assemble multiple polypeptide chains and to produce authentic glycosylation patterns.

Bacterial systems

The large-scale production of heterologous proteins was first achieved using bacterial cell cultures. Bacterial systems are simple and easy to scale up but as with all fermentation processes there are high capital and maintenance costs. Bacteria have been used with success for the production of simple eukaryotic proteins such as insulin and growth hormone [8–10]. However, more complex proteins tend to fold incorrectly and often aggregate as large insoluble complexes called inclusion bodies. This is due to the lack of eukaryote-like chaperons and the very slow rate at which disulphide bridges are formed in bacteria.

A further disadvantage of bacterial expression systems is that post-translational modification processes such as glycosylation do not take place. Bacterial systems are therefore suitable for the expression of aglycosylated antibody derivatives such as Fab fragments and scFvs [11, 12] but not for full-size immunoglobulins requiring intact effector functions. A full-size aglycosylated IgG has been expressed in *Escherichia coli*, and may be useful in cases where the effector functions are either unnecessary or detrimental [13].

Yeast systems

Many of the problems associated with bacteria are overcome through the use of yeast, which folds and assembles mammalian proteins correctly and also carries out glycosylation. However, the glycan chains synthesized

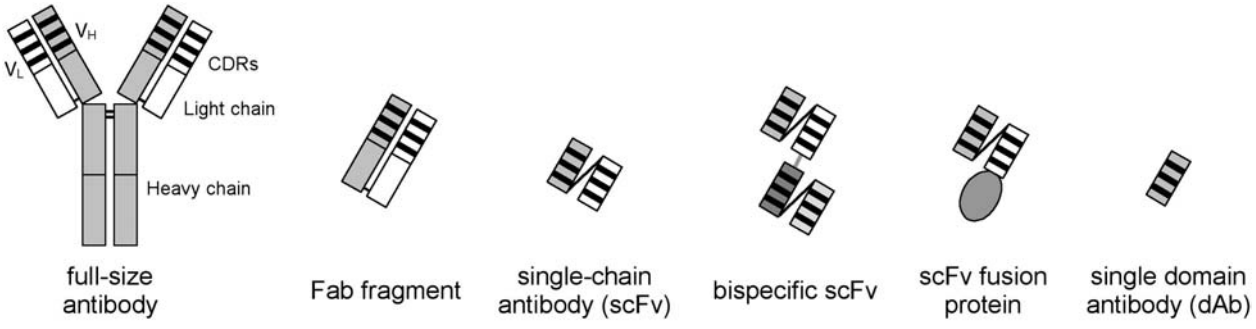

Figure 1. Different types of recombinant antibody produced in plants. V_H and V_L, antigen-binding domains of heavy and light chain; CDRs, complementarity-determining regions.

Table 1. Features of different systems for recombinant protein production.

Factor	Transgenic plants	Plant cell cultures	Bacteria	Yeast	Mammalian cell culture	Transgenic animals
Costs						
production costs	low	medium	low	medium	high	high
time effort	high	medium	low	medium	high	high
scale-up costs	low	high[1]	high[1]	high[1]	high[1]	high[1]
propagation	easy	easy	easy	easy	limited	possible
productivity	high	medium	medium	medium	medium	high
Quality						
product quality and homogeneity	high	high	low	medium	high	high
glycosylation	authentic[2]	authentic[2]	absent	incorrect	authentic[2]	authentic[2]
contamination risk	no	no	yes[3]	no	yes[4]	yes[4]
Practical application						
data monitoring	difficult	easy	easy	easy	easy	difficult
ethical concerns	medium	low	low	low	medium	high
GMP[5] conformity	difficult	possible	possible	possible	possible	possible
storage	cheap/room temperature	cheap/–20 °C	cheap/–20 °C	cheap/–20 °C	expensive/N_2	expensive/N_2

[1] Expensive media, expensive facilities for cultivation and livestock husbandry.
[2] Glycan chains produced in animal systems are authentic but may vary from those produced in humans. Plant glycans are similar to those of animals but have plant-specific groups such as β-xylose.
[3] Endotoxins.
[4] Residual viral sequences and oncogenes.
[5] Good manufacturing practice.

by yeast and mammalian cells are very different [14] so doubt has been expressed as to whether yeast-derived full-length antibodies would be functional. The yeast *Pichia pastoris* has been used to produce functional scFvs [12, 15].

Mammalian cell lines

Mammalian cells carry out authentic post-translational modifications and have for a long time been the system of choice for producing therapeutic human proteins [reviewed in ref. 16]. The down side is that mammalian cell cultures have high capital costs, they are expensive to maintain and scale up and the capacity for scale up is very limited. In common with transgenic animals (discussed below) there is also the danger that mammalian expression systems could harbour pathogenic organisms (viruses, prions) or oncogenic DNA sequences thus raising safety concerns when the recombinant protein is intended for therapeutic use in humans.

Many commercially available monoclonal antibodies that have been approved for use as therapeutics are currently produced in mammalian cell cultures [5]. The main systems are hybridoma cell lines, which are directly selected to produce full-size immunoglobulins of interest [17], and the popular general expression cell lines CHO and NS0 [16]. CHO and NS0 cells must be transfected with antibody-encoding genes or cDNAs and can therefore be used to produce full-size antibodies, scFvs and other derivatives. Insect cell lines, either directly transfected with an appropriate cDNA sequence or infected with recombinant baculovirus vectors have also been used to produce scFvs [18].

The mammalian cell lines discussed above are of rodent origin. Hybridomas and NS0 cells are murine whereas CHO derive from the Chinese hamster ovary. There are minor differences in glycosylation patterns between rodents and humans leading to slightly different glycan profiles on recombinant antibodies. For example, human antibodies contain only the sialic acid residue N-acetylneuraminic acid (NANA) while rodents produce a mixture of NANA and N-glycosylneuraminic acid (NGNA) [19]. Such differences have been addressed by the production of recombinant cell lines with modified glycosylation pathways [see e.g. ref. 20].

Transgenic animals

Despite advances in the efficiency of production, and scale up to 10,000-l fermentors, antibodies from mammalian cell lines remain among the most expensive to produce, carrying a price tag in the order of US$ 1000 per gram. An alternative to cell culture systems is the use of transgenic animals producing recombinant proteins in milk or other body fluids [reviewed in ref. 21]. Recombinant proteins can be harvested periodically, and the yields are potentially very high. However, the production of transgenic farm animals is a difficult process and in-

volves a long development phase with many regulatory hurdles. Scale up is slow, depending at the present time on the natural breeding cycle, and a founder herd carrying the transgene of interest must be maintained. As with mammalian cell lines, there are safety concerns about the transmission of pathogens or oncogenic DNA sequences.

The use of transgenic animals for antibody production has been investigated. Milk and serum have each been shown to support the assembly of multimeric proteins in a functional conformation [22]. Most conventional farm species have been considered as molecular farming expression hosts [23]. Mice are generally used as a test species for the production of proteins in milk because the technology for mouse transgenesis is well developed. For commercial production, cows, sheep and goats, which produce large volumes of milk, are preferable. Full-size recombinant antibodies have been made on a large scale using the goat system. A humanized version of the BR96 anti-lewisY monoclonal developed by Bristol Myers Squibb for cancer therapy was produced in goats. Expression levels ranged from 0.1 to 14 g/l; the antibody was functional and could be isolated and prepared to a purity of more than 99% [24]. Other companies developing commercial antibodies produced in transgenic animals include CellGenesys, Genzyme and Ligand.

Transformed plants and other plant systems

The idea of using plants to produce human proteins was initially greeted with great scepticism. However, plants offer a unique combination of advantages over traditional microbial and animal expression systems [1, 2]. We use the term *transformed plants* rather than transgenic plants because stable gene transfer is only one way of achieving antibody production in a whole-plant system. Other alternatives include transient expression, infection with recombinant viruses, and transformation of chloroplasts. Plant cell cultures can also be used to produce recombinant proteins. We discuss these individual methods in more detail below.

The advantages of transformed plants are summarized in table 1. The main advantage is that they can be used to produce any protein on an agricultural scale at perhaps 10% of the cost of other systems. These savings are possible because of the reduced capital costs compared to fermentation systems, and low running costs since the plants can be maintained and harvested using traditional, unskilled agricultural practices and existing infrastructure. Unlike fermentation systems and transgenic animals, scaling up production in plants is rapid and inexpensive. The capacity for scale up is limited only by the amount of land available. Unlike transgenic animals, there is no need to maintain a founder herd carrying the transgene, since plant material can be stored as seed and then sown as required.

Plant systems are also advantageous in terms of safety since they do not usually contain human or animal pathogens. This reduces the costs of processing and in some cases eliminates those costs altogether, since certain recombinant proteins can be administered directly in edible plant organs such as fruits and seeds. This applies particularly to subunit vaccines and antibodies for passive immunotherapy [25, 26]. The accumulation of recombinant proteins in seeds can be beneficial for other reasons. In cereals, proteins accumulating in seeds remain stable at ambient temperatures for months or years without loss of biological activity. Furthermore, the specific accumulation of proteins in seeds rather than vegetative organs can prevent high levels of the protein interfering with normal plant growth and development.

Extensive research has been carried out into the use of plants for the production of antibodies. The general eukaryotic protein synthesis pathway appears to be very well conserved between plants and animals, so plants can fold and assemble full-size antibodies [27] and secretory IgAs [28, 29]. In the latter case, four different subunits must assemble in the same plant cell to produce a functional product, even though two different cell types are required in mammals. The post-translational modifications carried out by plants and animals are not identical. There are minor differences in the structure of complex glycans, such as the presence of the plant-specific residues α1,3-fucose and β1,2-xylose [30]. However, studies of the immune response of mice to a systemically administered recombinant IgG (Guy's 13) isolated from plants showed that, although there were some differences in the glycan groups present on the recombinant antibody, neither the antibody nor the glycans were immunogenic in mice [31]. Clearly, the differences in protein glycosylation in plants and mammals did not provoke an immune response in this case, so the safe and functional expression of many therapeutic antibodies in plants seems possible. Several strategies have been developed to humanize the glycan patterns generated by transgenic plants, including the co-introduction and co-expression of mammalian glycosyltransferases along with the transgene of interest [32].

As well as full-size antibodies, various functional antibody derivatives have also been produced successfully in plants, including Fab fragments [33–35], scFvs [36–41], bispecific scFvs [42], single-domain antibodies [43] and antibody fusion proteins [44, 45].

Recombinant antibody production in transgenic plants

Overview

Molecular farming of antibodies in plants involves five essential steps:

1) obtaining the relevant cDNA encoding the antibody of interest;
2) inserting the cDNA into a plant expression construct;
3) transferring the expression construct into a suitable heterologous expression host and producing a functional recombinant protein;
4) scaling up production to commercial levels;
5) downstream processing and quality control.

Cloning and optimization of antibody transgenes

The traditional way to obtain an antibody transgene is selection of a hybridoma cell line showing appropriate antigen-binding activity. Complementary cDNAs encoding the desired antibody chains can then be isolated, subcloned in a suitable expression vector and modified if necessary to produce antibody derivatives [46, 47].

More recently, techniques such as phage display have been used to generate libraries comprising millions of different recombinant antibody fragments on the surface of nucleoprotein particles, physically coupled to the nucleic acid encoding them. Most phage display libraries have been constructed by cloning the natural immune repertoire into M13 or phage λ vectors. However, high-quality libraries in which the complementarity-determining regions of the antibodies are completely synthetic can be developed [48]. This enhances the production of therapeutic antibodies because preferred frameworks can be used and affinity maturation stimulated by the use of specific mutagenesis strategies. Another important application for phage display technology has been the humanization and affinity maturation of conventional murine antibodies [reviewed in ref. 49].

Design of the expression construct

Once a suitable cDNA is available, it is inserted into an expression construct designed to maximize recombinant antibody production. The expression construct contains various regulatory elements that control transcription and protein synthesis as well as sequences for targeting the recombinant protein to different plant cell compartments.

Transcription

High-level transcription in dicots is often achieved using the cauliflower mosaic virus 35S promoter (CaMV 35S). This is strong and constitutive, and its activity can be increased further by duplication of the enhancer region [50]. The CaMV 35S promoter is less active in monocots so alternatives are required. The maize ubiquitin-1 (*ubi*-1) and rice actin-1 (*act*-1) promoters are widely used because they are very active and show similar broad expression patterns [51]. Transcription is often stimulated by the presence of an intron, a phenomenon termed intron-mediated enhancement (IME), so introns are often included in the plant expression construct [see ref. 52]. Transcription may be restricted to certain tissues using organ- or tissue-specific promoters to avoid interference with vegetative growth [53]. Examples include the use of promoters specific for potato tubers [54] or cereal endosperm [55].

Protein synthesis

Several strategies are also available for increasing the rate of protein synthesis. These include replacing endogenous untranslated regions of the antibody genes with plant-specific translational enhancers, such as those found in the tobacco mosaic virus leader sequence and upstream of the petunia chalcone synthase gene [56, 57]. The translational start site and polyadenylation site of the construct are optimized for plants, and the coding region of the transgene may be modified if necessary to match the codon usage preference of the chosen expression host.

Protein targeting and modification

The most important factor in recombinant antibody production is the targeting of the recombinant protein, since this affects its folding, stability and post-translational modification, and determines whether accumulation of the protein will interfere with endogenous plant metabolism.

The use of fusion proteins targeting the same antibody to different cellular compartments has shown that the cytosol is not a suitable compartment in which to store recombinant antibodies and that the highest yields are achieved when an antibody is directed to the endoplasmic reticulum and retained there. Full-size recombinant antibodies do not accumulate in the plant cell cytosol because the heavy and light chains do not fold properly and do not assemble into the mature multimeric structure. They are consequently degraded and the final yields are low. The only forms of recombinant antibody that accumulate in the cytosol are single polypeptides, such as individual heavy chains or scFvs [58, 59]. In general, cytosolic antibodies do not accumulate beyond 0.1% total soluble protein although there are some notable exceptions [38, 60]. This suggests that the stability of a single-chain antibody depends on its intrinsic structure, which may determine its ability to form disulphide bridges in the reducing environment of the cytosol [61].

The addition of an N-terminal signal peptide will cause recombinant antibodies to be co-translationally imported into the endoplasmic reticulum. The targeting mechanism is apparently well conserved between animals and plants because the endogenous immunoglobulin signal peptides

function as efficiently as those derived from plant genes. In the absence of further targeting information, the expressed antibody will be secreted to the intracellular space beneath the plant cell wall (the apoplast). Antibodies targeted in this manner accumulate to higher levels than those expressed in the cytosol [39, 59, 62].

Antibodies accumulate to still higher levels if the fusion protein contains a C-terminal KDEL peptide, which is used by cells to retain a sub-population of proteins within the endoplasmic reticulum. The signal causes proteins in the secretory pathway to be retrieved from the Golgi apparatus and transported back to the endoplasmic reticulum so they are not lost in the continuous flow of proteins to the plasma membrane [63]. Accumulation levels are often two- to tenfold greater compared to an identical protein lacking the KDEL signal [41, 64]. The endoplasmic reticulum presumably provides a more stable environment for protein accumulation.

Gene transfer to plants

The next stage of the process involves transferring the gene expression construct to the expression host. Several considerations are important here. There is a choice between stable or transient transformation and between the use of whole plants or cell/tissue culture, and in each system, a species must be chosen as the expression host.

Stable gene transfer using whole plants

Stable transformation results in the permanent integration of an antibody-encoding transgene into the plant genome, such that it is passed to subsequent generations as a new genetic locus. The nuclear or plastid genomes can be modified in this way, resulting in mendelian or cytoplasmic inheritance of the transgene, respectively. Integration of transgenes into the chloroplast genome can generate higher expression levels than nuclear transgenes because of the potential for increasing transgene copy number and the lack of epigenetic silencing effects [65, 66]. However, this is an unsuitable strategy for the expression of full-size antibodies or Fab fragments because targeting to the secretory pathway is required for correct assembly and processing (see above).

Approximately 100 plant species can now be routinely transformed, in most cases using *Agrobacterium*-mediated gene transfer [67] or bombardment with DNA-coated particles [68]. However, the efficiency of these techniques varies and transforming and regenerating some species, such as field peas and wheat, is still technically demanding. Despite these complications, recombinant antibodies have been expressed in transgenic alfalfa [69], *Arabidopsis* [34, 70], pea [71, 72], petunia [60], potato [54], soybean [73], tobacco [58], rice [74] and wheat [75]. The expression of antibodies in transplastomic plants remains to be demonstrated.

The actual transfer of DNA into plants is rapid, but the selection of transformed cells and their regeneration into transgenic plant lines is time consuming. For example, 8–12 weeks are required to generate transgenic tobacco plants, and several months for transgenic cereals. This is the main disadvantage of stable transformation and it is general practice to test the activity and efficiency of expression constructs in transient expression systems, where gene expression can be confirmed within a few days, before going to the expense of stable transformation. Transient expression systems do not always predict the activity of an expression construct in stably transformed plants, but they do at least show that the construct can produce a functional protein.

Transient expression systems

Transient expression can be used to test the function of an expression construct before progressing to large-scale production in stable transgenic lines, but transient expression itself can be used for protein production. A useful method is *agroinfiltration*, in which recombinant *Agrobacterium tumefaciens* is infiltrated into plant tissue [76]. The T-DNA is transferred to the nucleus in a large number of plant cells resulting in the production of milligram amounts of recombinant protein within a few days. Fischer and colleagues [27] have used this method to express a tumour-specific scFv in tobacco leaves.

Viral vectors have also attracted interest because viral infections are rapid and systemic, and infected cells yield large amounts of virus and viral gene products [77, 78]. Since plant viruses do not integrate into the genome, there is no stable transformation and the transgene is not passed through the germ line. However, plant viruses often have a wide host range, are easily transmissible by mechanical inoculation and can spread from plant to plant, making possible rapid infection of large numbers of plants. Plant viral vectors have been used to express scFvs [79–81] and full-size antibodies [82]. In the latter case, two tobacco mosaic virus vectors were constructed carrying the heavy and light chains of the antibody. Tobacco plants were co-infected with the two vectors and both transgenes were expressed. Assembly of the antibody in planta was confirmed. The Large Scale Biology Corporation is carrying out field trials using tobacco mosaic virus vectors that produce hepatitis B surface antigen, scFvs and other recombinant proteins [81, 83].

Plant suspension cells for protein production

As an alternative to whole-plant expression systems, plant cell culture can be used for molecular farming. For pharmaceutical proteins such as antibodies, the increased capital and running costs of growing suspension cell lines in shaker flasks or fermentors may be ameliorated by the advantages in terms of the ease of transformation, the rapid production cycles and the high level of contain-

ment. Certainly, plant cell cultures are advantageous over animal cell cultures due to inexpensive media and improved safety (i.e. the absence of human and animal pathogens). Plant cells can be transformed by *Agrobacterium* [67, 84], particle bombardment [68], electroporation of protoplasts [85] or viral vectors [86]. The tobacco BY-2 cell line can be directly transformed by co-cultivation of suspension cells and *Agrobacterium* [87]. This has the advantage that transient expression of the foreign gene can be detected 2–3 days after co-cultivation.

Recombinant proteins expressed in plant cell suspension cultures may be secreted into the culture supernatant or retained within the cells. This depends on the nature of any targeting signals on the expression construct (see above) and the permeability of the plant cell wall to macromolecules [88]. Targeting signals can be used to direct the protein to the secretory pathway [89] or to intracellular organelles such as the chloroplast or vacuole [90]. As shown for transgenic plants, the cytosol is generally unsuitable for the accumulation of recombinant antibodies because incorrect folding and assembly lead to rapid degradation. In contrast, the retention of proteins in distinct intracellular organelles can preserve integrity by protecting the molecules from proteolysis [91]. Antibody derivatives such as Fab fragments and scFvs can pass through the plant cell wall and are secreted into the culture medium, but full-size antibodies tend to be retained in the apoplast [64]. Intracellular protein retention makes necessary the disruption of the cells prior to protein purification. This has several drawbacks, since it causes the release of phenolic substances or proteases that reduce protein yield. Thus, the preferred method is to target proteins for secretion, and capture them from the culture supernatant or release them from the cell by mild enzymatic cell wall digestion [64].

Plant suspension cells have the potential to become a valuable production system for recombinant antibodies because yields of up to 30 mg/l have been achieved in the case of a secreted single-chain antibody produced by cultured tobacco suspension cells [44]. This is described in a patent by Russell and Fuller where the Monsanto corporation is the assignee [92]. Furthermore, suspension cells can be grown in sealed reactors so that they are entirely contained and there is no risk to the environment. Thus, suspension cells provide an alternative to transgenic plants in those parts of Europe where environmental groups, the media and the general public have reacted with suspicion and aggression towards field trials. Tobacco suspension cultures have been used for the production of a number of pharmaceutical proteins, including antibodies and their derivatives [reviewed in ref. 93]) but rice suspension cultures are also gaining popularity [94, 95].

Choice of crop species

A large number of species are now amenable to molecular farming, including model plants (tobacco, *Arabidopsis*), cereal crops (rice, wheat, maize), legumes (pea, soybean, alfalfa) fruit crops (tomato, banana) and solanaceous species (potato). This, together with the number of different systems discussed above, makes the choice of host species a complex issue.

Many factors need to be taken into consideration. The yield of functional protein in a given species needs to be evaluated carefully, since this factor has to be weighed against the total biomass yield over a given planted area and any associated overhead costs. For example, tomatoes have a very high biomass yield per acre but they must be grown in greenhouses, which adds significantly to the cost. Even so, the advantages in terms of containment compared to field crops may need to be considered.

The system of choice has to be evaluated on a case-by-case basis according to the production area and the value of the recombinant protein. For highly valuable proteins, a considerable investment into set-up and running costs would be justified. For lower-value proteins, using existing agricultural and processing infrastructure would be more economical. The storage and distribution of the product is also an important factor. For example, the antibodies expressed in potato tubers and cereal grains are stable at room temperature for months or even years without loss of stability [62, 75]. The costs of grain storage and distribution are minimal compared those of freshly harvested tobacco leaves or tomato fruits, but the costs of extraction and purification are lower for watery plant material than desiccated seed. Another consideration is that the production of pharmaceutical proteins in edible crops will require intensive monitoring to avoid these products entering the food and feed chain. This makes non-food and feed crops such as tobacco attractive even though some varieties contain toxic metabolites that must be removed.

A valid comparison of different production hosts would require the same recombinant antibody to be expressed in several different species. We have performed such a study using a scFv antibody that recognizes the carcinoembryonic antigen (CEA), one of the best-characterized tumour-associated antigens. Anti-CEA antibodies are extensively used for tumour diagnosis and therapy and cost-effective systems for their production are needed. We have shown that the anti-CEA scFvT84.66 antibody fragment accumulates to the highest levels in the leaves and seeds of rice, followed by tobacco leaves, pea seeds and wheat [71, 75, 96]. Moreover, across all the species tested, accumulation of the scFv in the endoplasmic reticulum by means of the KDEL retrieval signal significantly improved protein levels compared to antibodies secreted into the apoplast (fig. 2).

The compromise between production costs and profit is likely to be a key issue in selecting the most suitable species for molecular farming because most pharmaceuticals will be produced by industry. We predict that these

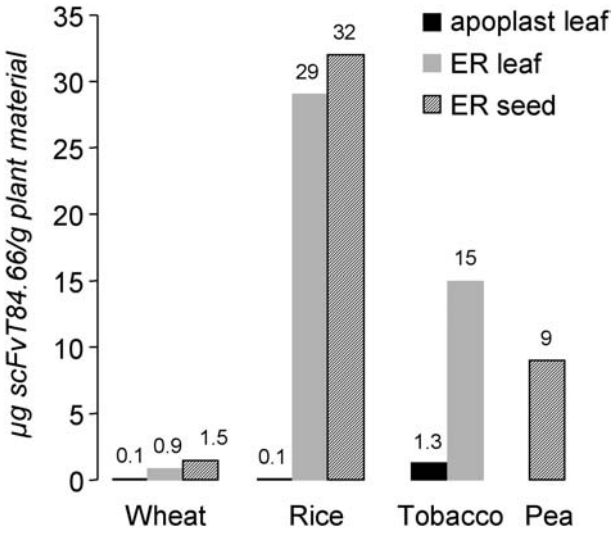

Figure 2. Maximum scFvT84.66 antibody production levels (µg/g plant material) in wheat and rice leaves and seeds, pea seeds and tobacco leaves. Accumulation of functional scFvT84.66 in the endoplasmic reticulum (ER) and apoplast of plant cells was analysed by ELISA [27, 71, 75, 96].

costs will dictate which crop or crops become generally accepted for recombinant antibody production.

Scaling production – determinants of absolute yields

The absolute yield of a given recombinant protein in a particular plant species and plant cell compartment is unpredictable, and while increasing the yields of recombinant protein is an important objective, most research in this area is speculative. Selected recombinant proteins already reach very high expression levels, while others hardly accumulate at all. For example, apoplast-targeted recombinant phytase accumulates to almost 14% total soluble protein (TSP) in tobacco leaves [97], and a eubacterial glucanase accumulates to 26% TSP in the apoplast of *Arabidopsis thaliana* [98]. The standard expression levels of recombinant antibodies in the apoplast and endoplasmic reticulum of stably transformed plants usually fall within the range 0.5–2% TSP [62]. This will likely improve because in the best cases, antibodies reach levels of nearly 7% TSP [41]. Further improvements are likely to come with the discovery or invention of improved promoters and other regulatory elements, and from the development of strategies to eliminate transgene silencing in transgenic plants.

Since the greatest cost in molecular farming comes not from protein production per se but from isolation and purification [99], the crops that will be favoured over the next decade may be those in which isolation and purification appear to be the most economical. In addition, products that need little or no purification or that can be directly consumed in transgenic grains, fruits or vegetables may be some of the first entries into the marketplace.

Downstream processing of recombinant antibodies from plant cells

Over 95% of the costs of producing a recombinant protein by molecular farming come from extracting the protein from the plant and purifying it [99]. Therefore, while improving yields remains an important objective, the development of optimized and economical methods for protein extraction is of much greater commercial interest.

Plants require different handling procedures early on in protein isolation compared to microbial and animal expression systems. If the recombinant polypeptide is secreted from suspension cells, it can be recovered after removal of cell material by filtration and clarification of the media before purification begins. If the protein is retained within the cells or produced in leaf tissue, a simple, efficient method has to be developed to disrupt the tissue and cells and release the protein. Mechanical cell disruption devices like bead-mills are efficient but introduce complications related to heat generation, lysis of subcellular organelles, liberation of noxious chemicals (such as alkaloids and phenolics) and generation of fine cell debris that can be difficult to remove. Protocols for the recovery of recombinant antibodies from animal cells, serum and microbial cells are refined and well described. However, there are few reports of purification methods for recombinant antibodies from plant suspension cells, leaves or seeds [90].

In this context, the development of transgenic plants that secrete recombinant proteins could be useful. Two recently described processes are rhizosecretion (secretion of proteins from roots) and phyllosecretion (secretion of proteins through guttation, the loss of water and dissolved materials from the aerial parts of plants). In both cases, transgenic tobacco plants have been produced in which recombinant proteins are targeted for secretion, and are recovered either from hydroponic medium or directly from guttation fluid. This potentially reduces the effort involved in processing, although the proteins are recovered at a low concentration [100–102].

We have investigated the criteria important for the recovery of full-size antibodies from suspension cells [64] and developed an affinity purification protocol exploiting protein-A-based matrices. We used partial enzymatic lysis to release full-size antibodies from the intercellular space of plant cells and this was the superior method for isolation of functional antibodies. As the initial step, Protein-A affinity chromatography was efficient for the removal of contaminants and achieved 100-fold concentration of the recombinant protein. Gel filtration was applied as a polishing step for the removal of recombinant antibody dimers and for exchange of the recombinant antibodies into a suitable storage buffer. This protocol allows more than 80% of a full-size IgG to be recovered from plant suspension cell cultures [64].

The economics of molecular farming in plants

From the point of view of industry, the key advantage of molecular farming in plants is the capacity for virtually limitless scale up with minimal associated costs. This will allow transgenic plants to be cultivated over large areas and the potential for profit will increase with scale.

There is a long-term demand for many proteins which is barely met by current production systems. Human serum albumin, for example, has an annual worldwide demand of over 500 tonnes. Currently, the protein is isolated at great expense from its natural source, blood. Transgenic plants could be used as an alternative, cheaper and safer production system limited only by the amount of plant biomass that could be harvested. High-intensity agriculture can produce surprisingly large amounts of biomass. For example, intensive cultivation of tobacco plants can yield 170 tonnes per hectare [103, 104]. Assuming that the levels of production seen at the laboratory scale could be maintained in the field and that for every 170 tonnes harvested, 100 tonnes are harvested leaves, a single hectare of tobacco could yield 50 kg of a secretory IgA per harvest [28]. Production costs of only US $ 40 per gram have been estimated [93] and with optimization of downstream processing methods this could be reduced to US $ 20. This compares favourably with animal culture systems which are more expensive by two orders of magnitude.

Long-term perspectives

As discussed in the Introduction, 20% of biopharmaceuticals under current development are antibodies and more than 200 are already at the clinical trials stage. These represent treatments for diseases ranging from multiple sclerosis to renal cancer. This demonstrates the importance of antibodies as a uniquely flexible tool for the treatment of disease. They are unlikely to be superseded in the coming years. A number of potential therapeutic antibodies have been produced in plants (table 2) and four case studies are described briefly below.

Table 2. Diagnostic and therapeutic antibodies produced in plants.

Antibody format	Antigen	Cellular location	Transformed	Maximum species	References expression level
dAb	substance P (neuropeptide)	apoplast	*Nicotiana benthamiana*	1% TSP leaves	43
IgG1, Fab	human creatine kinase	apoplast	*N. tabacum, Arabidopsis thaliana*	0.044% TSP leaves, 1.3% TSP leaves	33, 34
SIgA	streptococcal surface antigen (I/II)	apoplast	*N. tabacum*	500 µg/g FW leaves	28
scFv	human creatine kinase	Cytosol, apoplast	*N. tabacum*	0.01% TSP leaves	106
scFv-IT	CD-40	apoplast	*N. tabacum* cell culture	not reported	44
IgG1	herpes simplex virus 2	apoplast	*Glycine max*	not reported	73
IgG	colon cancer antigen	ER	*N. benthamiana*	not reported	82
IgG1	human IgG	apoplast	*Medicago sativa*	1% TSP	69
scFv, IgG1	carcinoembryonic antigen	apoplast, ER	*N. tabacum* (transient expression)	5 µg scFv/g leaves, 1 µg IgG/g leaves	27
scFv	38C13 mouse B cell lymphoma	apoplast	*N. benthamiana*	30.2 µg/g leaves	81
scFv	carcinoembryonic antigen	apoplast, ER	*Oryza sativa*	3.8 µg/g callus, 29 µg/geaves, l 32 µg/g seed	74, 75
scFv	carcinoembryonic antigen	apoplast, ER	*Triticum aestivum*	900 ng/g leaves, 1.5 µg/g seed	75
scFv	Carcinoembryonic antigen	ER	*Pisum sativum*	9 µg/g seed	71
IgG1	streptococcal surface antigen (I/II)	plasma membrane	*N. tabacum*	1.1% TSP leaves	107

dAb, single-domain antibody; FW, fresh weight; scFv-IT, scFv-bryodin-immunotoxin; SIgA, secretory IgA; TSP, total soluble protein; ER, endoplasmic reticulum.

1) A chimeric secretory IgG-IgA antibody produced in transgenic tobacco plants has been developed to prevent the oral bacterial infection that contributes to dental caries [105]. The antibody can stop recolonization of the buccal cavity by *Streptococcus mutans*, the organism responsible for tooth decay in humans. This results in the replacement of the pathogenic organism with endogenous flora. The antibody recognizes a surface adhesin which is essential for the bacteria to adhere to teeth. Phase II clinical trials of this antibody are underway [29].
2) Antibodies and antibody fragments specific for the human CEA have been produced in tobacco, rice, wheat and pea [27, 71, 75] (see fig. 2). Plant-produced anti-CEA antibodies are likely to allow the development of an inexpensive method for tumour detection and antibody-based cancer therapy.
3) A humanized antibody against herpes simplex virus 2 (HSV-2) has been produced in transgenic soybean and shown to be efficient in preventing vaginal HSV-2 transmission in mice. The ex vivo stability and in vivo efficacy of anti-HSV-2 antibodies produced in plants and mammalian cell culture were similar [73].
4) A plant virus transient expression system has been used to produce a tumour-specific vaccine for the treatment of malignancies. Tobacco plants were infected with a modified tobacco mosaic virus vector encoding an idiotype-specific scFv corresponding to the immunoglobulin from the 38C13 mouse B cell lymphoma. These plants secreted high levels of scFv protein to the apoplast. Mice vaccinated with the affinity-purified 38C13 scFv generated anti-idiotype immunoglobulins. These mice were protected from challenge by a lethal dose of the 38C13 tumour, similar to mice immunized with the native 38C13 IgM-keyhole limpet haemocyanin conjugate vaccine [81]. This rapid production system for tumour-specific protein vaccines may provide a viable strategy for the treatment of non-Hodgkin's lymphoma. The goal of the therapy is to create antibodies customized for each patient that will recognize unique markers on the surface of the malignant B cells and target the cells for destruction.

These case studies demonstrate that most therapeutic antibodies currently produced in alternative systems could be produced inexpensively in plants. This would reduce the cost of treatment and increase the number of patients with access to such medicines. This emphasizes the fact that molecular farming in plants can remove financial barriers to the wider use of antibodies in medicine and research. Thus, in the future, antibodies may find uses in the kinds of therapy where cost makes their current use prohibitive.

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