Chapter 3
Molecular Farming of Antibodies in Plants

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Abstract Biopharmaceuticals are produced predominantly in microbial or mammalian bioreactor systems. Over the last few years, however, it has become clear that plants have great potential for economical, large-scale biopharmaceutical production. Following the commercial release of several maize-derived technical proteins, the first plant-derived veterinary vaccine was approved in 2006. Plants offer the prospect of inexpensive production without sacrificing product quality or safety. The first therapeutic products for use in humans – mostly antibodies and vaccine candidates – are now at the clinical trials stage. In this chapter, we discuss the different plant-based production systems that have been used to synthesize recombinant antibodies and to evaluate the merits of plants compared with other platforms. Despite the currently unclear regulatory framework, the benefits of plant-derived systems are now bringing the prospect of inexpensive recombinant antibodies closer than ever before.

3.1 Introduction

Antibodies are multisubunit glycoproteins produced by the vertebrate immune system. They recognize and bind to their target antigens with great affinity and specificity, which allows them to be used for many applications, including the diagnosis, prevention, and treatment of human and animal disease (Andersen and Krummen, 2003; Chadd and Chamow, 2001; Fischer and Emans, 2000). It is estimated that approximately 1,000 therapeutic recombinant antibodies are under development, up to one-quarter of which may already be undergoing clinical trials. A large proportion of these antibodies recognize cancer antigens, but others have been developed for the diagnosis and treatment of infectious diseases, acquired disorders,

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and even transplant rejection (Gavilondo and Larrick, 2000). As well as having biomedical applications, antibodies can also be exploited to prevent diseases in plants (Schillberg et al., 2001), to detect and remove environmental contaminants, and for various industrial processes such as affinity purification and molecular targeting (Stoger et al., 2005b).

With such a diverse spectrum of uses, the potential market for antibodies is extremely large and there is considerable interest in high-capacity production technologies that are robust, economical, and safe. Over the last 15 years, plants have emerged as convenient, economical, and scalable alternatives to the mainstream antibody production systems which are based on the large-scale culture of microbes or animal cells (Chu and Robinson, 2001; Wurm, 2004). In this chapter, we discuss the advantages and disadvantages of plants for antibody production, the diverse plant-based systems that are now available, and factors governing the success of antibody production in plants. We begin, however, with a brief overview of recombinant antibody technology.

3.2 Recombinant Antibody Technology

The typical antibody format is the mammalian serum antibody, which comprises two identical heavy chains and two identical light chains joined by disulfide bonds (Fig. 3.1). Each heavy chain is folded into four domains, two on either side of a flexible hinge region, which allows the multimeric protein to adopt its characteristic shape. Each light chain is folded into two domains. The N-terminal domain of each of the four chains is variable, i.e., it differs among individual B cells due to unique

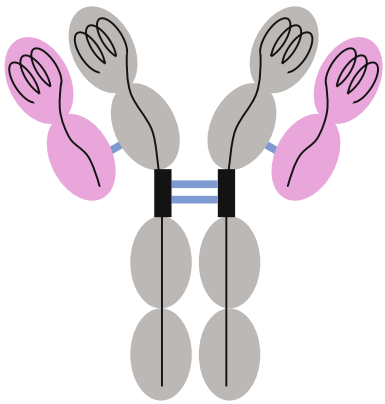

Fig. 3.1 Structure of a typical mammalian serum antibody, comprising two identical heavy chains (*gray*) and two identical light chains (*pink*). Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections*represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*

rearrangements of the germ-line immunoglobulin genes. This part of the molecule is responsible for antigen recognition and binding. The remainder of the antibody comprises a series of constant domains, which are involved in effector functions such as immune cell recognition and complement fixation. Below the hinge, in what is known as the Fc portion of the antibody, the constant domains are class-specific. Mammals produce five classes of immunoglobulins (IgG, IgM, IgA, IgD, and IgE) with different effector functions. The Fc region also contains a conserved asparagine residue at position 297 to which *N*-glycan chains are added. The glycan chains play an important role both in the folding of the protein and in the performance of effector functions (Jefferis, 2001).

Antibodies are also found in mucosal secretions, and these secretory antibodies have a more complex structure than serum antibodies. They are dimers of the serum-type antibody, the two monomers being attached by an additional component called the joining chain. There is also a further polypeptide called the secretory component, which protects the antibodies from proteases (Fig. 3.2).

Antibodies obtained from immunized animals are polyclonal, i.e., derived from many different B cells. The advantage of monoclonal antibodies, i.e., antibodies derived from a single clone of B cells, is that their binding specificity does not vary. The traditional source of monoclonal antibodies is murine B cells. To provide a constant source of the antibody, B cells of appropriate specificity are fused to immortal myeloma cells to produce a *hybridoma cell line*. However, the use of murine hybridoma-derived antibodies as therapeutics is limited because the murine components of the antibodies are immunogenic in humans, resulting in a so-called human antimouse antibody (HAMA) response. Therefore, numerous strategies

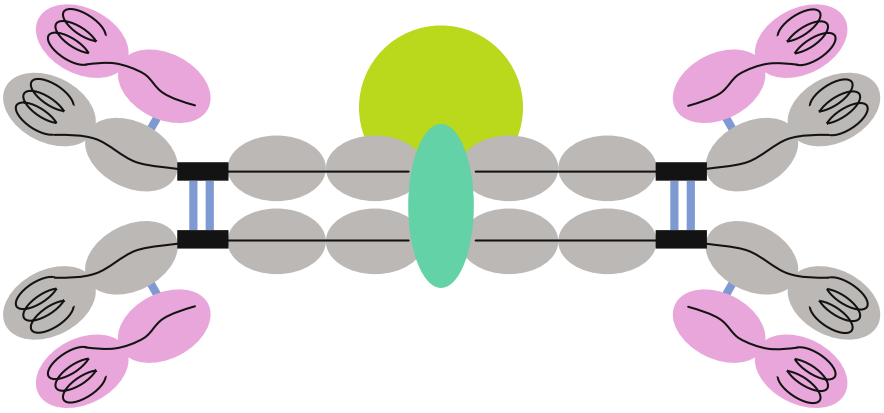

Fig. 3.2 Structure of a mammalian secretory antibody, comprising a dimer of the typical serum antibody and including two additional components, the joining chain (*blue disc*) and the secretory component (*green disc*). Heavy chains are shown in *gray* and light chains in *pink*. Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections*represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*

have been developed to humanize murine monoclonal antibodies (Kipriyanov and Little, 1999), culminating in the production of transgenic mice expressing the human immunoglobulin genes (Green, 1999). An alternative approach is to use phage display libraries based on the human immune repertoires. Phage display is advantageous because high-affinity antibodies can be identified rapidly, novel combinations of heavy and light chains can be tested, and the DNA sequence encoding the antibody is indirectly linked to the antibody itself (Griffiths and Duncan, 1998; Sidhu, 2000). This avoids the laborious isolation of cDNA or genomic immunoglobulin sequences from hybridoma cell lines.

The expression of serum-type or secretory-type antibodies as recombinant molecules requires the preparation and expression of two and four different transgenes, respectively. However, this is often an unnecessary complication, because in many cases, the effector functions conferred by the constant regions are neither required nor desired. The constant regions of native immunoglobulins are not required for antigen binding, and the variable regions of the heavy and light chains can interact perfectly well when joined on the same polypeptide molecule (Chadd and Chamow, 2001; Fischer and Emans, 2000). Smaller antibody derivatives, which still require two chains, include Fab and $F(ab')_2$ fragments (which contain only the sequences distal to the hinge region) and minibodies (which contain only part of the constant portion of the molecule). Other derivatives, such as large single chains, single-chain Fv fragments (scFvs), and diabodies, contain the variable regions of the heavy and light chains joined by a flexible peptide chain. Such derivatives are often more effective as drugs than full-length immunoglobulins because they show increased penetration of target tissues, reduced immunogenicity, and are cleared from tissues more rapidly. Another variant is the *camelid serum antibody*, which is unique in that it contains only heavy chains. A full-size camelid antibody can, therefore, be expressed from a single transgene. Further, more specialized derivatives include bispecific scFvs, which contain the antigen recognition elements of two different immunoglobulins and can bind to two different antigens, and scFv fusions, which are linked to proteins with additional functions. Examples of all these antibody derivatives are shown in Fig. 3.3.

3.2.1 Expression Systems for Recombinant Antibodies

Most of the recombinant full-length immunoglobulins being developed as pharmaceuticals are produced in mammalian cell cultures, a few in hybridoma lines, but most in immortalized lines that have been cleared by the FDA (Food and Drug Administration) and equivalent authorities in other countries. These lines include Chinese hamster ovary (CHO) cells, the murine myeloma cell lines NS0 and SP2/0, baby hamster kidney (BHK) and human embryonic kidney (HEK)-293 cells, and the human retinal line PER-C6 (Chu and Robinson, 2001). The main reason for this is the belief that mammalian cells yield authentic products, particularly in terms of glycosylation patterns. However, there are minor differences in glycan chain structure between rodent and human cells. For example, human antibodies contain only the

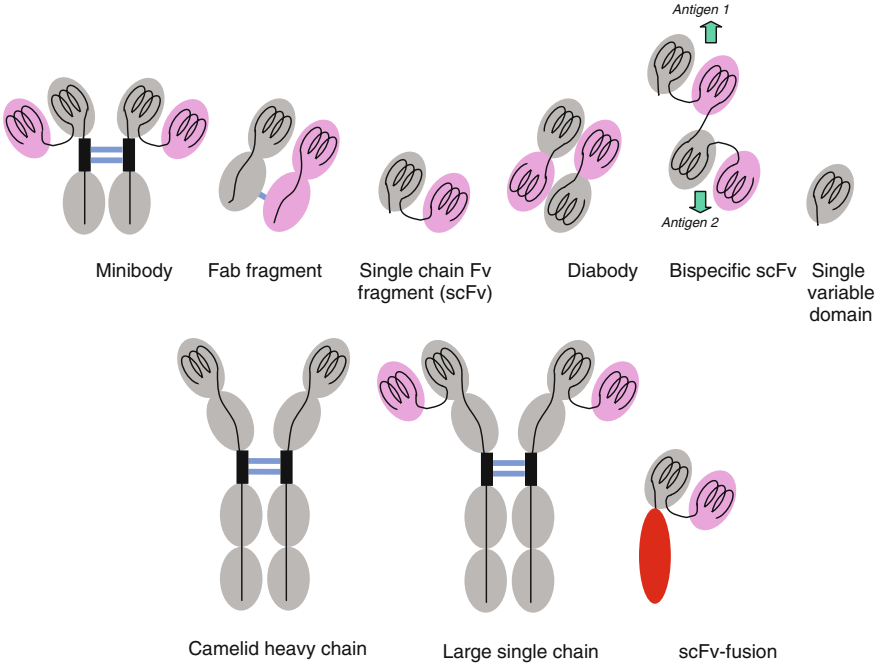

Fig. 3.3 Structure of recombinant antibody derivatives and atypical antibody formats, most of which have been expressed in plants. Heavy-chain derivatives are shown in *gray* and light-chain derivatives in *pink*. Solid *black lines* indicate continuation of the polypeptide backbone (*simple lines* indicate the constant parts of the antibody, *curly lines* indicate the variable regions, and *thick sections* represent the hinge region). Antibody domains are indicated by *colored circles*. Disulfide bonds are represented by *gray bars*. The *red disc* indicates a new functional protein domain in the scFv fusion protein

sialic acid residue *N*-acetylneuraminic acid (NANA), while rodents produce a mixture of NANA and *N*-glycosylneuraminic acid (NGNA) (Raju et al., 2000). There are many disadvantages to mammalian cell cultures, including the high setup and running costs, the limited opportunities for scale-up, and the potential contamination of purified recombinant antibodies with human pathogens. Bacterial fermentation systems are more cost-effective than mammalian cell cultures and are therefore preferred for the production of Fab fragments and scFvs, since these derivatives are not glycosylated. Even so, the yields of such products in bacteria are generally low because the proteins do not fold properly (Baneyx and Mujacic, 2004). The main reason for sticking to these systems is that they are well characterized and established and conform to the strict and extensive regulatory systems governing biopharmaceutical production.

Several alternative production systems have been explored, some of which are now well established while others are still experimental. In the former category, yeast and filamentous fungi have the advantages of bacteria (economy and robustness), but they do have the tendency to hyperglycosylate recombinant proteins

(Gerngross, 2004), while insect cells can be cultured in the same way as mammalian cells (although more cheaply) and also produce distinct glycan structures (Ikonomou et al., 2003). A more recent development is the production of antibodies in the milk of transgenic animals (Dyck et al., 2003). A disadvantage of animals, in common with cultured mammalian cells, is the existence of safety concerns about the transmission of pathogens or oncogenic DNA sequences. Finally, hen's eggs could also be used as a production system since they are protein-rich and already synthesize endogenous antibodies, but they remain a relatively unexplored potential expression system (Harvey et al., 2002). Plants offer a unique combination of advantages for the production of pharmaceutical antibodies (Twyman et al., 2003, 2005; Ma et al., 2003; Basaran and Rodriguez-Cerezo, 2008). Their main benefit is the low production costs, reflecting the fact that traditional agricultural practices and unskilled labor are sufficient for maintaining and harvesting antibody-expressing crops. Also, large-scale processing infrastructure is already in place for most crops. Scale-up is rapid and efficient, requiring only the cultivation of more land. There are minimal risks of contamination with human pathogens.

The general eukaryotic protein synthesis pathway is conserved between plants and animals. So plants can efficiently fold and assemble full-size serum immunoglobulins (as first demonstrated by Hiatt et al., 1989) and secretory IgAs (first shown by Ma et al., 1995). In the latter case, four different subunits need to assemble in the same plant cell to produce a functional product, even though two different cell types are required in mammals. The posttranslational modifications carried out by plants and animals are not identical to those in mammals, but they are very similar (certainly more so than in fungal and insect systems). There are minor differences in the structure of complex glycans, such as the presence in plants of the residues α-1,3-fucose and β-1,2 xylose, which are absent from mammals (Cabanes-Macheteau et al., 1999). These residues are immunogenic in several mammals, including humans, but curiously not in mice and only after multiple exposures in rats (Gomord et al., 2005; Faye et al., 2005). However, as discussed in more detail below, there are now many studies that show how the glycan profile of proteins produced in plants can be "humanized." As well as full-size antibodies, various functional antibody derivatives have also been produced successfully in plants, including Fab fragments, scFvs, bispecific scFvs, single-domain antibodies, and antibody fusion proteins (see Twyman et al., 2005).

3.2.2 Plant-Based Expression Platforms

The most widely used strategy for antibody production in plants is the nuclear transgenic system, in which the antibody transgenes are transferred to the plant nuclear genome. The advantages of this approach when used in our major terrestrial crop species include the following: (1) transformation is a fairly routine procedure in many plant species and can be achieved by a range of methods, the two most common of which are *Agrobacterium*-mediated transformation and the delivery of DNA-coated metal particles by microprojectile bombardment; (2) a stable transgenic line

can be used as a permanent genetic resource; (3) among the various plant systems, it is the simplest to maintain (once the producer line of transgenics is available) and is ultimately the most scalable; (4) it is possible to establish master seed banks. Disadvantages, compared to other plant systems, include the relatively long development time required for transformation, regeneration, analysis of transgenics, selection and bulking up of the producer line, the unpredictable impact of epigenetic events on transgene expression (e.g., posttranscriptional gene silencing and position effects), and the potential for transgene spread from some crops through outcrossing. A range of different crops have been explored for antibody production, and the main categories are described below.

Leafy crops have two major benefits: they have a large biomass, which translates to large product yields, and flowering can be prevented (e.g., genetically or by emasculation) to avoid the spread of transgenic pollen. On the other hand, leaf tissue is very watery such that proteins are expressed and accumulate in an aqueous environment in which they are subject to degradation. This means that antibody-containing leaves generally have to be processed soon after harvest or otherwise frozen or dried, which can add significantly to production costs. Tobacco (*Nicotiana tabacum* L.) has the longest history as a pharmaceutical production model crop system, having been used to express the very first plant-derived antibodies and many of the others since (Table 3.1). The major advantages of tobacco are the well-established technology for gene transfer and expression, the high biomass yield (over 100,000 kg/h for close cropped tobacco, since it can be harvested up to nine times a year), and the existence of large-scale infrastructure for processing that does not come into contact with the human or animal food chains. Particularly due to the yield potential and safety features, tobacco could be a major source of plant-derived recombinant antibodies in the future. Another leafy crop that has been evaluated for antibody expression is alfalfa (*Medicago sativa* L.). This has been developed as a production crop by the Canadian biotechnology company Medicago Inc., and they have secured a robust IP portfolio covering the use of expression cassettes for biopharmaceutical proteins in this species. Although not as prolific as tobacco, alfalfa nevertheless produces large amounts of leaf biomass and has a high leaf protein content. Alfalfa also lacks the toxic metabolites produced in many tobacco cultivars, which are often cited as a disadvantage, but instead it contains high levels of oxalic acid, which can affect protein stability. Alfalfa is particularly useful because it is a perennial plant that is easily propagated by stem cutting to yield clonal populations. Although alfalfa has been put on the biosafety "hit list" by the regulators because it outcrosses with wild relatives, this does not detract from the excellent properties of this species for antibody production under containment, as in greenhouses or programmed plant growth chambers. Alfalfa has been used for the production of a diagnostic IgG that recognizes epitopes specific to the constant regions of human IgG (Khoudi et al., 1999) and for several other antibodies in development by Medicago Inc.

The problem of protein instability in leafy tissue (see above) can be overcome by expressing antibodies in the dry seeds of cereals and grain legumes. Several different species have been investigated for antibody production including four major cereals (maize, rice, wheat, and barley) and two legumes (soybean and pea). The

Table 3.1 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 IP 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
HIV gp120 (2F5)	IgG	Tobacco, maize, tobacco suspension cells	Maximum yield ~75 μg/g seeds	Floss et al. (2008), Sack et al. (2007)
HIV gp120 (2G12)	IgG	Tobacco, maize	Maximum yield ~100 μg/g seeds	Rademacher et al. (2008), Ramessar et al. (2008c)
B-cell lymphoma, murine 38C13	scFv	Virus vectors in tobacco leaves	Maximum yield 30.2 μg/g leaves	McCormick et al. (1999)
Carcinoembryonic antigen	scFv, IgG1	Tobacco agroinfiltration	Directed to apoplast or ER. Maximum yields 5 μg scFv/g	Vaquero et al. (1999)
	dAb	Tobacco, agroinfiltration and transgenic plants	leaves, 1 μg IgG/g leaves	Vaquero et al. (2002)
	scFv	Rice, rice cell cultures	Directed to apoplast or ER. Maximum yields 3.8 μg/g callus, 29 μg/g leaves, 32 μg/g seed	Torres et al. (1999), Stoger et al. (2000)
	scFv	Wheat	Directed to apoplast or ER. Maximum yields 900 ng/g leaves, 1.5 μg/g seed	Stoger et al. (2000)
	scFv	Pea	Directed to rER. Maximum yield 9 μg/g seed	Perrin et al. (2000)
CD-40	scFv fusion	Tobacco suspension cells	Secreted into apoplast. Yield not reported	Francisco et al. (1997)
Colon cancer antigen	IgG	Virus vectors in tobacco leaves	Yield not reported	Verch et al. (1998)
Epidermal growth factor receptor (EGFR)	IgG	Tobacco	Aglycosylated antibody was directed to the ER and binds to EGFR expressed on the surface of human tumor cells	Rodriguez et al. (2005)
Human creatine kinase	IgG1, Fab	Tobacco and *Arabidopsis* leaves	Accumulated in nucleolus or apoplast. Maximum yield 1.3% TSP	De Neve et al. (1993), De Wilde et al. (1998)

Table 3.1 (continued)

Antigen	Antibody format	Production system	Comments	References
	scFv	Tobacco leaves	Directed to cytosol or apoplast. Maximum yield 0.01% TSP	Bruyns et al. (1996)
Rhesus D antigen	IgG1	*Arabidopsis* leaves	Reacted with RhD^+ cells in antiglobulin technique and elicited a respiratory burst in human peripheral blood mononuclear cells	Bouquin et al. (2002)
Ferritin	scFv	Tobacco leaves		Semenyuk et al. (2002)
Hepatitis B virus surface antigen	IgG	Tobacco leaves	Up to 25 mg antibody per kilogram biomass	Valdes et al. (2003[a],b)
	IgG	Tobacco suspension cells	Complement-dependent cytotoxicity demonstrated	Yano et al. (2004)
	scFv	Tobacco	Four different targeting constructs used, ER targeting achieved 0.22% TSP	Ramírez et al. (2002)
Herpes simplex virus 2	IgG1	Soybean	Secreted into apoplast. Yield not reported	Zeitlin et al. (1998)
HIV antibodies in blood	scFv fusion	Tobacco leaves, barley grains, potato tubers	Maximum yield 150 mg/g	Schunmann et al. (2002)
Human choriogonadotrophin	scFv, dAb, IgG	Tobacco leaves	Secreted into apoplast. Maximum yield 40 mg/kg fresh weight	Kathuria et al. (2002)
	IgG1, diabody	Tobacco and winter cherry leaves	Directed to apoplast or ER. Glycan patterns were analyzed	Sriraman et al. (2004)
Human IgG	IgG1	Alfalfa	Secreted into apoplast. Maximum yield 1% TSP	Khoudi et al. (1999)
Interleukin-4	scFv	Tobacco roots	Maximum yield 0.18% TSP	Ehsani et al. (2003)
Interleukin-6	scFv	Tobacco roots		Ehsani et al. (2003)
Protective antigen of *Bacillus anthracis*	IgG	*N. benthamiana*	Toxin activity was neutralized in vitro and in vivo	Hull et al. (2005)
Rabies virus	IgG	Tobacco	Directed to the ER. Activity of the rabies virus was neutralized. Glycan patterns were analyzed	Ko et al. (2003)
Salmonella enterica lipopolysaccharide	scFv	Tobacco	41.7 μg purified scFv per gram leaf tissue	Makvandi-Nejad et al. (2005)

Table 3.1 (continued)

Antigen	Antibody format	Production system	Comments	References
Streptococcal surface antigen (I/II)	sIgA	Tobacco leaves	Secreted into apoplast. Maximum yield 500 μg/g fresh weight	Ma et al. (1995)
	IgG1	Tobacco leaves	Directed to plasma membrane. Maximum yield 1.1% TSP in leaves	Vine et al. (2001)
	IgG1	Secretion from tobacco roots	Up to 11.7 μg per gram dry root weight per day	Drake et al. (2003)
Substance P	VH	Tobacco leaves	Secreted into apoplast. Maximum yield 1% TSP	Benvenuto et al. (1991)
Tetanus toxin C	IgG2a fused to tetanus toxin C	Tobacco	Animals immunized with recombinant immune complex without adjuvant were fully protected against lethal challenge	Chargelegue et al. (2005)
Tumor-associated antigen EpCAM	IgG	Tobacco	Secreted into the apoplast. Binding activity to colon cancer cells and tumor inhibition activity in nude mice	Ko et al. (2005)

idea is that such crops would be beneficial for production in developing countries, where on-site processing would not be possible and a cold chain could not be maintained. The accumulation of recombinant antibodies in seeds allows for long-term storage at ambient temperatures because the proteins accumulate in a stable form (Ramessar et al., 2008a). Seeds have the appropriate molecular environment to promote protein accumulation, and they achieve this through the creation of specialized storage compartments such as protein bodies and storage vacuoles that are derived from the secretory pathway. Seeds are also desiccated, which reduces the level of both nonenzymatic hydrolysis and protease degradation. It has been demonstrated that antibodies expressed in seeds remain stable for at least 3 years at ambient temperatures with no detectable loss of activity (Stoger et al., 2005a).

As well as their advantages in terms of product stability, seed expression might also be beneficial in terms of downstream processing. This is because seeds have a relatively simple proteome (therefore minimizing the likelihood that endogenous proteins would be copurified) and lack the phenolic compounds abundant in leaves that can interfere with affinity purification. The restriction of recombinant protein accumulation in seeds also helps to avoid any potentially negative effects on the growth and development of vegetative plant organs and on humans, animals, and microorganisms that interact with the plant or feed on its leaves.

Disadvantages of seed crops include the lower overall yields that have been obtained. The intrinsic yields are in a few cases higher than tobacco (e.g., on a kilogram-per-kilogram basis of harvested material, rice grains can accumulate more antibody than tobacco leaves; Stoger et al., 2002), but the vast abundance of harvested biomass per hectare from a tobacco crop far outweighs this. Also, seeds are regarded as viable genetically modified organisms in their own right. So while the transport of harvested transgenic tobacco leaves should not cause any problems, the transport of seeds could fall afoul of national and international regulations on the transport of GMOs (Sparrow et al., 2007; Spök et al., 2008); the seeds would have to be crushed to flour beforehand and this might offset the advantage of increased product longevity.

Maize (*Zea mays* L.) seeds have been investigated as an antibody production vehicle by Prodigene Inc. following successful demonstrations of the economical production of other valuable proteins using this system, including avidin and β-glucuronidase. Initial findings for the expression of a secretory IgA in maize showed that the four chains were expressed, directed to the cell wall matrix, and assembled correctly. The product accumulated to 0.3% of total soluble protein in the T1 seeds, and based on previous results, significant improvements were anticipated through selective breeding (Hood et al., 2002). An antibody derivative used for HIV diagnostics has been expressed in barley and has achieved a yield of 150 μg/g. More recently, the HIV-neutralizing antibody, 2G12, was produced in maize seeds with a yield >100 μg/g for use as a potential microbicide (Ramessar et al., 2008c).

Finally, antibodies have also been produced in soybean (*Glycine max* (L.) Merr.), although in this particular case, it was expressed constitutively and isolated from the leaves rather than from the seeds (Zeitlin et al., 1998). Soybean has been investigated

as a potential production crop by Prodigene and others because it is a self-fertilizing crop with a high biomass yield, but product yields have been low and the system has therefore been largely abandoned.

Recombinant antibodies have been produced in potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.), which also offer certain advantages over other crops. Proteins accumulating in potato tubers are generally stable, because, like the cereal seed endosperm, these are storage organs that are adapted for high-level protein accumulation. The potential of potato tubers for antibody production was first demonstrated by Artsaenko et al. (1998), who produced an scFv fragment specific for the inflammatory agent oxazolone. Potatoes have since been developed as a general production host for antibodies (De Wilde et al., 2002) as well as other biopharmaceuticals based on antibodies (Schunmann et al., 2002). Fruit crops have another potential advantage, i.e., antibody expression in organs that are consumed raw allows the direct oral administration of recombinant antibodies designed for passive immunotherapy, such as protection of the oral cavity against pathogens. Stoger et al. (2002) describe preliminary experiments in which scFv84.66, recognizing the carinoembryonic antigen (CEA), is expressed in tomato fruits, although the accumulation levels are rather low (0.3 μg/g fresh weight). Other advantages of tomato include the high biomass yields (about 68,000 kg/ha, approaching the yields possible in tobacco) and the increased containment offered by growth in greenhouses.

Instead of introducing transgenes into the nuclear genome, they can be targeted to the chloroplast genome using particle bombardment or another physical DNA delivery technique and ensuring the transgene is embedded in a chloroplast DNA homology region (Maliga, 2003, 2004; Bock, 2007). The main benefits of the chloroplast system are that there are thousands of chloroplasts in a typical leaf cell, yet only one nucleus; therefore, the number of transgene copies in the cell following plastid transformation and the establishment of homoplasmy is much higher, promising greater product yields. This is enhanced by the absence of epigenetic phenomena such as transgene silencing in the chloroplast genome. Chloroplasts, derived from ancient bacteria, also support operon-based transgenes, allowing the expression of multiple proteins from a single transcript. Finally, and perhaps most importantly from the regulatory perspective, chloroplasts are absent from the pollen of most of our food crops, which limits the potential for outcrossing (Daniell et al., 2005a).

There are two disadvantages to the chloroplast system: first, chloroplast transformation is not a standard procedure and is thus far limited to a relatively small number of crops (e.g., tobacco, tomato, potato, cotton, soybean, lettuce, cauliflower, and sugar beet; Daniell et al., 2005b; Lelivelt et al., 2005; Nugent et al., 2006; De Marchis et al., 2009); second, since chloroplasts are derived from ancient bacteria, they lack much of the eukaryote machinery for posttranslational modification, i.e., they are unable to synthesize glycan chains. For this reason, they would be suitable for the production of scFvs but not full-size immunoglobulins.

In the only published report thus far dealing with antibody expression in terrestrial plant chloroplasts, a camelid antibody fragment was expressed in tobacco using an inducible T7-promoter system. Transcripts could be detected but no protein

(Magee et al., 2004). However, antibodies have been expressed successfully in algal chloroplasts (see below).

Transient expression assays are generally used to evaluate the activity of expression constructs or to test the functionality of a recombinant protein before committing to the long-term goal of generating transgenic plants. However, transient expression can also be used as a routine production method if enough protein can be produced to make the system economically viable. The advantages of this approach include the minimal setup costs and the rapid onset of protein expression, but scaling-up is expensive and impractical. So this type of system is particularly useful for the production of high-value proteins such as therapeutic antibodies, which have specialized markets and are required in small amounts.

An example of a transient expression system is the agroinfiltration method, where recombinant *Agrobacterium tumefaciens* is infiltrated into tobacco leaf tissue under vacuum and milligram amounts of protein can be produced within a few weeks (Kapila et al., 1997). This system has also been developed in alfalfa by Medicago researchers (D'Aoust et al., 2004) and is applicable in many other leafy species. Although stable transformation occurs at very low efficiency, many cells are initially transiently transformed, only for the exogenous DNA to get diluted and degraded. However, before this happens, most cells contain the T-DNA and can express any transgenes carried therein. As extrachromosomal constructs, these unintegrated T-DNAs are free from position effects and epigenetic silencing phenomena that often reduce or abolish the expression of integrated nuclear transgenes.

A number of different antibodies and their derivatives have been produced by agroinfiltration, including the full-size IgG T84.66 along with its scFv and diabody derivatives (Vaquero et al., 1999, 2002) and a chimeric full-size IgG known as PIPP along with its scFv and diabody derivatives, which recognizes human chorionic gonadotropin (Kathuria et al., 2002).

Plant viruses are advantageous for the production of antibodies because viral genomes are easier to manipulate than plant genomes, and the infection of plants with recombinant viruses is a very simple process compared to the regeneration of transgenic plants (Yusibov et al., 2006; Yusibov and Rabindran, 2008). Potentially, plants carrying recombinant viruses can be grown on the same scale as transgenic plants, but with a much shorter development time. Viral infections are generally systemic, so infected plants carry the virus in all cells and can produce the antibody systemically, resulting in potentially very high yields. A further advantage of viruses is that mixed infections are possible, making it a simple process to express, for example, the multiple chains of a full-size immunoglobulin. Although the transgene is carried on a viral genome rather than on the plant genome, the expressed protein is processed in the same manner as it would be in transgenic plants, meaning that appropriate folding, targeting, and modification of antibodies are possible. The viral system is therefore uniquely simple, flexible, and efficient, and it has the potential for protein manufacture in both contained and open facilities (Canizares et al., 2005; Yusibov et al., 2006).

There are two types of expression systems based on plant viruses, one for full polypeptides and one for peptide epitopes displayed on the virion surface. Both have

been used to express antibodies. In the polypeptide expression system, the antibody is encoded by a discrete transgene and accumulates as a soluble protein within the plant cell. In the epitope display system, a small antibody derivative such as an scFv is expressed as a fusion with the viral coat protein in such a way that the antibody is displayed on the surface of the virus particle.

Tobacco mosaic virus (*TMV*) has a monopartite RNA genome of 6.5 kb encoding four proteins all of which are essential for systemic infection. The normal strategy for polypeptide expression is to place the transgene under the control of an additional coat protein promoter, although not a perfect copy of the endogenous coat protein promoter, as this is an unstable configuration that leads to transgene elimination (Donson et al., 1991). Many antibodies have now been expressed in TMV-infected plants. McCormick et al. (1999) produced an scFv fragment based on the idiotype of malignant B cells of the murine 38C13 B-lymphoma cell line. When administered to mice, the scFv stimulated the production of anti-idiotype antibodies capable of recognizing 38C13 cells, providing immunity against lethal challenge with the lymphoma. This has been developed into a personalized therapy for diseases such as non-Hodgkin's lymphoma, where antibodies capable of recognizing unique markers on the surface of any malignant B cells could be produced for each patient. Up to 15 such antibodies were tested in phase I and phase II clinical trials by the US biotechnology company Large Scale Biology Inc., before they went into liquidation. Additionally, Verch et al. (1998) produced a full-length IgG in transgenic tobacco plants by infecting them with two TMV vectors, one expressing the heavy chain and one the light chain. This study showed that viral coexpression was compatible with the correct assembly and processing of multimeric recombinant proteins.

Potato virus X (*PVX*), the type member of the Potexvirus family, has a 6.5-kb monopartite RNA genome rather like that of TMV. Also, like TMV, PVX vectors contain extra subgenomic promoters to drive transgene expression, but in this case, the lack of a closely related alternative means that transgene elimination by homologous recombination is unavoidable. PVX vectors have been used for the expression of several different antibodies, but none of medical relevance. Single-chain Fv antibodies have been expressed, specific for proteins from potato virus V (Hendy et al., 1999), tomato spotted wilt virus (Franconi et al., 1999) and against granule-bound starch synthase I (Ziegler et al., 2000).

In addition to the use of complete viruses carrying additional foreign genes, another strategy uses deconstructed viruses that cannot spread systemically in the plant. The magnifection strategy, developed by Icon Genetics (now part of Bayer CropSciences), renders the systemic spread of the virus unnecessary through the use of *A. tumefaciens* as a delivery vehicle (Marillonnet et al., 2005; Gleba et al., 2005). The bacterium delivers the viral genome to so many cells that local spreading is sufficient for the entire plant to be infected. Like the infection stage, systemic spread is a limiting function, often one of the primary determinants of host range. Taking the systemic spreading function away from the virus and relying instead on the bacterium to deliver the viral genome to a large number of cells allow the same viral vector to be used in a wide range of plants. The system has been used to express antigens and antibodies at high levels in tobacco and other plants (Gleba et al., 2004).

The above systems all involve the use of whole plants as the expression platform. Even if antibody production is limited to specific tissues, such as seeds or leaves, these are harvested from the whole plant at the beginning of downstream processing. An alternative is to culture the specific organs or cells that produce the antibody and either isolate the antibody from these cells or tissues or collect it from the culture medium. A number of different culture systems have been developed, although most research has focused on cell suspension cultures. In most cases where nuclear transgenic plants have been used for the production of recombinant antibodies, the product has been extracted from plant tissues. An alternative is to attach a signal peptide to the recombinant protein, thus directing it to the secretory pathway. In this way, the protein can be recovered from the root exudates or leaf guttation fluid, processes known, respectively, as *rhizosecretion* and *phyllosecretion* (Borisjuk et al., 1999; Komarnytsky et al., 2000). Although not widely used, the secretion of recombinant antibodies into hydroponic culture medium is advantageous because no cropping or harvesting is necessary. The technology is being developed by the US biotechnology company Phytomedics Inc. A monoclonal antibody was shown to be secreted into hydroponic culture medium resulting in a yield of 11.7 μg antibody per gram of dry root mass per day (Drake et al., 2003).

Other systems are based on the culture of plant organs. Hairy roots are neoplastic structures that arise following transformation of a suitable plant host with *Agrobacterium rhizogenes*. If the plant is already transgenic, or if the transforming *A. rhizogenes* strain is transgenic, and transfers the foreign gene to the host plant during the process of transformation, then hairy root cultures can be initiated, which will produce recombinant antibodies and secrete them into the growth medium (Sharp and Doran, 2001b). Hairy roots grow rapidly and can be propagated indefinitely in liquid medium. Thus far, hairy root cultures have been used to produce a relatively small number of antibodies (Sharp and Doran, 2001a), mainly because of the relative ease with which multisubunit proteins can be produced. The cultures can be initiated from transgenic plants already carrying multiple transgenes, wild-type plants can be infected with multiple *A. rhizogenes* strains, or established hairy root cultures can be supertransformed with *A. tumefaciens*. A clonal root system based on a similar principle has been developed as a commercial platform by the Fraunhofer Center for Molecular Biotechnology in Newark, Delaware. In this case, the root system is combined with the use of viral-derived vectors for high-yield antibody expression in sealed vessels. A tissue culture system has been developed from *shooty teratomas*, which are differentiated cell cultures produced by transformation with certain strains of *A. tumefaciens* (Subroto et al., 1996). Thus far, there has been only one report of pharmaceutical protein production in teratoma cultures, and the levels of antibody were very low (Sharp and Doran, 2001a,b).

As stated above, most of the work in this area has focused on suspension cell cultures, which are individual plant cells and small aggregates thereof growing in liquid medium in a fermenter (Hellwig et al., 2004; Doran, 2006). Suspension cell cultures are usually derived from callus tissue by the disaggregation of friable callus pieces in shake bottles and are later scaled up for fermenter-based production. Recombinant

antibody production is achieved by using transgenic explants to derive the cultures, or transforming the cells after disaggregation, usually by cocultivation with *A. tumefaciens*. Suspension cultures have the same advantages as the simple plants, i.e., controlled growth conditions, batch-to-batch reproducibility, containment, and production under GMP procedures. Many foreign proteins have been expressed successfully in suspension cells, including antibodies, enzymes, cytokines, and hormones (reviewed by Hellwig et al., 2004; Fischer et al., 1999). Tobacco cultivar "Bright Yellow 2" (BY-2) is the most popular source of suspension cells for molecular farming, since these proliferate rapidly and are easy to transform. However, rice (*Oryza sativa* L.) suspension cells have also been used to produce several antibodies (e.g., Torres et al., 1999).

Recombinant antibodies expressed in plant cell suspension cultures may be secreted into the culture supernatant or retained within the cells. Localization depends on the expression construct design (see below) and the permeability of the plant cell wall to the antibody. Targeting signals included in the expression construct can be used to direct the protein to the apoplast or to retain it within intracellular compartments. The fate of antibodies targeted for secretion depends to a large extent on their size: molecules of 20–30 kDa (the size range of scFvs) will generally pass through the plant cell wall and be secreted into the culture medium, whereas larger proteins (such as IgGs) will be retarded in a size-proportional manner. The inclusion of a C-terminal KDEL sequence results in higher levels of antibody accumulation in cultured cells because the biochemical environment of the endoplasmic reticulum favors stable protein folding and assembly while reducing the level of proteolytic degradation (see below). However, this also makes it necessary to disrupt the cells in order to isolate the protein, which requires additional processing time and causes the release of phenolic molecules that interfere with purification and reduce production yield. Thus, the preferred approach is to secrete the target proteins and capture them from the culture supernatant or release them from the cells by mild enzymatic digestion.

Single-celled plants and aquatic plants can be maintained in bioreactors, offering two advantages over terrestrial plants. First, the growth conditions can be controlled precisely, which means that optimal growth conditions can be maintained, batch-to-batch product consistency improved, and the growth cycle can conform to GMP. Second, growth in bioreactors offers complete containment. Although more expensive than agricultural molecular farming, the use of simple plants in bioreactors is not as expensive as cultured animal cells because the media requirements are generally very simple. Added to this, the proteins can be secreted into the medium, which reduces the downstream processing costs and allows the product to be collected in a nondestructive manner. A final, major advantage is the speed of production. The time from transformation to first product recovery is on the scale of days to weeks because no regeneration is required, and stable producer lines can be established in weeks rather than months to years because there is no need for crossing, seed collection, and the testing of several filial generations to check transgene stability. Three major bioreactor-based systems are currently under commercial development: algae, moss, and duckweed (*Lemna* spp.).

Thus far, a single report discusses the production of monoclonal antibodies in the chloroplast of the alga *Chlamydomonas reinhardtii* (Mayfield et al., 2003). Production costs appear similar to those of recombinant proteins produced in terrestrial plants, mainly due to the inexpensive media requirements. The medium does not cost very much to start with and in any case can be recycled for algal cultures grown in continuous cycles. Aside from the economy of producing recombinant proteins in algae, there are further attributes that make algae ideal candidates for recombinant protein production. First, transgenic algae can be generated quickly, requiring only a few weeks between the generation of initial transformants and their scale-up to production volumes. Second, both the chloroplast and the nuclear genome of algae can be genetically transformed, providing scope for the production of several different proteins simultaneously. In addition, algae have the ability to be grown on various scales, ranging from a few milliliters to 500,000 l in a cost-effective manner. These attributes, and the fact that green algae fall into the GRAS (generally regarded as safe) category, make *C. reinhardtii* a particularly attractive alternative to other plants for the expression of recombinant proteins. The production technology has been reviewed recently (Franklin and Mayfield, 2005; Mayfield and Franklin, 2005).

The moss *Physcomitrella patens* is a haploid bryophyte, which can be grown in bioreactors in the same way as algae, suspension cells, and aquatic plants. Like these other systems, it has the advantages of controlled growth conditions, synthetic growth media, and the ability to secrete recombinant proteins into the medium (Decker and Reski, 2004). The unique feature of this organism, relative to all other plants, is that it is amenable to homologous recombination (Schaefer, 2002). This means that not only can it be transformed stably with new genetic information but also that endogenous genes can be disrupted by *gene targeting*. The major application of gene targeting in molecular farming is the modification of the glycosylation pathway (by knocking out enzymes that add nonhuman glycan chains to proteins), thus allowing for the production of humanized glycoproteins (Faye et al., 2005).

The *P. patens* system is being developed by the German biotechnology company Greenovation Biotech GmbH, which is based in Freiburg. The company has developed transient expression systems that allow feasibility studies and stable production strains that can be scaled up to several thousand liters. The Lemna System is based on duckweed (*Lemna minor*) and has been developed by the US biotechnology company Biolex Inc. Lemna has a number of significant advantages for the production of recombinant pharmaceutical proteins (Gasdaska et al., 2003). Unlike transgenic terrestrial plants, this aquatic plant is cultured in sealed, aseptic vessels under constant growth conditions (temperature, pH, and artificial light). Only very simple nutrients are required (water, air, and completely synthetic inorganic salts), and under these conditions, the plant proliferates vegetatively and doubles its biomass every 36 h. This provides the optimal production environment for batch-to-batch consistency. Duckweed constitutes about 30% dry weight of protein, and recombinant proteins can either be extracted from wet plant biomass or secreted into the growth medium. Biolex Inc. has reported the successful expression of tens of proteins in this system, including several recombinant antibodies and enzymes (Gasdaska et al., 2003).

3.3 Optimizing Antibody Production in Plants

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 optimization 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 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 (Twyman et al., 2005). In cereals, the CaMV 35S promoter has a lower activity, and other promoters have been tested, such as the *maize ubiquitin-1* (*ubi-1*) *promoter* (Christensen and Quail, 1996). Some modified dicot promoters do work rather well in cereals, an example being the *pPLEX* series of constructs developed by Schunmann et al. (2003) adapted for use in monocots. The original pPLEX vectors were based on regulatory elements from subterranean clover stunt virus (SCSV). Modification was achieved by adding either the Ubi1 or Act1 introns, as well as GC-rich enhancer sequences from banana bunchy top virus (BBTV) or maize streak virus (MSV).

Regulated promoters can be used in preference to constitutive promoters to improve practicality and biosafety in addition to yields. 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 in the seeds, so the vegetative organs do not accumulate detectable levels of the recombinant protein. This increases the biosafety of the plants, since adventitious contact with nontarget organisms is unlikely (Commandeur et al., 2003). Among the many different seed-specific promoters that have been used (reviewed by Christou et al., 2004), the most impressive yields have been obtained with a novel seed-specific promoter from the common bean (*Phaseolus vulgaris* L.), which was used to express a single-chain antibody in *Arabidopsis thaliana* L. Heynh. In contrast to the CaMV 35S promoter, which resulted in antibody accumulation to 1% total soluble protein (TSP), the *bean arc5-I promoter* resulted in antibody levels in excess of 36% TSP in homozygous seeds, and the antibody retained its antigen-binding activity and affinity (De Jaeger et al., 2002).

The use of inducible promoters (Padidam, 2003) is also advantageous because recombinant protein synthesis can be delayed until just before harvest, or even after harvest, as is the case for the tomato hydroxy-3-methylglutaryl CoA reductase 2 (HMGR2) promoter developed by the now defunct CropTech Inc. as the MeGA promoter system (*mechanical gene activation*). The promoter used in this system is wound inducible, and gene expression is activated when the harvested tobacco leaves are shredded prior to protein extraction (Cramer et al., 1999). However, many endogenous inducible promoters show a degree of leakiness (background expression) and, in some cases, a low induction ratio. Recombinant systems such as those based on bacterial operons or animal hormones may be advantageous in these circumstances.

After promoter choice, the next most important aspect of construct design is the inclusion of sequences that control *subcellular 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 posttranslational modification (Ma et al., 2003; Schillberg et al., 2003). 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 (Zimmermann et al., 1998; Schillberg et al., 1999). The endoplasmic reticulum (ER) provides an oxidizing environment and an abundance of molecular chaperones, while there are few proteases. Proteins are directed to the secretory pathway using either a heterologous or an endogenous signal peptide, located at the N-terminus of the native protein. Such proteins are cotranslationally imported into the ER and are eventually secreted into 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 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 confined to the ER using an *H/KDEL C-terminal tetrapeptide tag* in addition to the signal peptide (Conrad and Fiedler, 1998). Accumulation levels are generally two- to ten-fold greater compared with an identical protein lacking the KDEL signal (Schillberg et al., 2002). 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 (Sriraman et al., 2003). Interestingly, recent experiments with antibodies expressed as fusion proteins with *elastin-like peptides* (*ELPs*) showed that the ELPs also had an impact on trafficking, albeit only in seeds. Two HIV-neutralizing antibodies expressed in tobacco seeds, namely 2F5 (Floss et al., 2008) and 2G12 (Floss et al., unpublished), were secreted into the apoplast when expressed as naked molecules bearing a KDEL tag, but initiated the formation of novel protein bodies that budded directly from the ER when expressed as ELP fusions. Aberrant antibody localization has also been reported in *Arabidopsis* seeds (Van Droogenbroeck et al., 2007).

Although the protein synthesis and folding pathways are highly conserved between plants and animals, there are some differences in the capacity for posttranslational 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 galactose and sialic acid residues that are found on many native human glycoproteins (Fig. 3.4). Since glycan structures can impact on the solubility, stability, immunogenicity, and biological activity of recombinant proteins, the "humanization" of glycan structures produced in plants has been an important topic of research and debate in the scientific community. There has been considerable interest in modifying the plant glycosylation pathway to humanize the glycan profile of recombinant proteins. Several changes in the pathway are required

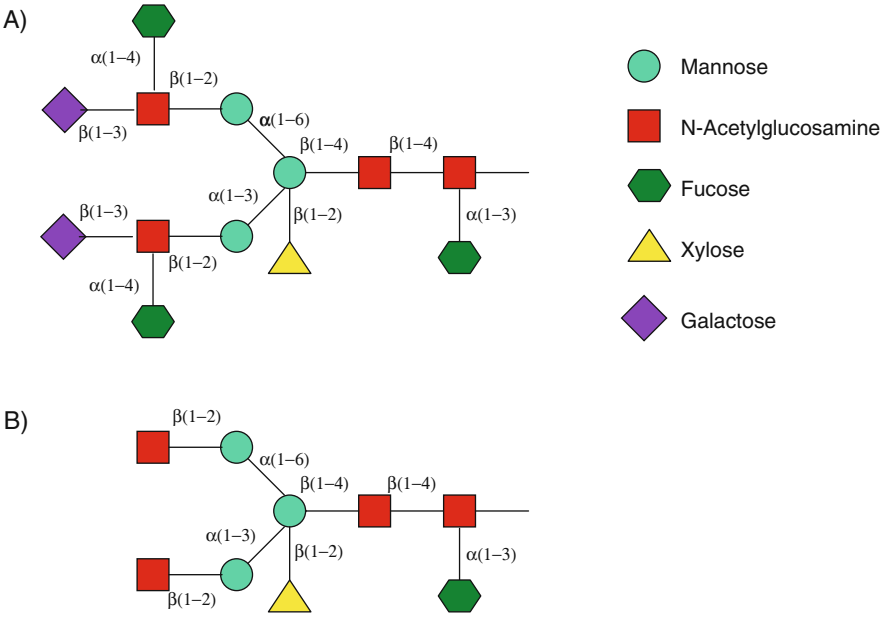

Fig. 3.4 Two glycan structures produced in plants. (**A**) Galactose-extended complex glycan. (**B**) Long-chain complex glycan. The xylose and α(1,3) fucose residues are not found in mammals

to produce proteins with typical human glycan structures (Warner, 2000; Gomord et al., 2005; Faye et al., 2005). Strategies used include the in vitro modification of plant-derived recombinant proteins by purified human β(1,4)-galactosyltransferase and sialyltansferase enzymes (Blixt et al., 2002) and the expression of human β(1,4)-galactosyltransferase in transgenic plants to produce recombinant antibodies with galactose-extended glycans (Bakker et al., 2001). 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 more recent report documenting sialylation in *A. thaliana* suspension cells has been challenged, although the subject remains a matter of controversy (Shah et al., 2003, 2004; Seveno et al., 2004). To remove the nonmammalian β(1→2) xylose and α(1→3) fucose residues, some researchers have explored the possibility of inhibiting the enzymes responsible for synthesizing these groups, while in one case, this goal has been achieved in whole *A. thaliana* plants by gene knockout techniques (Strasser et al., 2004). As discussed above, the moss *P. patens* can also be modified by gene targeting to eliminate these enzymes (Decker and Reski, 2004). Another approach is to prevent the glycoproteins passing through the Golgi so that only high-mannose glycans are added. This can be achieved simply by adding a KDEL C-terminal tag to the antibody, as demonstrated by Sriraman et al. (2004) and Triguero et al. (2005). This issue has been reviewed by Gomord et al. (2004).

3.3.1 Downstream Processing

Downstream processing, 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 (Drossard, 2003). In many cases, it is necessary to develop specific processing steps for each product, although certain classes of product can be isolated using a standardized approach (e.g., affinity chromatography to isolate recombinant antibodies; Stoger et al., 2005b). Several aspects of downstream processing have to be customized specifically for plant systems, including the removal of fibers, oils, and other by-products from certain crops and process optimization for the treatment of different plant species and tissues (Menkhaus et al., 2004; Nikolov and Woodard, 2004; Nikolov et al., 2009).

For the production of clinical-grade antibodies, downstream processing steps need to meet the standards that have been set for other biopharmaceutical production systems, including a strict regime of quality assurance and quality control to achieve approval by regulatory agencies (Fahrner et al., 2001). The initial stages of processing display the greatest variability and have to be optimized 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 treatments (grinding, milling, etc.). After cell disruption, clarification of the extract is often carried out by dead-end or cross-flow filtration, 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 specialization for plant-based production (Platis et al., 2008). 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 (Bai and Glatz, 2003; Menkhaus and Glatz, 2005). However, important exceptions include the use of *Protein A* or *Protein G affinity chromatography* for antibody purification and the use of affinity tags and their respective capture agents (e.g., His_6 and Ni-NTA resin), which are highly selective initial capturing methods.

3.3.2 Regulatory Landscape

One of the greatest uncertainties surrounding the use of plants for the production of pharmaceuticals is the regulatory landscape. While plants are grown in glasshouses and in enclosed bioreactors, the production of pharmaceuticals is regulated in the same way as 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 APHIS (Animal and Plant Health Inspection Service, a part of the USDA) 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 (FDA, 2002; CPMP, 2002, 2006). The impact of this is to suppress the market.

All recombinant pharmaceuticals, including those derived from plants, need to comply with the national and international GMP (good manufacturing processes) standards for product safety, quality, potency, and efficacy. However, it is not clear at which stage GMP requirements should come into effect when plants are used as the production system, since 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 (Sparrow et al., 2007; Spök et al., 2008). 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). Another important issue is the different regulatory structures for plant-derived pharmaceuticals and for genetically modified plants, in general, prompting calls for the harmonization of regulations internationally (Ramessar et al., 2008b).

3.4 Conclusions

The production of recombinant pharmaceuticals in plants is advantageous, theoretically offering unlimited production scales at unprecedented low manufacturing costs. We are beginning to overcome the technical limitations, such as low yields, instability, and nonauthentic glycan structures, that erect obstacles in the path toward commercialization. But more needs to be done to convince industry that plants represent a true alternative to CHO (Chinese hamster ovary) cells and bacteria. 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 IP (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 welcome announcement of the first approved plant-derived veterinary vaccine may open the way for antibodies, and in particular antibodies with therapeutic and diagnostic potential, to follow.

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