Molecular Farming

Plant-made Pharmaceuticals and Technical Proteins

Edited by
Rainer Fischer and Stefan Schillberg

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Preface

Mankind has used plants as a source of raw materials and medicines for thousands of years. From the earliest stages of civilization, plant extracts have been used to obtain technical materials and drugs to ease suffering and cure disease. Since the late seventies, many valuable therapeutic and diagnostic proteins have been discovered through molecular biology research and molecular medicine, but widespread use of these molecules has been hampered by production bottlenecks such as low yields, poor and inconsistent product quality and a shortage of production capacity. In the late 1980s, the application of recombinant DNA and protein technology in plants allowed the exploration of plant-based expression systems for the production of safer and cheaper protein medicines (Table 1). Over the last decade, plants have emerged as a convenient, safe and economical alternative to mainstream expression systems which are based on the large-scale culture of microbes or animal cells, or transgenic animals. The production of plant-made pharmaceuticals and technical proteins is known as *Molecular Farming (Molecular Pharming*^{TM}*)*. The objective is to harness the power of agriculture to cultivate and harvest plants or plant cells producing recombinant therapeutics, diagnostics, industrial enzymes and green chemicals.

Molecular Farming has the potential to provide virtually unlimited quantities of recombinant antibodies, vaccines, blood substitutes, growth factors, cytokines, chemokines and enzymes for use as diagnostic and therapeutic tools in health care, the life sciences and the chemical industry. Plants are now gaining widespread acceptance as a general platform for the large-scale production of recombinant proteins. The principle has been demonstrated by the success of a diverse repertoire of proteins, with therapeutic proteins showing the greatest potential for added value and technical enzymes the first to reach commercial status.

We are facing a growing demand for protein diagnostics and therapeutics, but lack the capacity to meet those demands using established facilities. Moreover, recombinant proteins will become more important as high throughput genomics, proteomics, metabolomics and glycomics projects spawn new product candidates, disease targets and eventually new remedies. A shift to plant bioreactors may therefore become necessary within the next few years. However, the production of pharmaceutical proteins in plants will only realize its huge potential if the products achieve consistent highest quality standards, enabling the provision of clinical grade proteins that will gain regulatory approval and can be used routinely in clinical trials and

treatments. The achievement of these goals is conditional on the development of technologies for improving yields, ensuring product sustainability and quality, including extraction and processing steps that comply with current good manufacturing practice (cGMP) standards. Moreover, there are several further challenges concerning the environmental impact, biosafety and risk assessment of *Molecular Farming*, which reflect the release of transgenic plants as well the safety of the plant-derived products themselves.

This book covers the most recent achievements and challenges of *Molecular Farming* technology written by experts working in this field. The first few chapters focus on the technological aspects of plant-based protein production, while the second part address the two major target product groups expressed in plant systems: pharmaceutical and technical proteins. Finally, issues concerning the production pipeline are discussed, including production and product safety, quantity and quality control.

We thank all the authors for their contributions and the time and effort they dedicated to compiling this book, which helped to make it a comprehensive and state-of-the-art overview of the technological, economical, commercial and regulatory aspects of *Molecular Farming*. We also gratefully acknowledge the help and support of Dr. Richard Twyman and the team at Wiley. Without all their help, this book would not have been possible.

Aachen, 2004 *Rainer Fischer* and *Stefan Schillberg*

Tab. 1 Key events in the history of *Molecular Farming*.

Year	*Highlight*	*Reference*
1986	First plant-derived recombinant therapeutic protein – human growth hormone in tobacco and sunflower [1)]	1
1989	First plant-derived recombinant antibody – full-size IgG in tobacco	2
1990	First native human protein produced in plants – human serum albumin in tobacco and potato	3
1992	First plant-derived vaccine candidate – hepatitis B virus surface antigen in tobacco	4
1992	First plant-derived industrial enzyme – α-amylase in tobacco	5
1995	Secretory IgA produced in tobacco	6
1996	First plant-derived protein polymer – artificial elastin in tobacco	7
1997	First clinical trial using recombinant bacterial antigen delivered in a transgenic potato	8
1997	Commercial production of avidin in maize	9
1999	First glycan analysis of plant-produced recombinant glycoprotein	10
2000	Human growth hormone produced in tobacco chloroplasts	11
2000	Triple helix assembly and processing of human collagen produced in tobacco	12
2001	Highest recombinant protein accumulation achieved in plants so far – 46.1% total soluble protein for *Bacillus thuringiensis* Cry2Aa2 protein	13
2001	First multi-component vaccine candidate expressed in potato – cholera toxin B and A2 subunits, rotavirus enterotoxin and enterotoxigenic *Escherichia coli* fimbrial antigen fusions for protection against several enteric diseases	14
2001	Glycan modification of a foreign protein produced in a plant host using a human glycosyltransferase	15
2003	Expression and assembly of a functional antibody in algae	16
2003	Commercial production of bovine trypsin in maize	17
2004	Genetic modification of the *N*-glycosylation pathway in *Arabidopsis thaliana* resulting in complex *N*-glycans lacking β1,2-linked xylose and core α1,3-linked fucose	18

[1)] Human growth hormone was expressed as fusion with the *Agrobacterium tumefaciens* nopaline synthase enzyme but only transcript was detectable

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1
Efficient and Reliable Production of Pharmaceuticals in Alfalfa

Marc-André D'Aoust, Patrice Lerouge, Ursula Busse, Pierre Bilodeau, Sonia Trépanier, Véronique Gomord, Loïc Faye and Louis-Philippe Vézina

1.1
Introduction

In 1986, it was shown that tobacco plants and sunflower calluses could express recombinant human growth hormone as a fusion protein [1]. Since then, a diverse range of plant systems has been used for the production of pharmaceuticals [2, 3]. We have developed a production system based on the leaves of alfalfa (*Medicago sativa* L.), a choice made originally because of the plant's many favorable agronomic characteristics. Alfalfa is a perennial plant, so vegetative growth can be maintained for many years. For molecular farming, this characteristic, combined with the ease of clonal propagation through stem cutting, makes alfalfa a robust bioreactor with regard to batch-to-batch reproducibility. Among perennial plants, legume forage crops such as alfalfa have the advantage of fixing atmospheric nitrogen, thus reducing the need for fertilizers. Moreover, as a feed fodder crop, alfalfa has benefited from important research aiming to increase leaf protein content, so that today's varieties produce as much as 30 mg total protein per gram fresh weight.

In addition to these appealing agronomic characteristics, biotechnological research has revealed additional benefits for the production of pharmaceuticals in alfalfa. Expression cassettes have been optimized for protein expression in alfalfa leaves. Methods for transient protein expression have been developed so that it is now possible to use agroinfiltration or the transformation of protoplasts for early-stage demonstration and validation steps. In addition, glycosylation studies have shown that alfalfa is capable of producing recombinant glycoproteins with homogenous (uniform) glycosylation patterns.

This chapter provides an overview of the tools that have been developed and optimized specifically for the production of pharmaceuticals in alfalfa, with the emphasis on recent technological breakthroughs. The ability of alfalfa leaves to produce complex recombinant proteins of pharmaceutical interest is discussed and illustrated with recent data obtained in our laboratories. Data are presented concerning the production and characterization of alfalfa-derived C5-1, a diagnostic anti-human

IgG developed by Héma-Québec (Québec, Canada) for phenotyping and cross matching red blood cells from donors and recipients in blood banks [4].

1.2 Alfalfa-specific Expression Cassettes

The first hurdle encountered during the development of alfalfa as a recombinant protein production system was the relative inefficiency of the available expression cassettes. A study in which a tomato proteinase inhibitor I transgene was expressed in tobacco and alfalfa under the control of the cauliflower mosaic virus (CaMV) 35S promoter showed that 3–4 times more protein accumulated in tobacco leaves compared to alfalfa leaves [5]. Despite the low efficiency of the CaMV 35S promoter in alfalfa, biopharmaceutical production using this system has been reported in the scientific literature. Such reports include expression of the foot and mouth disease virus antigen [6], an enzyme to improve phosphorus utilization [7] and the anti-human IgG C5-1 [8]. In this last work, the C5-1 antibody accumulated to 1% total soluble protein [8].

Given the relatively high level of C5-1 antibody detected in alfalfa leaves using the weak CaMV 35S promoter, it was expected that expression cassette optimization would lead to significantly higher yields. The first family of expression cassettes we developed was thus designed to achieve strong expression in the aerial parts of alfalfa plants. The MED-2000 series (patent pending) consists of strong, leaf-specific expression cassettes, and is based on regulatory sequences from the alfalfa plastocyanin gene. Using cassettes of this family to drive the *gusA* reporter gene, it was possible to achieve up to 14-fold the level of expression obtained in alfalfa leaves with the 35S promoter (Fig. 1.1). Interestingly, although the MED-2000 promoters were derived from alfalfa genomic sequences, they also produced up to 25-fold higher β-glucuronidase (GUS) activity than the 35S-*gusA*-*nos* construct in the leaves of transgenic tobacco plants.

Because pharmaceuticals are bioactive molecules, their accumulation in plant cells could have a deleterious effect on the growth and development of the host plant. Therefore, we have developed a second series of expression cassettes incorporating inducible promoter elements. The regulatory elements of the MED-1000 series expression cassettes are derived from the alfalfa nitrite reductase (NiR) gene [9]. The induction strategy used with these expression cassettes exploits the ability of alfalfa to grow abundantly in the absence of mineral nitrogen while fixing atmospheric nitrogen through its symbiosis with rhizobium, but also takes into account the fact that NiR genes are highly inducible by nitrate fertilizers [10, 11, 12].

We have demonstrated that the alfalfa NiR promoter is an excellent candidate for the inducible control of transgene expression in alfalfa leaves. As an example, a 3-kb genomic fragment corresponding to an alfalfa NiR promoter was isolated and fused to the *gusA* gene for analysis. We have shown that the promoter remains silent in nodulated plants grown in a nitrate-free medium. Upon the addition of nitrate, however, *gusA* gene expression is induced, and the reporter enzyme accumulates to a similar level to that observed in the leaves of 35S-*gusA* alfalfa plants (Fig. 1.1).

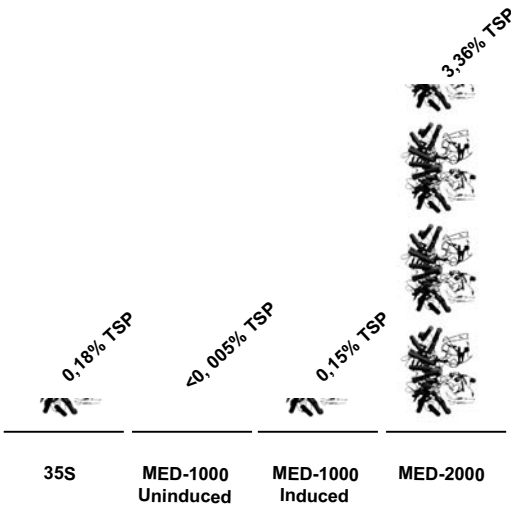

Fig. 1.1 Promoter activity in alfalfa leaves. Accumulation of β-glucuronidase achieved in transgenic alfalfa leaves expressing the *gusA* gene under the control of CaMV 35S and alfalfa promoters. %TSP, percentage of total soluble proteins.

1.3 Alfalfa Transformation Methods

Genetic transformation, which results in the stable integration of foreign DNA into the genome, is one of the key technologies underpinning the production of pharmaceuticals in alfalfa. Plant transformation at the industrial level must be optimized for efficiency, predictability and reproducibility in all aspects ranging from explant preparation to the physical conditions of DNA intake and the recovery of transgenic plants. This is an interesting challenge because plant transformation efficiency depends on many factors, including DNA conformation, explant type, plant species, plant genotype and the culture medium. In addition, the development of a plant-based expression platform to produce pharmaceuticals, nutraceuticals and industrial enzymes adds further requirements in terms of plant transformation. For example, a key issue in prototype development is the rapidity with which the ability of the system to produce a selected molecule can be tested, and this reflects the identification of optimal regulatory sequences to drive transgene expression. In order to address these various issues, we have adapted documented transformation methods and developed an alfalfa transformation portfolio ranging from proof-of-concept technology that allows rapid screening of target proteins, to stable expression in transgenic plants or cell cultures for sustainable commercial-scale production. Table 1.1 lists the characteristics of different transformation methods used with alfalfa.

As for many plants, alfalfa is amenable to transformation by various methods including *Agrobacterium*-mediated transfer, direct DNA transfer to protoplasts using polyethylene glycol, and particle bombardment (reviewed in [13]). In recent years, we have developed a medium-throughput system to manage the various activities related to plant transformation, from plant preparation through to transformation and regeneration. This allows us to maintain a continuous production schedule. The sys-

Tab. 1.1 Characteristics of alfalfa transformation methods

	Agrobacterium-mediated stable transformation – Plant	*Agrobacterium-mediated stable transformation – Cell culture*	*Transient protoplast transformation*	*Particle bombardment-based transient expression*	*Agrobacterium-mediated transient expression*
Plasmid type	Binary	Binary	pUC-based	pUC-based	Binary
Tissue	Leaves	Isolated cells	Protoplasts	Leaves	Leaves
Working conditions	Sterile	Sterile	Sterile	Sterile	Non-sterile
Integration in the genome	Yes	Yes	No	No	No
Timing	6 months minimum	5 weeks	2 days	2 days	5 days
Amount of protein produced	Micrograms or greater	Nanograms	Nanograms	Minimal	Micrograms
Complex protein assembly	Yes	Yes	Yes	No	Yes
Possibility to purify	Yes	Limited	Limited	No	Yes

tem allows the introduction of up to six constructs per week, which represents approximately 600 explants, and this capacity can easily be scaled up by increasing the number of staff and the availability of appropriate equipment. Thus far, more than 180 constructs have been integrated into alfalfa, and several thousand transgenic plants have been generated in our facilities. Given that 98% of the regenerated plants are PCR positive for the gene of interest, our medium-throughput system appears to work very efficiently.

In order to reduce the time required to confirm the accumulation of a given recombinant protein, we have developed a cell culture system in which transgenic alfalfa callus material produced at the proliferation step of *Agrobacterium*-based transformation is used to initiate cell cultures. These cell suspensions can be subcultured to sustain batch production of modest protein amounts. The protein blot shown in Fig. 1.2 demonstrates our ability to detect a recombinant protein in total

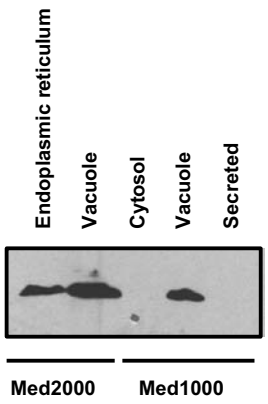

Fig. 1.2 Protein blot analysis of human therapeutic protease inhibitor (HTPI) produced in alfalfa cell cultures using different promoters and subcellular targeting peptides as shown. Equal amounts of total soluble proteins from cell cultures were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinyldifluoride (PVDF) membrane. Monoclonal anti-HTPI IgGs were used for detection.

soluble protein extracts from alfalfa cell cultures. It must be emphasized at this point that the recovered protein is most likely derived from several transformation events involving the same gene construct. This technique allows the detection of recombinant proteins 6–8 weeks after transformation, which is three times faster than the 20 weeks required to regenerate and screen transgenic plants following *Agrobacterium*-mediated transformation. This development has also shown that our alfalfa expression cassettes, although more adapted for leaf expression, provide adequate expression in cell cultures.

Although cell culture considerably reduces the time required to achieve proof-of-concept for new molecules, this time frame still needs to be reduced. In addition, there is some concern that the cell culture system might not correctly predict the ability of alfalfa to assemble complex proteins, and might not be a suitable guide for the selection of subcellular targeting strategies. We have therefore adapted several transient transformation methods to work with the alfalfa platform, including PEG-based protoplast transformation, particle bombardment and *Agrobacterium*-mediated transient transformation of leaves (agroinfiltration). The last method turned out to be particularly successful for the selection of optimal targeting strategies for a given candidate protein. Figure 1.3 shows that, for a given recombinant protein expressed in alfalfa leaves, the level of accumulation is dependent on the subcellular destination of the protein. More importantly, the figure shows that the relative protein accumulation in the different subcellular compartments is similar in leaves from agroinfiltrated and transgenic plants. In the case presented here, chloroplast targeting led to the highest accumulation both in agroinfiltrated leaves and transgenic plants, followed by targeting to the cytosol and mitochondria.

Agrobacterium-mediated transient gene expression has become the method of choice for rapid validation of gene constructs and targeting strategies in alfalfa leaves. It was adapted for alfalfa from a method published by Kapila *et al.* (1997) [14]. In this system, an *Agrobacterium* culture carrying the T-DNA of interest is forced to enter into the intercellular spaces of the leaves under high vacuum. Once the physi-

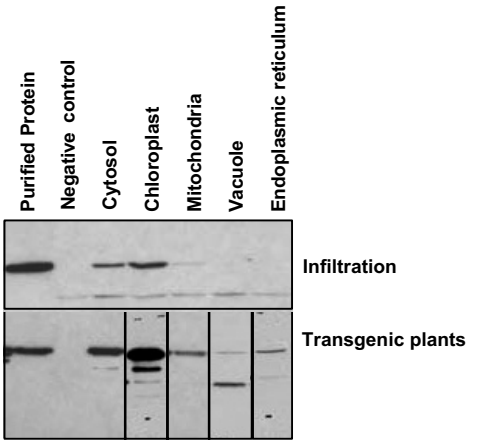

Fig. 1.3 Prediction of the most appropriate subcellular targeting strategies by agroinfiltration. The levels of an industrial enzyme (IE) are shown in agroinfiltrated and transgenic alfalfa leaves using different subcellular targeting peptides. Equal amounts of total soluble leaf proteins were separated by SDS-PAGE and blotted onto a PVDF membrane. Polyclonal anti-IE IgGs were used for detection.

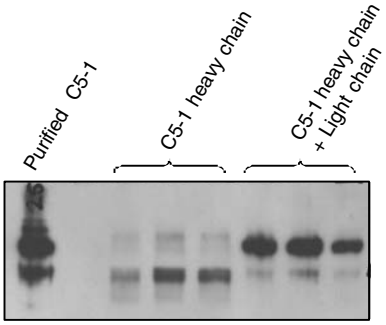

Fig. 1.4 Protein blot analysis of C5-1 assembly in agroinfiltrated alfalfa leaves. Total leaf soluble proteins, extracted 4 days after infiltration were separated by SDS-PAGE under non-reducing conditions and blotted onto a PVDF membrane. Polyclonal anti-mouse IgGs were used for detection. Purified C5-1 was mixed with total soluble proteins from control infiltrated alfalfa leaves and loaded as a standard.

cal barrier of the epidermis is crossed, the bacteria infect neighboring cells, transferring T-DNA copies into the nucleus. Although the T-DNA exists inside the nucleus only transiently, the genes present on the T-DNA are transcribed, leading to the production of the recombinant protein in each infected cell. The efficiency of this method is thus highly dependent on the ability to distribute the bacterial culture evenly inside the leaf tissue.

As well as its short time frame, agroinfiltration has several further advantages for recombinant protein production. The method allows the expression of multiple genes by infiltrating cells with a mixture of two or more *Agrobacterium* cultures (co-infiltration), thus eliminating the need to clone several genes within the same T-DNA. Agroinfiltration is also readily scalable. Routinely, 25 leaves are infiltrated for immunological verification of expression or the comparison of targeting strategies. However, after the selection of an ideal transgene construct, infiltration of 7500 leaves per week can be carried out by a limited number of staff, in a continuous process, for the production of micrograms of recombinant protein.

The production of C5-1 by co-infiltration illustrates the impressive capacity of this method. Results presented in Fig. 1.4 show that the production of C5-1 in detached alfalfa leaves was validated within 5 days from infiltration. In these experiments, different bacteria bearing the light- and the heavy-chain constructs were used to infect the cells. Most of the infected cells were occupied by both strains, and a protein corresponding to fully assembled C5-1 was detected in the infiltrated leaf extract. This result demonstrates the potential of agroinfiltration for testing the adequate expression and assembly of complex proteins in alfalfa leaves using different *Agrobacterium* strains.

1.4 Characteristics of Alfalfa-derived Pharmaceuticals

When recombinant proteins are produced in a heterologous system, there may potentially be differences between the final product and the natural molecule. Hence, for each new protein produced in alfalfa, a thorough analysis of the processing, folding, assembly and post-translational modification is conducted to ensure the conformity of the purified molecules. This section describes the analysis of alfalfa-derived

C5-1 antibodies to demonstrate the ability of alfalfa plants to produce large amounts of high-quality molecules for therapeutic or diagnostic applications.

Purified C5-1 has been obtained from alfalfa leaf extracts by affinity chromatography on either a human IgG-Sepharose column or a Streamline rProtein A-Sepharose column. Interestingly, the purified product obtained with these two methods differed significantly. As shown in Fig. 1.5 a, the antibody fraction obtained from the human IgG column contained a mixture of different intermediate assembly forms of the heavy (H) and light (L) chains, ranging from H2 to the fully assembled H2L2 form. In comparison, purification on rProtein A-Sepharose resulted in the isolation of H2L2 form alone (Fig. 1.5 b). This situation emphasizes the major impact that purification methods can have on the characteristics of the end product.

In some heterologous production systems, improper removal of the signal peptide may occur during the expression of secreted proteins, which would result in the addition or removal of amino acids at the N-terminal end. In most cases, these modifi-

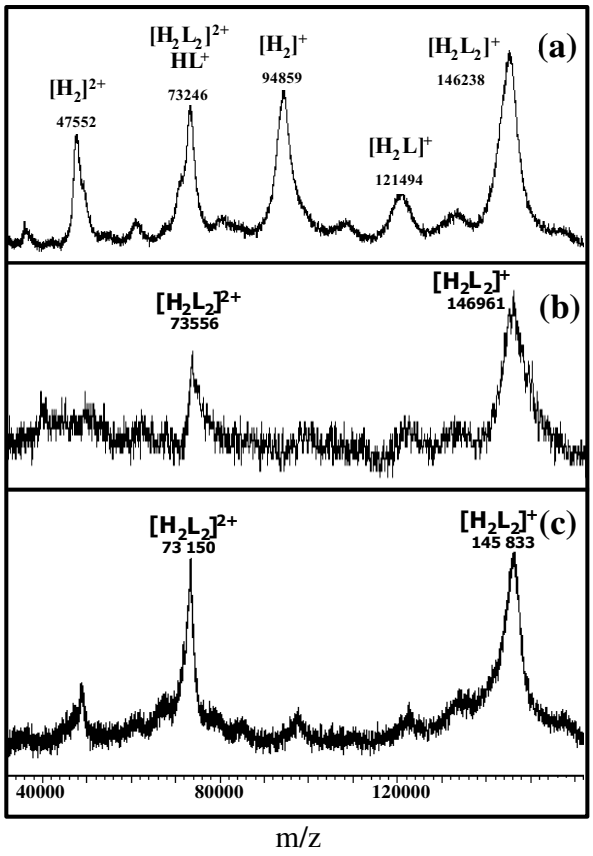

Fig. 1.5 MALDI-TOF mass spectra of purified alfalfa-derived C5-1 using **(a)** human IgG or **(b)** protein A. **(c)** Hybridoma-derived C5-1 as control. Used with permission from Ref 18.

cations are undesirable in a therapeutic context. For C5-1 expression in alfalfa, the natural sequence encoding the signal peptide was retained during the assembly of the expression cassettes. Although most examples show that mammalian signal peptides are correctly processed in plants, N-terminal amino acid sequencing was performed on the heavy chain of alfalfa-derived C5-1 in order to confirm the N-terminal integrity of the antibody. The N-terminal sequence of the heavy chain was confirmed as EIQLV, which is identical to that of the hybridoma-derived C5-1 and indicates the correct processing of the signal peptide in alfalfa.

N-glycosylation is another important issue when considering the conformity of therapeutic proteins produced in heterologous systems. Although every eukaryotic expression system N-glycosylates proteins targeted to the secretory pathway, each system links a different form of N-glycan to the recombinant protein. The glycans synthesized in a heterologous production system only rarely correspond to those found in the natural source of the protein. In this context, the ability of plants to perform complex glycosylation [15] represents an advantage over yeast and insect cells, and places the plant system in the group of Chinese hamster ovary cells (CHO) and murine myeloma cell lines (NSO). Importantly, however, the analysis of recombinant IgGs produced in tobacco indicates heterogeneity in the structure of N-glycans [16, 17].

In contrast, glycosylation analysis of alfalfa-derived C5-1 showed that a single, unique N-linked glycan form is found on the antibody (Fig. 1.5). The glycoform is representative of plant complex N-glycans, and includes core β(1,2)-xylose and α(1,3)-fucose. Figure 1.6 shows a comparison of N-glycan structures found on alfalfa- and mouse-derived C5-1. Homogenous N-glycosylation of a recombinant protein ensures batch-to-batch reproducibility, but also provides an ideal substrate for in vitro modification of the N-glycan. For example, it has been shown that incubating the purified alfalfa-derived C5-1 with β(1,4)-galactosyltransferase in the presence of UDP-galactose resulted in an efficient addition of β(1,4)-galactose to the terminal GlcNAc residues of the N-linked glycans [18].

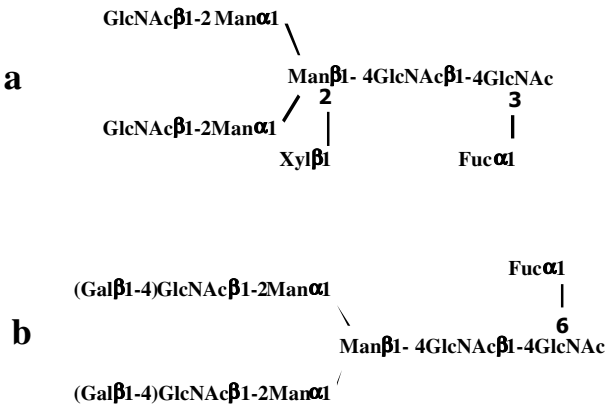

Fig. 1.6 Structure of N-glycans isolated from **(a)** alfalfa-derived C5-1 and **(b)** murine C5-1. Used with permission from Ref 18.