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review

Production and Applications of Plant-Produced Antibodies

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Summary

Plant biotechnology and antibody engineering have combined in recent years to provide many exciting new opportunities for plant molecular biology. Specifically, transgenic plants now have the potential to be used for generating large amounts of antibody for use in therapeutics and diagnostics. Alternatively, plant-produced antibodies can be used to alter existing plant biochemical pathways or to effectively immunize plants against pathogens or viral infections. Indeed, many different monoclonal antibodies and their derivatives have already been produced in plants for a variety of applications. However, before plant-produced antibodies can be safely utilized for immunotherapy or any other application, a number of concerns need to be addressed including the factors affecting subcellular localization of antibodies and the effects of plant glycosylation patterns on antibody activity and immunogenicity.

Keywords: monoclonal antibodies, transgenic plants, immunotherapy, plant immunization, intracellular immunomodulation

Introduction

Antibodies are extremely versatile molecules whose use for the treatment and prevention of disease has always been appealing. While there have been some problems with their application in many aspects of immunotherapy, the advent of monoclonal antibody (mAb) technology has rekindled the interest in using antibodies as therapeutic agents *(1)*. The high-affinity binding capacity of antibodies has also been used for the identification and purification of antigens in a wide range of diagnostic strategies, and more recently it has been demonstrated that antibodies can be used to intervene in specific intracellular biological processes *(2)*. That is, antibodies can at least temporarily alter cellular pathways by binding to their target antigen after being introduced into a cell. Indeed, it has been predicted that intracellular antibodies will make a significant impact on biological research, by providing a simple alternative to other, more technically difficult forms of gene inactivation *(2)*. Furthermore, this technology is not limited to mammalian or microbial systems, but can be applied to plant cells as well.

While recombinant DNA technology has been used extensively with microbial systems, it also offers the opportunity to alter plants genetically *(1)*. Indeed, genetic engineering provides a means of improving the agricultural value of plants, and offers an alternative means of studying basic processes of plant biology *(3)*. As well, transgenic plants can act as living bioreactors for the inexpensive production of important biological macromolecules.

Within the last decade, it has become apparent that the combination of antibody engineering and plant biotechnology has the potential to provide some very exciting opportunities. Indeed, antibodies expressed in plants can be used to alter the phenotype of the plant itself, or plants can be used as living factories for the large-scale production of antibodies. The aim of this review is to summarize much of the progress that has been made in the field of antibody engineering in plants, and to attempt to identify some of the aspects of the technology that need to be addressed before it becomes more widely used and accepted.

Antibodies and Their Derivatives

Of the many different classes of antibodies, and numerous antibody fragments, many of which are currently used in diagnostics, therapeutics and for pure research, those that are discussed in this review are shown schematically in Fig. 1. Immunoglobulin (Ig) molecules

typically have a molecular mass of around 150 kDa and are composed of two light and two heavy chains. The light chains have a molecular mass of about 25 kDa while the heavy chains are about 50 kDa each.

An antibody molecule contains a number of distinct regions. Antigen-binding (F_{ab}) fragments are generated from whole antibodies by digestion with the proteolytic enzyme papain and consist of the variable domains of the protein (VH_1 and VL_1) as well as a constant domain from each chain (CH_1 and CL_1). The variable domains of the light and heavy chains are located at the terminus of the F_{ab} fragment and together form the antigen-binding site of the antibody (Fig. 1A). It is this segment of the antibody molecule that is most divergent between antibody classes. The complement-binding (F_c) portion of an antibody is comprised exclusively of constant domains of the heavy chain (CH_2 and CH_3) (Fig. 1A). This portion of the antibody does not react with antigens, but is often immunogenic itself when used for immunotherapy *(4)*. Other antibodies of interest are those that occur in secretions of the digestive and respiratory tracts, specifically IgM and IgA class antibodies. Immunoglobulin A molecules often occur as dimers linked together by a secretory component (SC) and a short joining (J) chain (Fig. 1B). There are also a number of useful small molecules that can be derived from whole antibodies. The F_v (variable) fragment is an antibody fragment consisting only of the variable domains of the light and heavy chains, while a single chain F_v (scF_v) fragment consists of the variable domains linked together by a short peptide linker (Fig. 1C). The binding affinity of scF_v molecules for specific epitopes is similar to their parental antibodies, but their small size and undemanding folding requirements give them an advantage over whole antibodies where the F_c portion of the molecule is not needed *(5)*.

Monoclonal Antibodies

Monoclonal antibodies are produced by hybridoma cell lines derived from the fusion of an immortalized myeloma cell and an antibody-producing B cell *(6)*. Each hybridoma cell line produces a single type of antibody. Recently, monoclonal antibodies have been produced by a number of different cell systems after transformation

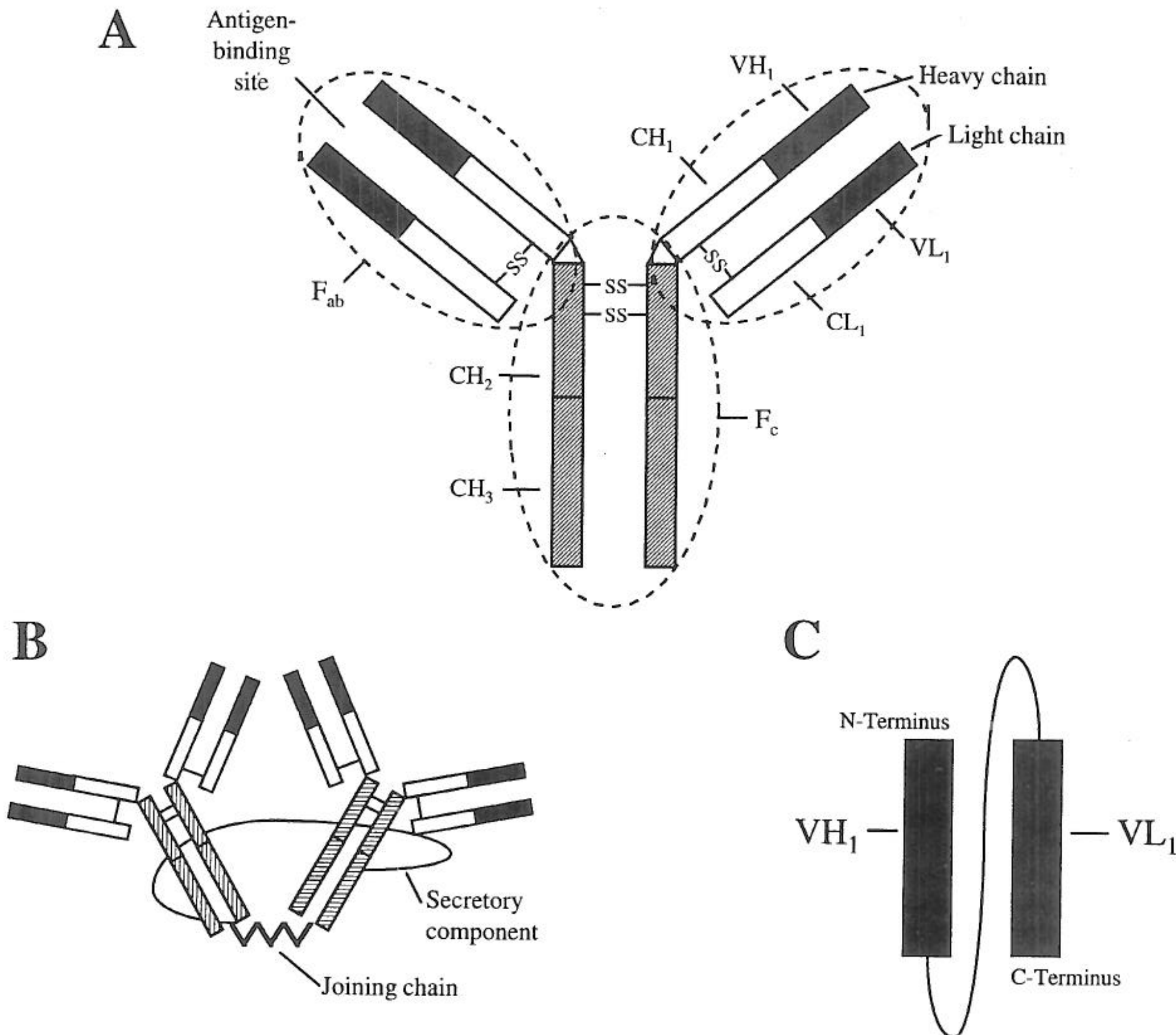

Fig. 1. Diagrammatic representation of antibody molecules. (A) Whole immunoglobulin molecule showing F_{ab} and F_c fragments; (B) secretory dimeric immunoglobulin; (C) single chain F_v (scF_v) antibody fragment.

with light and heavy chain genes. The tremendous potential of monoclonal antibodies for use as therapeutic and diagnostic tools has long been evident. However, use of immunoglobulins has proved difficult for many of these applications. Problems have included eliciting an immune response in the host and an inability to penetrate the target cell, due to the large size of whole immunoglobulin molecules. Recently, a number of workers have developed experimental approaches to deal with these problems including 'humanizing' antibodies and using smaller antibody fragments to reduce host immune responses and increase cell penetration *(7)*. Human therapeutics and diagnostics have been identified as two of the biggest potential areas for biotechnology commercialization. Sales of biotechnology therapeutic agents are projected to reach $30 billion (U.S.) by the year 2000 and monoclonal antibodies head the list of products that are projected to be used in human therapeutics and diagnostics *(8)*. Consequently, at the present time monoclonal antibodies outnumber all other products being explored by biotechnology companies for the treatment and prevention of disease. For example, endoplasmic reticulum (ER)-targeted intracellular antibodies have been used successfully to prevent the transport of growth-factor receptors to the plasma membrane, essentially providing an alternative to gene inactivation strategies *(2)*. In addition, many potential strategies for the treatment of cancerous tumours and even inhibition of HIV-1 replication center around the use of monoclonal antibodies *(2,9)*.

Antibodies also have a great deal of potential for use in basic research. The binding of antibodies to their antigens is a very powerful tool that may be used to inactivate the antigens *in vivo*. It is this principle that is the basis for many therapeutic applications. However, if the antibody is directed against an endogenous antigen, this technology can also be used to inactivate or alter any number of normal biological processes, providing an alternative method of producing knock-out mutants. Of course, antibodies are also essential components of immunological isolation and detection methods.

Traditional Methods of Production

As the therapeutic and diagnostic use of monoclonal antibodies expands there will be an increased demand for their safe and cost-effective production. Certainly, many different production systems have and will be explored. Systems currently used to produce monoclonal antibodies include cultured lymphoid cell lines, yeast (both *Saccharomyces cerevisiae* and *Pichia pastoris*) cells, *Trichoderma reesei* (Ascomycetes), insect cells, monkey (COS) and chinese hamster ovary cells, as well as *Escherichia coli* and more recently phage-display libraries *(10,11)*. Cloning has proven difficult in many of these systems, and a great deal of variability in expression levels has been achieved, even with the most successful cell lines. However, more recent developments have caused transgenic plants and plant cell cultures to be explored as potential antibody expression systems. The quickly expanding area of plant biotechnology has made this a possibility worthy of consideration, and it has been gaining attention since its first successful report in 1989 *(12)*.

Plants as Bioreactors

Advances in plant biotechnology have made it possible to consider the use of transgenic plants as a cost-effective alternative for the large-scale production of biological macromolecules of non-plant origin such as lipids, carbohydrates and proteins that have traditionally been produced microbially *(1,13,14)*. Indeed, the possibility of using plants as bioreactors for the large-scale production of both protein and non-protein products is currently being explored *(15)*. Heterologous biomolecules may also be used to alter the phenotype of the plant itself *(16)*. Phenotypic modifications of plants using biotechnology will soon lead to the development of more hardy transgenic crops as well as plants with enhanced nutritional quality due to altered protein and lipid compositions *(17)*.

The use of plants for production of proteins is particularly attractive, which is reflected by the fact that recombinant proteins make up most of the biomolecules currently under study for production in transgenic plants or already licensed for pharmaceutical use *(14)*. Examples of proteins already being produced on a large scale in plants included monoclonal antibodies, enzymes, hormones, cytokines, plasma proteins and vaccines *(14)*.

Plant-Produced Antibodies

Of the heterologous proteins currently being expressed in plants, monoclonal antibodies are attracting the most attention. This is due, in part, to the fact that there are many potential uses for these so-called plantibodies. They have the potential to be produced on a large scale for therapeutic or diagnostic use *(16)* and also provide the opportunity to manipulate agronomic crop plant traits or reduce pathogenic infections through antibody-mediated modifications of antigen activity *in planta (18)*. In addition, antibodies produced in plants may be used for basic plant research, knocking out or altering existing biochemical pathways by binding a specific antigen. Antibodies may also be used for a process known as biofiltration, where the binding and retention capacity of the antibodies is used to isolate and process environmental contaminants *(18)*.

Several groups have successfully produced full-length antibodies in higher plants *(12,19–25)*, while other groups have had success producing smaller antibody derivatives such as F_{ab} and single-chain F_v molecules *(5,20,24,26–30)*. The various antibodies and antibody fragments that have been expressed in plants are summarized in Table 1. Indeed, the use of plants to produce all kinds of antibodies and their fragments for many different applications is quickly growing in popularity.

Assembly, Targeting and Secretion of Whole Antibodies in Plants

Full-length antibodies were first expressed in plants by Hiatt and co-workers in 1989. Separate transgenic tobacco lines were produced which expressed either the light (κ) or heavy (γ) chains of an immunoglobulin G

Table 1. Summary of Antibodies and Antibody Fragments Produced in Plants

Host Plant	Antibody Molecule	Antigen	Organ/Subcellular Location	Maximum Accumulation Level	Ref.
Tobacco	IgG	Phosphonate ester	Leaf/ER	1.3% TLP	*(12)*
Tobacco	IgM	(4-hydroxy-3-nitrophenyl)acetyl	Leaf/ER and chloroplast	NR	*(19)*
Tobacco	VH Domain	Substance P (neuropeptide)	Leaf/ER and apoplast	1% TSP	*(25)*
Tobacco	IgG	Phosphonate ester	Leaf/ER and apoplast	0.8% TLP	*(32)*
Algae (*Acetabularia mediterranea*)	IgM	(4-hydroxy-3-nitrophenyl)acetyl	Cytoplasm	NR	*(36)*
Tobacco	scF_V	Phytochrome	Seed	0.06–0.1% TSP	*(5)*
Tobacco	scF_V	Artichoke Mottled Crinkle Virus	Leaf/Cytoplasm	0.1% TSP	*(27)*
Tobacco	scF_V	Phytochrome	Leaf/Apoplast	0.5% TSP	*(26)*
Tobacco and *Arabidopsis*	IgG and F_{ab}	Human creatine kinase	Leaf/Nucleolus	1.3% TSP	*(20)*
Tobacco	IgG	Cell-surface antigen SA I/II of *S. mutans*	Leaf/Apoplast	10 μg/mL plant extract	*(21)*
Tobacco	IgG and F_{ab}	Fungal cutinase	Root/Apoplast	1.1% TSP	*(22)*
Tobacco	scF_V	Oxazolone	Seed/Protein bodies	0.67% TSP	*(29)*
Tobacco	scF_V	Abscisic acid	Leaf/ER	4.8% TSP	*(28)*
Tobacco	IgA-G hybrid	Cell-surface antigen SA I/II of *S. mutans*	Leaf/Cytoplasm and apoplast	500 μg/g fresh weight	*(23)*
Tobacco	IgG	Tobacco Mosaic Virus	Leaf/Apoplast	3 μg/mg TPP	*(25)*
Tobacco	scF_V	Cutinase	Leaf/ER and apoplast	1% TSP	*(30)*
Arabidopsis	IgG and F_{ab}	Human creatine kinase	Leaf/Intercellular spaces	13% TSP	*(24)*

Abbreviations: ND, Not determined; NR, Not reported; TLP, Total leaf protein; TSP, Total soluble protein; TPP, Total Plant Protein.

(IgG) molecule, both with and without their natural, mammalian leader sequences. These transgenic tobacco lines were then sexually crossed, to yield plants that expressed both κ- and γ-chains simultaneously. The ability of the resulting IgG molecules to bind the appropriate antigen showed that assembly of light and heavy chains did indeed occur and was extremely efficient. In fact, the plant-derived antibody bound antigen equally well as the hybridoma-produced monoclonal antibody (mAb). However, this was true only in those plants that included the mammalian leader sequence with both the light and heavy chain genes. The apparent dependence for proper assembly on a leader sequence highlighted the sequence's importance for assembly and accumulation of functional antibodies in tobacco and suggested that antibody assembly took place in a similar manner as in B lymphocytes *(12)*. While the mechanism of antibody assembly in mammalian B lymphocyte cells is only partially understood, it is known that both light and heavy chains are synthesized as precursor proteins. These precursors are targeted to the lumen of the endoplasmatic reticulum (ER) where the signal sequences are cleaved and chaperonin molecules direct the assembly of functional immunoglobulins *(32)*. Hiatt *et al. (12)* suggested that the association of immunoglobulin with the ER indicated the antibody was to be secreted from the plant cell. However, they did not discount the possibility that assembly could also take place spontaneously in other cellular compartments, given sufficient levels of each chain.

If plants are to be used for the large-scale production of antibodies, a quick and efficient method of extraction is required. Secretion of antibodies to the extracellular space may allow for gentler extraction and perhaps easier purification of the antibodies. Therefore, in an attempt to optimize secretion of plant-produced antibodies Hein *et al. (33)* further evaluated the dependence of mAb assembly on the N-terminal leader sequence. Expression of IgG in plants transformed with γ- and κ-chain genes, each containing their native mouse signal sequence, was compared to IgG expression in plants that were transformed with genes containing a yeast pre-pro sequence in place of the native signal *(33)*. Antibody accumulated to significant levels regardless of the origin of the signal sequence used, although plants expressing a leaderless transcript did not accumulate any assembled IgG. The aim of this study was to maximize antibody accumulation in plants, and indeed, if high per-plant yields can be combined with efficient purification procedures, antibody-producing crop plants growing in agricultural proportions could produce a significant amount of antibody. A promising and potentially powerful purification scheme uses the binding affinity of staphylococcal protein A with the F_c region of IgG antibodies *(33)*. This approach needs only minor modifications to become widely used on a large scale.

Immunoglobulin classes other than IgG have also been successfully produced in plants. For example, Düring *et al. (19)* used an expression cassette containing both the light and heavy chain genes of an IgM molecule to produce full-size antibody in tobacco. This approach eliminated the need to perform sexual crosses of transgenic plants. Chimeric light and heavy chain genes were constructed using the mature genes for the antibody, each linked to the coding sequence for the barley α-amylase signal peptide. The chimeric genes were each placed under the control of an active plant promoter, linked on a plasmid, and integrated into the genome of tobacco using *Agrobacterium*-mediated gene transfer *(19)*. Immunogold labeling was used to determine the cellular location of the resulting IgM molecules. As was the case for IgG, the majority of the gold particles accumulated in the ER. However, some particles were detected in the cytoplasm and surprisingly, considerable signal was also localized

in the thylakoid membranes of chloroplasts. The use of a plant-derived ER targeting sequence and the fact that there was significant accumulation of antibody in the ER supports the contention that transport to the ER lumen is necessary for proper assembly of full-length antibodies, as is the case for mammalian antibodies *(12)*. ER localization also supports the contention that antibodies are involved in the secretory pathway of plants *(12,19)*. However, the cytoplasmic and chloroplastic antibody seen by Düring *et al. (19)* would not be part of the secretion pathway, and certainly this distribution pattern was not expected. Nevertheless, the most important factor in the accumulation of functional, full-length antibody in transgenic plants appears to be the presence of an ER signal peptide, regardless of its origin *(19,34)*.

Additional support for the hypothesis that full-length antibodies are secreted by plant cells was recently reported in an immunolocalization study, where it was established that both full-length IgG molecules and F_{ab} fragments accumulate in the intercellular spaces of mesophyll cells of transgenic *Arabidopsis thaliana* leaves *(24)*. This study provided the first conclusive evidence that full-sized plant-produced antibodies do indeed accumulate in the intercellular space and not simply the apoplast. The finding that the antibodies were not retained by the cell walls, which are believed to have exclusion limits of between 17 and 60 kDa *(24,35,36)*, was particularly surprising. Indeed, the results of De Wilde *et al. (24)* suggest that the mesophyll cell walls of *Arabidopsis* are permeable to intact IgG molecules, which have a molecular weight of 146 kDa. These findings are of particular interest to any application where extracellular antigen binding is desired.

The finding that some plant-produced mAb accumulated in the cytoplasm of transgenic tobacco cells *(19)* prompted Stieger *et al. (38)* to investigate more closely the possibility that antibodies are able to self-assemble in the cytoplasm. An ability to direct antibody accumulation in the cytoplasm would provide many interesting possibilities for use *in planta*. To obtain cytosolic expression, leaderless chimeric genes were injected into the nuclei of the alga, *Acetabularia mediterranea (38)*. Immunofluorescence confirmed that the antibodies formed antigenic binding sites in the cytoplasm. However, the relatively high reducing conditions of the cytoplasm do not allow formation of disulfide bonds, which are characteristic of all antibodies *(38)*. It was speculated that the antibodies folded correctly in the algal cytoplasm without forming disulfide bonds, and relied simply on hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions for proper folding. If indeed cytoplasmic accumulation of fully functional antibodies can be achieved, this technology would be significant for plant molecular biologists and plant breeders. However, significant cytosolic accumulation of full-size mAb has not yet been reported in higher plants, and more work needs to be done before such accumulation patterns are consistently obtained in systems other than algae.

Antibody Fragments

Small size and undemanding folding requirements make single-chain F_v molecules very attractive antibody derivatives for expression in plants. The binding capacity of scF_v molecules is similar to their parental antibodies, but their small size gives them an advantage over whole antibodies where the F_c portion of the molecule is not needed *(5)*. Single-chain F_v fragments are especially attractive for intracellular immunomodulation of plant cell components due to their less demanding assembly requirements in the reducing environment of the cytoplasm.

Immunomodulation

Owen *et al. (5)* were able to successfully express an scF_v in tobacco in an effort to modify plant metabolism. A synthetic gene was used to produce an anti-phytochrome scF_v molecule. Germination of seeds expressing the anti-phytochrome scF_v was reduced by approximately 40% as compared to controls *(5)*; presumably due to binding of phytochrome by the scF_v, which affected the regulatory molecule's ability to function properly. This was the first report of successfully altering plant metabolism using a plant-produced mAb against an endogenous antigen. This approach could also be applied to many other metabolic pathways and could be used to study plant metabolism, or perhaps enhance crop value by blocking undesired pathways.

In a similar study, transgenic tobacco plants expressing an anti-abscisic acid (ABA) scF_v were used to obtain wilty, ABA-deficient-like plants *(29)*. These plants targeted the scF_v for expression in the ER by incorporating a four amino acid sequence (Lys, Asp, Glu, Leu) into the antibody fragment that causes proteins to be retained in the ER and therefore prevents secretion. High expression levels were achieved in the leaves of the transgenic tobacco (up to 4.8%) and the scF_v purified from the leaves was shown to bind ABA *in vitro*. Although the total ABA levels in the anti-ABA plants was higher than in controls, the plants expressing the scF_v did show ABA-deficient symptoms including wilty leaves. It is believed the ER-retained scF_v molecules may act as an ABA-sink, causing free ABA levels in the cytoplasm to be lower than in controls *(29)*. Additionally, this study showed that the use of stably expressed antibodies to produce loss-of-function mutants is not restricted to targeting antigenic proteins, but can target any biomolecule that can act as an antigen.

It has also been shown that functional scF_v molecules can be secreted to the apoplast and therefore be available to bind apoplastic antigen *(27)*. Indeed, the ability to bind and modulate extracellular as well as intracellular antigens is very desirable. Moreover, it is possible that secreted scF_v may become available to bind plant pathogens in the apoplast. If this technology was used with plant cell cultures, it might provide large volumes of secreted scF_v which could be easily extracted from the growth medium. Finally, these results suggest that it may be possible to direct scF_v molecules expressed in transgenic plants to subcellular locations other than the cytoplasm, ER and apoplast (27).

Targeting of Antibody Fragments

There is still some uncertainty as to whether cytosolic expression and accumulation of scF_v fragments is in-

deed possible in plant cells. A study by Schouten *et al.* *(31)* therefore examined functional expression of antibodies targeted to different subcellular compartments. In this study, transgenic plants containing scF_v genes targeted for expression in the cytoplasm were unable to accumulate any detectable scF_v *(31)*. On the other hand, plants expressing scF_v targeted to the ER accumulated significant amounts of the protein. Interestingly, when an ER retention signal (amino acids Lys, Asp, Glu, Leu) was included in either of the antibody gene constructs, accumulation of scF_v increased significantly *(31)*. The ER retention signal may somehow protect the cytosolic scF_v from proteolytic degradation, or it may confer protection to the protein through an interaction with the cytosolic side of the ER *(31)*. Nevertheless, this work demonstrated that the exact requirements for antibody expression and accumulation in plants remain elusive *(31)*.

In another report, it was demonstrated that active scF_v molecules can be targeted to an organ other than leaves *(30)*. Specifically, scF_v was shown to accumulate in developing and mature tobacco seeds. The antibody accumulated to 0.67% of the total soluble protein in the seeds, and was stably stored for one year at room temperature *(30)*. This system therefore offers high expression levels along with long-term storage of the protein and does not appear to have a negative influence on plant growth or seed development. The exact cellular location of the antibody was not determined, although the authors felt it may have accumulated in protein bodies within the seeds *(30)*. Determining the exact cellular location of the stored antibody and transferring the system to a more edible crop, such as corn or peanut, could make this strategy extremely valuable for the commercial production of scF_v molecules *(30)*. In fact, this system may be even more valuable if long-term stable expression could be achieved for full-length antibodies as well.

Following conflicting literature reports, the overall accumulation and assembly patterns of whole antibodies and their F_{ab} fragments were studied in both *Arabidopsis thaliana* and tobacco *(20)*. The assembly patterns for the complete antibody differed in both species. *Arabidopsis* contained primarily full-size antibodies, whereas tobacco had an abundance of smaller 50 kD fragments. Also, F_{ab} fragments accumulated to much higher levels in *Arabidopsis* leaves (1.3%) than in tobacco leaves (0.044%). In addition, when immunolocalization was used to detect the subcellular location of the antibodies, antibody was surprisingly detected in the nucleolus of meristematic tissue *(20)*. This finding adds to the uncertainty surrounding the subcellular targeting of plant-produced antibodies, as the nucleolus is not part of the secretory pathway that many plant-produced antibodies are believed to use.

Pathogen and Viral Protection

The prospect of using whole antibodies to obtain disease resistance against plant pathogens has also been examined. Using *Agrobacterium*-mediated transformation to introduce the light and heavy chain genes, high expression levels (1.1% of total soluble root protein) of functional, full-length antibodies were achieved in the apoplast of roots *(22)*. Use of this approach with the appropriate antibody could eventually confer pathogen resistance upon plant roots *(22)*.

In an effort to confer viral resistance to plants, an scF_v antibody directed against the artichoke mottled crinkle virus was expressed in the cytoplasm of tobacco *(28)*. These transgenic plants were specifically protected from virus attack, in that they had a reduced incidence of infection and showed delayed symptom development *(28)*. The success of this approach to attenuate viral infection was dependent on stable antibody expression in the cytoplasm. This was achieved using scF_v molecules rather than full-length antibodies, since as indicated above, full-length antibodies must be targeted to the ER for secretion in order to get stable accumulation in higher plants *(28,32)*. Indeed, tobacco mosaic virus (TMV) infectivity has also been successfully reduced in transgenic tobacco plants secreting a full-size anti-TMV antibody *(25)*. Plants secreting the anti-TMV antibody showed up to a 70% reduction in the number of necrotic lesions from this virus *(25)*. This type of »engineered intracellular immunization« approach seems to have great potential for supplying plants with viral and perhaps pest resistance *(28)*. However, before it becomes a commercially usable or valuable approach, the mechanism(s) of action should be more fully elucidated.

Human Immunotherapy

The immunotherapeutic potential of full-length plant-produced antibodies has received considerable attention in recent years *(32,34)*. It has been suggested that so-called plantibodies are more desirable for human use than microbially produced antibody proteins because they undergo eukaryotic rather than prokaryotic post-translational processing and human glycosylation patterns are more closely matched by plants than by bacteria, making plants a more attractive production system for antibodies. Indeed, many of the N-linked glycans involved in glycosylation are the same with respect to both size and extent of branching in plants and animals *(33,34)*. As well, transgenic plantibodies are thought to be processed in a similar manner as mammalian glycoproteins *(33)*. However, post-translational glycosylation of monoclonal antibodies by plants remains a concern for those antibodies that may be used for human immunotherapy. Furthermore, there are some differences in the carbohydrates involved and the pattern of glycosylation in plants *(34)*, which is the cause of some concern that the plant-specific glycans may increase the immunogenicity of plantibodies used for human immunotherapy. While this may seem unlikely, given humans' daily exposure to plant glycoproteins in food, pollen and personal care products *(32)*, if plant-produced antibodies are to be used for human immunotherapy, plant-specific glycosylation is a concern that needs to be addressed. If plant glycosylation does present a problem, it might be circumvented by removing the glycosylation site(s) from the antibody molecule, removing the glycan itself from the plant or perhaps by generating a plant mutant lacking the glycosylating enzyme *(34)*.

Perhaps the most promising application for plant-produced antibodies in immunotherapy, is in passive immunization, e.g. against *Streptococcus mutans*, the most common cause of tooth decay *(21)*. Large doses of antibody are required in multiple applications for topical passive immunotherapy and transgenic antibody-producing plants are thought to be one of the sources that can supply the large quantities of safe, cost-effective antibodies needed. The predominant antibody found in human mucosal secretions, such as saliva, is the secretory form of immunoglobulin A (SIgA) *(37)*. Secretory IgA is a good candidate for use in passive topical immunization against oral bacteria such as *S. mutans*, or other pathogens of the digestive tract, due to its resistance to proteolytic digestion *(21,39)*. Even so, a hybridoma-derived IgG antibody (Guy's 13) was first used successfully in a local passive immunization study on human subjects *(40)*. This study suggested that passive immunization using monoclonal antibodies might be an effective strategy for the prevention of colonization of teeth by *S. mutans*, and the subsequent development of tooth decay. Ma *et al.* *(21)* later produced a hybrid IgA-IgG molecule in plants, and showed that it caused aggregation of *S. mutans* in culture, which appears to be how the antibody prevents colonization of the bacteria *in vivo*. Some non-essential constant regions of the Guy's 13 heavy chain were replaced with IgA constant regions, making the hybrid antibody less prone to proteolytic digestion in human secretions. Incorporation of IgA constant regions did not alter the antigen recognition capacity of the recombinant antibody *(21)*.

More recently, simultaneous expression and assembly of four separate protein chains has been achieved in tobacco to form functional, dimeric secretory antibody *(23)*. The resulting secretory antibody (SIgA-G) which binds the same cell-surface antigen (SA I/II) of *S. mutans* as the IgA-IgG hybrid molecule previously produced *(21)*, consists of a monoclonal κ chain, a hybrid IgG-IgA heavy chain, a joining (J) chain from mouse and a secretory component (SC) from rabbit *(23)*. Successive sexual crosses between transgenic plants, each expressing one of the protein chains, resulted in the simultaneous expression and assembly of all four peptides. The resulting SIgA-G molecule matches the most common form of IgA found in mammals, which is a dimer associated with a small polypeptide J chain and a secretory component *(23)*. In mammalian systems production of monoclonal SIgA requires two different cell types, while functional expression of this secretory molecule was achieved in single tobacco leaf cells. Moreover, the nature of the association of the J chain and SC with IgA-G (to form SIgA-G) was confirmed to be similar to their association with IgA in mammals. The binding specificity of the plant-produced SIgA-G was also similar to the native SIgA antibody *(23)*. In addition, the yield of SIgA-G in tobacco was much higher than achieved for IgA-G in the previous study *(21)*, which indicated that secretory IgA-G was more resistant to proteolysis and suggested that its production could more easily be scaled up to agricultural proportions *(23)*. Preliminary results also suggest that the development of plants capable of producing native SIgA may be possible, which could have a significant impact on passive immunotherapeutic applications *(23)*.

Although this approach seems to have great potential for success, no antibodies produced in plants have been approved for clinical use. A major hurdle has been the cost of antibody production in plants. However, it has been estimated that antibodies expressed in soybean at a level of 1% of total protein, could be produced for as little as $100 per kg of antibody *(18)*, a cost that should decrease as purification procedures are improved. Large quantities of antibody are required to overcome the rapid rate of clearance when used for passive topical immunotherapy and plant agriculture could provide the cost-effective, large-scale production system needed for such treatments *(32)*. Furthermore, if alternative delivery systems could be developed, extensive purification schemes could be avoided. For instance, it has been shown that full-length antibodies and scF_v molecules can be assembled and accumulated in the roots of transgenic tobacco *(22)* and tobacco seeds *(30)*, respectively. If this technology could be utilized to obtain stable accumulation of antibodies in more edible plant organs such as potato tubers, carrots or peanuts, it might allow for long-term storage and easy delivery of antibodies for immunotherapeutic applications.

Further Considerations

In order for any application of plant-produced antibodies to be most effective, optimal expression and accumulation conditions need to be determined. These conditions will vary depending on the antibody being produced, the species of plant being used and the end use of the antibody. Subcellular targeting as well as which tissue the antibody will be expressed in need to be considered. Perhaps the biggest consideration is whether or not the antibody will be secreted. This will affect accumulation levels as well as the ease with which the antibody can be extracted and purified from the plant. An issue that has not attracted a great deal of attention in the literature is that of codon usage. Especially if the transgenic plants are to be used for the large-scale production of antibodies, differences in codon usage between plant and mammalian cells may well affect antibody expression and accumulation levels. That is, in situations where the highest expression levels are required, differences in codon usage could limit the ability of plants to accumulate the desired amount of antibody.

Glycosylation patterns are a very important aspect of the technology to consider when plant-produced antibodies are for animal or human use. Indeed, even small differences in glycosylation patterns can potentially alter the activity and/or immunogenicity of any protein *(14)*. Therefore, it may be necessary to 'humanize' any such antibodies or perhaps use plant hosts altered in their capacity for glycosylation for the production of these antibodies.

The down-stream processing of plant-produced antibodies has also not received a great deal of attention. Strategies for the extraction and purification of antibodies from plants that yield fully functional molecules in a safe, cost-effective manner will need to be explored as we get closer to the possibility of utilizing these recombinant proteins. It might even be possible to correct im-

proper glycosylation of antibodies at this stage of purification. It is also possible that for some forms of immunotherapy, purification may not be necessary as the antibody-expressing tissue may be directly consumed. For example, this is the ultimate goal of most strategies that aim to use the plant-produced antibodies for passive, topical immunotherapy.

Conclusions

In recent years, plant biotechnology has made agriculture a more attractive alternative for the large-scale production of biomolecules such as proteins. The ability to produce antibodies and antibody fragments in plants presents some very interesting possibilities. Since the first monoclonal antibody was expressed in tobacco *(12)*, a number of different antibodies have been produced in plants and these antibodies have been used for a variety of purposes. Full-length antibodies have been expressed in plants with the aim of producing large quantities for use in human immunotherapy. Indeed, the production of multimeric secretory antibodies *(23)* and the ability to store antibodies long-term in seeds or other organs *(30)* indicates this goal may soon be realized, provided that the potential problem of plant-specific glycosylation can be solved.

The use of antibodies *in planta* for immunomodulation of plant processes is another exciting area of research. Plant-produced antibodies might be used to change the appearance of a plant or alter plant metabolism by altering existing biochemical pathways. For these purposes antigen binding *in vivo* is required, so the cellular location of the plant-produced antibody is crucial. Since the F_c portion of the antibody is not necessarily required for this application, easily assembled scF_v molecules have been used to achieve phenotypic modifications when intracellular antigen-binding is required. Phytochrome-mediated germination has been altered *(5)* and ABA-deficient mutants have been developed *(28)* using scF_v molecules.

Antibodies expressed in plants may also serve to effectively immunize the host plant against pathogen or viral attack. Indeed, tobacco has already been effectively immunized against virus attack using both intracellular scF_v molecules and secreted full-size antibodies *(25,27)*. The possibility of using scF_v molecules to interfere with pathogenesis or viral attack in the apoplast also exists *(22,27)*.

Clearly though, the effects of cellular targeting on expression levels and therefore activity of antibodies in plants are not yet fully elucidated. Although some groups have reported successful expression of scF_v molecules in the cytoplasm *(5,28)*, others failed to get any antibody accumulation unless an ER retention signal was included *(31)*, and only recently has the exact extracellular location of secreted antibodies been determined *(24)*. Surprisingly, it was also revealed that whole antibodies secreted from the ER are not retained by plant cell walls *(24)*. Furthermore, it was reported that full-length antibodies unexpectedly accumulated in chloroplasts and the nucleolus *(19,20)*. Therefore, while the production of antibodies in plants offers many exciting possible applications, such as in passive immunization of large human populations and altering plant metabolism, many unanswered questions about the technology remain.

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Proizvodnja i primjena antitijela dobivenih iz biljaka

Sažetak

Povezivanje biotehnologije biljaka i inženjeringa antitijela posljednjih je godina pridonijelo znatnom napretku molekularne biologije biljaka. Transgenske biljke mogu se danas koristiti za proizvodnju velike količine antitijela koja se primjenjuju u terapeutske i dijagnostičke svrhe. Antitijela proizvedena u biljkama mogu se također koristiti za mijenjanje postojećih biokemijskih putova u biljkama ili za učinkovitu imunizaciju biljaka prema patogenima ili virusnim infekcijama. Mnoga različita monoklonska antitijela i njihovi derivati već se proizvode u biljkama za raznoliku primjenu. Međutim, prije nego što se antitijela mogu sa sigurnošću primijeniti u imunoterapiji ili za druge svrhe, treba razmotriti utjecaj pojedinih činitelja u biljci na njihovu subcelularnu lokalizaciju te način glikozilacije na aktivnost i imunogenost antitijela.