Plant Physiol. (1995) 109: 341–346

Update on Biotechnology

Plant Antibodies for Immunotherapy

Julian K.-C. Ma* and Mich B. Hein

Department of Immunology, United Medical and Dental Schools, Guy's Hospital, London SE1 9RT, United Kingdom (J.K.-C.M.); and Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037 (M.B.H.)

The use of transgenic plants for the expression of therapeutic recombinant proteins is gradually gaining acceptance in the scientific community (Moffat, 1995). There are many attractions to this technology, including the possibility of manufacture on an agricultural scale for bulk production and to reduce costs, as well as the range of plant hosts available and the stability of transgenic lines in seeds that might allow export of transgenic crops (containing, for example, medicinal compounds) to deprived areas of the world. These could then be grown within the existing agricultural infrastructure rather than require the funding of new pharmaceutical factories. From a technical viewpoint, the general capabilities and adaptability of plants are exciting. For example, the processing and assembly of proteins in plants appear to match closely that found in mammalian cells, which might be a particular advantage over the more commonly used microbial expression systems. In addition, because transformation techniques result in the stable integration of the foreign DNA into the plant genome, genetic recombination by sexual crossing of transgenic plants is a simple method to introduce new genes or to accumulate multiple foreign genes into plants.

The original description of antibodies produced in plants demonstrated the principle of co-expression of two recombinant gene products in plants that were correctly folded and assembled into a molecule that was functionally identical with its mammalian counterpart (Hiatt et al., 1989). Several groups have since expressed antibody molecules in plants, either to modify or improve plant performance and characteristics or with a view to harnessing plants as bioreactors for large-scale production of antibodies. In this *Update* we shall review briefly the developments in the field of antibody engineering in transgenic plants and address the potential of plant antibodies for immunotherapy.

STRATEGIES FOR ANTIBODY PRODUCTION IN PLANTS

A number of transformation techniques have been used to introduce antibody genes into plant cells. Currently, the most convenient are *Agrobacterium* and shotgun microprojectile bombardment-mediated transformation. The vectors based on the tumor-inducing plasmid of *Agrobacterium tumefaciens*, which contain selectable plant markers, promoters upstream from polylinkers, and *Escherichia coli* and *Agrobacterium* origins of replication, are generally quite large, and the initial strategy to produce antibody in plants was to express each immunoglobulin chain separately in different plants and to introduce the two genes together in the progeny plant by cross-pollination of the individual heavy- and light-chain-expressing plants. Most groups have used the constitutive 35S promoter from cauliflower mosaic virus. With this promoter, a variety of cell types are expected to express the recombinant protein, although there is evidence for differential expression related to developmental stage and tissue type. In any strategy in which multiple gene expression is required for assembly of a complex multimeric protein, it is important to use the same or similar promoters for each gene to maximize the likelihood of co-expression in single cells (Ma et al., 1995).

The use of cross-pollination of individually transformed plants expressing antibody light or heavy chains involves two generations of plants to generate an antibody-producing plant (Hiatt et al., 1989; Ma et al., 1994). The yield of recombinant antibody is consistently high by this technique, between 1 and 5% of total plant protein. Others have used double-transformation techniques to introduce both the heavy- and light-chain genes into the same plant cell simultaneously (De Neve et al., 1993). Although reducing the time required to produce an antibody-secreting plant, the transformation may be less than optimal and more screening may be required, since there can be individual variability of the expression of either gene. The yield of antibody was lower with this technique, amounting to only 0.055% of total soluble protein, and the efficiency of assembly also appeared to be reduced. An alternative strategy is to introduce the light- and heavy-chain genes on a single T-DNA (During et al., 1990; van Engelen et al., 1994). This is a more rapid technique and avoids the problem of segregation in the progeny plants, but the promoter and terminator need to be chosen carefully to coordinate expression of the two transgenes. Expression levels of up to 1.1% of total protein have been reported (van Engelen et al., 1994).

Expressing recombinant proteins in transgenic plants is not a rapid procedure, requiring 2 to 3 months in the case of tobacco. This does not compare at all favorably with other expression systems; therefore, if the recombinant an-

* Corresponding author; e-mail j.ma@umds.ac.uk; fax 44–171–407–6693.

Abbreviations: BiP, immunoglobulin heavy chain-binding protein; J chain, joining chain; SC, secretory component; SIgA, secretory IgA.

tibody is being produced for uses such as human immunotherapy rather than for modification of plant performance, there must be compelling reasons for the choice of plants as the expression system.

ANTIBODIES IN PLANTS

In designing therapeutic antibodies for production in recombinant expression systems, it is important to understand the mechanisms of action of the antibody. Although the primary function of antibodies is to bind antigen, this in itself is not necessarily a protective function. A proportion of antibodies have a direct neutralizing effect, for example in blocking bacterial or viral adherence or blocking active sites of virulence factors, such as enzymes. By mimicking critical epitopes, some antibodies such as the catalytic antibodies can enhance biochemical reactions. In these cases, it can be sufficient to produce the antigen-binding domain of the antibody molecule alone for the recombinant protein to be active. Examples are the single-chain Fv molecules, which consist of the variable domains of the light and heavy chains joined by a peptide linker (Fig. 1), and Fab fragments. However, it is important to remember that these types of molecules have only single antigen-binding sites and therefore reduced binding avidity, as compared with the parental whole molecule counterparts that bind antigen bivalently.

In other examples, the therapeutic action of the antibody is dependent on aggregation of cells or bacteria, in which case the hinge region of the antibody molecule is required to link two antigen-binding domains [such as in the $F(ab')_2$ fragment]. However, in the majority of cases, the interaction between antigen and antibody in vivo would be of little significance if secondary effector functions were not invoked (Fig. 1). These include the ability to activate complement and bind to phagocytes, functions that are located in the Fc region of antibody molecules. Other important features of the Fc region are the hinge region and the sites involved in glycosylation, placental transfer, and association with J chain and SC (in IgA and IgM antibodies). The presence of the binding site for staphylococcal protein A is also a useful feature for purification purposes.

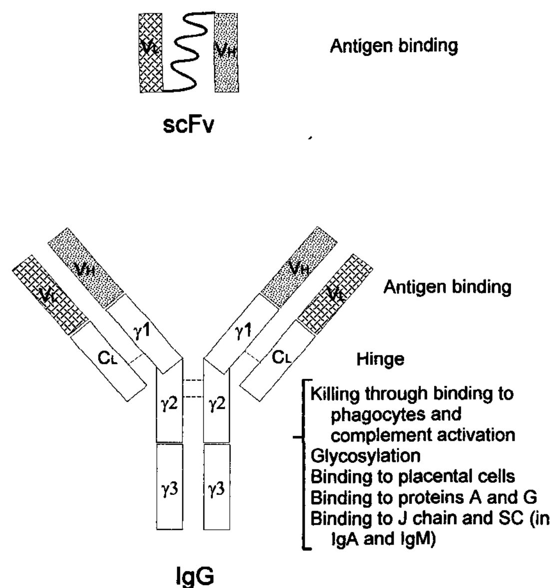

Figure 1. Comparison between functions of single-chain Fv (scFv) antibody fragments and whole molecule IgG antibodies. Whereas scFv molecules can only bind antigen monovalently, IgG binds more avidly and bivalently because there are two antigen-binding sites. In addition, the constant region of the antibody contains functional domains that have a number of important secondary effector mechanisms.

For immunotherapy in mammals, consideration needs to be given to the potential benefits and harmful effects of the Fc in the recombinant antibody. Thus, in some cases it might be beneficial to retain the ability to activate the complement pathway and to enhance target killing, whereas in others, it may be detrimental to induce inflammation through complement activation. Similarly, as will be discussed later, the consequences of plant glycosylation of a mammalian protein may be harmful. Thus, by careful planning one can utilize one of the major benefits of using a recombinant expression system to produce antibody: the ability to design antibody molecules with precise requirements by genetic manipulation to incorporate or eliminate functional regions.

An important advantage in plants is the ability to assemble full-length heavy chains with light chains to form full-length antibody with intact Fc regions. In contrast, in *E. coli*, the largest antibody fragment that can be reliably assembled is the monovalent Fab fragment. Several groups have reported the production of full-length antibodies in plants (Hiatt et al., 1989; During et al., 1990; Ma et al., 1994; van Engelen et al., 1994). The antibodies are functional in terms of antigen recognition and binding, which is a critical and sensitive test for correct assembly. In mammalian plasma cells, the mechanism of assembly of these molecules is only partially understood. The immunoglobulin light and heavy chains are synthesized as precursor proteins, and the signal sequences direct translocation into the lumen of the ER. Within the ER, the signal peptides are cleaved and at least two stress proteins, BiP/GRP78 and GRP94, function as chaperones, which bind to unassembled heavy and light chains and direct folding and assembly within the ER (Gething and Sambrook, 1992; Melnick et al., 1992). In plants, the same pathway is utilized, and assembled antibody has been detected within the ER by electron microscopic immunogold labeling (During et al., 1990). The need for signal peptides to direct the nascent light and heavy chains to the ER has been demonstrated, because assembly of antibody only occurred if both chains were synthesized with a signal sequence (Hiatt et al., 1989), although the origin of the signal sequence does not appear to be critical, since plant, mouse, and yeast sequences can be used (During et al., 1990; Hein et al., 1991). Within the ER, the correct proteolytic processing of the signal sequence was confirmed by N-terminal sequencing of the light chain (Hein et al., 1991).

ER resident chaperones homologous to BiP and GRP94 have been described in plants (Fontes et al., 1991; Walther-

Larsen et al., 1993). BiP in tobacco is encoded by a multigene family and is closely related to mammalian BiP (Denecke et al., 1991). It is expressed abundantly in tissues with high proportions of dividing cells. Expression of the GRP94 homolog identified in barley was associated with stress induced by infection or heat shock. Although the mechanism of antibody assembly in plants has yet to be determined, in the presence of these homologous chaperonins it would be surprising if the mechanisms controlling protein folding and assembly in plants and mammals were not broadly similar. Thus, it is consistent with the mammalian model for light- and heavy-chain assembly that assembly of a complete tetrameric antibody in plants entails an increase in protein accumulation, indicating that the native conformation of immunoglobulin molecules is an important determinant of stability (Hiatt et al., 1989).

As in mammalian cells, following assembly and post-translational processing, antibodies are secreted by plant cells (Hein et al., 1991). This has been demonstrated by pulse-chase-labeling experiments, using protoplasts isolated from regenerated transgenic plants to demonstrate the secretion of the antibody through the cell membrane, as well as callus cells to demonstrate that antibody secretion can occur across the plant cell wall. Accumulation of secreted antibody into the apoplasm could be advantageous, since this is a large, stable, extracellular aqueous environment in which there is minimal hydrolytic processing. For example, the accumulation levels of a single-chain Fv antibody fragment in plants were considerably higher when targeted for secretion rather than expressed within the cytoplasm (Firek et al., 1993). An additional advantage is that extraction of antibodies from apoplastic fluid is simpler and can be achieved under more mild conditions than those required for proteins located elsewhere. Extracellular secretion is not the only option for a full-length antibody. We have also found that by the addition of membrane-spanning and intracellular regions derived from the murine membrane immunoglobulin receptor, recombinant full-length antibody can be retained within the cell membrane (J.K.-C. Ma, M.B. Hein, and C. van Dolleweerd, unpublished data).

The ability of plant cells to produce full-length antibodies and to complement mammalian protein assembly can be used to produce antibody molecules with altered Fc-mediated properties. This is facilitated by the domain structure of immunoglobulin chains, which allows individual domains to be "cut and spliced" at the gene level. For example, the carboxyl-terminal domains of an IgG antibody heavy chain have been modified by replacing the Cγ2 and Cγ3 domains with Cα2 and Cα3 domains of an IgA antibody, while maintaining the correct assembly of the functional antibody in plants (Ma et al., 1994). These alterations have no effect on the antigen binding or specificity but may modify the protective functions of the antibody that are mediated through the Fc region discussed earlier. The number of constant region domains in the immunoglobulin molecule can also be changed. For example, a chimeric IgA/G heavy chain was constructed that consisted of var-Cγ1-Cγ2-Cα2-Cα3 domains, which increases the length of the heavy chain by one domain. Again, this has no effect on the ability of the heavy chains to interact with each other or with light chains, and it has no effect on antigen recognition. However, this engineered plant antibody now incorporates Fc functions of both IgG and IgA molecules, namely the Cγ2 domain, which could bind complement, neutrophils, and staphylococcal protein A, and the Cα3 domain, which includes the binding site for J chain and SC.

In extending these findings, we have recently described the assembly of an antibody molecule of therapeutic importance that has uniquely been produced in plants (Ma et al., 1995). This is the multimeric SIgA molecule that is the predominant form of immunoglobulin found in mucosal secretions, such as the gastrointestinal tract. Like serum antibody, SIgA is composed of heavy and light chains arranged in the usual manner, but there are two modifications that enhance its activity in the mucosal environment (Fig. 2). First, two immunoglobulin units are dimerized by a small polypeptide J chain, which increases the avidity of binding and enhances the potential for bacterial aggregation. Second, a fourth polypeptide SC is also associated with the dimeric antibody complex, which confers a degree of resistance against proteolysis, an important property for antibodies existing within the harsh environment of the gastrointestinal tract. Until now, attempts to produce monoclonal SIgA have been frustrated by the complexity of this molecule, which in mammals requires both a plasma cell, which secretes dimeric IgA, consisting of monomeric IgA antibody units associated with J chain, as well as epithelial cells expressing the SC precursor molecule. Normally, the SC precursor on the epithelial basolateral surface binds the dimeric IgA, initiating a process of endocytosis, transcy-

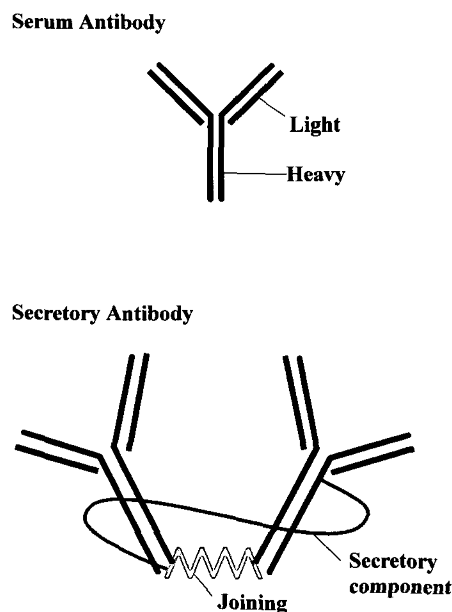

Figure 2. Serum antibody (IgG) and secretory antibody (SIgA). Monomeric forms of immunoglobulin have a basic structure consisting of two heavy chains and two light chains. SIgA consists of two monomeric immunoglobulin units, dimerized by a J chain and protected from proteolysis by SC.

tosis, and proteolysis, which leads ultimately to release of the SIgA complex into the secretion.

Four transgenic *Nicotiana tabacum* plants were generated to express a murine monoclonal antibody κ chain, the hybrid IgA-G antibody heavy chain described above, murine J chain, and rabbit SC. A series of sexual crosses was performed between these plants and filial recombinants to generate plants in which all four protein chains were expressed simultaneously (Fig. 3). In the final SIgA plant, the four recombinant proteins were assembled into a functional, high molecular weight secretory immunoglobulin extremely efficiently. A major difference in plants was that single cells were able to assemble the secretory antibodies unlike in mammals. This approach demonstrates not only the fidelity of plant assembly mechanisms compared with those in mammals in constructing the dimeric IgA molecule but also the overall flexibility of plant cells, and it indicates that they might possess the requisite mechanisms for assembly and expression of other complex recombinant protein molecules.

Other examples of the potential to cut and splice antibody domains at the gene level are the development of chimeric immunoconjugates, which could be produced in plants, or of antibodies modified to contain sites for convenient chemical cross-linking to chemotherapeutic drugs or diagnostic reagents. Considerable effort has been expended in the development of immunoconjugates, and a variety of toxins has been evaluated as partners with antibodies and antibody-derived binding domains. In addition

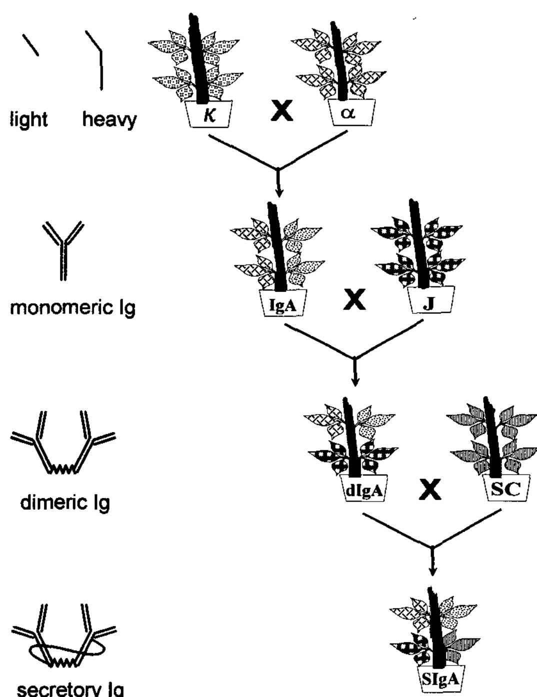

Figure 3. Assembly of SIgA in plants. The genes encoding the four polypeptides of SIgA are accumulated in a single plant by successive cross-fertilization of single transgenic plants and their filial recombinants. Molecular structure of the antibody molecules in each generation are depicted on the left. Ig, Immunoglobulin; dIgA, dimeric IgA.

to the microbial toxins, plants are themselves the source of a number of toxins, such as ricin, abrin, saporin, and trichosanthrin, all of which have been used as chemical or genetic fusion partners in immunoconjugate preparations. Because of their ability to assemble antibody-derived molecules efficiently, plants may provide an advantage in view of the relatively low efficiency of production for some recombinant immunotoxins in other expression systems, such as *E. coli* (Choe et al., 1994). This, and the ability to produce plant antibodies at nearly any scale, should now provide impetus to evaluate immunoconjugate production in plant systems.

Many other, smaller forms of engineered antibody molecules have been described in the literature and most of these have also been produced in plants. These include a single-domain antibody in tobacco (Benvenuto et al., 1991), single-chain Fv molecules (Owen et al., 1992), and Fab production in tobacco and Arabidopsis (De Neve et al., 1993). Compared with full-length antibody, these types of molecule have the advantage that requirements for assembly are thought to be less demanding. Since processing through the ER is not essential, the antibody fragments can be targeted intracellularly if required or, if secretion into the extracellular space is preferred, this can be achieved by the inclusion of a signal sequence. In either case, glycosylation does not occur because the normal antibody glycosylation site is absent.

IMMUNOTHERAPY

The immunotherapeutic potential of antibodies for humans has been appreciated for many years, and yet to date, only three antibodies are approved for clinical use by the U.S. Food and Drug Administration. Clearly, there are problems in developing therapeutic monoclonal antibodies, irrespective of the expression system used for their production. Transgenic plants may help to overcome some of these problems, especially with regard to the requirement for large quantities of antibody at low cost. Large amounts of monoclonal antibodies are required for in vivo passive immunization to administer sufficient amounts of antibody at the site of disease and to overcome the rapid rate of clearance from the body and because repeated treatments are usually required. It has been estimated that by expressing antibody in soybean at a level of 1% of total protein, 1 kg of antibody could be produced for approximately \$100 (Hiatt, 1990). This will certainly be further reduced as improved vectors and purification procedures are developed.

Plant antibodies may be of particular benefit in the area of topical immunotherapy. A number of groups (Lehner et al., 1985; Bessen and Fischetti, 1988; Ma et al., 1990) have demonstrated that topically applied antibodies can prevent colonization by pathogenic bacteria, as well as modify the resident bacterial flora in a highly specific manner. For example, in the case of dental caries, topically applied monoclonal antibody that was raised against a cell surface adhesin of *Streptococcus mutans* prevented infection by the bacteria and reduced the levels of disease in animal models and humans (Lehner et al., 1985; Ma et al., 1990). Although

the optimal dose of monoclonal antibody was not determined, to overcome the dilution effect of saliva in the human trial, approximately 2.5 mg of purified antibody was applied on six occasions per individual, which indicates the importance of a source of large quantities of antibody.

SIgA is the predominant form of antibody in the oral cavity and at all mucosal sites in humans, and it is predicted that SIgA would be more effective than IgG class monoclonal antibodies at these sites, by virtue of increased avidity of antigen binding, as well as the increased resistance to proteolysis. The normal protective role of SIgA in secretions has largely been ignored for many years, despite the fact that the majority of infectious agents are initially encountered at mucosal sites. This has partly been due to the difficulty in inducing high titer and long-lasting SIgA responses by active immunization (Russell and Wu, 1991). An alternative is passive immunization with monoclonal SIgA, whereby high concentrations of specific antibody could be administered regularly without the need to induce either mucosal or systemic immune responses. Until now, insufficient quantities of SIgA were available, because assembly of the antibody molecule could only be achieved with some difficulty (Winner et al., 1991), but with transgenic plants this approach can now be contemplated. Because of the increased stability of SIgA, treatment of all mucosal sites, including the gastrointestinal tract, can be envisioned. The use of edible plants or of targeting antibody expression to seeds, tubers, or fruit for oral delivery is also a compelling possibility. Antibody delivery through foodstuffs has been successful in the oral cavities of rats (Michalek et al., 1987; Hamada et al., 1991), and the availability of functional antibody in palatable plant preparations could reduce or possibly eliminate the need for purification of the plant antibody prior to treatment.

Immunotherapy can also be aimed at the plants themselves. To this end, antibodies or antibody fragments can be targeted to the cytoplasm of plant cells, chloroplasts, cell membranes, or the apoplastic space, and these recombinant antibody molecules can be effective in insect or disease resistance. Tavladoraki et al. (1993) reported the cytoplasmic expression of a single-chain Fv in transgenic tobacco that protected against infection by artichoke mottled crinkle virus. Transgenic plants had significantly lower levels of virus accumulation, a reduced incidence of infection (approximately 50–60%), and a marked delay in the appearance of symptoms compared with control plants. Voss et al. (1994) also reported the production of transgenic tobacco expressing a full-length antibody specific for tobacco mosaic virus. These plants showed a reduction in their susceptibility to viral infection, and the reduction in symptoms was correlated with the amount of antibody produced in individual transgenic plants. It is important to remember that antibody-mediated protective mechanisms in plants are limited to those that rely directly on the binding to the antigen and no secondary effector functions can be elicited. Thus, the epitope specificity of the antibody is critical. Furthermore, the antibody fragments that have been expressed in these studies bind antigen monovalently and, as discussed earlier, the reduced binding energy of these types of molecules as compared with full-length antibodies may be an important consideration for future studies.

Intracellular expression of antibody molecules can also be used to modulate the metabolism of the expressing cell (Tang et al., 1991). In plants it may be possible to alter plant metabolism by expressing appropriate catalytic antibodies or enzyme-/hormone-inactivating antibodies. A good target would be the plant hormones that control growth and development. This approach has been successfully carried out by Owen et al. (1992), who reported the production of transgenic tobacco plants transformed with gene sequences encoding an anti-phytochrome single-chain Fv antibody. Phytochrome is a family of cytoplasmic proteins that act as photoreceptors and are involved in the photocontrol of tobacco seed germination. Seeds from the transgenic plant line expressing antibody were reported to show aberrant light-mediated germination behavior, because levels of germination were reduced by approximately 40%.

REGULATORY ISSUES

Although the potential use of plants to manufacture functional antibodies in bulk was first demonstrated more than 6 years ago, plant antibody development has proceeded at what can only be described as a slow pace, perhaps because the recombinant antibody industry was built on a foundation of large-scale cellular fermentation technology rather than an agricultural base. Given the absence of plant-derived monoclonal antibodies from clinical trials for human or animal application, none of the issues related specifically to plant-derived antibodies have been formally addressed at the regulatory level and therefore few have been dealt with rigorously at the experimental level.

However, plant-derived antibodies targeted for clinical uses are expected to meet the minimum standards of performance and safety of antibodies derived from other nonmammalian systems. An advantage is that plant-derived antibodies are not exposed to, or generated in, the presence of animal agents, products, or adventitious organisms. Because the protein backbones, processing and assembly, are known to be preserved with high fidelity in plants, in all likelihood, plant antibody evaluation will be focused in the direction of equivalency of function as compared to the parental mammalian antibody source from which the genes are derived.

Other concerns may be directed toward the glycosylation pattern, the one area of divergence shown to exist among mammalian, bacterial, yeast, and plant systems (Sturm et al., 1987; Faye et al., 1989). This is an important issue for all production systems and not just for plants. Some carbohydrate moieties may be unique to plants and the exposure to these carbohydrates, although unlikely to be novel for humans, given the daily exposure to plant glycoproteins in food, personal care products, and pollen, needs to be considered. Thus, in the context of large quantities of antibodies required for some therapies, sensitization to plant antigens may be an issue of concern for some future applications. Some plant secondary metabolites may also

pose a potential concern, since they possess recognized biological functions. Most of these molecules would not be co-purified with the recombinant proteins from plants but may require monitoring. The choice of plant material for the source of antibodies is relevant to these issues, and precedents for other plant-derived medicinal compounds would therefore be useful in guiding the evaluation of plant antibodies for human or veterinary uses.

SUMMARY

The original report of Hiatt (1989) initiated a wave of excitement at the realization that a complex mammalian protein such as immunoglobulin could be assembled within a plant cell. The general reaction was one of amazement, but interest in exploiting the possibilities arising from the discovery, for example to make antibodies of therapeutic value, has taken a considerable time to develop. In the meantime, other recombinant expression systems and traditional cell culture techniques have advanced and overcome some of their problems, particularly those associated with yields. Plants, however, still offer unique advantages, especially in their ability to match the protein assembly capabilities of mammalian cells (as demonstrated by the assembly of SIgA molecules), as well as to provide antibodies in bulk at low cost. In addition, the area of "immunization" of plants holds great promise and will surely be a field of enormous growth for the future.

Received May 19, 1995; accepted June 12, 1995.
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