

Review

Plant biopharming of monoclonal antibodies

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Available online 19 April 2005

Abstract

Recent advances in molecular biology and plant biotechnology have shifted the concept of growing crops as a food source to serving as a bioreactor for the production of therapeutic recombinant proteins. Plants are potential biopharming factories because they are capable of producing unlimited numbers and amounts of recombinant proteins safely and inexpensively. In the last two decades, plant production systems have been developed for monoclonal antibody production, which has been useful in passive immunization of viral or bacterial diseases. Recently, a recombinant monoclonal antibody for rabies prophylaxis was produced in transgenic plants. Rabies virus epidemics remain still problematic throughout the world, and adequate treatment has been hampered by the worldwide shortage and high cost of prophylactic antibodies such as HRIG. Successful mass production of this monoclonal antibody in plants might help to overcome these problems. An effective plant production system for recombinant biologicals requires the appropriate heterologous plant expression system, the optimal combination of gene expression regulatory elements, control of post-translational processing of recombinant products, and efficient purification methods for product recovery. This review discusses recent biotechnology developments for plant-derived monoclonal antibodies and discusses these products as a promising approach to rabies prophylaxis and the consequence for global health benefits.

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Keywords: Rabies; Monoclonal antibody; Biopharming; Glycosylation

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1. Introduction

Appropriate administration of rabies immunoglobulin (RIG) is essential and critical for human rabies post-exposure prophylaxis (PEP) in situations of severe exposure to rabies virus. However, the dramatic worldwide shortage of

these immunoglobulins derived from immunized horses and humans, as well as the risk of adverse reactions associated with equine anti-rabies immunoglobulin (ERIG) and the high cost of human anti-rabies immunoglobulin (HRIG), have hampered efforts to reduce the number of human deaths from rabies exposure. Recent advances in molecular immunology and plant biotechnology have led to the possibility for large-scale production of recombinant monoclonal antibody (mAb) in plants as a safe and inex-

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pensive alternative to mammalian-derived RIG (Ko et al., 2003).

Plants have been genetically modified to produce valuable proteins, showing enormous bioreactor potential for expressing and assembling mAbs for human and animal disease therapy (Daniell et al., 2001; Ma et al., 2003). Plants have several advantages, which include the lack of animal pathogenic contaminants, low cost of production, and ease of agricultural scale-up compared to other currently available conventional production systems. We recently reported the first plant-derived full-size recombinant mAb for the PEP against rabies (Ko et al., 2003), suggesting the use of plants as an economically feasible production system of anti-rabies virus mAb. In light of the clear consequential treatment failure when immunoglobulin is omitted from post-exposure therapy (PET) (Wilde et al., 1999), countries with chronic shortages of HRIG and ERIG would benefit greatly from plant bioreactors to produce anti-rabies mAbs easily, safely, and economically.

This review discusses recent advances in plant expression technology and issues that are developing for their application to mAb production. Advantages and potential bottlenecks in the therapeutic application of plant-derived products are considered.

2. Transgenic plants: expression of plant-derived recombinant mAb

Two general methods that are considered to be state-of-the-art to introduce transgenes encoding a suitable antibody into plants are *Agrobacterium*-mediated transformation and particle bombardment. *Agrobacterium*-mediated transformation is used to transfer foreign genes into the genome in the plant nucleus where individual genes encoding the respective heavy and light chains are co-expressed to produce full-size mAb by sequential crossing of the transgenics (Hiatt et al., 1989; Khoudi et al., 1999; Ma et al., 1995). A single plant binary vector carrying genes encoding heavy and light chains under two different promoters has been used to express both genes and to assemble functional full-size anti-rabies virus-specific monoclonal antibody (Ko et al., 2003). The particle bombardment approach can serve to insert genes into the genome of the plant nucleus or the plastid genome, depending on the vector construct (Daniell, 2002a). Chloroplast transgenic plants have been obtained for stable expression of mAb in the chloroplast genome (Daniell, 2002a). Chloroplasts can process foreign proteins with disulfide bridges, a function required for proper folding of proteins. The chloroplast transgenic system is suitable for scFv antibody (Daniell, 2002a), but not for the full-size mAb since chloroplasts lack the glycosylation processing machinery required for the proper assembly and functionality of the antibody.

An alternative strategy for expression of transgenes in plants is transient transformation. Agroinfiltration (Vaquero et al., 2002) or plant viral vector systems (Scholthof et al.,

1996; Verch et al., 1998) can be used for the transient expression of both heavy and light chain genes and the assembly of full-size mAb in tobacco plants. Agroinfiltration is generally applied to test the activity and efficiency of expression constructs within a few days before plants undergo stable transformation (transgenic plants) for large-scale production. Plant virus expression systems are potentially more efficient than the establishment of transgenic plants since viral infections are rapid and systemic, resulting in high yields of virus and viral gene products. However, virus expression systems require virus transcript inoculation due to the temporary nature of gene expression and are often associated with very high mutation and deletion rates that can affect the foreign gene during plant RNA virus replication (Smith et al., 1997). In contrast, transgenic plants provide stable gene insertion and easy propagation through in vitro tissue culture or generation of seedlings (Koprowski and Yusibov, 2001). The stable expression and standardized production process for mAb production are essentially known in agricultural plant cultivation as “biopharming.” Stable expression implies stable transformation, where transgenes encoding multimeric antibody domains are permanently integrated into the plant genome and expressed over generations. Despite the disadvantages and advantages outlined above, the transformation methods of choice must take into account the host species and the transgenes of interest. *Agrobacterium*-mediated transformation is a simple method for most dicotyledon species, whereas particle bombardment is a suitable method for some cereals (Ma et al., 2003). Because *Agrobacterium*-mediated transformation has the advantage of producing plants with low copy numbers and stable expression over generations, much effort has been applied to improve *Agrobacterium*-mediated transformation for monocotyledon species (rice, wheat, maize, barley, and sorghum) (Cheng et al., 2004). Many factors influencing transformation of monocotyledonous plants such as plant genotype, explant type, *Agrobacterium* strain, and co-cultivation conditions have been investigated (Cheng et al., 2004).

Various non-plant expression systems exist for monoclonal antibody production, such as bacteria, yeast, insect, and mammalian cell cultures, and transgenic animals (Table 1). Traditional production systems, such as mammalian cell cultures, have been used to produce antibodies; however, the demand for therapeutic and diagnostic mAbs has increased to such an extent that these reagents can be produced only by using often costly production systems. Compared with those systems, plant expression systems have several advantages, such as well-established cultivation and down-processing of plant products, easily scaled-up industrial production levels, low health risks from human pathogen and toxin contamination, and efficient post-translational glycosylation modifications of mAb components (Bakker et al., 2001; Ko et al., 2003; Lerouge et al., 2000; Ma et al., 1998). Thus, plant expression “biopharming” systems represent a cheaper and safer alternative method for antibody production, which is especially relevant in developing countries

Table 1
Full-size monoclonal antibodies recently produced in transgenic plants

Plant	Antibody type (target)	Purpose	References
Tobacco	IgG (low molecular weight phosphonate ester)	Catalytic antibodies	Hiatt et al. (1989)
Tobacco	IgG (nematode)	Plant pathogen resistance	Baum et al. (1996)
Tobacco	sIgA/G (<i>Streptococcus mutans</i>)	Therapeutic (topical)	Ma et al. (1998)
Soybean, rice	IgG (herpes simplex virus)	Therapeutic (topical)	Zeitlin et al. (1998)
Tobacco	IgG (colon cancer)	Therapeutic (systemic injection)	Verch et al. (1998); Ko et al. (2004)
Alfalfa	IgG (human IgG)	Diagnostic	Khoudi et al. (1999)
Tobacco	IgG (rabies virus)	Therapeutic: first IgG expressed in plant showing therapeutic activity (systemic injection)	Ko et al. (2003)
Tobacco	IgG (hepatitis B virus)	Immunopurification of hepatitis B surface antigen	Valdes et al. (2003)
Tobacco	IgG (hepatitis B virus)	Therapeutic	Yano et al. (2004)

where sufficient resources to produce and distribute antibody for rabies post-exposure treatment are limited. The low cost of production, rapid scale-up, simple distribution by seeds, and ease of handling are key issues in suggesting this plant-derived anti-rabies virus mAb as a promising candidate for PET. Indeed, the choice of expression system depends mainly on the fidelity and production cost of the antibody molecule.

3. Forms of recombinant antibodies expressed in plants

Many different antibody forms have been expressed successfully in plants (Fig. 1). These include full-size antibodies Table 1, large single-chain antibodies (Mayfield et al., 2003), camelid heavy-chain antibodies (Jobling et al., 2003), Fab fragments (Peeters et al., 2001), scFvs (Conrad and Fiedler, 1998), bispecific Fvs (Fischer et al., 1999), diabodies (Kathuria et al., 2002), and minibodies (Hudson and Souriau, 2003). The choice among the forms depends on the requirement for therapy or diagnosis of disease. For instance, the Fc domain of the full-size or large single-chain antibody is required for effector function (Burton, 2002; Jefferis et al., 1998). When the form of antibodies is determined, several choices can be made regarding expression systems, transformation methods, and subcellular targeting. For proper function in therapy, diagnosis, or prevention of disease, antibodies must be properly folded and assembled. The whole process of functional antibody formation requires mainly disulfide bond formation (Schouten et al., 2002) and glycosylation (Rudd et al., 2001). In plants, the endoplasmic reticulum (ER) is an important site of the major biofunctions of synthesis, assembly, and glycosylation of proteins (Helenius and Aebi, 2001). Full-size antibodies, large single-chain antibodies, and camelid heavy-chain antibodies, which contain an Fc region carrying glycosylation sites, should be targeted to the ER for glycosylation, disulfide bonding and proper assembly. Structures with disulfide bonds such as minibodies and Fab fragments, which do not contain glycosylation sites, can be targeted to subcellular compartments where only disulfide bonding occurs. Bispecific scFv, diabodies, and minibodies

do not require targeting to the ER or chloroplast, since they do not contain glycosylation and disulfide bond sites. Some light chains have glycosylation sites, which affect antibody binding activity (Tachibana et al., 1997). In that case, ER subcellular targeting should be considered. Despite the glycosylation or disulfide bond requirement, antibodies are often targeted to subcellular compartments or the apoplastic space since most proteins are more stable in the subcellular compartment than in the cytosol (Conrad and Fiedler, 1998). Highest accumulation of full-size antibodies has been obtained by targeting to the apoplastic space and high-level accumulation of scFv antibodies was obtained when the antibodies were retained in ER (Conrad and Fiedler, 1998; Fischer et al., 1999). Chloroplasts are also able to process correct folding and formation of disulfide bonds, with a several hundred-fold accumulation of recombinant proteins (Ruf et al., 2001). Targeted transgene integration into the chloroplast genomes has some advantages including no “position effect” (Daniell, 2002b), no gene silencing (Daniell and Dhingra, 2002), high expression/accumulation (Staub et al., 2000), and minimized environmental concerns (Daniell, 2002b). Chloroplasts are appropriate subcellular compartments only for certain antibodies that do not require glycosylation.

4. Optimization of plant-derived mAb in transgenic plants

The transgenic plant expression system provides the advantage of low overall cost only when the product per unit of plant biomass is high (Table 2). There are two important key steps in achieving high yields: (1) introduction of the gene encoding antibody into the plant expression system and (2) isolation of the antibody product. For the first step, several factors related to expression levels should be considered, including insertion and transcription of transgenes, translation of mRNA to protein, and post-translational events. These factors can be optimized to enhance protein expression by the inclusion of appropriate combination of gene regulatory elements, i.e. promoters and polyadenylation sites, in the plant expression vector. The cauliflower mosaic virus

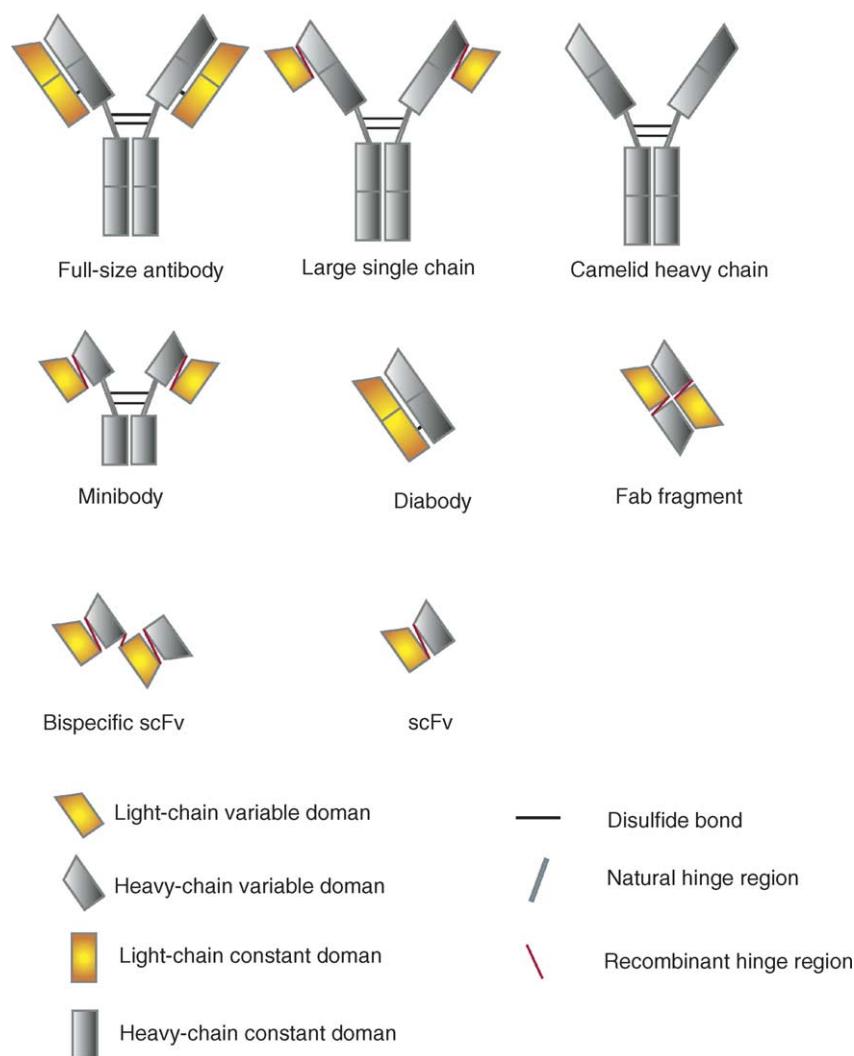


Fig. 1. Different forms of antibodies expressed in transgenic plants.

35S (CaMV35S) promoter has been used to enhance the strong constitutive expression of antibodies. The CaMV35S promoter is often used in dicotyledons, while the maize ubiquitin-1 promoter is preferable in monocotyledons (Christensen and Quail, 1996; Hiatt et al., 1989; Ma et al., 1995). For assembly of a full-size antibody, two promoters are required for the expression of both light and heavy-chain genes. Expression of different promoters avoids

homology-based silencing by the same promoter (De Neve et al., 1999; De Wilde et al., 2000). The combination of the potato proteinase inhibitor II (*pin2*) promoter used to control the light chain gene and the 35S promoter controlling the heavy-chain gene resulted in expression of both genes and assembly of both protein domains in a functional anti-rabies monoclonal antibody in transgenic tobacco (Ko et al., 2003). Tissue-specific or inducible promoters can also provide

Table 2

Comparison of various expression systems for recombinant pharmaceutical proteins (combined from Schillberg et al., 2003; Ma et al., 2003)

Features	Bacteria	Yeasts	Plant cell cultures	Transgenic plants	Mammalian cell cultures	Transgenic animals
Production cost	Low	Medium	Medium	Low	High	High
Timescale for production	Short	Medium	Medium	Short	Long	Very long
Propagation	Easy	Easy	Easy	Very easy	Medium	Medium
Distribution	Difficult	Difficult	Difficult	Easy	Very difficult	Easy
Quality	Low	Medium	High	High	High	High
Safety	Low	High	High	High	Low	Low
Glycosylation	Incorrect ^a	Incorrect	Differences	Differences	Minor differences	Minor differences
Ease of glycosylation modification	Very difficult	Medium	Easy	Easy	Difficult	Very difficult

^a See references, Benz and Schmidt (2002) and Szymanski et al. (2003).

advantages, such as preventing adverse effects on the growth (Stoger et al., 2000) and development of the plant or the environment (Cramer et al., 1999).

Transgene expression is affected by the translation rate (Datla et al., 1993; Ko et al., 2000). The untranslated leader sequence of alfalfa mosaic virus mRNA 4 increases transgene expression several-fold due to enhanced translation efficiency of transcripts (Datla et al., 1993). The translation rate can also be maximized by codon usage in some transgenes for protein synthesis and removal of undesired sequences (Kozziel et al., 1996). Antibody folding, assembly, and post-translational modifications affecting stability as well as accumulation also strongly depend on the subcellular compartment of plant cell chosen for expression (Conrad and Fiedler, 1998). Generally, the secretory pathway-targeted antibody is more stable and accumulates to higher levels than the cytosol-localized antibody (Schillberg et al., 1999). Antibody fused to the N-terminal signal peptide is targeted to the default secretory pathway. The ER is the site of assembly, folding, as well as glycosylation, which is essential for the functionality of full-size antibodies (Helenius and Aebi, 2001; Rudd et al., 2001). ER has an oxidizing environment and few proteases, leading to greater stability of antibodies as compared to the apoplastic space or cytosol (Conrad and Fiedler, 1998). When attached to the C-terminus, the ER retention signal H/ KDEL retains the antibody in the ER (Ko et al., 2003; Sharp and Doran, 2001; Tekoah et al., 2004). Such retention of proteins usually increases the production level compared to that without KDEL in transgenic plants (Conrad and Fiedler, 1998; Sharp and Doran, 2001).

5. Downstream processing

Among the factors affecting the production cost of plant biopharming, purification of the plant-produced mAb is the most significant (Evangelista et al., 1998). Thus, the commercial viability of biopharming depends on efficient purification procedures. Immunoprecipitation and chromatography represent two general purification methods. For mAb, affinity purification on protein G or protein A is the most useful method. Recently large-scale purification has been developed using a recombinant protein A streamline chromatography (Valdes et al., 2003). Despite the high level of expression in the targeted tissue, the concentration of mAb vary with the age of the plant (Valdes et al., 2003). Thus, the time of harvest must also be considered since it affects the purification starting volume of leaves carrying antibody. Plant cultivation-to-purification processing procedures should be standardized to ensure the same final product for therapeutic or diagnostic purposes. Plant-derived recombinant vaccines can be administered orally by raw or partially processed fruits and vegetables, and topically applied mAb requires only partial purification (Ma et al., 2003). For rabies mAb, purity is an important issue since the antibody is systemically administered for rabies post-exposure prophylaxis (Ko et al.,

2003; Koprowski and Black, 1952). In biopharming, utilizing leafy plants such as tobacco to produce mAbs has several advantages over other plants. These include highly efficient transformation and regeneration, a relatively short period for mass production of seeds, high leaf biomass yield, and easy processing for protein purification. However, tobacco plants contain S-(–)-nicotine as the predominant alkaloid which is highly addictive (Birtwistle and Hall, 1996; Rincon et al., 1999; Uematsu et al., 2001) and could hinder the potential of plant biopharming. We recently modified immunoprecipitation and column affinity purification methods and confirmed by gas chromatography and mass spectrometry at a detection of 5 pg that the nicotine is absent in the purified antibody produced from tobacco leaves (Ko et al., 2004). Antibody which can also be expressed in seed and fused with oilbodies (oleosin-fusion technology) facilitates purification (Kuhnel et al., 2003). To avoid disruption of plant cells, root-secreted mAb (rhizosecretion) can be applied without extracting antibody from the plant (Drake et al., 2003; Gaume et al., 2003). Full-size antibodies with a signal sequence targeting the protein to the ER are secreted into the apoplastic space (Hiatt et al., 1989). scFvs and full-size mAbs with a signal sequence in transgenic *Nicotiana* can be naturally rhizosecreted (Drake et al., 2003; Firek et al., 1993; Magnuson et al., 1996), indicating these antibodies cross the cell wall despite the fact that their molecular size exceeds that of the plant cell pores (Carpita et al., 1979). The rhizosecretion method using root cultures is a viable alternative to agricultural production or cell culture for the generation of mAb in transgenic plants.

6. Post-translational modification for authenticity of plant-derived mAb

Most therapeutic proteins are glycoproteins, and *N*-glycosylation is often crucial for their stability, folding, and biological activity (Lerouge et al., 2000). Despite the advantage of plant cell glycosylation machinery over bacterial systems with respect to glycoprotein expression, glycan structures generated by plants and animals differ (Cabanès-Macheteau et al., 1999; Tekoah et al., 2004) (Fig. 2). Plant glycans contain β (1,2)-xylose residues and α (1,3)-fucose residues linked to the proximal *N*-acetylglucosamine, which are absent in mammals (Cabanès-Macheteau et al., 1999) (Fig. 2). The altered glycan structure in plants can potentially change the activity or longevity of antibodies compared with their mammalian-derived counterparts (Ko et al., 2003; Rudd et al., 2001; Wright and Morrison, 1997). The potential allergic responses against plant-specific glycans might hamper plant production of mAb, even though these glycan residues are present in every normal plant glycoproteins found in the human diet. Some mAbs are directly administered into the bloodstream, which can be more sensitive than the mucosal or other routes to the immune response. As a result, these foreign glycan structures represent one of the most important issues affecting the use and acceptance of plant-

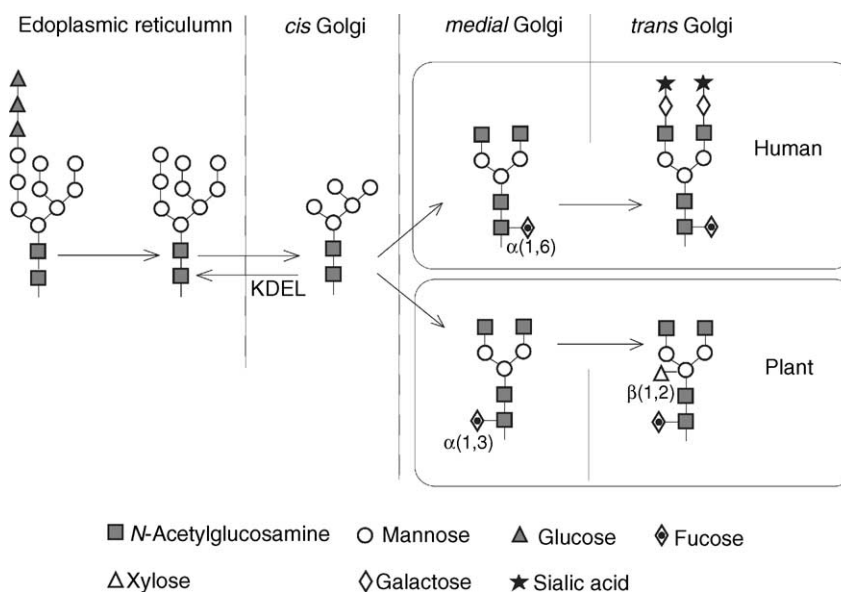


Fig. 2. Schematic representation of the glycosylation pathway of glycoproteins in plants and humans. In ER, glycan structure is attached to Asn-X-Ser/Thr sequence in glycoproteins and three glucoses are removed from the attached glycan. The glycoproteins then move to the Golgi apparatus where mannoses are trimmed and the sugar residues are sequentially added (Wright and Morrison, 1997). The glycosylation process that takes place in the ER and *cis* Golgi is conserved between plants and humans, whereas the medial and *trans* Golgi-generated glycosylation processing is highly diverse. When the process stops at *cis* Golgi, glycoproteins with oligomannose type *N*-glycans are yielded. When the KDEL sequence is attached to the C-terminus of glycoproteins, it allows glycoproteins to be retained in or returned to the ER. Plant glycans contain $\beta(1,2)$ -xylose residues and $\alpha(1,3)$ -fucose residues linked to the proximal *N*-acetylglucosamine (Cabanes-Macheteau et al., 1999) whereas human glycans contain $\alpha(1,6)$ -fucose, galactose, and sialic acid (Helenius and Aebi, 2001).

derived mAb. Strategies to avoid these concerns include the humanization of plant *N*-glycans by expression of human $\beta(1,4)$ -galactosyltransferase and sialyltransferase in transgenic plants (Bakker et al., 2001), as well as the generation of oligomannose glycan structure by retaining the recombinant antibody in the ER to avoid plant-derived specific glycan structures (Ko et al., 2003). In the latter strategy, a KDEL sequence is fused to the heavy chain to retain the mAb in ER where only mannose attachment occurs (Ko et al., 2003; Tekoah et al., 2004) (Fig. 2). We developed this modification of glycosylation on antibody using KDEL, especially for oligomannose type mAb for use in rabies post-exposure prophylaxis. Whereas the oligomannose-type rabies mAbs have a shorter half-life than the mammalian-derived product, the immunological protection against rabies is not adversely affected (Ko et al., 2003). Instead, a shorter half-life may offer a certain advantage to current commercial antibody-vaccine prophylaxis because of a lower probability of interference between passive and active immunity. The modified glycosylation does not affect the activity of the mAb, suggesting the promise of glycosylation modification using plant expression systems.

7. Conclusion

With currently available technologies, monoclonal antibody production that is achieved using bacterial, mam-

malian, yeast, or insect, or insect cell culture methods, as a replacement for those produced in humans and animals, is often not sufficient for use in passive immunological treatment of infectious diseases and cancers. There is a particularly severe shortage of rabies-specific antibodies worldwide (Wilde et al., 1999). Production of inexpensive and safe plant-derived mAb holds the promise that sufficient mAb can be produced for effective rabies PEP in developing countries where the antibody is most urgently needed. Feasible distribution of plants producing mAbs to the regions with urgent demand for disease treatment, and easy purification and administration of plant-derived mAb to patients are now realistic goals. In light of their advantages with respect to productivity, safety, cost, and timelines, plants represent a viable alternative to mammalian and prokaryotic expression systems. Easy scale-up of production is a major advantage of transgenic plant systems, but the full advantage of plant systems will be realized when techniques are developed to increase yields in expression systems and to produce and purify safe and functional mAb with authentic glycan structures.

Acknowledgements

This work was supported by the United States Department of Agriculture.

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