Production of Antibodies in Plants: Approaches and Perspectives

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Contents

Abstract Advances in molecular biology, immunology, and plant biotechnology have changed the paradigm of plant as a food source to so-called plant bioreactor to produce valuable recombinant proteins. These include therapeutic or diagnostic monoclonal antibodies, vaccines, and other biopharmaceutical proteins. The plant as a bioreactor for the production of therapeutic proteins has 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 systems. Thus, plants are considered to be a potential alternative to compete with other systems such as bacteria, yeast, or insect and mammalian cell culture. Plant production systems, particularly therapeutic antibodies, are very attractive to pharmaceutical companies to produce the antibodies in demand. Currently, we have successfully

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developed a plant system for production of anti-rabies monoclonal antibody and anti-colorectal cancer monoclonal antibody. The effective plant production system for recombinant antibodies requires the appropriate plant expression machinery with optimal combination of transgene expression regulatory elements, control of posttranslational protein processing, and efficient purification methods for product recovery. However, there are several limitations that have to be resolved to establish the efficient plant system for antibody production. Here, we discuss the approaches and perspectives in plant systems to produce monoclonal antibody.

Introduction

Over the 30 years since Kohler and Milstein (1975) reported production of monoclonal antibody (mAb) using hybridoma cell line, many mAbs have been produced in diverse expression systems (Chadd and Chamow 2001; Ma et al. 2003; Ko and Koprowski 2005). Antibodies have been developed mainly for tumor therapy (Harris 2004), but it is expanding to other diseases such as pandemic infectious diseases and bioterrorism agents (Casadevall et al. 2004). Antibody itself has a number of advantages such as low toxicity and high specificity, and it can be structurally modified to different forms to be feasible for diverse approaches. The antibodies can be directly armed with radionuclides, toxins, or cytokine for control of tumor or infected cells. In 2001, the antibody market value was US $2 billion. The monoclonal antibody market was one of the fastest growing therapeutic protein areas between 2003 and 2004 (Gomord et al. 2004). The antibody market value was nearly $5 billion in 2005. In 2010, the market will be expanded to more than $30 billion. Antibodies are produced in hybridoma systems or other mammalian culture systems. However, the limited capacity of these systems and their high production cost hamper the attempts to meet such increasing demand of antibodies. Thus, many companies are interested in alternative economically feasible production approaches. Advanced immunology and bioengineering led us to produce monoclonal antibody in variable expression systems, such as *Escherichia coli*, yeast, insect, and mammal cells (Chadd and Chamow 2001). Since expression of antibody in transgenic plants was first described by (Duering 1988; Hiatt et al. 1989), different antibodies and their derivatives have been expressed in plant systems (Fig. 1). The use of transgenic plants for the production of monoclonal antibody has many potential economic and safety advantages (Ma et al. 2003;

Fig. 1 (continued) Mayfield et al. 2003). The different forms of antibodies can be chosen to express in plant on intended antibody-based applications such as therapy and diagnosis. When ideal properties for applications is mainly high affinity for the targeted antigen such as certain molecules to detect, pathogens to inhibit infection, or toxins to neutralize by physically blocking ligand-receptor interactions, antibody derivatives without Fc regions are desirable. In other words, when the antibody-dependent cellular cytotoxicity (ADCC) where the Fc region of the antibody is essential, a full-size or large Fc region is chosen

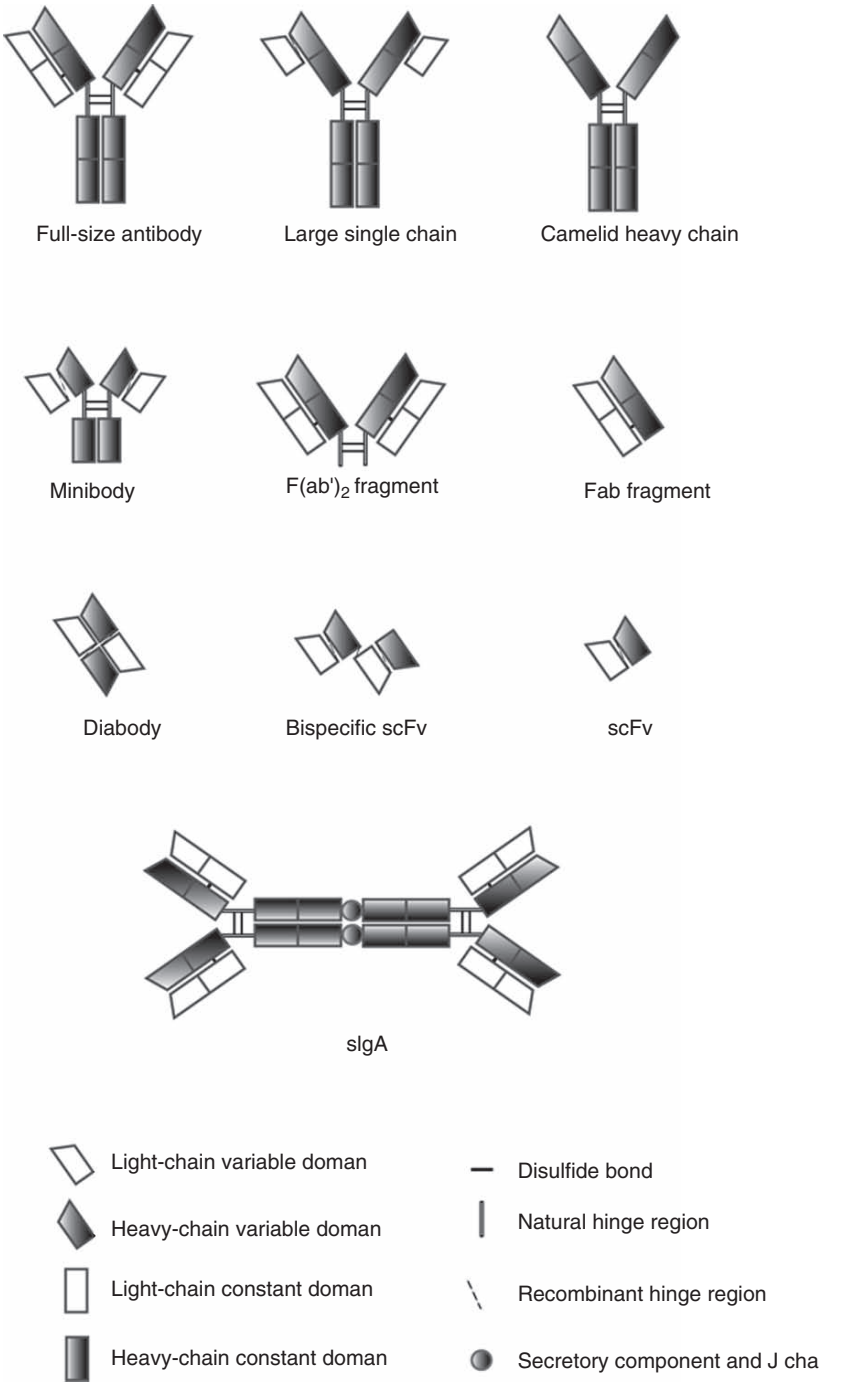

Fig. 1 Various forms of recombinant antibodies expressed in plants. Many forms of antibodies have been expressed in plant systems, including full-size antibody (Hiatt et al. 1989) and its derivatives (Conrad and Fiedler 1998; Fischer et al. 1999; Peeters et al. 2001; Jobling et al. 2003;

Gomord et al. 2004; Ko and Koprowski 2005), including large scale-up, the ease to manipulation, and lack of human pathogenic contaminants. There is no doubt about the capability of the plant expression system for production of antibody since plants are advanced eukaryotic organisms that are able to perform posttranslational modifications. Plant cells correctly assemble and fold antibodies with disulfide bridges and *N*-glycosylation similar to the parental antibody produced in mammalian cell. Although the plant system has economic and safety advantages over other systems, there are several obstacles such as plant-specific *N*-glycosylation, purification costs, environmental impact, and public acceptance of plant-made therapeutic proteins. To obtain full advantage of the plant expression system, it is essential to understand the limitations of current approaches to using plant systems for antibodies and developing novel technology to overcome the remaining hurdles. This chapter discusses current approaches and perspectives on plant systems to produce antibodies to resolve these problems and shows the potential for the use of plants as bioreactors.

Antibody and Its Therapeutic Activity

IgG antibodies are large glycoprotein molecules composed of four polypeptides: two heavy chains and two light chains assembled by disulfide bonds and attached with *N*-glycans (Fig. 2). The heavy and light chains are composed of the variable and constant regions. The amino-terminal domain of light and heavy chains is variable in sequence and therefore termed V_L and V_H, whereas the domain with the constant sequence of each chain is termed C_L and C_H. The variable and constant regions of antibody have two distinctive functions: one is to bind foreign agents

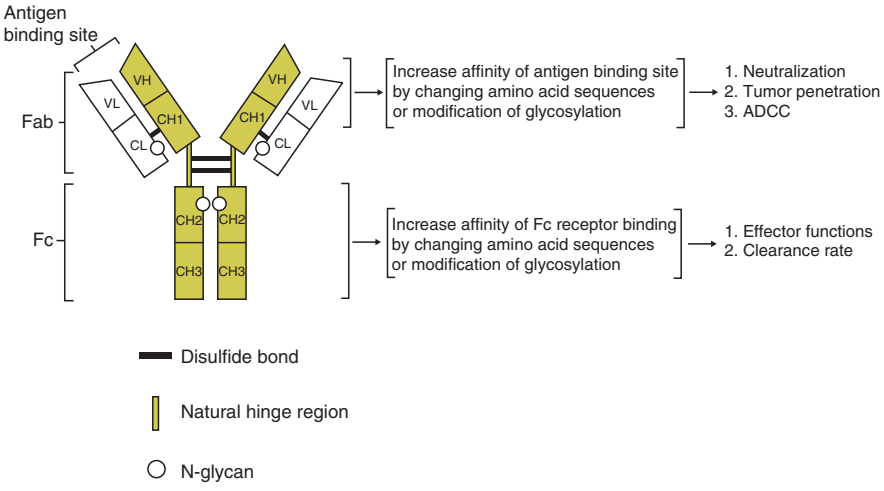

Fig. 2 Antibody structure and its tailor-made functional components

specifically and the other is to recruit various cells and molecules to destroy the foreign agents and pathogens as the antibody binds them. These biological activities of the antibodies allow a wide range of potential applications for immunotherapy and diagnosis.

Antibody-Based Therapies for Cancer

Antibodies have Fab and Fc regions with a specific ability to recognize tumor antigens highly expressed by tumor cells and to recruit immunological effector cells such as natural killer cells and macrophages to disrupt tumor cells, respectively (Herlyn et al. 1980; Houghton and Scheinberg 2000). Thus, antibody therapy is considered a major protein therapeutic for cancer. The mechanisms of this destruction are mainly antibody-dependent cellular cytotoxicity (ADCC), by which immune cells are recruited to kill target tumor cells and complement-dependent cytotoxicity (CDC) (Fig. 3). In some cases, mAbs bound to tumor cells may generate transmembrane signals that directly alter or control tumor growth, potentially leading to growth arrest and apoptosis (Vietta and Uhr 1994; Vuist et al. 1994). Unlabeled antibodies with no attachment of any drug or radioactive material show significant efficacy in treatment of breast cancer, colorectal cancer, non-Hodgkin's

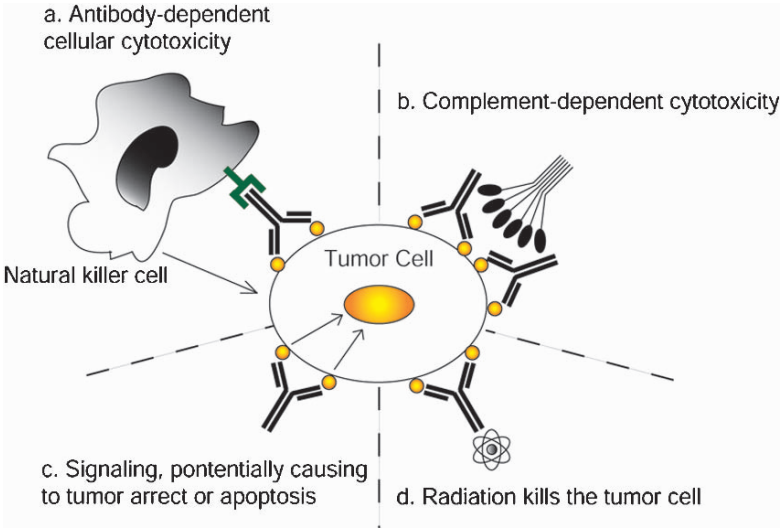

Fig. 3 **a-b** Mechanisms of anti-tumor activity by antibodies. Monoclonal antibodies recognize antigens on the tumor cells. **a**: Monoclonal antibody binds to Fc receptors on the effector cells, recruiting the effector cells to destroy tumor cells. **b**: Monoclonal antibody bound to antigen activates complement deposition, leading to the lysis of tumor cells. **c**: Monoclonal antibody bound to tumor cells generates transmembrane signals directly altering or controlling tumor growth. **d**: Monoclonal antibody can be armed with a radioisotope, other chemical drugs, or toxins to deliver their cytotoxic agents

Table 1 Antibodies with approval for treatment of cancer. (Modified from Carter 2001; von Mehren et al. 2003; Harris 2004; Gomord et al. 2004)

Antibody name/type	Tumor types	Antigen category/name	Year approved
Edrecolomab/muIgG2a	Dukes C CRC	Growth factor receptors/ EpCAM (GA733–2)	1995
Rituximab/chIgG$_1$	Lymphocytic leukemia	Hematopoietic/CD20	1997
Trastuzumab/huIgG$_1$	Breast cancer	Growth factor receptors/ HER2/Neu	1998
Gemtuzumab ozogamicin/humIgG$_4$	Acute myelocytic leukemia (AML)	Hematopoietic/CD33	2000
Alemtuzumab/huIgG$_1$	Chronic lymphocytic leukemia (CLL)	Hematopoietic/CD52	2001
Ibritumomab tiuxetan/ muIgG$_1$	Non-Hodgkin lymphoma	Hematopoietic/CD20	2002
Tositumomab/muIgG2a	Non-Hodgkin lymphoma	Hematopoietic/CD20	2003
Cetuximab/chIgG$_1$	Colorectal cancer	Growth factor receptors/ HER1	2004
Bevacizumab/humIgG$_1$	Colorectal cancer	Angiogenesis and stromal antigen/VEGF	2004

Table 2 Human infectious diseases which antibodies have been developed to control. (From Ma et al. 1995; Ko Ko et al. 2003; Mett et al. 2005)

Disease	Microorganism	Target
Antrax*	*Bacillus anthracis*	Protective antigen
Botulism	*Clostridium Botulinum*	Botulinum neurotoxins
Ebola virus	Ebolar virus	Ebola glycoprotein
Rabies*	Rabies virus	Virus glycoproteins
RSV infection	Respiratory syncytial virus	F glycoproteins
Smallpox	Variola major	14-kDa protein encoded by the A27 gene
West Nile virus	West Nile virus	Viral envelope (E) protein
Dental caries*	*Streptococcus mutans*	Streptococcal antigen I/II

*Monoclonal antibodies for these diseases have been expressed in transgenic plants (Ma et al. 1995; Ko et al. 2003; Mett et al. 2005)

lymphoma, and chronic lymphocytic leukemia through these anti-cancer mechanisms (Table 1). Monoclonal antibodies conjugated to radioactive materials, toxins, and chemotherapy drugs also show efficacy in non-Hodgkin's lymphoma and acute myeloid leukemia (Fig. 3). Thus, antibodies are considered smart guided missiles. Several anti-cancer mAbs have been produced in plant systems (Verch et al. 1998; Vaquero et al. 2002; Ko et al. 2005).

Passive Immunization for Infectious Diseases

Passive antibody therapies for infectious diseases caused by the viral, bacterial, fungal, and parasitic microbes are currently gaining interest in research and clinical fields (Casadevall et al. 2004) (Table 2). Although many vaccines against infectious diseases

have been developed and used, the limited vaccine production and immunization against currently emerging infectious diseases hinders protection of unexpectedly infected patients. The increase in drug-resistant microorganisms is driving the development of antibody-based immunotherapeutic applications for the prevention and treatment of infectious diseases.

Advanced cloning of human antibodies from combinatorial libraries can rapidly select mAbs carrying intended affinity against infectious diseases (Burton and Barbas 1994; Winter et al. 1994), giving advantages over vaccines and antimicrobial drugs. The advantage of antibody-based therapy is the high specificity and thus low toxicity compared to conventional drugs. In contrast to anti-cancer immunotherapy, which relies on identifying self-antigens highly expressed in tumor cells, antibody-based passive immunization for infectious diseases benefits from recognizing pathogen-originated antigens, which are largely different from those of the hosts. In infectious diseases caused by a microorganism such as a virus, the high specificity to a certain virus strain does not always provide an advantage since a single highly specific antibody is not satisfactory to control microorganisms with high antigenic variation. The emergence of virus mutants or different virus strain variants encourages the use of low specific antibody activity with binding specificity against broad virus strains. Antibody-based therapies with the multiple low and broad specific antibodies provide a synergistic or additive effect to control viral diseases by variant virus strains (Nosanchuk et al. 2003; Casadevall et al. 2004). In principle, the cocktails of antibodies that are specific for different virus strains is thought to efficiently control virus escaping, as a combination of antiretroviral drugs against HIV-1 infection (Prosniak et al. 2003; Montefiori 2005; Trkola et al. 2005). In the case of bacterial toxin, combining multiple monoclonal antibodies with different targets related to pathogenesis increased the potency of in vivo botulinum toxin neutralization (Nowakowski et al. 2002). Additional advantages of using antibodies are easy modification of the antibody to its derivative structures (Fig. 1). The biological mechanisms of the therapeutic antibody are mainly distinguished by the involvement of the Fc region (Fig. 4). The mechanisms such as toxin or virus neutralization and direct antimicrobial functions have an Fc-independent action, whereas antibody-dependent cellular cytotoxicity have an Fc-dependent action. In the former mechanism, the binding of antibody to a target antigen via the binding region of Fab is sufficient to mediate antimicrobial effects. In contrast, the latter mechanism requires an intact molecule of antibody carrying both Fab and Fc structures. Thus, different forms of antibody derivatives can be chosen for the intended antimicrobial mechanisms (Ko and Koprowski 2005). Neutralizing activity that causes the loss of virus infectivity is essential for the antiviral activity of antibodies. In HIV-1 virus, a combination of three human neutralizing mAb can delay viral rebound after cessation of antiretroviral treatment (Trkola et al. 2005), suggesting that antibodies that neutralize HIV-1 in vitro can suppress the virus in infected individuals. The potency of neutralization is enhanced by increased functional affinity of the mixture antibodies. However, it does not necessarily mean that neutralization is the major antiviral mechanism of protective activity (Fig. 4).

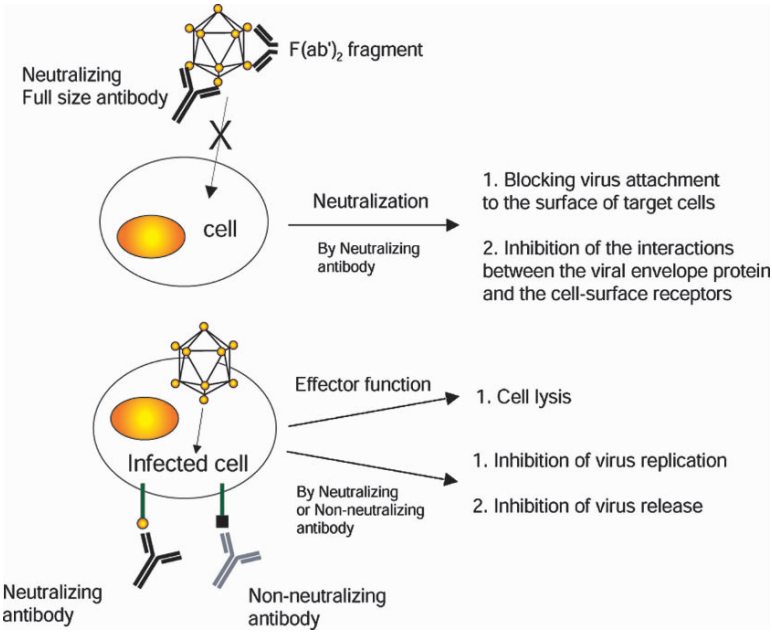

Fig. 4 Mechanisms of antiviral activities of antibodies before and after virus infection. Neutralization is mediated by antibody that binds to surface molecules on viral particle and thus blocking virus entry before virus infection. Antibodies specific for spike proteins of an enveloped virus or other antigens presented by infected cells trigger Fc-mediated effector mechanisms to eliminate infected cells. Neutralizing and non-neutralizing antibodies specific for virus proteins processed in infected cell can give antiviral activity against infected cells, such as cell lysis and inhibiting virus replication and release

Neutralizing IgG1 antibodies with poor effector functions is often ineffective at protection but IgG2a antibodies highly triggering effector functions with the same specificity are effective (Schlesinger et al. 1993), indicating that the mechanisms other than neutralization such as effector functions can be important for protection by neutralizing or non-neutralizing antibodies.

Tailor-Made Antibodies to Improve Efficacy

Antibody-based immunotherapies require particular antibody mechanisms to obtain therapeutic efficacy, as described above. When antibodies are applied to inhibit the activity of certain molecules, pathogens, or toxins by physically blocking ligand–receptor interactions, the ideal properties will be only high affinity of the variable binding region for the targeted antigen. On the other hand, when the

desired therapeutic mechanism replies effector functions such as ADCC, complement action, and phagocytosis, both the binding property of mAbs to a target antigen and efficient interactions between the antibody Fc region and Fc receptors are required. In this case, both Fab antigen-binding and Fc constant regions play an equally important role (Fig. 2).

Today, by using advanced molecular biology and immunology techniques, it is possible to redesign the desired properties such as antigen specificity, antigen-binding affinity, and Fc receptor-binding affinity (Fig. 2). To generate mAbs with high antigen specificity and antigen-binding affinity, the mAb antigen-binding site is altered by site-directed mutagenesis (Schier et al. 1996) or random mutagenesis with yeast surface display or phage display (Colby et al. 2004; Brockmann et al. 2005) or glycosylation on variable region of the mAb is modified (Tachibana et al. 1992; Coloma et al. 1999). Glycosylation of Asn^{58} of the V_H of anti-dextran mAb increased the affinity of the antibody for antigen approximately tenfold while carbohydrate at Asn^{60} of the V_H only increased the affinity threefold (Wright et al. 1991), indicating that the glycosylation position affects affinity of variable antibody regions. The structure of the V region carbohydrate can be associated with differences in binding specificity and affinity (Matusuuchi et al. 1981; Tachibana et al. 1992). The enhanced affinity improves antibody neutralizing activity, which is important for inhibition of virus infection (Burton 2002). Thus, simple modification of the glycosylation position might improve the antibody's neutralizing activity. In tumor immunotherapy, the high affinity for tumor antigen enhances cytotoxicity of bispecific antibody against both Fcγ RIII and cancer antigen (McCall et al. 2001). The high affinity bispecific antibodies are retained longer by tumor cells, thus allowing more time for the leukocytes to bind to the available anti-Fcγ RIII binding domain of the bispecific antibody, enhancing its cytotoxicity. However, high affinity may not always be helpful when rapid and complete tumor penetration is essential for immunotoxins since the higher the affinity with the higher antigen density, the greater the binding site barrier, which severely hinders the diffusion of antibodies throughout the tumor mass (Juwied et al. 1992; Weiner and Carter 2005). In this case, relatively low-affinity IgG that relies on a high surface density of antigen on tumor cells can be tuned up to enhance both targeting selectivity and antibody diffusion (Carter 2001).

When the therapy requires the expanded presence of antibody in the blood stream, a slow blood clearance rate is required. The antibody's blood clearance rate can be optimized to enhance antibody efficacy by modification of sequences or glycosylation of mAb Fc regions that affect antibody structural confirmation (Vaccaro et al. 2005; Weiner and Carter 2005; Fig. 2). On the other hand, in immunotoxin-based therapy, rapid clearance of immunotoxins is preferable for reduction of the side effect caused by sustained existence of circulating immunotoxins in body. In glycosylation, lack of galactose decreases the half-life through binding of agalactosyl IgG to mannose-binding protein or mannose receptors (Malhotra et al. 1995; Wright and Morrison 1998). In plants, the half-life of high mannose-type mAb with the heavy chain fused to KDEL, the endoplasmic reticulum (ER)

retention motif retaining mAb in ER has rapid blood clearance, indicating that modification of glycosylation in plants might be a useful strategy to regulate the blood clearance rate (Ko et al. 2003).

Also, the altered glycosylation pattern on Fc regions of mAb increases ADCC by enhancing the interaction between Fc regions and Fc receptors (Shields et al. 2002). For example, Umana et al. (1999) showed that high levels of bisected, nonfucosylated glycan in the Fc region enhanced antibody-dependent cellular cytotoxicity. When major effector functions of a given mAb are mainly mediated by ADCC, the lack of fucosylation might increase the mAb anti-tumor activity by enhancing ADCC activity (Herlyn et al. 1986; Shields et al. 2002). In anti-colorectal cancer mAb CO17–1A, mAb-mediated tumor inhibition is due mainly to ADCC activity (Herlyn et al. 1980). Thus, the ADCC increased by using an altered glycosylation pattern on the Fc region of mAb is beneficial for mAb CO17-1A. The use of a plant system offers scalability and shorter timelines to produce antibody. In addition, simple manipulation of plant glycosylation machinery through subcellular localization of antibody and blocking/insertion enzymes related to glycosylation can refine the plant system to enhance the tailor-made quality of antibody. This issue will be discussed later. To maximize the advantages of the plant system, before the transgenes of antibodies are integrated into plant genome, tuning up the intended biological characteristics of antibody should be considered by designing an antibody structure and modifying the sequences and glycan composition.

Current Approach to Express Antibodies in Plants

In plants, to achieve the expression of antibodies, the cDNAs of heavy and light chains of monoclonal antibody from a hybridoma cell are cloned and transferred into the plant genome. Two general methodological categories to transfer the cDNAs to be expressed in plant cells are stable and transient expression. For stable expression, *Agrobacterium*-mediated transformation or particle bombardment are mainly applied as a representative method to stably insert DNA fragments encoding both heavy and light chains to the nuclear or other compartment genomes in plants. The heavy and light chain genes can be introduced to plant cells at the same time by using a single transformation event with one plant expression binary vector (Ko et al. 2003). Often, the genes are separately inserted and expressed in plant lines and co-expressed by crossing these individual lines (Ma et al. 1995; Fig. 5a). Transgenic lines that contain nucleus carrying both inserted genes are further self-crossed to obtain homozygous transgenic plants expressing monoclonal antibody. The advantage of this approach is that lines with high expression of heavy or light chains can be screened and crossed to generate transgenic lines expressing both heavy and light chains with other antibody elements for secretory IgA (Ma et al. 1995; Fig. 5c). This conventional crossing approach can be applied to express multiple monoclonal antibodies and modify glycosylation (Fig. 5b and d, respectively).

The gene can be inserted into chloroplast genome to generate so-called chloroplast transgenic plants in which chloroplasts express and properly fold functional

antibodies with disulfide bonds (Daniell 2002a). The advantages of chloroplast genome-based transgene expression are the no-position effect, no gene silencing, and high expression and accumulation. Although expression of the heterologous transgenes is high in chloroplast, it might not be suitable to express monoclonal antibody, which requires glycosylation for the full biological activity since chloroplasts lack glycosylation machinery in nature. Thus, the possibility of establishing glycosylation pathways in the chloroplast compartment has been investigated by incorporating the multiple genes for glycosylation processing (H. Daniell, personal communication). If the equipping glycosylation machinery in the chloroplast com-

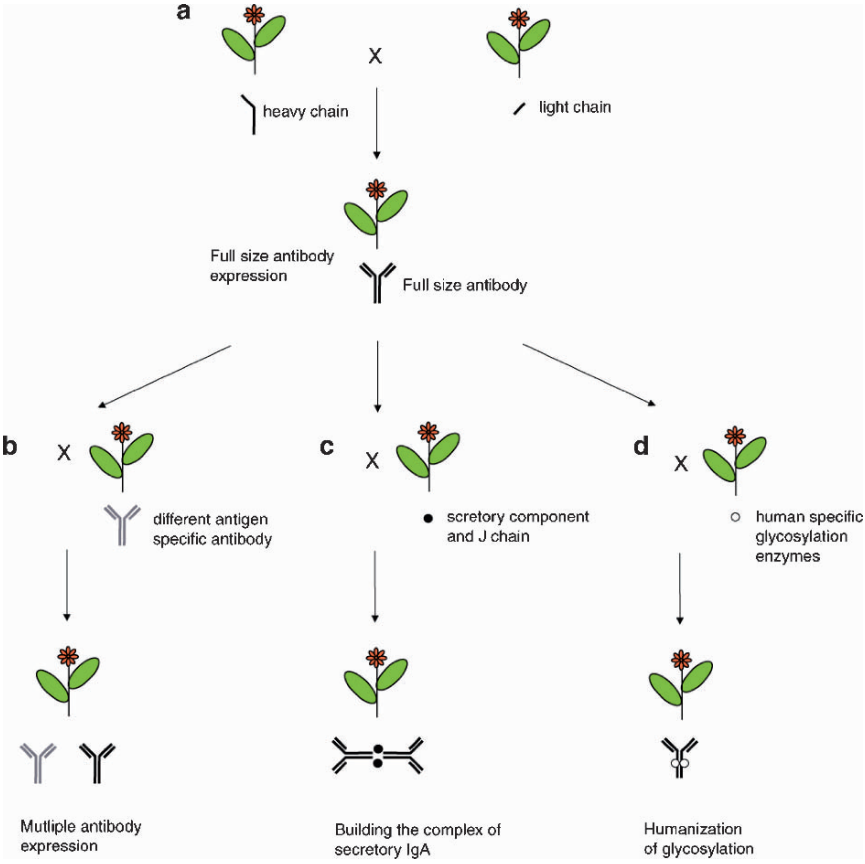

Fig. 5 Diverse uses of plant crossing. Crossing between transgenic lines carrying light and heavy chains, respectively, can generate a transgenic line expressing full-size antibody. This crossing strategy can be efficiently applied to express multiple monoclonal antibodies and a highly complex form of antibody such as a secretory IgA. Glycosylation of monoclonal antibody can be modified by co-expression of antibody and a human-specific glycosylation enzyme such as a human galactosyltransferase in a single transgenic line obtained from crossing lines expressing antibody and the human enzyme (Bakker et al. 2001). This easy manipulation by crossing is another advantage over the other production systems

partment is possible, the chloroplast transgenic plants can become ideal choices for production of most monoclonal antibodies.

For transient expression, agroinfiltration (Vaquero et al. 2002) and recombinant plant viruses (Verch et al. 1998) are mainly used to produce antibodies without generating transgenic plants. Agroinfiltration is generally used as an evaluation method to determine the efficiency of expression cassettes and the activity of recombinant proteins over a short period time before generating transgenic plants. This agroinfiltration method can be used successfully for large-scale production systems. Full-size monoclonal antibodies have been efficiently produced by agroinfiltration systems (Kathuria et al. 2002; Vaquero et al. 2002; Hull et al. 2005).

Plant viruses have been used as protein-expression vectors carrying transgenes both for vaccine delivery and therapeutic protein production (Koprowski and Yusibov 2001). The advantage of this viral vector system is high level of expression, since the virus infection is rapid and systemic to whole plant tissue after inoculation, and easy purification of virus particles. As a production system, the antibodies have been expressed in *N. benthamiana* using the Tobacco Mosaic Virus (TMV) vector (Verch et al. 1998; McCormick et al. 1999). For full-size monoclonal antibody expression, two TMV vectors carrying the heavy and light chain of CO17–1A monoclonal antibody and anti-colorectal cancer antibody were co-infected to *N. benthamiana* (Verch et al. 1998). The expressed light and heavy chains were correctly assembled to full-size monoclonal antibody. For single-chain antibody (scFv), this virus system has a rapid reproduction of customized scFv for cancer patients who usually have unique epitopes to be targeted. This approach could provide personalized immunotherapies for diseases such as non-Hodgkin's lymphoma using easy virus vector-producing scFv and transient expression in a short period of time (McCormick et al. 1999).

This viral vector system requires virus transcript inoculation on each plant tissue since systemic movement of viruses is often limited depending on the size of the integrated transgenes. Also, high mutation and deletion rates are often obtained on the foreign gene during plant RNA virus replication (Smith et al. 1997). The main cause of low systemic infectivity is often the large size fused to coat protein, which hampers efficient viral particle cell-to-cell movement. Marillonnnet et al. (2005) recently developed systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Agrobacterium*-mediated delivery of RNA viral vectors as DNA precursors results in simultaneous gene amplification in all leaves of a plant. This method relies on *Agrobacterium*-infiltration delivering viral vectors. Thus, high expression of heterologous genes in individual bacterial infected cells and consequently no requirement of systemic viral particle movement resolve the gene size-limitation problem and provide synchronous and faster expression. This transient expression process yields as much as 4 g of recombinant protein (green fluorescent protein) per kilogram of fresh leaf biomass in *N. benthamiana* and up to 2.5 g/kg of tobacco (*N. tabacum*), can be applied to other plant species (Gleba et al. 2005; Marillonnnet et al. 2005).

Other Applications of Antibodies Expressed in Plants

Antibody is biologically active protein with its specific binding properties to target antigens resulting in physically or biologically blocking the activities of the antigens. These unique biological activities are applied to other applications such as the sensitive detection and removal of environmental contaminants and industrial purification and processes. For agricultural research, the applications include engineering antibody-mediated resistance to plant disease and immunomodulation of physiological processes (Stoger et al. 2002). In this section, we discuss potential approaches for agricultural applications rather than medical applications using antibody expressed in transgenic plants.

Disease Resistance in Plants

Plant pathogens are a great and growing threat to crop production worldwide. The current homogenous plant cultivation system is vulnerable to the outbreak of epidemics. Conventional plant breeding using sexual crossing of crops is often limited with a narrow choice of resistance genes in plants that are transferred to their elite line. However, plant genetic engineering can generate transgenic plants resistant to plant pathogens by expression of antimicrobial proteins, pathogen-related proteins, or antisense RNAs against pathogenesis obtained from interspecies. Expression of antibody in plants is another novel approach to obtain disease-resistant plants. Application of antibody to control pathogens in plants relies on its interaction with pathogens and antigens involved in pathogenesis. Expression of several antibodies in plants has been described to reduce infection and symptoms caused by viruses, insects, and animals. This antibody-based disease resistance approach is dependent on the precise localization of antibodies in specific subcellular compartments of plant cells or specific tissues. When scFv against TMV is expressed in the cytosol, it effectively confers resistance despite a lower level of expression than that of secreted antibody (Voss et al. 1995). In contrast to TMV, Mollicutes such as phytoplasma responsible for more than 300 diseases of vegetable, ornamental, and perennial plants are strictly localized in the sieve tubes of the phloem tissue. Transgenic tobacco shoots expressing phytoplasma-specific scFvs secreted to the apoplast grew free of disease symptoms (Le Gall et al. 1998), indicating secreted scFv is more effective than cytosol localized scFv. Baum et al. (1996) proposed that antibody-based resistance can also be applied to obtain plants that are resistant to nematodes. The plant nematode's stylet secretions are essential for the initial steps of pathogenesis. Thus, anti-stylet secretion-specific antibody was expressed and secreted into the intercellular space of plant cells. However, secreted antibody did not effectively reduce nematode infection (Baum et al. 1996). It seems that the enzyme produced from the nematode cannot be inactivated by antibodies at apoplast, proposing that cytosolically localized antibody might provide enhanced resistance since the

nematode stylet directly injects pathogenesis-related enzymes into cells at the early stage of infection (Baum et al. 1996). Baum et al. (1996) proposed that in the case of root-knot nematodes, a cellulase from *Meloidogyne incognita* is a potential antigen to be selected as a target for antibody since this enzyme plays an essential role in the migration of the nematode inside the plant (Vrain 1999). Another antibody-mediated approach is the use of recombinant antibodies fused to antimicrobial agents expressed in plants to deliver the antimicrobial proteins to the infection site, where the pathogens are populated at the early stage of infection (Schillberg et al. 2001). An antibody-mediated strategy to obtain disease resistance will be appreciated as an alternative approach to control plant disease free of the many limitations if localization of targeted antibody can be regulated with its high expression level.

Antibody-Mediated Metabolic Engineering in Plants

Antibody-mediated metabolic engineering focuses on development of plant varieties with greater yields of specific products (such as carbohydrates, proteins, and oils), and improved tolerance to environmental stress. Immunomodulation manipulates plant cellular metabolism by antibody-mediated alteration of the protein function in plants. Jobling et al. (2003) described the first application of immunomodulation to efficiently inhibit enzyme activity involved in starch biosynthesis in the potato. In this study, they confirmed that expression of plastid-targeted starch-branching enzyme A neutralizing scFv increases accumulation of high-amylose potato starch in the potato. Antibody-mediated metabolic engineering can be applied to understand changes of molecular structures and protein–protein interactions involving stress tolerance. The conformational dynamism and aggregate state of small heat shock proteins (sHSPs) are essential for the function of this protein on heat stress in plants (Miroshinichenko et al. 2005). The sHSPs are important for thermotolerance of plant cells from the detrimental effects of heat stress. Ectopic expression of scFv antibodies against cytosolic sHSPs was used to generate sHSP loss-of-function mutants, which is the lack of the sHSP assembly in vivo, resulting in a lower survival rate in plant cells under heat-stress conditions. He showed that the ability of sHSPs to assemble into heat-stress granulars (HSGs) as well as the HSG disintegration is a prerequisite of survival of plant under the heat stress conditions. This approach could be applied with direct mutagenesis and homology-dependent gene used to understand important metabolisms in the plant.

Antibody-Mediated Phytoremediation

Phytoremediation is an approach which extracts, sequesters, or detoxifies pollutants in soils and surface waters by using plants (Drake et al. 2002). The most advanced strategy in this field is using metal-absorbing plants to clean up areas polluted with

lead, cadmium, and copper. Several plant species, particularly in the *Brassicaceae*, are efficient metal-absorbing plants. Transgenic plants expressing protein detoxifying metal pollutants enhance tolerance of metal pollutants and accumulate greater amounts of these metals, suggesting its potential usage for phytoremediation (Song et al. 2003). The majority of environmental pollutants are pesticides, endocrine-disrupting chemicals that are implicated in reproductive system disorders in exposed animal species. Drake et al. (2002) proposed that the phytoremediation capabilities of plants could be extended to these types of chemicals to neutralize by generating transgenic plants expressing an antibody specific to such chemicals. Two strategies of rhizosecretion-mediated binding and sequestration in leaf tissue could potentially be used in the phytoremediation of any pollutant. It is possible to express a monoclonal antibody neutralizing biologically active pollutants and immune complex formation in situ on the plasma membrane in leaves (Drake et al. 2002).

Plant Species to Produce Monoclonal Antibody

Diverse plant species have been transformed for production of recombinant antibodies (Schiermeyer et al. 2004). The choice of plant species is crucial for successful plant biopharming for antibody production since each plant species has unique characteristics that affect antibody expression, product storage, downstream processes, and the quality of the final antibody products (Table 3). The yield of functional antibodies is the first standard to be considered for the choice of plant species. In tobacco, the main advantages are the high biomass yield and the rapid scale-up by prolific seed production compared to other plant species. In addition, tobacco is a non-food, non-feed plant which can attenuate biosafety concerns. Currently, well established tobacco rhizosecretion systems where antibodies are targeted to the secretory pathway for ease of purification (Borisjuk et al. 1999) or a magnifection system where the TMV agroinoculation delivery vectors enhance the expression level makes this plant species attractive. However, tobacco generates a heterogeneous *N*-glycosylation profile of antibodies reflecting the heterogeneous distribution of antibody in the secretory pathway, which may make it difficult to

Table 3 Plant species used for antibody production

Species	Expression tissues	Storage cost	Other culture system	Glycosylation	Protein expression
Alfalfa	Leaf	High	Difficult	Homologous	High
Algae	Total tissue	High	Easy	Heterologous	Medium
Arabidopsis	Leaf	High	Difficult	Heterologous	Medium
Maize	Seed	Low	Difficult	Heterologous	High
Rape	Seed	Low	Difficult	Heterologous	Low biomass
Rice	Seed	Low	Difficult	Heterologous	Medium
Soybean	Seed	High	Difficult	Heterologous	Low biomass
Tobacco	Leaf	High	Easy	Heterologous	Medium

control standardized antibody quality (Cabanes-Macheteau et al. 1999; Ko et al. 2003, 2005). In contrast, alfalfa produces a relatively homologous *N*-glycan structure with its highly efficient folding and/or secretion machinery and fixes atmospheric nitrogen (D'Aoust et al. 2004), which is an advantage in terms of the cultivation of legumes over other plant species. Although alfalfa contains oxalic acid compounds, which affects downstream processing and produces lower amounts of leaf biomass than tobacco, the high protein level in alfalfa leaf tissues maximizes accumulation of recombinant antibodies per plant biomass.

One of disadvantages of these leafy crops comes from the storage and distribution of the plant leaf product, which is instable unless the leaf tissue is frozen or processed. Antibodies expressed in corn seeds are stable at room temperature for more than 3 years without loss of activity (Stoger et al. 2000). Thus, seeds are advantageous in terms of the cost of grain storage and distribution. However, in contrast to leaf plant products such as tobacco, after harvesting of corn seeds for antibody purification, large volumes of waste tissues such as stems and leaves remain, which might have an impact on the environment and generate extra costs downstream. Although the costs of extraction and purification for corn seeds might be higher than plant leaf materials, a well-established food-processing facility may be able to rapidly start up downstream processing. Several other cereal and legume crops have been used for antibody production. Usage of these food crops for therapeutic antibody production may raise concerns about environmental or biosafety issues because of the potential risk of transgene flow to the nontransgenic food crops and contamination of food products by outcrossing (Ko et al. 2003; Ma et al. 2003; Stoger et al. 2005). As plants for pharmaceutical protein production, non-food and non-feed plants might be the choice to avoid transgene contaminants (Ma et al. 2003). There is no ideal choice of plant species since each of the plants has its advantages and disadvantages. Thus, the choice of host plants should be carefully determined.

Purification of Plant-Derived Antibody

The plant system has many advantages compared with other systems for the biopharming of antibodies. One of main advantages of the plant system is the low cost of production. However, downstream processes such as extraction and purification of proteins, a key step, represents more than half of total production cost. Even if the low cost of upstream production is achieved, the total production cost might not be satisfactory without reducing this downstream cost. Thus, efficient purification procedures for plant production systems are as important as effective expression levels. In a plant expression system, the plant tissue and cells should be disrupted to release antibodies for purification since antibody is expressed and localized within the cells. In addition, antibodies should be recovered with a removal of cell debris, noxious chemicals, and contaminants. Currently, for purification of antibody expressed in plants, an affinity purification protocol exploiting protein A-based matrices is mainly used. Large-scale purification has

been using protein A streamline chromatography (Valdes et al. 2003). However, the cost of the protein A is prohibitive and fine plant cell debris is difficult to remove from the plant leaf extracts before applying to protein A affinity chromatography, often blocking the column process, consequently reducing durability of protein A column. Although several affinity tagging systems such as the histine tag and intein fusion expression have been applied for purification of recombinant proteins, these systems do not resolve the problems caused by plant cell debris. Therefore, oleaginous plants such as a rapeseed oil are useful hosts for monoclonal antibody production and purification since the oil bodies can be applied to simplify the first steps of antibody isolation (Boothe et al. 1997; Seon et al. 2002). Transgenic plant expresses both protein A fused to oleosin anchored into oilbodies and monoclonal antibody in two distinct cellular compartments of the seed (Seon et al. 2002). The antibody is bound to the protein A-fused oleosin during seed grinding and can be recovered from oil bodies carrying the protein A and antibody complex using a simple extraction procedure (Seon et al. 2002). Another approach is so called ZERA technology (Eraplantech, www.eraplantech.com). Zera technology uses proline-rich (gamma)-zeins, which are maize storage proteins that accumulate inside large vesicles called protein bodies. When recombinant proteins fused to zeins are localized in endoplasmic reticulum, a high amount of the fusion proteins is accumulated to form stable bodies, which allows the production of recombinant proteins through their deposition on protein bodies of plant cells. Zera assemble heavy protein bodies in the endoplasmic reticulum (ER) of plant cells to highly accumulate and form supermolecular aggregates of polyproine structures and thus eases their recovery by simple homogenization and centrifugation. This technology has been successfully applied to produce calcitonin in tobacco plants. At present, ERA Plantech aims to validate and optimize ZERA technology to ensure its viability and competitiveness to produce a wide range of peptides and proteins such as antibodies.

Glycosylation

Antibodies are glycoproteins with specific glycoforms that are involved in their folding, stability, and activity. The altered glycosylation can influence biological characteristics such as antigen binding activity, affinity of Fc receptors, stability, and immunogenicity. Thus, the authentic *N*-glycosylation pattern on antibodies produced from any production system is necessary to obtain their intended therapeutic effect similar to the parental antibody. Plants have *N*-glycosylation capability similar to those of mammalian cells. However, *N*-glycosylation patterns processed in plant cells differ from those of mammals and humans (Fig. 6).

In plants, *N*-linked glycans contain β(1,2)-xlyose and α(1,3)-fucose instead of α(1,6)-fucose in mammals (Fig. 6). Furthermore, the plant *N*-glycan rarely carries galactose and lacks sialic acid (Fig. 6). These plant-specific glycans are considered to be potential antigenic and/or allergenic epitopes (Bakker et al. 2001). Although

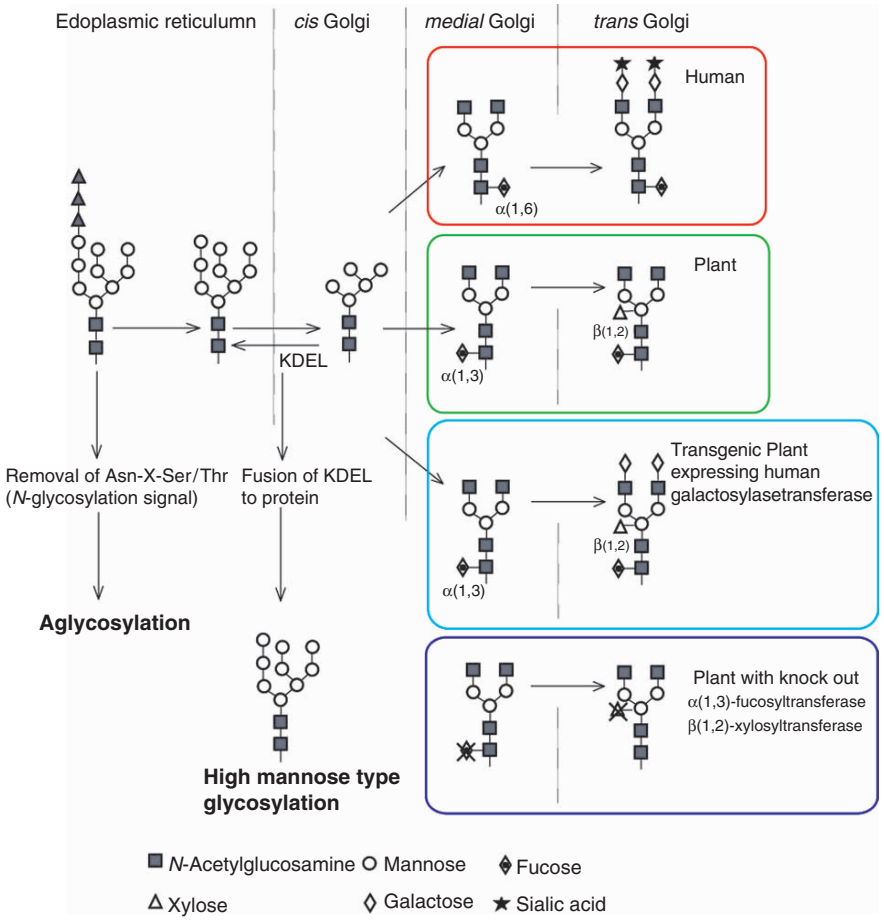

Fig. 6 *N*-glycosylation processing of glycoproteins in human and plant. *N*-glycosylation in the endoplasmic reticulum (ER) starts with transferring oligosaccharide precursor to Asn-X-Ser/Thr glycosylation signals on proteins. This precursor is processed by glycosidases and glycosyltransferases in the ER and the Golgi apparatus. Glycosylation processing in the ER is conserved in the plant and animal kingdoms and restricted to high mannose-type *N*-glycans, whereas the further glycosylation process in Golgi apparatus generates highly diverse matured *N*-glycan structures

plant-specific glycosylated antibody is not immunogenic in mice (Chargelegue et al. 2000), one of the major safety issues of plant-produced therapeutics remains these plant-specific *N*-glycosylation patterns. These potential concerns could become a drawback of the plant production system. Several research groups have studied removing plant-specific glycans, the potential antigenic components (Wenderoth and von Schaewen 2000; Ko et al. 2003; Finnem et al. 2005). One simple approach is aglycosylation to obtain antibodies with no *N*-glycosylation by

mutating Asn-X-Ser/Thr sites, which are signals for *N*-glycosylation (Nuttall et al. 2005; Fig. 6). This approach is effective if the intended antibody activity is not affected by aglycosylation. Another approach is to retain the antibodies in ER to avoid plant-specific glycan residues such as β-(1,2)-xylose and α-(1,3)-fucose (Ko et al. 2003; Fig. 6). Glycosylation processing in the ER is conserved in the plant and animal kingdom and restricted to high mannose-type *N*-glycans, whereas the further glycosylation process in the Golgi apparatus where additional glycans are added for glycan maturation is highly diverse. When the ER-retention signal KDEL/HDEL fused to proteins, the signal retains proteins in the ER (Nuttall et al. 2002). We found that the KDEL fused to the heavy chains effectively generated 90% of the high mannose-type glycosylated antibodies in a total population of antibody produced in plants. The remaining 10% of the antibody population was GlcNAc2Man3GlcNAc2 (4.3%) and GlcNAc2Man3(xylose)GlcNAc2 (5.7%) without α(1,3)-fucose residues, which were often added on the trans-Golgi side (Fitchette-Laine et al. 1994; Ko et al. 2003). In this study, the KDEL signal fused to heavy chain does not completely retain antibodies to ER from Gogi, resulting in heterologous forms of glycan structures. Gomord et al. (2004) proposed fusion of KDEL to both light and heavy chains to increase high retrieval efficiency to obtain a homogenous population of high mannose-type glycan on full-size monoclonal antibody. When compared with mammal-derived antibodies, antibodies with high mannose-type glycans were rapidly cleared in vivo (Ko et al. 2003). This rapid clearance rate of antibodies may benefit rabies postexposure prophylaxis where vaccines are applied 10 days after passive immunization with antibodies. The dual effect of rabies postexposure treatment with both antibody and vaccine may often cause interference between passive and active immunization because of larger persistence of antibody in the circulation (Koprowski and Black 1952; Schumacher et al. 1992; Lang et al. 1998). Thus, the short half-life of antibodies may reduce potential interference between the two types of immunization. However, this strategy to generate high mannose-type glycosylated antibodies is not universally applicable because of its lower stability in vivo unless the high clearance rate is beneficial for therapeutic application.

Another approach to eliminate plant-specific glycan residues is to knock out the expression of glycosyltransferases involving β-(1,2)-xylosylation and α-(1,3)-fucosylation in Gogi apparatus enzymatic machinery (Gomord et al. 2004; Fig. 6). The *N*-glycan processing enzymes, β-(1,2)-xylosytransferase and α-(1,3)-fucosyltransferase, have been identified and cloned from different plant species. The expression of these enzymes has been inhibited using antisense technology. In moss (*Physcomitrella patens*), homologous recombination for gene targeting has been used to knock out expression of the α-(1,3)-fucosyltransferase and β-(1,2)-xylosyltransferase, eliminating plant-specific glycoepitopes without any effect on protein secretion (Koprivova et al. 2004). However, so far the approach has only been able to decrease the enzyme activities in *N*-glycan biosysnthesis in plants (Wenderoth and von Schaewen 2000).

In addition to eliminating plant-specific sugar, humanization of *N*-glycosylation is also essential for the production of authentic glycosylated antibodies in plants.

The strategy to humanize plant *N*-glycans is to express mammalian glycosyltransferases, which would complete *N*-glycan maturation in the plant's Golgi apparatus. The expression of human β(1,4)-galactosyltransferase in transgenic tobacco plants produces 30% galactosylated *N*-glycan in an antibody population, whose level is comparable to hybridoma cells (Bakker et al. 2001; Fig. 6). Addition of sialylation into plant *N*-glycosylation machinery is another important humanization process that can affect biological activity and in vivo half-life in the human body. Shah et al. (2003) reported the presence of sialylated glycoconjugates in suspension-cultured cells of *Arabidopsis thaliana* and suggested that a genetic and enzymatic basis for sialylation exists in plants. In contrast, Senveno et al. (2004) argued that sialylation does not exist in plants and proposed that obtaining sialylated glycan in plant cells requires the input and expression of at least five heterologous genes involving in entire sialylation pathway. Furthermore, these enzymes should be active and correctly targeted in Golgi compartment in plant cells. In nature, it seems that efficient sialylation processes are absent from plant cells. Thus, the humanization of antibodies with sialylation in plant systems, if necessary, remains a major challenge in producing authentic glycosylated antibodies in plant systems. New technology for the humanization of *N*-glycosylation will open up plant systems that efficiently produce authentic antibodies with enhanced bioactivities for human immunotherapy in the near future.

Conclusion

Plants have many advantages over other existing systems to produce monoclonal antibody. The advantages are recognized and appreciated with no doubt on the ability to produce functional antibodies, whereas the drawbacks related to the limited expression level, the authentic quality of antibody, downstream processing cost, public acceptance, and environmental concern hold their steps to utilization of plant systems as the commercial systems. A growing demand for therapeutic and diagnostic antibodies and the lack of current production facilities to meet the demand will be encountered. Thus, it is necessary to establish an alternative antibody production platform. New technologies are currently being developed to overcome the limitations of plant systems for antibody production.

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