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Expression of Recombinant Vaccines and Antibodies in Plants

Kisung Ko

Plants are able to perform post-translational maturations of therapeutic proteins required for their functional biological activity and suitable *in vivo* pharmacokinetics. Plants can be a low-cost, large-scale production platform of recombinant biopharmaceutical proteins such as vaccines and antibodies. Plants, however, lack mechanisms of processing authentic human *N*-glycosylation, which imposes a major limitation in their use as an expression system for therapeutic glycoproducts. Efforts have been made to circumvent plant-specific *N*-glycosylation, as well as to supplement the plant's endogenous system with human glycosyltransferases for non-immunogenic and humanized *N*-glycan production. Herein we review studies on the potential of plants to serve as production systems for therapeutic and prophylactic biopharmaceuticals. We have especially focused on recombinant vaccines and antibodies and new expression strategies to overcome the existing problems associated with their production in plants.

Introduction

Vaccines and antibodies are a common therapeutic modality for prophylaxis or treatment of several infectious diseases and cancers. Such biopharmaceutical proteins require industrial-scale production due to a strong market demand. Traditionally, microbial fermentation and mammalian cells have been used to produce recombinant proteins.[1] Human pathogen contamination and high production costs hamper animal-based expression systems, compelling alternative production platform development. For instance, according to a World Health Organization survey, human mortality from endemic canine rabies was estimated to be 55,000 deaths per year during the last several decades.[1] Anti-rabies equine immunoglobulin is now defunct, precipitating a worldwide shortage of anti-rabies immunoglobulin. This has particularly impacted developing countries since concerns about the safety of animal-derived products (a source of animal pathogens) have been raised. In general, control of the lethal rabies virus in both humans and animals requires post-exposure prophylaxis (PEP) through the combined administration of both vaccine and immunoglobulin (RIG). Thus, a shortage of both vaccines and immunoglobulins in developing countries has put rabies patients in danger. In addition to the shortage, parenteral vaccine administration *via* needle injection has increased treatment cost, which is another difficulty in economically challenged developing countries.

Dr. Hilary Koprowski and his co-workers recognized that the needle injection method was a hurdle for global vaccine application and accordingly developed an oral spray for polio vaccination and subsequently conducted an oral vaccination trial in the Belgian Congo.[2] Since then, the oral spraying method has become the standard administration model for polio vaccines, which eventually contributed to the eradication of polio worldwide. Vaccines are generally produced in animal organs or cells, with the inherent risk of animal pathogen contamination. Recent advances in the field of molecular immunology and biotechnology have created opportunities for the use of recombinant vaccines and monoclonal antibodies (MAbs) in the prevention and control of infectious disease or cancer.[2,3] Plant genetic engineering can now be applied to express valuable recombinant biopharmaceutical proteins on an industrial scale. The advantages of plant expression systems include large-scale production capacity, lack of animal pathogen contamination, and low cost of biomass production, compared to mammalian systems.[4–6] Plants are additionally suitable for extensive post-translational modifications of proteins required for full biological activity, including *N*-glycosylation, in a manner similar to mammalian expression systems.[4] Koprowski anticipated that global use of vaccines would require low costs, ease of maintenance, and efficient distribution.[2] Thus, he initiated the development of the plant expression system as a viable alternative platform for the production of oral vaccines and therapeutic antibodies for infectious diseases and cancer. Since then, successful diverse plant-derived vaccines for infectious diseases such as rabies,[7] HIV,[8] severe acute respiratory syndrome (SARS),[9] smallpox,[10] and hepatitis B[11] have been reported. Monoclonal antibodies for

Department of Medicine, Therapeutic Protein Engineering Lab, College of Medicine, Chung-Ang University, Seoul, Korea.

neutralizing viruses and controlling cancer cell growth have been successfully produced in plants,[12–15] including highly valuable glycoproteins for immunotherapeutic actions such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and others.[16] Herein we have primarily discussed the requirements for efficient production of therapeutic recombinant vaccines and antibodies by plant molecular biofarming, which harmonizes regulatory factors for transcriptional, translational, and post-translational events in gene expression.

Expression of Recombinant Antibodies in Plants

Hiatt and colleagues[17] reported expression, folding, and assembly of bioactive full-length heavy- and light-chain monoclonal antibodies in the tobacco plant. In general, the heavy- and light-chain genes are either expressed separately in individual plants or expressed in a single plant. The F1 generation is obtained by cross-pollination of two transgenic plants.[17–19] Another strategy to express both the heavy- and light-chain genes in a single plant is the co-transformation of two gene expression vectors or single-transformation of a single expression vector carrying two gene expression cassettes.[12–14,20] Depending on specific requirements, different strategies can be employed for higher expression levels or establishing transgenic lines within a short period of time. Regardless of the strategies employed, careful selection for expression of regulatory elements, including the promoter and terminator, is essential to avoid unbalanced expression of both light- and heavy-chain genes and their gene silencing.[21] Complete antibodies such as IgG, IgA, and even IgM have been successfully expressed in plants.[12,22,23] Furthermore, multiple monoclonal antibodies can be expressed in a single plant via cross-fertilization between individual transgenic plants that express different target-binding monoclonal antibodies.[24] The chimeric IgG1/IgA and Fab and single-chain variable fragment (scFV) genes have been successfully expressed in plants.[25–29] Native, full-size antibodies of animal origin and recombinant antibodies/fragments expressed in plants differ in their biological activities because of their size and glycan structures. Thus, size-based selection of recombinant antibodies and post-translational modification regulated subcellular targeting, including glycan structures, are key issues in plant-based antibody expression. Diverse forms of antibodies such as full-size, large single-chain, camelid heavy-chain, Fab fragments, scFvs, and biospecific antibodies have been expressed for therapeutic or diagnostic purposes in various plant-based expression systems.[30]

In plants, recombinant therapeutic glycoproteins are properly folded and assembled, which typically includes *N*-glycosylation processing and core glycostructures similar to mammals.[31] Thus, plants are capable of producing human glycoproteins.[32] Recombinant therapeutic antibodies for the treatment of infectious diseases and cancer have been produced in transgenic plant systems (Table 1).[2,33] Glycosylation of antibodies is carried out in a different manner in plant cells than in mammalian cells.[34,35] Glycosylation modifies biological activities of antibodies by regulating stability, CDC, and ADCC, as well as immunogenicity and allergenicity in animals and humans.[32] Nevertheless, antibodies expressed in plants have similar antigen binding as their counterparts expressed in mammalian systems.[33,36]

Table 1. Recombinant Vaccines and Antibodies Expressed in Plants by Dr. Koprowski

Antigen/Antibody	*Host plant*	*Reference*
Rabies virus and HIV-1 virus antigenic peptide	*Nicotiana tabacum*	Yusibov et al., 1997
Rabies virus peptide CPDrg24	*Spinacea oleraceae*	Modelska et al., 1998
Rabies virus peptide CPDrg24	*Nicotiana benthamiana*	Modelska et al., 1998
Rabies glycoprotein and nucleoprotein	*Nicotiana benthamiana*	Yusibov et al., 1998
Rabies glycoprotein and nucleoprotein	*Nicotiana tabacum*	Yusibov et al., 1998
Hepatitis B antigen	*Lupinus luteus*	Kapusta et al., 1999
Hepatitis B antigen	*Lactuca sativa*	Kapusta et al., 1999
Human respiratory syncytial virus vaccine	*Nicotiana tabacum*	Belanger et al., 2000
Rabies peptide	*Glycine max*	Fleysh et al., 2001
Rabies peptide	*Glycine max*	Brodzik et al., 2002
Rabies peptide	*Nicotiana tabacum*	Brodzik et al., 2002
Anti-rabies virus (human antibody)	*Nicotiana tabacum*	Ko et al., 2003
Tumor-associated colorectal cancer antigen	*Nicotiana benthamiana*	Verch et al., 2004
Anti-colon cancer (murine antibody)	*Nicotiana tabacum*	Ko et al., 2005
Severe acute respiratory syndrome (SARS)	*Nicotiana tabacum*	Pogrebnyak et al., 2005
HIV (Type 1) tat protein	*Spinacea oleraceae*	Karasev et al., 2005
Anthrax protective antigen (PA-D4s)	*Nicotiana benthamiana*	Brodzik et al., 2005
Anti-breast cancer (murine antibody)	*Nicotiana tabacum*	Brodzik et al., 2006
Smallpox B5 antigenic domain (pB5)	*Nicotiana tabacum*	Golovkin et al., 2007
Smallpox B5 antigenic domain (pB5)	*Brassica oleracea*	Golovkin et al., 2007
Tumor-associated colorectal cancer antigen	*Nicotiana tabacum*	Brodzik et al., 2008
Hepatitis B core protein	*Nicotiana tabacum*	Bandurska, et al., 2008
Diphtheria-Tetanus-Pertussis (DTP)	*Nicotiana tabacum*	Brodzik et al., 2009
Avian flu H5/HA1 variant antigens	*Nicotiana tabacum*	Spitsin et al., 2009
Anthrax toxin receptor (ATR/CMG2)	*Nicotiana tabacum*	Andrianov et al., 2010
Anti-rabies virus (human antibody)	*Nicotiana tabacum*	Lee et al., 2013

Glycoengineering is an effective tool to knock-out plant-specific glycan transferase genes and knock-in mammalian transferase genes to add sialic acid, fucose, and galactose to the *N*-glycans. Plant production systems can be engineered to be similar to mammalian expression systems in terms of the glycosylation, thus avoiding the concerns of plant-specific glycans.[32] Plants have additional limiting factors such as low level recombinant protein expression. Yield of recombinant antibodies in plants may be improved by several approaches: the choice of plant species to be transformed, the transformation method, codon optimization, design of recombinant gene expression cassette for subcellular localization of recombinant antibodies, choice of specific plant tissues to be harvested, and the timing of biomass harvesting.[33] The localization of accumulated protein to subcellular compartments, such as ER, chloroplast, mitochondria, and vacuole, is important to ensure correct folding and assembly and consequently for protein stability and biofunctionality.[37]

Plant specific tissues including leaf, seed, and root are harvested to purify recombinant therapeutic proteins.[38,39] Generally, antibodies with an Fc region carrying glycosylation sites are targeted to the ER for glycosylation, which ensures avoidance of plant-specific glycan residues, as well as proper folding, assembly, and enhanced accumulation of recombinant antibodies within the ER.[13] Tobacco, alfalfa, and legumes including foliage vegetables can be chosen to express exogenous proteins in fresh plant leaf tissue, whereas corn and rapeseed can be used to accumulate the proteins in dry tissue such as seeds using high protein seed tissue-specific promoters such as the beta-phaseolin promoter of common bean and the oleosin promoter of *Brassica* species.[40–42] In plants, recombinant proteins can be localized to plant subcellular compartments such as the nucleus, plastids, and mitochondria using specific signal peptide sequences in the recombinant gene expression cassette. Transcription and post-translational modification in plants control both expression and harvesting levels of recombinant therapeutic proteins from the plant biomass, which are essential features for a suitable alternative expression system as compared to animal cell–based production systems.[33] Koprowski and his colleagues have achieved marked progress in expression of recombinant vaccines in plants (Table 1).

Expression of Recombinant Vaccines in Plants

Successful plant-derived recombinant vaccines are achievable through harmonization of plant genetic engineering with molecular immunology. There are two administration strategies: intravenous usage of recombinant vaccine protein purified from plant biomass and oral administration of the edible part of plants expressing recombinant vaccine protein without downstream purification processes. There is a concern regarding retention of immunocompetence in recombinant vaccine proteins purified from plant tissues as reflected by levels of specific antibody production in animals. Plant-derived oral vaccines can induce a mucosal and humoral immune response in the intestine. Plant-derived oral vaccines have distinct advantages over traditional vaccination, including lower cost due to convenient storage and easy usage without a needle.[2] Koprowski and colleagues reported successful plant-derived recombinant vaccines for bacterial and viral diseases and cancer (Table 1).

Plant-derived oral vaccines against hepatitis B virus (HBV) and human immunodeficiency viruses (HIV) induce *in vivo* immune responses in animal models.[43] The cholera toxin B subunit (CTB) fused to three copies of tandemly repeated diabetes-associated autoantigen (the B chain of human insulin) expressed in low-nicotine tobacco retain GM1–ganglioside receptor binding specificity, a potentially effective strategy to prevent and treat autoimmune diabetes by inducing oral tolerance.[44] Parenteral immunization with plant-derived recombinant virus-like particles carrying the chimeric hybrid tobacco mosaic virus (TMV) coat protein and a 13 amino acid sequence of the murine zona pellucida ZP3 protein induced an immune response to the ZP3 epitope in mice.[45] In order to optimize plants as bioreactors for recombinant vaccine production, several concerns need to be addressed, including the loss of immunogenicity or degradation in the gastrointestinal microenvironment, risks of unwanted plant biochemical contaminants (such as nicotine and other alkaloids), and low expression levels of recombinant vaccine proteins. For instance, alkaloids can be removed through a purification process prior to usage.[46]

Glycomodification to Avoid Potential Immunogenicity of *N*-glycans in Plant-derived Pharmaceuticals

Both plant and mammalian *N*-glycans share a common core structure, $Man_3GlcNAc_2$.[47] In plants, as in mammals, most secreted proteins are *N*-glycosylated. Depending on which additional sugars are attached to the core structure, plant *N*-glycans can be classified into three groups: oligomannosidic, paucimannosidic, and complex type *N*-glycans.[48]

Oligomannose type *N*-glycans have five to nine mannose residues attached to 2 *N*-acetylglucosamines $(GlcNAc)_2$ in the endoplasmic reticulum (ER), which are highly conserved in both plants and humans. Further, glycosylation processes in the Golgi complex differ between plants and humans. In plants, the α(1,3)-fucose (Fuc) residue is attached to the proximal GlcNAc and/or β(1,2)-xylose (Xyl) and is linked to the β-mannose (Man) residue of the core structures, yielding plant specific mature *N*-glycans in the Golgi complex. In contrast, human *N*-glycans harbor only α(1,6)-Fuc on the core glycan structure without any Xyl residues and with a terminal sialic acid attached to β(1,4)-galactose (Gal) residues on the $GlcNAc_2$.[49,50]

Plant-specific paucimannosidic type *N*-glycans are considered to be typical vacuolar *N*-glycans. They are modified oligosaccharides containing only an α(1,3)-Fuc linked to the GlcNAc and/or a β(1,2)-Xyl attached to the β-Man residue of $Man_3GlcNAc_2$ or $Man_2GlcNAc_2$.[51–54] The maturation of plant N-glycan structures within the Golgi complex differs from human *N*-glycosylation, which hampers production and commercialization of recombinant therapeutic glyoproteins of human origin in plants.

Advancements in glycoengineering of plant biopharmaceutical proteins can lead to increases in the biopharmaceutical market, which is approximately $9–11 billion annually in the United States.[55] The worldwide pharmaceutical market is estimated to grow to $1.3 trillion by the year 2020.[36] In 2005, Koprowski and colleagues[2] emphasized the practice, development, and likely future trend in the plant molecular biopharmaceutical industry.

In plants, unlike in humans, matured *N*-glycan structures carry plant-specific immunogenic β(1,2)-Xyl and α(1,3)-Fuc residues and lack terminal β(1,4)-Gal and sialic acid residues. Thus, glycomodification should include removal of non-mammalian glycan residues and concomitant addition of terminal sialic acid residue, thus increasing immunogenic potential and longevity of proteins in humans, respectively. Knock-out and RNAi approaches can be used to modulate the *N*-glycosylation processing for removal of plant-specific glycan epitomes.[56–60] In addition, a knock-in strategy can be applied to express human β(1,4)-galactosyltransferase for elongation with β(1,4)-galactose in plants, which is essential for carrying sialic acid residues.[35,61] The most terminal glycan residues of mammalian glycoproteins are sialic acids linked to terminal β(1,4)- or β(1,3)-Gal residues.

Terminal sialic acids affect biological activity and longevity of most therapeutic glycoproteins.[62–64] However, plant cells do not carry heterologous enzyme genes for sialylation of glycoproteins.[65,66] Thus, plant *N*-glycomodification to obtain sialic acid residue-terminated glycans is via expression of exogenous enzymes required to catalyze sialic acid synthesis, transport into the Golgi apparatus, and transfer of sialic acid to a β(1,4)-galactosylated glycan. Diverse strategies exist to reduce the potential allergenicity or immunogenicity of plant specific *N*-glycans on recombinant therapeutic proteins.[30] One approach is to eliminate the *N*-glycosylation sites by point mutation of an amino acid, thus preventing glycosylation of the recombinant protein. However, this strategy does not work for therapeutic glycoproteins, which require *N*-glycosylation for *in vivo* stability and biological activities. Gomord and colleagues[30] have reported that the addition or removal of *N*-glycan residues from several therapeutic recombinant proteins improves their half-life and biological activity.

The second approach is to retain recombinant proteins in the ER before transferring them to the Golgi complex where plant-specific glycan structures mature. Recombinant proteins retained in the ER contain oligomannose-type *N*-glycans, which are commonly found in both plants and mammals, and thus are probably not immunogenic in humans or animals.[57,67–71] Protein localization to the ER of eukaryotic cells can be achieved via the addition of the retention motif: KDEL or HDEL to the C-terminal end of a secretory protein.[56] In tobacco plants, antibodies containing KDEL sequences fused to the C-terminal end of their heavy chains were oligomannose *N*-glycans, with six to nine mannose residues (90%).[12] The antibody fused with KDEL retention signals on both the heavy and light chains were 100% oligomannose *N*-glycans.[72] Koprowski and colleagues reported that plant-derived antibodies with oligomannose *N*-glycans (90%) were less stable compared to their human counterparts in mice, indicating that oligomannose *N*-glycan structures can affect their biological properties.[12] The *in vivo* half-life of antibodies with the oligomannosidic groups in mice was likewise reduced relative to mammalian-derived antibodies, probably due to endocytosis and degradation following binding to mannose receptors, as previously observed for oligomannose glycosylated antibodies expressed in CHO cells.[73,74] The rapid disappearance of the antibody can reduce both active and passive immunity in humans, as in the interference between vaccine and antibody application for rabies prophylaxis.

The third approach is the inhibition of Golgi glycosyltransferase expression using knockout mutants, preventing the synthesis and maturation of plant-specific complex *N*-glycans. The *Arabidopsis thaliana cgl* mutant deficient in the glycosyltransferase I can accumulate mainly oligomannose structures $Man_5GlcNAc_2$ carrying terminal GlcNAc residues on both α(1,3)- and α(1,6)-linked Mans without the allergenic and immunogenic plant specific α(1,3)-Fuc and β(1,2)-Xyl.[57,75] In general, the glycosyltransferase deficiency is lethal in mice.[76] However, the knockout plant mutants show no adverse impact on plant development or morphology. This glycan structure has the advantage of easily adding Gal and sialic acid residues to the terminal GlcNAc for humanization of *N*-glycosylation in plants. Indeed, expression of the genes encoding transferases for galactose and sialic acid residues was attempted in *Arabidopsis thaliana* producing anti-HIV virus antibodies.[77] *N*-glycosylation analysis revealed terminal sialic acids attached to the Gal with the absence of α(1,3)-Fuc and β(1,2)-Xyl residues in the plant-derived antibody. Evidently, plants can be a suitable alternative expression system for therapeutic glycoproteins with human-like glycosylation patterns.

Koprowski and his colleagues at the Biotechnology Foundation Laboratories at Thomas Jefferson University and The Wistar Institute were the major group to introduce plants as an alternative platform for the production of functional therapeutic proteins, mainly due to the rapid progress in plant biotechnology and genetic engineering enabling human-like glycosylation patterns in therapeutic glycoproteins. The advantages of plant-based production systems over animal-based ones for therapeutic and prophylactic biopharmaceuticals include lower production costs, reduced production time and effort to obtain plant biomass, lack of mammalian pathogen contaminants, and ease of scalability.[1,33,78–80]

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Address correspondence to:
Prof. Kisung Ko
Department of Medicine
College of Medicine
Chung-Ang University
Seoul, 156-756
Korea

E-mail: ksko@cau.ac.kr