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(54) Title: COMPOSITIONS AND METHODS FOR PRODUCING POLYPEPTIDES WITH A MODIFIED GLYCOSYLATION PATTERN IN PLANT CELLS

(57) Abstract: A method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell is provided. The method comprising expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that the at least one glycosidase and the polypeptide-of-interest are co-localized to the subcellular compartment of the plant or plant cell, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

COMPOSITIONS AND METHODS FOR PRODUCING POLYPEPTIDES WITH A MODIFIED GLYCOSYLATION PATTERN IN PLANT CELLS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to compositions and methods for producing polypeptides with a modified glycosylation pattern in plant cells.

Plants have great potential as hosts for the production of mammalian therapeutic proteins including enzymes, growth factors, structural protein such as collagen, chimeric proteins such as Enbrel and multimeric proteins such as antibodies.

The benefits of using plants for the production of recombinant pharmaceuticals include large scale production, reduced costs for production, maintenance and delivery as well as eliminating the risk of the resultant product containing possibly harmful contaminants such as viruses or prions that are pathogenic to humans and other mammals. Plants, like other heterologous expression systems including mammalian cells, bacteria, yeast, and insects, exhibit differences in glycosylation.

In plants, as in other eukaryotes, most of the soluble and membrane bound proteins that are synthesized on polyribosomes associated with the endoplasmic reticulum (ER) are glycoproteins, including those proteins which will later be exported to the Golgi apparatus, lysosomes, plasma membrane or extracellular matrix. The glycans attached to glycoproteins contain a variety of sugar residues linked in linear or branched structures that can assume many different conformations. These glycans can play a fundamental role in promoting correct protein folding and assembly and, as a consequence, enhance protein stability. They may also contain targeting information, or may be directly involved in protein recognition. The three main posttranslational modifications of proteins that involve carbohydrates are N- and O-linked glycosylation and the insertion of glycosyl phosphatidyl inositol anchors.

The N-linked glycosylation mechanisms in mammalian and plant systems have been conserved during evolution. However, differences are observed in the final steps of oligosaccharide trimming and glycan modification in the Golgi apparatus. In contrast to bacteria, having no N-linked glycans, and yeast, having polymannose glycans, plants produce glycoprotein multimers with complex N-linked glycans having a core substituted by two N-acetylglucosamine (GlcNAc) residues. These glycoprotein multimers are also observed in mammals. See, for example, Kornfeld and Kornfeld,

Ann. Rev. Biochem. 54:631 (1985). Plant and animal glycopolypeptide multimers contain different terminal carbohydrates that are directly linked to the outer branches of the oligosaccharides present. Animal glycopolypeptide multimers, including mammalian glycopolypeptide multimers, have sialic acid present as a terminal carbohydrate residue, while plant glycopolypeptide multimers do not. The terminal core is substituted by β 1,2-linked xylose (Xyl) and α 1,3-linked core fucose (Fuc) instead of α 1,6-linked core fucose as occur in mammals. Furthermore, plant glycoproteins lack the characteristic galactose (Gal)- and sialic acid-containing complex N-glycans (N-acetylneuraminic-α-2-6/3Gal β 1-4) found in mammals.

Plant-derived recombinant proteins hold a risk of severe immunogenicity due to the presence of the foreign sugar residues, i.e., α-1,3 fucose and β-1,2 xylose residues. In order to reduce immunogenicity, a number of platform technologies have been developed, some are described in Naoko Yamane-Ohnuki and Mitsuo Satoh MAbs. 2009 May-Jun; 1(3): 230–236, Strasser et al., Plant Biotechnology Journal (2008) 6, pp. 392–402; Matsuo et al., Journal of Bioscience and Bioengineering (2014) 118, 4, pp. 448-454; Matsuo Plant Biotechnol. J., 9, 264-281 (2011), as well as in US 20030159178, US 20120079627, 20070089201 and WO 01/29242.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell, the method comprising expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that the at least one glycosidase and the polypeptide-of-interest are co-localized to the subcellular compartment of the plant or plant cell, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a method of producing a polypeptide-of-interest, the method comprising:

(a) expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that the at least one glycosidase and the polypeptide-of-

interest are co-localized to the subcellular compartment of the plant or plant cell; and subsequently

(b) isolating the polypeptide-of-interest.

According to some embodiments of the invention, the plant or plant cell transformed to express at least one glycosidase in the subcellular compartment further comprises reduced level or activity of at least one glycosyl transferase as compared to a plant or plant cell of the same species expressing wild-type levels or exhibiting wild-type activity of the at least one glycosyl transferase.

According to some embodiments of the invention, the glycosyl transferase comprises Beta-(1-2)-xylosyltransferase and/or Alpha-(1, 3)-fucosyltransferase.

According to some embodiments of the invention, the plant or plant cell transformed to express at least one glycosidase in the subcellular compartment further comprises a nucleic acid sequence encoding a fusion polypeptide comprising a cell wall binding peptide translationally fused to an affinity moiety for binding the polypeptide of interest.

According to an aspect of some embodiments of the present invention there is provided an isolated polypeptide comprising a cell wall binding peptide translationally fused to a heterologous affinity moiety.

According to some embodiments of the invention, the cell wall binding peptide is a cellulose binding domain (CBD).

According to some embodiments of the invention, the affinity moiety is for binding an antibody.

According to some embodiments of the invention, the affinity moiety is for binding an enzyme growth factor or structural protein.

According to some embodiments of the invention, the affinity moiety for binding the antibody comprises protein A/G/L.

According to some embodiments of the invention, the isolated polypeptide is as set forth in SEQ ID NO: 10.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding the polypeptide.

According to some embodiments of the invention, the isolated polynucleotide is as set forth in SEQ ID NO: 9.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising the isolated polynucleotide and a cis-acting regulatory element for directing expression of the polypeptide in a plant cell.

According to some embodiments of the invention, the nucleic acid construct comprises an additional nucleic acid sequence encoding at least one glycosidase.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell comprising the polynucleotide of nucleic acid construct described herein.

According to some embodiments of the invention, the transgenic plant or plant cell is transformed to express as least one glycosidase in a subcellular compartment.

According to some embodiments of the invention, the transgenic plant of plant cell comprises reduced level or activity of at least one glycosyl transferase as compared to a plant or plant cell of the same species expressing wild-type levels or exhibiting wild-type activity of the at least one glycosyl transferase.

According to some embodiments of the invention, the glycosyl transferase comprises Beta-(1-2)-xylosyltransferase and/or Alpha-(1, 3)-fucosyltransferase.

According to an aspect of some embodiments of the present invention there is provided a method of producing a transgenic plant or plant cell, the method comprising expressing in the plant or plant cell at least two glycosidases such that the at least two glycosidases are co-localized to a subcellular compartment of the plant or plant cell.

According to some embodiments of the invention, the expressing the at least two glycosidases comprises:

(a) expressing a first glycosidase of the at least two glycosidases in the subcellular compartment of a first plant;

(b) expressing a second glycosidase of the at least two glycosidases in the subcellular compartment of a second plant; and

(c) crossing the first plant and the second plant.

According to some embodiments of the invention, the expressing the at least two glycosidases comprises:

(i) introducing into the plant or plant cell a nucleic acid construct comprising a nucleic acid sequence encoding the at least two glycosidases, wherein each of the at least two glycosidases is translationally fused to a signal peptide for co-localization in the subcellular compartment of the plant or plant cell; or

(ii) introducing into the plant or plant cell a nucleic acid construct system comprising:

a first nucleic acid construct comprising a nucleic acid sequence encoding a first glycosidase;

a second nucleic acid construct comprising a nucleic acid sequence encoding a second glycosidase,

wherein each of the first glycosidase and the second glycosidase is translationally fused to a signal peptide for co-localization in the subcellular compartment of the plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a method of producing a transgenic plant or plant cell, the method comprising expressing in the plant or plant cell at least one glycosidase and an affinity moiety to a polypeptide-of-interest, wherein the affinity moiety is translationally fused to a cell wall binding peptide.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding at least one glycosidase;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding an affinity moiety to a polypeptide-of-interest,

wherein the affinity moiety is translationally fused to a cell wall binding peptide.

According to some embodiments of the invention, the expressing the nucleic acid sequence encoding the polypeptide-of-interest comprises crossing:

(i) a first transgenic plant transformed to express the at least one glycosidase; and

(ii) a second transgenic plant transformed to express the polypeptide of interest.

According to some embodiments of the invention, the first plant is transformed to express an affinity moiety translationally fused to a cell wall binding peptide, wherein the affinity moiety is for binding the polypeptide of interest.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding at least two glycosidases, wherein each of the at least two glycosidases is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a first glycosidase of at least two glycosidases;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding a second glycosidase of the at least two glycosidases,

wherein each of the first glycosidase and the second glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to some embodiments of the invention, the signal peptide is a vacuolar signal peptide or an apoplast signal peptide.

According to some embodiments of the invention, the signal peptide is a vacuolar signal peptide or an apoplast signal peptide fused at an N-terminus of the first glycosidase and the second glycosidase.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell transformed to express at least two glycosidases in a subcellular compartment in a co-localized manner.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest and at least one glycosidase, wherein each of the polypeptide of interest and the at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding and at least one glycosidase,

wherein each of the at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a glycosidase translationally fused to a signal peptide for localization in a subcellular compartment of interest.

According to an aspect of some embodiments of the present invention there is provided an isolated polypeptide comprising an amino acid sequence of a protein A/G/L tranlationally fused to a heterologous transmembrane domain.

According to some embodiments of the invention, the tranlationally fused is via a linker.

According to an aspect of some embodiments of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding the polypeptide.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell comprising the nucleic acid construct as described herein.

According to an aspect of some embodiments of the present invention there is provided a method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell, the method comprising introducing into a plant or plant cell the nucleic acid construct or the nucleic acid construct system as described herein, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a method of producing a polypeptide-of-interest, the method comprising:

(a) introducing into a plant or plant cell the nucleic acid construct or the nucleic acid construct system as described herein; and subsequently

(b) isolating the polypeptide-of-interest.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell recombinantly expressing:

(i) a polypeptide of interest; and

(ii) at least one glycosidase

wherein each of the polypeptide of interest and the at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of the plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell comprising the nucleic acid construct or nucleic acid construct system as described herein.

According to some embodiments of the invention, the at least two glycosidases comprise a fucosidase and a xylosidase.

According to some embodiments of the invention, the at least one glycosidase is selected from the group consisting of a fucosidase and a xylosidase.

According to some embodiments of the invention, the subcellular compartment is selected from the group consisting of a vacuole, an apoplast, an endoplasmic reticulum and golgi.

According to some embodiments of the invention, the subcellular compartment is a vacuole.

According to some embodiments of the invention, the plant or plant cell is a tobacco plant or plant cell.

According to some embodiments of the invention, the plant cell is a root cell.

According to some embodiments of the invention, the signal peptide is selected from the group consisting of a vacuolar targeting signal, an endoplasmic targeting signal, an apoplast targeting signal, a mitochondria targeting signal and a plastid targeting signal.

According to some embodiments of the invention, the plant or plant cell transformed to express at least one glycosidase in the subcellular compartment, is further transformed to express an additional glycosidase in the subcellular compartment.

According to some embodiments of the invention, the signal peptide is translationally fused at a C-terminus of the polypeptide of interest or the glycosidase.

According to an aspect of some embodiments of the present invention there is provided an isolated polypeptide produced according to the method described herein.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a glycosidase translationally fused to a signal peptide for localization in a subcellular compartment of interest.

According to an aspect of some embodiments of the present invention there is provided a transgenic plant or plant cell comprising the nucleic acid construct as described herein.

According to some embodiments of the invention, the polypeptide-of interest is a human polypeptide.

According to some embodiments of the invention, the polypeptide-of interest is a pharmaceutical.

According to some embodiments of the invention, the polypeptide-of interest is selected from the group consisting of an antibody, a vaccine, an enzyme, a growth factor, a hormone and a structural protein.

According to some embodiments of the invention, the polypeptide-of interest is an antibody or an antibody fragment.

According to some embodiments of the invention, the antibody is bevacizumab or adalimumab.

According to an aspect of some embodiments of the present invention there is provided a seed of the transgenic plant described herein.

According to some embodiments of the invention, the seed is a hybrid seed.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the

drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-B are schematic illustrations showing cloning of the light and heavy chains of Avastin and Humira in the Rubisco-vac cassette in pUC18 vector. Figure 1A - The DNA fragments encoding the heavy and light chains of the mAbs (SEQ ID NOs: 1, 3, 5 and 7) and pUC18 plasmid bearing the Rubisco-vac expression cassette were restricted by MunI and NotI. Figure 1B - Four different pUC18 plasmids were created: pUC18 Rb-Humira heavy chain, pUC18 Rb-Humira light chain, pUC18 Rb-Avastin heavy chain and pUC18 Rb-Avastin light chain.

FIGs. 2A-C are schematic illustrations of the cloning of pBINPLUS-Humira coding the light chain and heavy chain on a single plasmid. Figure 2A - Rubisco-vac cassette with the Humira light chain was cloned to pBINPLUS vector with HindIII restriction enzymes. Figure 2B - Rubisco-vac cassette with the heavy chain of Humira was cloned to pBINPLUS Rb-Humira light chain vector with EcoRI and SacI enzymes. Figure 2C – the final construct of pBINPLUS Humira.

FIGs. 3A-C are schematic illustrations of the cloning of pBINPLUS-Avastin coding the light chain and heavy chain on a single plasmid. Figure 3A - Rubisco-vac cassette with the Avastin heavy chain was cloned to pBINPLUS vector with HindIII restriction enzymes. Figure 3B - Rubisco-vac cassette with the light chain of Avastin was cloned to pBINPLUS Rb-Avasin heavy chain vector with EcoRI and SacI enzymes. Figure 3C - the final construct of pBINPLUS Avastin.

FIGs. 4A-B are schematic illustrations showing the construction of the Humira double cassette in pUC18. The synthetic DNA fragment (SEQ ID NO: 25) including Rubisco terminator, Vacuolar SP1 (SEQ ID NO: 18), Humira heavy chain (SEQ ID NO: 15), Rubisco promoter, Vacuolar SP2 (SEQ ID NO: 19), Humira light chain (SEQ ID NO: 16) was cloned into Rubisco cassette by NcoI, NotI in pUC18 creating double cassette with both mAb chains.

FIGs. 5A-C are schematic illustrations showing cloning of CBD-PrtA (SEQ ID NO: 9) into pBINPLUS plasmid. Figure 5A - DNA including 35S promoter, a coding

region of vacuole signal, CBD and proteinA and Nos terminator was cloned to pUC18 plasmid. Figure 5B - 35S cassette with CBD-PrtA was cloned to pBINPLUS plasmid by EcoRI and pBINPLUS CBD-PrtA was created (Figure 5C).

FIGs. 6A-C are schematic illustrations showing cloning of Xylosidase and Fucosidase into pBINPLUS plasmid. Figure 6A - DNA encoding xylosidase (2344 bp, SEQ ID NO: 11) or fucosidase (1564 bp, SEQ ID NO: 13) was restricted by MunI and NotI and cloned into 35S cassette after CBD-PrtA was cut out by the same enzymes. Figure 6B - Xylosidase or Fucosidase in 35S cassette was cloned in pBINPLUS vector using SdaI and SacI restriction enzymes. Figure 6C - Two plasmids were constructed: pBINPLUS Xylosidase (15496 bp) and pBINPLUS Fucosidase (14716 bp).

FIGs. 7A-B are Western blot images showing that CBD-PrtA (SEQ ID NO: 10) is expressed in the tobacco plants. Figure 7A shows screening recombinant tobacco by Western blot with anti-CBD antibody. Figure 7B – shows a Slot blot: the amount of commercial Humira is indicated was added to the pellet from 100 mg of WT and CBD-PrtA expressing tobacco tissue. After the pellet was incubated and washed for several times, the antibody was eluted by a mild acid and applied on the nitrocellulose membrane. The mAb was detected by anti-human IgG-AP.

FIG. 8 is a bar graph showing glycosidases activity measured in tobacco plants expressing the Fucosidase (left side) and Xylosidase (right side). The product of the enzymatic reaction (4-methylumbelliferone) was measured at pH 10. Released 4-methylumbelliferone is measured using an excitation wavelength of 355 nm with emission at 460 nm.

FIG. 9 is an image showing stable expression and purification of adalimumab expressed in the apoplast of tobacco plant.

FIG. 10 is a graph showing TNF- binding by adalimumab which was expressed in the apoplast of tobacco plant and purified therefrom, as tested by ELISA assay.

FIGs. 11A-B are schematic illustrations showing construction of the expression cassettes encoding light and heavy chains of the indicated antibodies with a Cell apoplast signal peptide. The mAb (Avastin/Humira) chains were inserted into the expressing cassette including Rubisco promoter, Cell signal peptide and Rubisco terminator. Resultant constructs are: pBINPLUS Rubisco Cell humira heavy chain, pBINPLUS Rubisco Cell humira light chain, pBINPLUS Rubisco Cell avastin heavy

chain, pBINPLUS Rubisco Cel1 avastin light chain. The cassettes with the heavy and the light chain of the same mAb were co-transformed into tobacco plants to get the expression of the full mAb in the apoplast.

FIGs. 12A-D are schemes and purification results using a Transmembrane bound protein A expressed on the cell membrane of cells expressing the polypeptide of interest of some embodiments of the invention.

FIG. 13 shows xylose and fucose excision of plant derived Adalimumab. Abbreviation: 2h, 3h and 4h - treatment length in hours (2,3 and 4 hours respectively); N – not treated Adalimumab; C – commercial Humira. 3 different sets of antibodies: Anti-Xylose, Anti-Fucose and Anti-Human IgG were used for detection.

FIG. 14 shows a Western blot performed with anti-Human IgG showing bands at approximately 55 KDa corresponding to adalymumab Heavy chain and at approximately 25 KDa corresponding to adalymumab Light chain.

FIG. 15 shows the results of an ELISA assay for the in-vitro bioactivity of plant derived Adalimumab (PDA) performed on TNF-α pre-coated ELISA plates that were incubated with plant derived adalimumab from 3 (1-3) different transgenic tobacco plant lines. Binding of the mAb to the target was then detected by using anti-human IgG-HRP. PDA average concentration shown in ng (mAb)/mg (fresh leaves).

FIG. 16 shows a TNFa neutralization using PDA as compared to Commercial Humira (shown in circles, Test Reference) VS. Plant derived adalimumab (shown in squares, Test Item) bioactivity of neutralization of rhTNF-α was tested in L929 cell line.

FIGs. 17A-C show the results of SDS-PAGE Western Blotting. Figure 17A - anti protein A staining; Figure 17B - anti human IgG staining of pellet; Figure 17C - anti human IgG staining of soluble fraction. Com - commercial Humira control; PDA - plant derived adalimumab. Sample preparation was made in 2 different buffers: Binding buffer and Grinding buffer.

FIGs. 18A-D show the cloning of GMD RNAi into pUC18 plasmid. Figure 18A - pUC18 plasmid containing 35S promotor, Cel1 signal peptide, gene insert that will be replaced by GMD RNAi encoding DNA and Nos terminator; Figure 18B - Step 1: GMD anti-sense encoding DNA (423 bp) was inserted by restriction with NotI and BamHI; Figure 18C - Step 2: β-Xylose Transferase (XylT) intron encoding DNA (242

bp) was inserted by restriction with BamHI and MfeI; Figure 18D - Step 3: GMD sense encoding DNA (442 bp) was inserted by restriction with MfeI and NcoI.

FIGs. 19A-B show the cloning of the GMD RNAi. Figure 19A. pBINPLUS vector; 35S GMD RNAi cassette (1747 bp) was cloned using HindII and SacI restriction enzymes to form Figure 19B. pBIN 35S GMD RNAi plasmid (14094 bp).

FIGs. 20A-D show the cloning of XylT into pBINPLUS plasmid. Figure 20A., pUC18 plasmid RUBISCO promotor, Cel1 signal peptide, Adalimumab Heavy Chain encoding DNA and RUBISCO terminator; Figure 20B., Replacing Adalimumab Heavy Chain encoding DNA (1362 bp) with XylT (617 bp) by NcoI and NotI restriction enzymes; Figure 20C., pBINPLUS plasmid; Figure 20D., RUBISCO XylT cassette (2569 bp) was cloned in pBINPLUS vector using HindII restriction enzyme to form pBINPLUS RUBISCO XylT plasmid (14965 bp).

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to compositions and methods for producing polypeptides with a modified glycosylation pattern in plant cells.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Plants are attractive hosts for the production of recombinant pharmaceuticals by avoiding the risk of animal-derived viral infection and cost-effectiveness of biopharmaceutical production. Higher plants have similar N-glycosylation pathways compared to mammals, and mainly generate complex-type glycans. with an α-1,3 fucose residue attached to the innermost GlcNAc, a β-1,2 xylose residue attached to the junction mannose of the tri-mannosyl core, neither of which is found in humans. The immunogenicity of the non-human glycosylation, α-1,3 fucosylation and β-1,2 xylosylation, is of concern to regulatory authorities.

Hence, an industrially applicable protein production process that provides consistent yields of fully non-fucosylated and/or non-xylosylated protein therapeutics with fixed quality has become a key goal in the successful development of next-generation therapeutic agents.

The present inventors now offer a novel platform for protein production in plant cells in which the recombinant polypeptide of interest is expressed such that it co-localizes with at least one glycosidase to a subcellular compartment of the plant cell. The polypeptide thus produced carries no α-1,3 fucose or β-1,2 xylose on N-glycans. The process is simple and cost-effective since, it does not require post production processing by exposing the expressed polypeptide to in vitro enzymatic processing. A further advantage of this process is in its directed nature, that is, the plant's glycosylation machinery is unaffected and hence the plants vigor and viability are uncompromised.

As is illustrated hereinbelow and in the Examples section which follows, the present inventors have implemented this platform for the production of two FDA-approved monoclonal antibodies, Avastin® (bevacizumab) and Humira® (adalimumab). The present inventors have co-expressed these antibodies in the vacuole or apoplast of tobacco to-colocalize with recombinantly expressed xylosidase and fucosidase and showed elevated levels of glycosidase activity in these plants.

Specifically, quantification of apoplast targeted adalimumab, also referred to herein as a specific configuration plant-derived Adalimumab (PDA) as assayed by ELISA showed that 4.9 mg PDA/kg leaves was obtained. Both heavy and light chain antibody subunits were detected by Western Blotting (WB), and at the correct ratio. The plants were F1 generation of the transformed plant, and it is expected that the yields are substantially increased by homozygotization.

CBD-Protein A based purification of plant derived Adalimumab from a double transgenic plant expressing both proteins was proven to be feasible. Suitable reaction conditions were found in which both CBD binds cellulose and protein A binds the antibody Fc region. The antibody is therefore effectively maintained in cellulose containing the insoluble fraction immediately after the plant tissue grinding stage. Binding is strong enough to allow washing of the insoluble fraction bound protein without losses.

Expression of recombinant fucosidase and Xylosidase successfully removed the xylose and Fucose residues from the recombinant antibody e.g., Adalimumab. Increased reduction of Fuc and Xyl concentration was observed when both Xylosidase and fucosidase were applied together.

Overall, no significant difference was detected between the test (adalimumab) and reference (Humira) antibodies. The adalimumab activity assay showed that up to a concentration of 62.5 ng/ml, cells protected by Humira were slightly more viable than cells protected with PDA (not significant). When the concentration was further elevated to 125 and afterwards to 250 ng/ml the plant adalimumab appeared to give better results.

Thus according to as aspect of the invention there is provided a method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell, the method comprising expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that said at least one glycosidase and the polypeptide-of-interest are co-localized to said subcellular compartment of the plant or plant cell, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

As used herein the term "modifying" refers to changing the native post-translational (in-vivo) glycosylation of a polypeptide as compared to same when expressed in a plant cell which comprises a wild-type glycosylation pathway.

According to a specific embodiment, modifying refers to a reduced or complete elimination of at least one glycoside species, e.g., β-1,2-linked xylose (Xyl) or α-1,3-linked core fucose (Fuc). Reduced glycoside species refers to at least 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % or even 100 % (i.e., complete elimination) of the glycoside species from the polypeptide of interest following in vivo expression as described herein.

As used herein, the term "glycoside" refers to any compound containing a carbohydrate molecule (sugar), particularly any such natural product in plants, convertible by hydrolytic cleavage, into a sugar and a non-sugar component.

According to a specific embodiment, the glycoside comprises β-1,2-linked xylose (Xyl) or α-1,3-linked core fucose (Fuc).

Thus according to a specific embodiment, modifying the glycosylation pattern results in a polypeptide which lacks Fuc or Xyl, also termed as unfucosylated or unxylosylated polypeptide, respectively.

According to another specific embodiment, modifying the glycosylation pattern results in a polypeptide which lacks Fuc and Xyl, also termed as unfucosylated and unxylosylated polypeptide, respectively.

According to another embodiment, modifying may also comprise the post-translation processing of the polypeptide to include glycoside species which are absent from plant cells such as characteristic galactose (Gal)- and sialic acid-containing complex N-glycans (N-acetylneuraminic-α-2-6/3Gal β 1-4).

A "glycosylation pattern" refers to a single (e.g., Fuc) or a plurality of glycoside species (e.g., Fuc, Xyl and optionally sialic acid or galactose) and their relative abundance on the polypeptide or preparation thereof.

The term '"plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers and root stocks), and plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantee, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising Acacia spp., Acer spp., Actinidia spp., Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chacoomeles spp., Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Dibeteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehraffia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalypfus spp., Euclea schimperi, Eulalia vi/losa, Pagopyrum spp., Feijoa sellowlana, Fragaria spp., Flemingia spp, Freycinetia banksli, Geranium thunbergii, GinAgo biloba, Glycine javanica, Gliricidia

spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemaffhia altissima, Heteropogon contoffus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hypeffhelia dissolute, Indigo incamata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesli, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago saliva, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativam, Podocarpus totara, Pogonarthria fleckii, Pogonaffhria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys vefficillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp., Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, Brussels sprouts, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash tea, trees. Alternatively algae and other non-Viridiplantae can be used for the methods of some embodiments of the invention.

According to a specific embodiment the plant or plant cell is a tobacco plant or plant cell (e.g., *N. tabacum* and *N. benthemiana*).

According to a specific embodiment the plant cell is a root cell such as selected from the group consisting of Agrobacterium rihzogenes transformed root cell, celery cell, ginger cell, horseradish cell and carrot cell.

According to a specific embodiment the plant or plant cell is a duckweed plant or plant cell (e.g., lemna).

According to other specific embodiments, the plant or plant cell is of a, maize, alfalfa, Arabidopsis, tomato, collard, lettuce, tobacco, soybean, rice and potato.

As used herein the term "glycosidase" refers to an enzyme which cleaves O, S or N-linked glycosyl compounds e.g., E.C. 3.2.1 e.g., mannosidase, fucosidase and xylosidase.

The enzyme may be naturally occurring (e.g., plant, bacterial or fungal) or synthetic.

As used herein the term "fucosidase" refers to EC 3.2.1.111 1,3-α-L-fucosidase.

According to a specific embodiment, the alpha-1,3/4-fucosidase [Streptomyces sp.] is Sequence ID: gb|AAD10477.1| (SEQ ID NOs: 13,14).

As used herein the term "xylosidase" refers to Beta (1-2) Xylosidase (®-D-xylanxylohydrolase, EC 3.2.1.37) cleaves xylose linked β (1-2). According to a specific embodiment, the enzyme is exo-1,4-beta-xylosidase xlnD [Aspergillus niger CBS 513.88] Sequence ID: ref|XP_001389416.1| (SEQ ID NO: 11,12).

According to a specific embodiment, the plant cell is transformed with the at least one glycosidase (e.g., at least two glycosidase i.e., non-identical, wherein each glycosidase is directed at a different glycosyl compound e.g., α-1,3 Fuc and β-1,2 Xyl).

Thus, according to an aspect of the invention, there is provided a method of producing a transgenic plant or plant cell, the method comprising expressing in the plant or plant cell at least one glycosidase in a subcellular compartment or at least two glycosidases, in the latter case the at least two glycosidases are co-localized to a subcellular compartment of the plant or plant cell.

As used herein the term "subcellular compartment of a plant cell" refers to any compartmentalized region of the cell in which the polypeptide of interest can accumulate, such as, as an end product. According to a specific embodiment, the subcellular compartment is of the endomembrane system. Examples of subcellular compartments include, but are not limited to, the vacuole, apoplast, endoplasmic reticulum (ER), golgi, protein bodies derived from the ER and the vacuole, as well as oil bodies. According to a specific embodiment, the proteins are accumulated in the subcellular organelle following (e.g., apoplast, oil bodies) or concomitantly (e.g., ER, golgi and vacuole) with post-translational processing (i.e., glycosylation).

Of note, the selection of the sub-cellular compartment will much depend on the type of polypeptide and activity of the end-product.

For example, human collagen production in plant cells requires hydroxylation on prolines by the human enzyme, to ensure activity of the end product. WO2006/035442 teaches co-expression of the collagen and prolyl-4-hydroxylase (P4H) in a subcellular compartment such as the vacuole or apoplast. In such a case, the glycosidase (e.g., fucosidase and/or xylosidase) is expressed in the vacuole or apoplast as well, to ensure co-localization with the expressed collagen.

In an alternative example, mannose-terminated glycans are thought to be the dominant complex glycans of vacuolar glycoproteins and are considered pertinent for the activity of lysosomal proteins facilitating improved uptake and lysosomal delivery of the proteins administered to the patients (see e.g., WO2004/096978). In such a case the glycosidase (e.g., fucosidase and/or xylosidase) is expressed in the vacuole as well, to ensure co-localization with the expressed polypeptide (e.g., high mannose protein, e.g., lysosomal protein).

According to a specific embodiment, accumulation of the glycosidase (and the polypeptide of interest, hereinafter "the proteins") in a subcellular compartment is achieved by the inclusion of a signal sequence for targeting the expressed protein to a subcellular compartment such as the vacuole, endoplasmic reticulum, golgi, mitochondria and apoplast.

A signal peptide, signal sequence, localization sequence or a sorting sequence (all interchangeably used) is a nucleotide sequence, translated to give an amino acid sequence, which is used by a cell to direct the protein or polypeptide of interest to be placed in a particular place within or outside the eukaryotic cell. Many signal sequences are known in the art. See, for example Becker et al., Plant Mol. Biol. 20:49 (1992), Close, P. S., Master's Thesis, Iowa State University (1993), Knox, C., et al., "Structure and Organization of Two Divergent Alpha-Amylase Genes from Barley", Plant Mol. Biol. 9:3-17 (1987), Lerner et al., Plant Physiol. 91:124-129 (1989), Fontes et al., Plant Cell 3:483-496 (1991), Matsuoka et al., Proc. Natl. Acad. Sci. 88:834 (1991), Gould et al., J. Cell. Biol. 108:1657 (1989), Creissen et al., Plant J. 2:129 (1991), Kalderon, et al., A short amino acid sequence able to specify nuclear location, Cell 39:499-509 (1984), Steifel, et al., Expression of a maize cell wall hydroxyproline-rich glycoprotein gene in early leaf and root vascular differentiation, Plant Cell 2:785-793 (1990).

According to a specific embodiment the signal sequence is heterologous to the protein.

As used herein "translational fusion" refers to an in-frame fusion of the nucleic acid sequence(s) encoding the targeting sequence and the nucleic acid sequence encoding the protein (i.e., glycosidase or the polypeptide-of-interest) such that a single polypeptide is expressed which comprises both the targeting sequence(s) as well as the protein. The in-frame fusion may be a direct fusion or via a linker (i.e., a nucleic acid sequence encoding an amino acid linker). The linker and/or the signal peptide may be cleavable.

According to a specific embodiment, the proteins (e.g., glycosidase(s) and polypeptide of interest) are expressed in an endomembrane system, which includes the endoplasmic reticulum (ER), the vacuole, and protein bodies derived from ER or vacuoles.

For expression in the endomembrane system the proteins comprise (by translational fusion) an N-terminal signal peptide which is critical for the entry of secreted proteins and all luminal proteins that are subsequently trafficked to the various endomembrane compartments. N-terminus signal peptides are typically interchangeable. The signal is not a defined sequence but rather a pattern or motif that typically comprises one or more positively charged amino acid residues at the N-terminus, followed by a stretch of 6-12 hydrophobic amino acids and a cleavage site. This signal peptide is typically 20-30 amino acids long. Signal peptide prediction tools and databases are publicly available www(dot)cbs(dot)dtu(dot)dk/services/SignalP/; links cited in www(dot) signalpeptide(dot)de/index.php?m=links) to identify putative signal peptides and the signal peptide cleavage site.

For expression of heterologous proteins, in this case e.g., the polypeptide-of-interest and the glycosidase, plant-specific signal peptides (SPs) are often used. Common plant signal sequences include the signal peptides from tobacco extensin, PR-S, and osmotin, the barley α-amylase SP and the potato patatin SP.

In many cases, the signal peptide from the heterologous protein (e.g., a human polypeptide-of-interest) efficiently targets its protein to the plant ER and is recognized by the plant signal peptidase to create the precise N-terminus of the matural product seen in its native organism, such as human IL-2, interferon-β, and β-casein; fungal phytase;

and xylanase. However, it is possible to enhance expression at the subcellular localization of interest by the use of a plant signal peptide.

Accumulation in the ER

A specific protein motif is typically required to retain proteins within the ER. Exemplary sequences include the most widely used motif, KDEL, SEKDEL or HDEL, all being ER retention motifs. Proteins having C-terminal KDEL or HDEL interact with the KDEL receptor, a transmembrane protein that functions in vesicular trafficking primarily between the ER and the Golgi.

Accumulation in ER-derived protein bodies (PBs)

Proteins directed to the ER may either retain in the ER or bud-off into discrete organelles. Proteins stored in ER-derived PBs versus vacuole-derived PBs differ in their glycan composition (routing through the golgi facilitates processing of high-mannose N-glycans to complex glycans).

Exemplary signals for ER-derived PBs include, the proline-rich N-terminal domain of the γ-zein (maize storage protein), which includes a highly repetitive sequence (VHLPPP)n that forms an amphipathic polyproline helix and is critical for zein protein aggregation at the ER membrane (Kogan et al., 2001, J. Mol. Biol. 312:907-913). Mainieri et al. (2004) Plant Physiol. 136:3447-3456, demonstrated that the fusion of 89 amino acid residues of γ-zein is sufficient to mediate the assembly of a target protein into PBs. A synthetic sequence consisting of (PPPVHL)8 has been developed as a targeting tag (termed Zera®) to facilitate assembly and recovery of recombinant proteins (Torrent et al., 2009 BMC Biology 7, 5).

Accumulation in the vacuole or vacuole-derived protein bodies (PBs)

Vacuolar targeting of a protein encoded by a nuclear gene requires dual targeting signals. First, an ER signal sequence (as described above) is required for entry into the endomembrane system. A second signal is active after the protein has progressed through the ER and Golgi network where it is carried in vesicles to the vacuole. Receptors for these sequences allow binding and delivery to the organelle. Vacuolar targeting signals are less tightly defined compared to the N-terminal ER-signal peptides and have been identified at the C-terminus (C-terminal pro-peptide, CTPP; e.g., barley lectin, phaseolin, tobacco chitinase) and the N-terminal region of the "mature" protein (N-terminal pro-peptide, NTPP, located immediately upstream of the ER signal

sequence; e.g., sporamin, aleurain) as well as internal domains that direct vacuolar targeting (e.g., phytohemagglutinin, legumin, ricin). The NTPP and CTPP are typically removed by proteases within the vacuole. In some cases (e.g., the A-B plant toxins such as ricin and abrin), the internal vacuolar targeting sequences are also removed within the vacuole as part of protein processing. All three types of vacuolar targeting signals (C, N, and internal) have been shown to be necessary and sufficient to sort model proteins from the default secretion route to the vacuole.

Accumulation in the apoplast

Secretion is the default pathway of the plant endomembrane system and without addition of specific signals for sorting or retention, the proteins (e.g., at least one glycosidase and polypeptide-of-interest) are secreted to the extracellular space and typically accumulate within the apoplast-the region between the plasma membrane and the cell wall. Since the diffusion through the cell matrix is size delimiting, this strategy is used when the polypeptide of interest is large enough not to diffuse out of the cell wall. Alternatively or additionally the polypeptide-of-interest and optionally the glucosidase is immobilized to the apoplast, or to the cell wall by a heterologous polypeptide expressed in the plant cell comprising a cell wall binding peptide translationally fused to a heterologous affinity moiety.

Accumulation in the cell wall

In order to be accumulated in the cell wall each of the polypeptide of interest and the at least one glycosidases may be expressed in translational fusion with a cellulose binding domain pfam00942: **CBM_3**.

Accumulation in oil bodies

Oil bodies are organelles that encompass oils (e.g., triglycerides) in a single layer phospholipid membrane that contains the highly hydrophobic protein oleosin. Heterologous proteins have been expressed as oleosin fusions. Oleosins, low molecular mass (Mr 16–24 kDa) polypeptides, consist of a hydrophobic domain flanked by two hydrophilic domains. Oleosins are initially targeted to the ER membranes although both C- and N-termini remain in the cytosol and the proteins are subsequently transferred to the oil bodies. Thus, the fused polypeptide of interest and glycosidase essentially coats the oil bodies and is positioned on the cytosolic face. To allow post-translational modifications, the targeting the protein of interest and the glycosidase is effected

through the endomembrane systems, e.g., with an ER retrieval domain as well, then retrieving the protein onto oil body surfaces through binding with an anti-oleosin single chain antibody (scFv). Thus, the product is trafficked and accumulated within the endomembrane system for post-translational processing, but associates with the oil bodies upon cell breakage, providing the advantages of oil-body-based flotation centrifugation, combining the benefits of both systems.

Specific embodiments of the sorting approaches which can be used in accordance with the present teachings are summarized in Table 1, below.

Table 1

Target Organelle	Location in the protein (e.g., polypeptide of interest and/or glucosidase)	Nature of signal	Signal removed
Endomembrane system (lumen)	N-ter	1-3 basic aa followed by 6-12 hydrophobic aa	Yes
ER retention	C-ter	KDEL; HDEL; SEKDEL	No
Vacuole	N-ter C-ter Internal	NPR-consrved domain No consensus identified No consensus identified	Yes Yes Varies

According to a specific embodiment, for cell wall expression, the barley alpha-amylase signal sequence is used (Rogers, J. C. 1985. Two barley alpha-amylase gene families are regulated differently in aleurone cells. J. Biol. Chem. 260: 3731-3738).

According to a specific embodiment, a signal peptide for apoplast secretion is the cel-1 signal peptide (SEQ ID NO: 21, 22).

Targeting the enzyme to the vacuole is another embodiment. Signal sequences to accomplish this are well known. For example, Raikhel U.S. Pat. No. 5,360,726 shows a vacuole signal sequence as does Warren et al. at U.S. Pat. No. 5,889,174. Vacuolar targeting signals may be present either at the amino-terminal portion, (Holwerda et al., The Plant Cell, 4:307-318 (1992), Nakamura et al., Plant Physiol., 101: 1-5 (1993)), carboxy-terminal portion, or in the internal sequence of the targeted protein. (Tague et al., The Plant Cell, 4:307-318 (1992), Saalbach et al. The Plant Cell, 3:695-708 (1991)). Additionally, amino-terminal sequences in conjunction with carboxy-terminal sequences are responsible for vacuolar targeting of gene products (Shinshi et al. Plant Molec. Biol. 14:357-368 (1990)).

According to a specific embodiment, a signal peptide for vacuolar accumulation is the SP (SEQ ID NO: 20) encoded by SP (SEQ ID NO: 17), SP1 (SEQ ID NO: 18) or SP2 (SEQ ID NO: 19), as described by Wei et al. (2004) Plant Biotechnol. J Fluorescent Screening of Transgenic Arabidopsis Seeds without Germination Plant Physiol. Jun 2004; 135(2): 709–714. THE paper mentions Cel1 signal peptide.

In order to optimize product yield, multiple SPs are tested for initial assessment of production strategies. For pharmaceutical applications, precise cleavage of the signal peptide — whether from animal, fungal, or plant sources — is often required and generally confirmed by N-terminal sequencing of the final purified product.

As used herein the term "polypeptide of interest" refers to at least one (e.g., 2, 3, 4, more) recombinant polypeptide which modified glycosylation is of value. Such a polypeptide can be widely employed in research and industrial settings, for example, for production of therapeutics, vaccines, diagnostics, collectively termed as pharmaceuticals and many other applications of interest.

According to a specific embodiment, the polypeptide-of-interest is a multimeric protein e.g., collagen, or antibody (i.e., heavy chain and light chain).

According to a specific embodiment, the polypeptide of interest is a human polypeptide.

According to a specific embodiment, the polypeptide of interest is a naturally-occurring polypeptide.

According to a specific embodiment, the polypeptide of interest is a synthetic polypeptide.

According to a specific embodiment, the polypeptide of interest is a chimeric polypeptide.

The polypeptide of interest may be endogenous or exogenous to the plant cell. The polypeptides may be intracellular polypeptides (e.g., a cytosolic protein), transmembrane polypeptides, or secreted polypeptides.

Exemplary therapeutic proteins that can be produced by employing the subject compositions and methods include but are not limited to human hormones (e.g., insulin, growth hormone, insulin-like growth factor 1, follicle-stimulating hormone, and chorionic gonadotropin), hematopoietic proteins (e.g., erythropoietin, C-CSF, GM-CSF, and IL-11), thrombotic and hematostatic proteins (e.g., tissue plasminogen activator and

activated protein C), immunological proteins (e.g., interleukin), antibodies and other enzymes (e.g., deoxyribonuclease I). Exemplary vaccines that can be produced by the subject compositions and methods include but are not limited to vaccines against various influenza viruses (e.g., types A, B and C and the various serotypes for each type such as H5N2, H1N1, H3N2 for type A influenza viruses), HIV, hepatitis viruses (e.g., hepatitis A, B, C or D), Lyme disease, and human papillomavirus (HPV). Examples of heterologously produced protein diagnostics include but are not limited to secretin, thyroid stimulating hormone (TSH), HIV antigens, and hepatitis C antigens.

According to other embodiments, examples of the polypeptide of interest include, but are not limited to cytokines, chemokines, lymphokines, ligands, receptors, hormones, enzymes, structural proteins, antibodies and antibody fragments, and growth factors. Non-limiting examples of receptors include TNF type I receptor, IL-1 receptor type II, IL-1 receptor antagonist, IL-4 receptor and any chemically or genetically modified soluble receptors. Examples of enzymes include acetlycholinesterase, lactase, activated protein C, factor VII, collagenase (e.g., marketed by Advance Biofactures Corporation under the name Santyl); agalsidase-beta (e.g., marketed by Genzyme under the name Fabrazyme); dornase-alpha (e.g., marketed by Genentech under the name Pulmozyme); alteplase (e.g., marketed by Genentech under the name Activase); pegylated-asparaginase (e.g., marketed by Enzon under the name Oncaspar); asparaginase (e.g., marketed by Merck under the name Elspar); and imiglucerase (e.g., marketed by Genzyme under the name Ceredase). Examples of specific polypeptides or proteins include, but are not limited to granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor (CSF), interferon beta (IFN-beta), interferon gamma (IFNgamma), interferon gamma inducing factor I (IGIF), transforming growth factor beta (IGF-beta), RANTES (regulated upon activation, normal T-cell expressed and presumably secreted), macrophage inflammatory proteins (e.g., MIP-1-alpha and MIP-1-beta), Leishmnania elongation initiating factor (LEIF), platelet derived growth factor (PDGF), tumor necrosis factor (TNF), growth factors, e.g., epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, (FGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-2 (NT-2), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4),

neurotrophin-5 (NT-5), glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), TNF alpha type II receptor, erythropoietin (EPO), insulin and soluble glycoproteins e.g., gp120 and gp160 glycoproteins. The gp120 glycoprotein is a human immunodeficiency virus (WIV) envelope protein, and the gp160 glycoprotein is a known precursor to the gp120 glycoprotein. Other examples include secretin, nesiritide (human B-type natriuretic peptide (hBNP)) and GYP-I.

Other products may include GPCRs, including, but not limited to Class A Rhodopsin like receptors such as Muscatinic (Muse.) acetylcholine Vertebrate type 1, Musc. acetylcholine Vertebrate type 2, Musc. acetylcholine Vertebrate type 3, Musc. acetylcholine Vertebrate type 4; Adrenoceptors (Alpha Adrenoceptors type 1, Alpha Adrenoceptors type 2, Beta Adrenoceptors type 1, Beta Adrenoceptors type 2, Beta Adrenoceptors type 3, Dopamine Vertebrate type 1, Dopamine Vertebrate type 2, Dopamine Vertebrate type 3, Dopamine Vertebrate type 4, Histamine type 1, Histamine type 2, Histamine type 3, Histamine type 4, Serotonin type 1, Serotonin type 2, Serotonin type 3, Serotonin type 4, Serotonin type 5, Serotonin type 6, Serotonin type 7, Serotonin type 8, other Serotonin types, Trace amine, Angiotensin type 1, Angiotensin type 2, Bombesin, Bradykffin, C5a anaphylatoxin, Finet-leu-phe, APJ like, Interleukin-8 type A, Interleukin-8 type B, Interleukin-8 type others, C-C Chemokine type 1 through type 11 and other types, C--X--C Chemokine (types 2 through 6 and others), C-X3-C Chemokine, Cholecystokinin CCK, CCK type A, CCK type B, CCK others, Endothelin, Melanocortin (Melanocyte stimulating hormone, Adrenocorticotropic hormone, Melanocortin hormone), Duffy antigen, Prolactin-releasing peptide (GPR10), Neuropeptide Y (type 1 through 7), Neuropeptide Y, Neuropeptide Y other, Neurotensin, Opioid (type D, K, M, X), Somatostatin (type 1 through 5), Tachykinin (Substance P(NK1), Substance K (NK2), Neuromedin K (NK3), Tachykinin like 1, Tachykinin like 2, Vasopressin/vasotocin (type 1 through 2), Vasotocin, Oxytocin/mesotocin, Conopressin, Galanin like, Proteinase-activated like, Orexin & neuropeptides FF, QRFP, Chemokine receptor-like, Neuromedin U like (Neuromedin U, PRXamide), hormone protein (Follicle stimulating hormone, Lutropin-choriogonadotropic hormone, Thyrotropin, Gonadotropin type I, Gonadotropin type II), (Rhod)opsin, Rhodopsin Vertebrate (types 1-5), Rhodopsin Vertebrate type 5, Rhodopsin Arthropod, Rhodopsin Arthropod type 1, Rhodopsin Arthropod type 2,

Rhodopsin Arthropod type 3, Rhodopsin Mollusc, Rhodopsin, Olfactory (Olfactory 11 fam 1 through 13), Prostaglandin (prostaglandin E2 subtype EP 1, Prostaglandin E2/D2 subtype EP2, prostaglandin E2 subtype EP3, Prostaglandin E2 subtype EP4, Prostaglandin F2-alpha, Prostacyclin, Thromboxane, Adenosine type 1 through 3, Purinoceptors, Purinoceptor P2RY1-4,6,11 GPR91, Purinoceptor P2RY5,8,9,10 GPR35,92,174, Purinoceptor P2RY12-14 GPR87 (JDP-Glucose), Cannabinoid, Platelet activating factor, Gonadotropin-releasing hormone, Gonadotropin-releasing hormone type I, Gonadotropin-releasing hormone type II, Adipokinetic hormone like, Corazonin, Thyrotropin-releasing hormone & Secretagogue, Thyrotropin-releasing hormone, Growth hormone secretagogue, Growth hormone secretagogue like, Ecdysis-triggering hormone (ETHR), Melatonin, Lysosphingolipid & LPA (EDG), Sphingosine 1-phosphate Edg-1, Lysophosphatidic acid Edg-2, Sphingosine 1-phosphate Edg-3, Lysophosphatidic acid Edg4, Sphingosine 1-phosphate Edg-5, Sphingosine 1-phosphate Edg-6, Lysophosphatidic acid Edg-7, Sphingosine 1-phosphate Edg-8, Edg Other Leukotriene B4 receptor, Leukotriene B4 receptor BLT1, Leukotriene B4 receptor BLT2, Class A Orphan/other, Putative neurotransmitters, SREB, Mas proto-oncogene & Mas-related (MRGs), GPR45 like, Cysteinyl leukotriene, G-protein coupled bile acid receptor, Free fatty acid receptor (GP40, GP41, GP43), Class B Secretin like, Calcitonin, Corticotropin releasing factor, Gastric inhibitory peptide, Glucagon, Growth hormone-releasing hormone, Parathyroid hormone, PACAP, Secretin, Vasoactive intestinal polypeptide, Latrophilin, Latrophilin type 1, Latrophilin type 2, Latrophilin type 3, ETL receptors, Brain-specific angiogenesis inhibitor (BAI), Methuselah-like proteins (MTH), Cadherin EGF LAG (CELSR), Very large G-protein coupled receptor, Class C Metabotropic glutamate/pheromone, Metabotropic glutamate group I through III, Calcium-sensing like, Extracellular calcium-sensing, Pheromone, calcium-sensing like other, Putative pheromone receptors, GABA-B, GABA-B subtype 1, GABA-B subtype 2, GABA-B like, Orphan GPRC5, Orphan GPCR6, Bride of sevenless proteins (BOSS), Taste receptors (TiR), Class D Fungal pheromone, Fungal pheromone A-Factor like (STE2,STE3), Fungal pheromone B like (BAR,BBR,RCB,PRA), Class E cAMP receptors, Ocular albinism proteins, Frizzled/Smoothened family, frizzled Group A (Fz 1&2&4&5&7-9), frizzled Group B (Fz 3 & 6), fizzled Group C (other),

Vomeronasal receptors, Nematode chemoreceptors, Insect odorant receptors, and Class Z Archaeal/bacterial/fungal opsins.

Bioactive peptides may also be produced. Examples include: BOTOX, Myobloc, Neurobloc, Dysport (or other serotypes of botulinum neurotoxins), alglucosidase alfa, daptomycin, YH-16, choriogonadotropin alfa, filgrastim, cetrorelix, interleukin-2, aldesleukin, teceleulin, denileukin diftitox, interferon alfa-n3 (injection), interferon alfa-nl, DL-8234, interferon, Suntory (gamma-1a), interferon gamma, thymosin alpha 1, tasonermin, DigiFab, ViperaTAb, EchiTAb, CroFab, nesiritide, abatacept, alefacept, Rebif, eptoterminalfa, teriparatide (osteoporosis), calcitonin injectable (bone disease), calcitonin (nasal, osteoporosis), etanercept, hemoglobin glutamer 250 (bovine), drotrecogin alfa, collagenase, carperitide, recombinant human epidermal growth factor (topical gel, wound healing), DWP401, darbepoetin alfa, epoetin omega, epoetin beta, epoetin alfa, desirudin, lepirudin, bivalirudin, nonacog alpha, Mononine, eptacog alfa (activated), recombinant Factor VIII+VWF, Recombinate, recombinant Factor VIII, Factor VIII (recombinant), Alphnmate, octocog alfa, Factor VIII, palifermin, Indikinase, tenecteplase, alteplase, pamiteplase, reteplase, nateplase, monteplase, follitropin alfa, rFSH, hpFSH, micafungin, pegfilgrastim, lenograstim, nartograstim, sermorelin, glucagon, exenatide, pramlintide, iniglucerase, galsulfase, Leucotropin, molgramostirn, triptorelin acetate, histrelin (subcutaneous implant, Hydron), deslorelin, histrelin, nafarelin, leuprolide sustained release depot (ATRIGEL), leuprolide implant (DUROS), goserelin, somatropin, Eutropin, KP-102 program, somatropin, somatropin, mecasermin (growth failure), enlfavirtide, Org-33408, insulin glargine, insulin glulisine, insulin (inhaled), insulin lispro, insulin deternir, insulin (buccal, RapidMist), mecasermin rinfabate, anakinra, celmoleukin, 99 mTc-apcitide injection, myelopid, Betaseron, glatiramer acetate, Gepon, sargramostim, oprelvekin, human leukocyte-derived alpha interferons, Bilive, insulin (recombinant), recombinant human insulin, insulin aspart, mecasenin, Roferon-A, interferon-alpha 2, Alfaferone, interferon alfacon-1, interferon alpha, Avonex' recombinant human luteinizing hormone, dornase alfa, trafermin, ziconotide, taltirelin, diboterminalfa, atosiban, becaplermin, eptifibatide, Zemaira, CTC-111, Shanvac-B, HPV vaccine (quadrivalent), octreotide, lanreotide, ancestirn, agalsidase beta, agalsidase alfa, laronidase, prezatide copper acetate (topical gel), rasburicase, ranibizumab, Actimmune, PEG-Intron, Tricomin, recombinant house dust

mite allergy desensitization injection, recombinant human parathyroid hormone (PTH) 1-84 (sc, osteoporosis), epoetin delta, transgenic antithrombin III, Granditropin, Vitrase, recombinant insulin, interferon-alpha (oral lozenge), GEM-21S, vapreotide, idursulfase, omnapatrilat, recombinant serurn albumin, certolizumab pegol, glucarpidase, human recombinant C1 esterase inhibitor (angioedema), lanoteplase, recombinant human growth hormone, enfuvirtide (needle-free injection, Biojector 2000), VGV-1, interferon (alpha), lucinactant, aviptadil (inhaled, pulmonary disease), icatibant, ecallantide, omiganan, Aurograb, pexigananacetate, ADI-PEG-20, LDI-200, degarelix, cintredelinbesudotox, Favld, MDX-1379, ISAtx-247, liraglutide, teriparatide (osteoporosis), tifacogin, AA4500, T4N5 liposome lotion, catumaxomab, DWP413, ART-123, Chrysalin, desmoteplase, amediplase, corifollitropinalpha, TH-9507, teduglutide, Diamyd, DWP-412, growth hormone (sustained release injection), recombinant G-CSF, insulin (inhaled, AIR), insulin (inhaled, Technosphere), insulin (inhaled, AERx), RGN-303, DiaPep277, interferon beta (hepatitis C viral infection (HCV)), interferon alfa-n3 (oral), belatacept, transdermal insulin patches, AMG-531, MBP-8298, Xerecept, opebacan, AIDSVAX, GV-1001, LymphoScan, ranpirnase, Lipoxysan, lusupultide, MP52 (beta-tricalciumphosphate carrier, bone regeneration), melanoma vaccine, sipuleucel-T, CTP-37, Insegia, vitespen, human thrombin (frozen, surgical bleeding), thrombin, TransMID, alfimeprase, Puricase, terlipressin (intravenous, hepatorenal syndrome), EUR-1008M, recombinant FGF-I (injectable, vascular disease), BDM-E, rotigaptide, ETC-216, P-113, MBI-594AN, duramycin (inhaled, cystic fibrosis), SCV-07, OPI-45, Endostatin, Angiostatin, ABT-510, Bowman Birk Inhibitor Concentrate, XMP-629, 99 mTc-Hynic-Annexin V, kahalalide F, CTCE-9908, teverelix (extended release), ozarelix, rornidepsin, BAY-504798, interleukin4, PRX-321, Pepscan, iboctadekin, rhlactoferrin, TRU-015, IL-21, ATN-161, cilengitide, Albuferon, Biphasix, IRX-2, omega interferon, PCK-3145, CAP-232, pasireotide, huN901-DMI, ovarian cancer immunotherapeutic vaccine, SB-249553, Oncovax-CL, OncoVax-P, BLP-25, CerVax-16, multi-epitope peptide melanoma vaccine (MART-1, gp100, tyrosinase), nemifitide, rAAT (inhaled), rAAT (dermatological), CGRP (inhaled, asthma), pegsunercept, thymosinbeta4, plitidepsin, GTP-200, ramoplanin, GRASPA, OBI-1, AC-100, salmon calcitonin (oral, eligen), calcitonin (oral, osteoporosis), examorelin, capromorelin, Cardeva, velafermin, 131I-TM-601, KK-220,

T-10, ularitide, depelestat, hematide, Chrysalin (topical), rNAPc2, recombinant Factor V111 (PEGylated liposomal), bFGF, PEGylated recombinant staphylokinase variant, V-10153, SonoLysis Prolyse, NeuroVax, CZEN-002, islet cell neogenesis therapy, rGLP-1, BIM-51077, LY-548806, exenatide (controlled release, Medisorb), AVE-0010, GA-GCB, avorelin, AOD-9604, linaclotid eacetate, CETi-1, Hemospan, VAL (injectable), fast-acting insulin (injectable, Viadel), intranasal insulin, insulin (inhaled), insulin (oral, eligen), recombinant methionyl human leptin, pitrakinra subcutancous injection, eczema), pitrakinra (inhaled dry powder, asthma), Multikine, RG-1068, MM-093, NBI-6024, AT-001, PI-0824, Org-39141, Cpn10(autoimmune iseases/inflammation), talactoferrin (topical), rEV-131 (ophthalmic), rEV-131 (respiratory disease), oral recombinant human insulin (diabetes), RPI-78M, oprelvekin (oral), CYT-99007 CTLA4-Ig, DTY-001, valategrast, interferon alfa-n3 (topical), IRX-3, RDP-58, Tauferon, bile salt stimulated lipase, Merispase, alaline phosphatase, EP-2104R, Melanotan-II, bremelanotide, ATL-104, recombinant human microplasmin, AX-200, SEMAX, ACV-1, Xen-2174, CJC-1008, dynorphin A, SI-6603, LAB GHRH, AER-002, BGC-728, malaria vaccine (virosomes, PeviPRO), ALTU-135, parvovirus B19 vaccine, influenza vaccine (recombinant neuraminidase), malaria/HBV vaccine, anthrax vaccine, Vacc-5q, Vacc-4x, HIV vaccine (oral), HPV vaccine, Tat Toxoid, YSPSL, CHS-13340, PTH(1-34) liposomal cream (Novasome), Ostabolin-C, PTH analog (topical, psoriasis), MBRI-93.02, MTB72F vaccine (tuberculosis), MVA-Ag85A vaccine (tuberculosis), FARA04, BA-210, recombinant plague F1V vaccine, AG-702, OxSODrol, rBetV1, Der-p1/Der-p2/Der-p7 allergen-targeting vaccine (dust mite allergy), PR1 peptide antigen (leukemia), mutant ras vaccine, HPV-16 E7 lipopeptide vaccine, labyrinthin vaccine (adenocarcinoma), CML vaccine, WT1-peptide vaccine (cancer), IDD-5, CDX-110, Pentrys, Norelin, CytoFab, P-9808, VT-111, icrocaptide, telbermin (dermatological, diabetic foot ulcer), rupintrivir, reticulose, rGRF, P1A, alpha-galactosidase A, ACE-011, ALTU-140, CGX-1160, angiotensin therapeutic vaccine, D-4F, ETC-642, APP-018, rhMBL, SCV-07 (oral, tuberculosis), DRF-7295, ABT-828, ErbB2-specific immunotoxin (anticancer), DT3SSIL-3, TST-10088, PRO-1762, Combotox, cholecystokinin-B/gastrin-receptor binding peptides, 111In-hEGF, AE-37, trasnizumab-DM1, Antagonist G, IL-12 (recombinant), PM-02734, IMP-321, rhIGF-BP3, BLX-883, CUV-1647 (topical), L-19 based radioimmunotherapeutics

(cancer), Re-188-P-2045, AMG-386, DC/1540/KLH vaccine (cancer), VX-001, AVE-9633, AC-9301, NY-ESO-1 vaccine (peptides), NA17.A2 peptides, melanoma vaccine (pulsed antigen therapeutic), prostate cancer vaccine, CBP-501, recombinant human lactoferrin (dry eye), FX-06, AP-214, WAP-8294A (injectable), ACP-HIP, SUN-11031, peptide YY [3-36] (obesity, intranasal), FGLL, atacicept, BR3-Fc, BN-003, BA-058, human parathyroid hormone 1-34 (nasal, osteoporosis), F-18-CCR1, AT-1100 (celiac disease/diabetes), JPD-003, PTH(7-34) liposomal cream (Novasome), duramycin (ophthalmic, dry eye), CAB-2, CTCE-0214, GlycoPEGylated erythropoietin, EPO-Fc, CNTO-528, AMG-114, JR-013, Factor XIII, aminocandin, PN-951, 716155, SUN-E7001, TH-0318, BAY-73-7977, teverelix (immediate release), EP-51216, hGH (controlled release, Biosphere), OGP-I, sifuvirtide, TV4710, ALG-889, Org-41259, rhCC10, F-991, thymopentin (pulmonary diseases), r(m)CRP, hepatoselective insulin, subalin, L19-IL-2 fusion protein, elafin, NMK-150, ALTU-139, EN-122004, rhTPO, thrombopoietin receptor agonist (thrombocytopenic disorders), AL-108, AL-208, nerve growth factor antagonists (pain), SLV-317, CGX-1007, INNO-105, oral teriparatide (eligen), GEM-OS1, AC-162352, PRX-302, LFn-p24 fusion vaccine (Therapore), EP-1043, S pneumonia pediatric vaccine, malaria vaccine, Neisseria meningitidis Group B vaccine, neonatal group B streptococcal vaccine, anthrax vaccine, HCV vaccine (gpE1+gpE2+MF-59), otitis media therapy, HCV vaccine (core antigen+ISCOMATRIX), hPTH(1-34) (transdermal, ViaDerm), 768974, SYN-101, PGN-0052, aviscumnine, BIM-23190, tuberculosis vaccine, multi-epitope tyrosinase peptide, cancer vaccine, enkastim, APC-8024, GI-5005, ACC-001, TTS-CD3, vascular-targeted TNF (solid tumors), desmopressin (buccal controlled-release), onercept, and TP-9201.

In certain embodiments, the heterologously produced protein is an enzyme or biologically active fragments thereof. Suitable enzymes include but are not limited to: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. In certain embodiments, the heterologously produced protein is an enzyme of Enzyme Commission (EC) class 1, for example an enzyme from any of EC 1.1 through 1.21, or 1.97. The enzyme can also be an enzyme from EC class 2, 3, 4, 5, or 6. For example, the enzyme can be selected from any of EC 2.1 through 2.9, EC 3.1 to 3.13, EC 4.1 to 4.6, EC 4.99, EC 5.1 to 5.11, EC 5.99, or EC 6.1-6.6. According to a specific embodiment,

the enzyme is a high mannose enzyme such as a lysosomal protein, e.g., glucocerebrosidase and alpha-galactodsidase.

As used herein, the term "antibody" refers to a substantially intact antibody molecule. The term refers to a monospecific antibody as well as bi- and tri-specific antibodies.

As used herein, the phrase "antibody fragment" refers to a functional fragment of an antibody (such as Fab, F(ab')2, Fv or single domain molecules such as VH and VL) that is capable of binding to an epitope of an antigen.

Exemplary antibodies produced in the cells of the present invention include, but are not limited to, abciximab (ReoPro^{RTM}), adalimumab (Humira^{RTM}), alemtuzumab (Campath^{RTM}), basiliximab (Simulect^{RTM}), bevacizumab (Avastin^{RTM}), cetuximab (Erbitux^{RTM}), daclizumab (Zenapax^{RTM}), dacetuzumab, eculizumab (Soliris^{RTM}), efalizumab (Raptiva^{RTM}), Edrecolomab (Panorex^{RTM}), epratuzumab, ibritumomab (Zevalin^{RTM}), tiuxetan, infliximab (Remicade^{RTM}), muromonab-CD3 (OKT3), natalizumab (Tysabri^{RTM}), omalizumab (Xolair^{RTM}), palivizumab (Synagis^{RTM}), panitumumab (Vectibix^{RTM}), ranibizumab (Lucentis^{RTM}), gemtuzumab ozogamicin (Mylotarg^{RTM}), oregovomab (OvaRex^{RTM}), rituximab (Rituxan^{RTM}), tositumomab (Bexxar^{RTM}), trastuzumab (Herceptin^{RTM}), MetMAb, ocrelizumab, pertuzumab, Raptiva^{RTM} (efalizumab), hu M195Mab, MDX-210, BEC2, anti-Abeta, anti-CD4, anti-IL-13, anti-oxLDL, trastuzumab-DM1, apomab, rhuMAb beta7, rhuMAb IFNalpha, GA101, anti-OX40L, ipilimumab, Valortim, ustekinumab, golimumab, ofatumumab, zalutumumab, tremelimumab, motavizumab, mitumomab, ecromeximab, ABX-EGF, MDX010, XTL 002, H11 SCFV, 4B5, XTL001, MDX-070, TNX-901, IDEC-114, and any antibody fragments specific for antigens including but not limited to complement C5, CBL, CD147, gp 120, VLA4, CD11a, CD18, VEGF, CD40L, anti-Id, ICAM1, CD2, EGFR, TGF-beta2, TNF-alpha, TNF receptor, E-selectin, FactII, Her2/neu, F gp, CD11/18, CD14, CD80, ICAM3, CD4, CD23, beta.2-integrin, alpha4beta7, CD52, CD22, OX40L, IL-5 receptor, GM-CSF receptor, GM-CSF, HLA-DR, oxLDL, CD64 (FcR), TCR alpha beta, CD3, Hep B, CD 125, DR5,EpCAM, gpIIbIIIa, IgE, beta 7 integrin, CD20, IL1beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL10, IL13, IL-12/IL-23, IL-1 5, IFN-alpha, IFN-beta, IFN-gamma, VEGFR-1, platelet-derived growth factor receptor .alpha. (PDGFRalpha), vascular adhesion protein 1 (VAP1), connective tissue

growth factor (CTGF), Apo2/TRAIL, CD25, CD33, HLA, F gp, IgE, CTLA-4, IP-10, anti-C. difficile Toxin A and Toxin B, B. anthracis PA, respiratory syncytial virus (RSV), mannose receptor/hCG.beta, integrin receptors, PD1, PDL-1, CD 19, CD70, and VNR integrin.

Exemplary structural proteins that can be produced according to the present teachings include, but are not limited to collagen, procollagen, albumin, fibrinogen or derivatives of same.

For multimeric protein production is may be desired to express all subunits on a single nucleic acid construct to ensure stoichiometric production. However, expression from a plurality of nucleic acid constructs (construct system) in a single plant cell or a plurality of cells may also be achieved.

The proteins as described herein are encoded by isolated polynucleotide(s) for recombinant expression in plant cells. Each of the open reading frames encoding the proteins (e.g., polypeptide of interest and at least one glycosidase) is translationally fused to a signal peptide such as described above. Although both proteins are targeted to the same subcellular compartment, the signals need not be the same.

The phrase “an isolated polynucleotide” refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence (i.e. comprising ribonucleotides), a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence (i.e. comprising deoxyribnonucleotides) and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exon sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing

therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

Exemplary nucleic acid sequences encoding for the proteins of the invention include, but are not limited to SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 20, 30.

For heterologous expression, nucleic acid sequences encoding each of the above-mentioned polypeptides (as well as other polypeptides, such as described further below), are ligated into a nucleic acid construct or construct systems.

As used herein, the qualifier "heterologous" when relating to the proteins of the invention, indicates that the proteins are encoded by a nucleic acid sequence(s) which are foreign to (non-naturally occurring within) the expressing cell.

According to an embodiment of the invention, there is provided a nucleic acid construct comprising a nucleic acid sequence encoding at least two glycosidases, wherein each of the at least two glycosidases is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an additional or alternative embodiment of the invention, there is provided a nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a first glycosidase of at least two glycosidases;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding a second glycosidase of the at least two glycosidases,

wherein each of the first glycosidase and the second glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an additional or alternative embodiment of the invention, there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest and at least one glycosidase, wherein each of the polypeptide of interest and the at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an additional or alternative embodiment of the invention, there is provided a nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest (at least one subunit or more, e.g., 2, 3);

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding and at least one glycosidase,

wherein each of the at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

According to an additional or alternative embodiment of the invention, there is provided a nucleic acid construct comprising a nucleic acid sequence encoding a glycosidase translationally fused to a signal peptide for localization in a subcellular compartment of interest.

According to a specific embodiment, each of the above mentioned nucleic acid constructs or nucleic acid construct systems, may comprise additional nucleic acid sequences or constructs such as those encoding additional glycosidases, or post-translational modification enzymes which include, but are not limited to, prolyl 4-hydroxylase or a subunit thereof, lysyl oxidase, lysyl hydroxylase, C-proteinase, N-proteinase, PACE, γ-glutamyl carboxylase, N-acetylglucosaminaltransferases, N-acetlygalactosaminyltransferases, N-acetlygalactosaminyltransferases, sialyl-transferases, fucosyltransferases, galactosyltransferases, mannosyltransferases, sulfotransferases, glycosidases, acetyltransferases, and mannosidases, as taught in WO/2001/029242.

Alternatively or additionally to improve the afucosylation and axylosylation, the plant or plant cell may comprise a reduced level or activity of at least one glycosyl transferase such as compared to a plant or plant cell of the same species expressing wild-type levels or exhibiting wild-type activity of said at least one glycosyl transferase.

According to a specific embodiment, the glycosyl transferase comprises Beta-(1-2)-xylosyltransferase and/or Alpha-(1, 3)-fucosyltransferase.

Methods of reducing expression or activity of glycosyl transferases are described in details in WO/2001/029242. Generally methods of suppressing gene expression in plants are well known in the art and include, but are not limited to, siRNA, dsRNA, antisense, hnRNA and chimeric nucleases such as comprising DNA-binding domain of a meganuclease DNA-binding domain, a leucine zipper DNA-binding domain, a

transcription activator-like (TAL) DNA-binding domain, a recombinase, a CRISPR-Cas9 and a zinc finger protein DNA-binding domain.

According to a specific embodiment, the target for silencing is GDP-D-mannose 6,6-dehydrase gene(s).

According to a specific embodiment, the target for silencing is Xylose transferase (XylT).

Additional teachings for silencing of gene involved in protein fucosylation/xylosylation can be found in Matsuo et al. 2014 J. Bioscience and Bioengineering 9:264-281.

Alternatively or additionally, the plant is transformed with a polynucleotide, which confers a culturing or agricultural effective trait, e.g insect resistance, disease resistance, herbicide resistance, increased yield, increased tolerance to environmental stress, increased or decreased starch, oil or protein content, for example.

Alternatively or additionally, the plant is transformed with a polynucleotide which simplify the isolation of the polypeptide of interest. According to an embodiment, the plant thus expresses a nucleic acid sequence encoding a fusion polypeptide comprising a cell wall binding domain (e.g., CBD) translationally fused to a (e.g., heterologous, chimeric protein) affinity moiety for binding the polypeptide of interest.

Examples of cellulose binding domains which can be used in accordance with the present teachings are those provided in the Examples section as well as from the following protein sources (see also WO2009/069123):

β-glucanases (avicelases, CMCases,cellodextrinases)

exoglucanses or cellobiohydrolases

cellulose binding proteins

xylanases

mixed xylanases/glucanases

esterases

chitinases

β-1,3-glucanases

β-1,3-(β-1,4)-glucanases

(β-)mannanases

β-glucosidases/galactosidases

cellulose synthases

Yet alternatively or additionally, the plant or plant cell may be transformed with a nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence having an affinity towards the polypeptide of interest (e.g., in the case of an antibody, protein A/G/L) tranlationally fused to a heterologous transmembrane domain. Thus following homogenization, the polypeptide of interest may bind the membrane-bound affinity moiety.

The fusion of the affinity moiety to the transmembrane domain may be direct or via a linker (e.g., SEQ ID NO: 31).

Thus, either during culturing (e.g., when the polypeptide of interest and the glycosidase are directed to the apoplast), or following culturing and lysis of the cells, the polypeptide of interest will bind the affinity moiety and will immobilize to the insoluble fraction.

The affinity moiety may be any amino acid sequence which has a specific affinity (and not to plant cell proteins) e.g,. above 10^{-4} M or 10^{-6} M to the polypeptide of interest. According to an exemplary embodiment the affinity moiety is protein A, G or L. According to a specific embodiment, the affinity moiety is protein A.

These expression products may or may not co-localize with the polypeptide of interest.

Constructs useful in the methods according to some embodiments of the invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The nucleic acid constructs, may be proprietary or commercially available, suitable for transforming into plants and suitable for expression of the proteins in the transformed cells. The genetic construct can be an expression vector wherein said nucleic acid sequence is operably linked to one or more regulatory sequences allowing expression in the plant cells.

In a particular embodiment of some embodiments of the invention the regulatory sequence is a plant-expressible promoter.

As used herein the phrase "plant-expressible" refers to a promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell,

tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ. Examples of preferred promoters useful for the methods of some embodiments of the invention are presented in Table 2, 3, 4 and 5.

Table 2

Exemplary constitutive promoters for use in the performance of some embodiments of the invention

Gene Source	*Expression Pattern*	*Reference*
Actin	constitutive	McElroy et al., Plant Cell, 2: 163-171, 1990
CAMV 35S	constitutive	Odell et al., Nature, 313: 810-812, 1985
CaMV 19S	constitutive	Nilsson et al., Physiol. Plant 100:456-462, 1997
GOS2	constitutive	de Pater et al., Plant J Nov;2(6):837-44, 1992
ubiquitin	constitutive	Christensen et al., Plant Mol. Biol. 18: 675-689, 1992
Rice cyclophilin	constitutive	Bucholz et al., Plant Mol Biol. 25(5):837-43, 1994
Maize H3 histone	constitutive	Lepetit et al., Mol. Gen. Genet. 231: 276-285, 1992
Actin 2	constitutive	An et al., Plant J. 10(1);107-121, 1996

Table 3

Exemplary seed-preferred promoters for use in the performance of some embodiments of the invention

Gene Source	*Expression Pattern*	*Reference*
Seed specific genes	seed	Simon, et al., Plant Mol. Biol. 5. 191, 1985; Scofield, etal., J. Biol. Chem. 262: 12202, 1987.; Baszczynski, et al., Plant Mol. Biol. 14: 633, 1990.
Brazil Nut albumin	seed	Pearson' et al., Plant Mol. Biol. 18: 235- 245, 1992.
legumin	seed	Ellis, et al. Plant Mol. Biol. 10: 203-214, 1988
Glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987
Zein	seed	Matzke et al. Plant Mol Biol, 143).323-32 1990
napA	seed	Stalberg, et al., Planta 199: 515-519, 1996
wheat LMW and HMW, glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2,
Wheat SPA	seed	Albanietal, Plant Cell, 9: 171-184, 1997

wheat a, b and g gliadins	endosperm	EMBO3:1409-15, 1984
Barley ltrl promoter	endosperm	
barley B1, C, D hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750- 60, 1996
Barley DOF	endosperm	Mena et al., The Plant Journal, 116(1): 53- 62, 1998
Biz2	endosperm	EP99106056.7
Synthetic promoter	endosperm	Vicente-Carbajosa et al., Plant J. 13: 629-640, 1998
rice prolamin NRP33	endosperm	Wu et al, Plant Cell Physiology 39(8) 885- 889, 1998
rice -globulin Glb-1	endosperm	Wu et al., Plant Cell Physiology 398) 885-889, 1998
rice OSH1	emryo	Sato et al., Proc. Nati. Acad. Sci. USA, 93: 8117-8122
rice alpha-globulin REB/OHP-1	endosperm	Nakase et al. Plant Mol. Biol. 33: 513-S22, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum gamma- kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	emryo	Postma-Haarsma et al, Plant Mol. Biol. 39:257-71, 1999
rice oleosin	Embryo and aleuton	Wu et at, J. Biochem., 123:386, 1998
sunflower oleosin	Seed (embryo and dry seed)	Cummins, etal., Plant Mol. Biol. 19: 873- 876, 1992

Table 4

Exemplary flower-specific promoters for use in the performance of the invention

Gene Source	*Expression Pattern*	*Reference*
AtPRP4	flowers	salus. medium.edu/mmg/tierney/html
chalene synthase (chsA)	flowers	Van der Meer, et al., Plant Mol. Biol. 15, 95-109, 1990.
LAT52	anther	Twell et al. Mol. Gen Genet. 217:240-245 (1989)
apetala- 3	flowers	

Table 5

Alternative rice promoters for use in the performance of the invention

PRO #	*gene*	*expression*
PR00001	Metallothionein Mte	transfer layer of embryo + calli
PR00005	putative beta-amylase	transfer layer of embryo
PR00009	Putative cellulose synthase	Weak in roots
PR00012	lipase (putative)	

PR00014	Transferase (putative)	
PR00016	peptidyl prolyl cis-trans isomerase (putative)	
PR00019	unknown	
PR00020	prp protein (putative)	
PR00029	noduline (putative)	
PR00058	Proteinase inhibitor Rgpi9	seed
PR00061	beta expansine EXPB9	Weak in young flowers
PR00063	Structural protein	young tissues+calli+embryo
PR00069	xylosidase (putative)	
PR00075	Prolamine 10Kda	strong in endosperm
PR00076	allergen RA2	strong in endosperm
PR00077	prolamine RP7	strong in endosperm
PR00078	CBP80	
PR00079	starch branching enzyme I	
PR00080	Metallothioneine-like ML2	transfer layer of embryo + calli
PR00081	putative caffeoyl- CoA 3-0 methyltransferase	shoot
PR00087	prolamine RM9	strong in endosperm
PR00090	prolamine RP6	strong in endosperm
PR00091	prolamine RP5	strong in endosperm
PR00092	allergen RA5	
PR00095	putative methionine aminopeptidase	embryo
PR00098	ras-related GTP binding protein	
PR00104	beta expansine EXPB1	
PR00105	Glycine rich protein	
PR00108	metallothionein like protein (putative)	
PR00110	RCc3 strong root	
PR00111	uclacyanin 3-like protein	weak discrimination center / shoot meristem
PR00116	26S proteasome regulatory particle non-ATPase subunit 11	very weak meristem specific
PR00117	putative 40S ribosomal protein	weak in endosperm
PR00122	chlorophyll a/lo-binding protein precursor (Cab27)	very weak in shoot
PR00123	putative protochlorophyllide	Strong leaves

	reductase	
PR00126	metallothionein RiCMT	strong discrimination center shoot meristem
PR00129	GOS2	Strong constitutive
PR00131	GOS9	
PR00133	chitinase Cht-3	very weak meristem specific
PR00135	alpha- globulin	Strong in endosperm
PR00136	alanine aminotransferase	Weak in endosperm
PR00138	Cyclin A2	
PR00139	Cyclin D2	
PR00140	Cyclin D3	
PR00141	Cyclophyllin 2	Shoot and seed
PR00146	sucrose synthase SS1 (barley)	medium constitutive
PR00147	trypsin inhibitor ITR1 (barley)	weak in endosperm
PR00149	ubiquitine 2 with intron	strong constitutive
PR00151	WSI18	Embryo and stress
PR00156	HVA22 homologue (putative)	
PR00157	EL2	
PR00169	aquaporine	medium constitutive in young plants
PR00170	High mobility group protein	Strong constitutive
PR00171	reversibly glycosylated protein RGP1	weak constitutive
PR00173	cytosolic MDH	shoot
PR00175	RAB21	Embryo and stress
PR00176	CDPK7	
PR00177	Cdc2-1	very weak in meristem
PR00197	sucrose synthase 3	
PRO0198	OsVP1	
PRO0200	OSH1	very weak in young plant meristem
PRO0208	putative chlorophyllase	
PRO0210	OsNRT1	
PRO0211	EXP3	
PRO0216	phosphate transporter OjPT1	
PRO0218	oleosin 18kd	aleurone + embryo
PRO0219	ubiquitine 2 without intron	

PRO0220	RFL	
PRO0221	maize UBI delta intron	not detected
PRO0223	glutelin-1	
PRO0224	fragment of prolamin RP6 promoter	
PRO0225	4xABRE	
PRO0226	glutelin OSGLUA3	
PRO0227	BLZ-2_short (barley)	
PR00228	BLZ-2_long (barley)	

Nucleic acid sequences of the polypeptides (e.g., glycosidase and polypeptide of interest) of some embodiments of the invention may be optimized for plant expression. Examples of such sequence modifications include, but are not limited to, an altered G/C content to more closely approach that typically found in the plant species of interest, and the removal of codons atypically found in the plant species commonly referred to as codon optimization.

The phrase "codon optimization" refers to the selection of appropriate DNA nucleotides for use within a structural gene or fragment thereof that approaches codon usage within the plant of interest. Therefore, an optimized gene or nucleic acid sequence refers to a gene in which the nucleotide sequence of a native or naturally occurring gene has been modified in order to utilize statistically-preferred or statistically-favored codons within the plant. The nucleotide sequence typically is examined at the DNA level and the coding region optimized for expression in the plant species determined using any suitable procedure, for example as described in Sardana *et al.* (1996, Plant Cell Reports 15:677-681).

Thus, some embodiments of the invention encompasses nucleic acid sequences described hereinabove; fragments thereof, sequences hybridizable therewith, sequences homologous thereto, sequences orthologous thereto, sequences encoding similar polypeptides with different codon usage, altered sequences characterized by mutations, such as deletion, insertion or substitution of one or more nucleotides, either naturally occurring or man induced, either randomly or in a targeted fashion.

Plant cells may be transformed stably or transiently with the nucleic acid constructs of some embodiments of the invention.

The plant cells may be transformed with the nucleic acid sequence (construct or construct system) encoding the at least one glycosidase (e.g., at least two glycosidases) followed by transformation with the nucleic acid construct encoding the polypeptide-of-interest. Alternatively, plant cells may be transformed with the nucleic acid sequence (construct or construct system) encoding the at least one glycosidase (e.g., at least two glycosidases) and the polypeptide-of-interest. Alternatively, plant cells may be transformed with the nucleic acid sequence (construct or construct system) encoding the at least one glycosidase (e.g., at least two glycosidases) following transformation with the nucleic acid construct encoding the polypeptide-of-interest.

In stable transformation, the nucleic acid molecule of some embodiments of the invention is integrated into the plant genome and as such it represents a stable and inherited trait. In transient transformation, the nucleic acid molecule is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledonous plants, collectively termed herein as transforming, introducing, infecting (Potrykus, I., Annu. Rev. Plant. Physiol., Plant. Mol. Biol. (1991) 42:205-225; Shimamoto et al., Nature (1989) 338:274-276).

The principle methods of causing stable integration of exogenous DNA (i.e., heterologous) into plant genomic DNA include two main approaches:

(i) Agrobacterium-mediated gene transfer: Klee et al. (1987) Annu. Rev. Plant Physiol. 38:467-486; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) direct DNA uptake: Paszkowski et al., in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. et al. (1988) Bio/Technology 6:1072-1074. DNA uptake induced by brief electric shock of plant cells: Zhang et al. Plant Cell Rep. (1988) 7:379-384. Fromm et al. Nature (1986)

319:791-793. DNA injection into plant cells or tissues by particle bombardment, Klein et al. Bio/Technology (1988) 6:559-563; McCabe et al. Bio/Technology (1988) 6:923-926; Sanford, Physiol. Plant. (1990) 79:206-209; by the use of micropipette systems: Neuhaus et al., Theor. Appl. Genet. (1987) 75:30-36; Neuhaus and Spangenberg, Physiol. Plant. (1990) 79:213-217; glass fibers or silicon carbide whisker transformation of cell cultures, embryos or callus tissue, U.S. Pat. No. 5,464,765 or by the direct incubation of DNA with germinating pollen, DeWet et al. in Experimental Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S. H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA (1986) 83:715-719.

The Agrobacterium system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the Agrobacterium delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. Horsch et al. in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the Agrobacterium delivery system in combination with vacuum infiltration. The Agrobacterium system is especially viable in the creation of transgenic dicotyledonous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following stable transformation plant propagation is exercised. The most common method of plant propagation is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the transformed plant be

produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed plant be regenerated by micropropagation which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original transgenic or transformed plant. The advantages of cloning plants are the speed of plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased so that it can be grown in the natural environment.

Although stable transformation is presently preferred, transient transformation of leaf cells, meristematic cells or the whole plant is also envisaged by some embodiments of the invention.

Transient transformation can be effected by any of the direct DNA transfer methods described above or by viral infection using modified plant viruses.

Viruses that have been shown to be useful for the transformation of plant hosts include CaMV, TMV and BV. Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (BGV), EP-A 67,553 (TMV), Japanese Published

Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. et al., Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants, is described in WO 87/06261.

Construction of plant RNA viruses for the introduction and expression of non-viral exogenous nucleic acid sequences in plants is demonstrated by the above references as well as by Dawson, W. O. et al., Virology (1989) 172:285-292; Takamatsu et al. EMBO J. (1987) 6:307-311; French et al. Science (1986) 231:1294-1297; and Takamatsu et al. FEBS Letters (1990) 269:73-76.

When the virus is a DNA virus, suitable modifications can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

Construction of plant RNA viruses for the introduction and expression in plants of non-viral exogenous nucleic acid sequences such as those included in the construct of some embodiments of the invention is demonstrated by the above references as well as in U.S. Patent No. 5,316,931.

In one embodiment, a plant viral nucleic acid is provided in which the native coat protein coding sequence has been deleted from a viral nucleic acid, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral nucleic acid, and ensuring a systemic infection of the host by the recombinant plant viral nucleic acid, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native nucleic acid sequence within it, such that a protein is produced. The

recombinant plant viral nucleic acid may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or nucleic acid sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) nucleic acid sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one nucleic acid sequence is included. The non-native nucleic acid sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral nucleic acid is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral nucleic acid is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral nucleic acid. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native nucleic acid sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that said sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral nucleic acid is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral nucleic acid to produce a recombinant plant virus. The recombinant plant viral nucleic acid or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral nucleic acid is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) (isolated nucleic acid) in the host to produce the desired protein.

In addition to the above, the nucleic acid molecule of some embodiments of the invention can also be introduced into a chloroplast genome thereby enabling chloroplast expression.

A technique for introducing exogenous nucleic acid sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the exogenous nucleic acid is introduced via particle bombardment into the cells with the aim of introducing at least one exogenous nucleic acid molecule into the chloroplasts. The exogenous nucleic acid is selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the exogenous nucleic acid includes, in addition to a gene of interest, at least one nucleic acid stretch which is derived from the chloroplast's genome. In addition, the exogenous nucleic acid includes a selectable marker, which serves by sequential selection procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the exogenous nucleic acid. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane.

Thus, according to a specific embodiment, expressing the at least two glycosidases comprises introducing into the plant or plant cell a nucleic acid construct comprising a nucleic acid sequence encoding the at least two glycosidases, wherein each of the at least two glycosidases is translationally fused to a signal peptide for co-localization in the subcellular compartment of the plant or plant cell. The plant or plant cell may be further transformed with the polypeptide of interest. Alternatively or additionally, the plant cell is transformed with the polypeptide of interest and a glycosidase.

Thus, a single plant (whether transgenic or not) is transformed with nucleic acid construct or construct systems as described herein.

However, as the present teachings, relate to the expression of a plurality of transgenes, the transgenic plants or plant cells can be generated by crossing plants each

expressing an individual transgene (or more) so as to obtain a hybrid product which comprises the plurality of transgenes.

Thus, according to a specific embodiment, expressing the transgenes (e.g., two glycosidases, glycosidase and polypeptide of interest or two glycosidases and polypeptide of interest) is effected by the art of crossing and selection.

Thus, expressing the at least two glycosidases comprises:

(a) expressing a first glycosidase of the at least two glycosidases in the subcellular compartment of a first plant;

(b) expressing a second glycosidase of the at least two glycosidases in the subcellular compartment of a second plant; and

(c) crossing the first plant and the second plant.

Alternatively, a first plant expressing at least one glycosidase (e.g., at least two glycosidases) in a subcellular compartment is crossed with a second plant expressing the polypeptide of interest.

Alternatively, a first plant expressing a polypeptide fusion comprising a cell wall binding peptide translationally fused to a heterologous affinity moiety and optionally at least one glycosidase (e.g., at least two glycosidases) in a subcellular compartment is crossed with a second plant expressing the polypeptide of interest.

Each of these plants may further comprise a nucleic acid sequence for downregulating an activity of fucosyl transferase or xylosyl transferase in the plant cell.

Crossing and breeding can be accomplished by any means known in the art for breeding plants such as, for example, cross pollination of the first and second plants that are described above and selection for plants from subsequent generations which express both the first and second enzymes. The plant breeding methods used herein are well known to one skilled in the art. For a discussion of plant breeding techniques, see Poehlman (1987) Breeding Field Crops. AVI Publication Co., Westport Conn. Many crop plants useful in this method are bred through techniques that take advantage of the plant's method of pollination.

According to a specific embodiment, following transformation the plant or plant cell is selected for the highest level of expression of the polypeptide of interest as well as the level/activity of the glycosidase(s), and it is thus useful to ascertain expression levels in transformed plant cells, transgenic plants and tissue specific expression.

One such method is to measure the expression of the polypeptide-of-interest as a percentage of total soluble protein. One standard assay is the Bradford assay which is well known to those skilled in the art (Bradford, M. 1976. Anal. Biochem. 72:248). The biochemical activity of the recombinant protein should also be measured and compared with a wildtype standard. The activity of polysaccharide degrading enzymes, i.e., glycsidases, can be determined by the methods well known in the art such as Fuc-Mu (4-Methylumbelliferyl α-L-fucopyranosidase) and Xyl-Mu (4-Methylumbelliferyl-b-D-xylopyranosidase), for testing fucosidase and xylosidase activity, respectively.

Other assays for glycosidase activity are known in the art and can be used to detect enzyme activity in extracts prepared from callus, leafs, fruits and seeds. See, Coughlan et al. ((1988) J. Biol. Chem. 263:16631-16636) and Freer ((1993) J. Biol. Chem. 268:9337-9342). In addition, western analysis and ELISAs can be used to assess protein integrity and expression levels.

Thus, the present teachings provide for transgenic plants or plant cells e.g., a transgenic plant or plant cell transformed to express at least two glycosidases in a subcellular compartment in a co-localized manner, or a transgenic plant or plant cell transformed to express at one glycosidase (e.g., at least two glycosidases) and a polypeptide of interest in a subcellular compartment in a co-localized manner.

Alternatively or additionally, there is provided a transgenic plant or plant cell comprising the nucleic acid construct or nucleic acid construct system as described herein.

As used herein a transgenic plant or plant cell refers to a plant or plant cell which comprises a heterologous nucleic acid sequence which translates to at least one of glycosidase and a polypeptide of interest.

One transformed the plant cells are cultured or the plants are grown under conditions which are suitable for transgene expression so as to produce the polypeptide of interest.

Thus, according to an aspect of the invention, there is provided a method of producing a polypeptide-of-interest, the method comprising:

(a) expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that said at least one glycosidase and the polypeptide-of-

interest are co-localized to said subcellular compartment of the plant or plant cell; and subsequently

(b) isolating the polypeptide-of-interest.

Alternatively or additionally, there is provided a method of producing a polypeptide-of-interest, the method comprising:

(a) introducing into a plant or plant cell the nucleic acid constructs as described herein; and subsequently

(b) isolating the polypeptide-of-interest.

Thus, plant cells can be cultured cells, cells in cultured tissue or cultured organs, or cells in a plant. In some embodiments, the plant cells are cultured cells, or cells in cultured tissue or cultured organs. In yet further embodiments, the plant cells are any type of plant that is used in gene transference. The plant cell can be grown as part of a whole plant, or, alternatively, in plant cell culture.

According to some aspects of the invention, the plant cells are grown in a plant cell suspension culture. As used herein, the term "suspension culture" refers to the growth of cells separate from the organism. Suspension culture can be facilitated via use of a liquid medium (a "suspension medium"). Suspension culture can refer to the growth of cells in a three dimensional culture in liquid nutrient media, for example, but not limited to, growth in suspension culture in a bioreactor. Methods and devices suitable for growing plant cells of the invention in plant cell suspension culture are described in detail in, for example, PCT WO2008/135991, U.S. Patent No. 6,391,683, U.S. Patent Application No. 10/784,295; International Patent Publications PCT Nos.WO2004/091475, WO2005/080544 and WO 2006/040761, all of which are hereby incorporated by reference as if fully set forth herein. Also contemplated are hairy root cultures grown in suspension culture, in some embodiments, in bioreactors.

Thus, the invention encompasses plants or plant cultures expressing the nucleic acid sequences, so as to produce the recombinant polypeptide-of-interest. Once expressed within the plant cell or the entire plant, the level of the polypeptide-of-interest encoded by the nucleic acid sequence can be determined by methods well known in the art such as, described hereinabove or well known in the art.

The polypeptide of interest is then isolated from the plant or plant cell. The degree of isolation depends on the subcellular compartment as well as the intended use.

Typically, a cell extract which comprises the polypeptide of interest is produced. The extract is usually subject to clarification to remove host cell contaminants and culture remnants.

Following clarification, the clarified extract can be further processed, used as is, or stored for future use. According to some embodiments of some aspects of the invention, following extraction and clarification of the extract, the polypeptide-of-interest can be further isolated, also termed as purified. Purification may be carried out by chromatography, for example, ion-exchange, size filtration, HPLC, or ultra-filtration, counter-current dialysis, affinity purification, immune-purification, and the like. Thus, in some embodiments, polypeptide-of-interest is a purified polypeptide, characterized by a purity of at least 85%, at least 87%, at least 90%, at least 91%, at least 91.5%, at least 92%, at least 92.5%, at least 93%, at least 93.1%, at least 93.2%, at least 93.3%, at least 93.4%, at least 93.5%, at least 93.6%, at least 93.7%, at least 93.8%, at least 93.9%, at least 94%, at least 94.5%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.1%, at least 99.2%, at least 99.3%, at least 99.4%, at least 99.5%, at least 99.6%, at least 99.7%, at least 99.8%, at least 99.9%, in a range of at least 92.0-99.8%, or at least 95-99%, or at least 97-99%, or at least 98-99.5 or 100% purity. In some embodiments, purity of the polypeptide-of-interest is measured by HPLC.

Purity of the plant expressed polypeptide-of-interest can be expressed as a weight percent of the total, or as the weight percent of impurities. In various embodiments, the cumulative weight percentage of all proteins other than the polypeptide-of-interest in the composition used in the methods of the present invention is less than 10%, 5%, less than 1%, and in some embodiments, less than 0.5%, 0.4%, 0.3%, 0.2%, even in some embodiments less than 0.1%. In particular embodiments, the composition completely lacks host cell proteins other than the polypeptide-of-interest. Thus, as a weight percentage of protein, the compositions administered in the methods of the present invention typically comprise at least 90%, 91%, 92%, 93%, 94%, at least 95%, 96%, 97%, 98%, 99%, and in some embodiments, at least 99.5%, the polypeptide-of-interest or active portion thereof.

In some embodiments the plant-expressed polypeptide-of-interest composition comprises impurities derived from the plant host cell, such as, but not limited to nucleic acids and polynucleotides, amino acids, oligopeptides and polypeptides, glycans and

other carbohydrates, lipids and the like. In some embodiments the host-cell derived impurities comprise biologically active molecules, such as enzymes. Host cell proteins can be monitored, for example, by HPLC, using host cell protein-specific antibodies raised against plant cell fractions from polypeptide-of-interest-null plant cells cultured under similar conditions, and other assays known in the art.

The polypeptide is characterized by reduced immunogenicity in human subjects as compared to the same protein produced in a plant system having a wild-type glycosylation system.

The polypeptide thus produced can be used *per se* or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

Alternatively, it can be packed in a kit or article of manufacture for research, cosmetic or clinical applications.

It is expected that during the life of a patent maturing from this application many relevant polypeptide-of-interest as well as glycosidases will be developed and the scope of these terms is intended to include all such new technologies *a priori*.

The terms "comprises", "comprising", "includes", "including", “having” and their conjugates mean "including but not limited to".

The term “consisting of” means “including and limited to”.

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such

as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided

separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); “Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and

Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXMAPLE 1

Cloning of Avastin (bevacizumab) and Humira (adalimumab) to the binary pBINPLUS vector for tobacco transformation mediated by agrobacterium

Restriction enzymes were purchased from Thermo Scientific.

For expression in the vacuole, a Rubisco-vac cassette was used comprising the Rubisco promoter (SEQ ID NOs: 26), vacuolar signal peptide (SEQ ID NOs: 17 and 20) and Rubisco terminator (SEQ ID NOs: 27).

I. First, the commercial plasmid (pUC57 from Genscript) bearing synthetic genes was restricted by MunI and NotI enzymes to create four DNA inserts coding for: Humira heavy chain (SEQ ID NOs: 1 and 2), Humira light chain (SEQ ID NOs: 3 and 4), Avastin heavy chain (SEQ ID NOs: 5 and 6) and Avastin light chain (SEQ ID NOs: 7 and 8). Then the inserts were ligated into the pUC18 plasmid containing Rubisco-vac cassette also restricted by MunI and NotI (Figures 1A-B) to create four different constructs: pUC18 Rb-Humira heavy chain, pUC18 Rb-Humira light chain, pUC18 Rb-Avastin heavy chain and pUC18 Rb-Avastin light chain, where vacuolar targeting signal is positioned N-terminally to the coding sequence (SEQ ID NO: 17).

II. At the next step, the binary vectors pBINPLUS with both, heavy and light chains with Rubisco promoter and terminator, of every mAb were constructed: pBINPLUS-Humira (Figures 2A-C) and pBINPLUS-Avastin (Figures 3A-C).

To create pBINPLUS-Humira, a two steps ligation was applied. At the first step, the pUC18 Rb-Humira light chain was restricted by HindIII and cloned to the

pBINPLUS vector restricted by HindIII creating a pBINPLUS Rb-Humira light chain plasmid. At the second step, the pUC18 Rb-Humira heavy chain was restricted by EcoRI and SacI restriction enzymes and cloned with the same enzymes to the pBINPLUS Rb-Humira light chain to create the pBINPLUS Humira with both chains (Figure 2C).

The construction of pBINPLUS-Avastin was performed similarly to that of pBINPLUS-Humira (Figures 3A-C). First, Rb-Avastin heavy chain was cloned into pBINPLUS vector by HindIII creating pBINPLUS Rb-Avasin heavy chain (Figure 3B). Then, Rb-Avastin light chain was cloned to pBINPLUS Rb-Avastin heavy chain by EcoRI and SacI restriction (Figure 3C).

As shown in Figures 2C and 3C each of the coding sequences (heavy chain and light chain) is translationally fused at the N-terminus to the Rubisco-derived vacuolar signal peptide.

III. Rubisco-vac cassette with Humira (codon optimized by Entelechon) - Rubisco promoter, vacuolar signal peptide (SEQ ID NOs: 18, 19 and 20) and Rubisco terminator.

DNA sequence of Humira heavy and light chains was optimized by Leto optimization software (by Entelechon) and the genes (SEQ ID NOs: 15 and 16) were cloned into the Rubisco cassette with Leto optimized vacuolar signals (SEQ ID NOs: 18 and 19). Synthetic DNA fragment including Vacuolar SP1 (seq. 18, 20), Humira heavy chain (SEQ ID NOs 15, 2), Rubisco terminator, Rubisco promoter, Vacuolar signal SP2 (seq. 19, 20),Humira light chain (seq. 16, 2) was inserted into Rubisco expression cassette using NcoI and NotI enzymes thus creating an expression cassette in pUC18 with both Humira chains (SEQ ID NO: 25)(Figures 4A-B). The double cassette is cloned into pBINPLUS.

IV. 35S-vac cassette – CaMV 35S promoter, vacuolar signal peptide (SEQ ID NOs. 17 and 20) and NOS (nopaline synthase) terminator (SEQ ID NO: 28).

The genes coding Humira heavy (SEQ ID NO: 1) and light chains (SEQ ID NO: 3) were cloned into 35S-vac cassette, where they were fused in frame to the vacuolar signal peptide regulated by 35S promoter. The expression cassettes were constructed in pUC18 and then transformed into binary plasmid pBINPLUS (SEQ ID NOs: 1 and 3).

Constructs pUC18 35S-vac-Humira heavy chain, pUC18 35S-vac-Humira light chain, pBIN 35S-vac-Humira heavy chain, pBIN 35S-vac-Humira light chain were created.

V. For apoplast expression, the Rubisco-Cel1 cassette was used including Rubisco promoter, short Cel1 signal peptide (SEQ ID NOs: 21 and 22) and Rubisco terminator.

In the mAb containing Rubisco cassette the vacuolar signal peptide (Figures 11A-B) was replaced by Cel1 signal peptide by NcoI/MunI restriction and further ligation, in order to create constructs expressing mAbs chains in the apoplast. The expression cassettes were constructed in pUC18 and then transformed into binary plasmid pBINPLUS. Constructs pUC18 RBc-Cel1-Humira heavy chain, pUC18 RBc-Cel1-Humira light chain, pBIN RBc-Cel1-Humira heavy chain, pBIN RBc-Cel1-Humira light chain were created (SEQ ID NOs: 1 and 3).

VI. 35S-Cel1 cassette – CaMV 35S promoter, Cel1 signal peptide (SEQ ID NOs: 23 and 24) and NOS (nopaline synthase) terminator (SEQ ID NO: 28).

The genes coding Humira heavy and light chains were cloned into 35S-Cel1 cassette, where they were fused in frame to the Cel1 signal peptide and downstream to 35S promoter. The expression cassettes were constructed in pUC18 and then transformed into the binary plasmid pBINPLUS. Constructs pUC18 35S-Cel1-Humira heavy chain, pUC18 35S-Cel1-Humira light chain, pBIN 35S-Cel1-Humira heavy chain, pBIN 35S-Cel1-Humira light chain were designed (SEQ ID NOs: 1 and 3).

EXAMPLE 2

Cloning of CBD-PrtA, β-xylosidase, α-fucosidase to the binary pBINPLUS vector for tobacco transformation mediated by agrobacterium

I. For vacuolar expression: 35S-Vac cassette – CaMV 35S promoter, Vacuolar signal peptide (SEQ ID NOs: 17 and 20) and NOS (nopaline synthase) terminator (SEQ ID NO: 28).

The pUC57 with the expression cassette of CBD-PrtA (SEQ ID NOs: 9 and 10).

Gene fusion of CBD coding domain (SEQ ID NO: 29) and of Protein A (SEQ ID NO: 30) coding domain under the 35S promoter, vacuole signal peptide and Nos terminator was restricted by PstI, SacI and cloned to pUC18 plasmid (Figures 5A-B). The expression cassette in pUC18 was restricted by EcoRI and cloned into pBINPLUS

(Figures 5B-C). The genes encoding xylosidase (XlnD from *A. niger* SEQ ID NOs: 11, 12) and fucosidase (α-1,3/4-fucosidase from *Streptomyces sp.* SEQ ID NOs: 13, 14) restricted by MunI and NotI were cloned into expression cassette instead of CBD-PrtA (Figures 6A-C). The constructs of pUC18 35S-Xylosidase and pUC18 35S-Fucosidase were made. The expression cassettes were cloned to pBINPLUS binary plasmid by SdaI and SacI restriction to create pBINPLUS 35S-Xylosidase and pBINPLUS 35S-Fucosidase plasmids (each being directed to the vacuole via a Vac SP).

II. For apoplast expression: 35S-Cel1 cassette – CaMV 35S promoter, Cel1 signal peptide (SEQ ID NOs: 23 and 24) and NOS (nopaline synthase) terminator (SEQ ID NOs: 28).

Two constructs for apoplast expression of xylosidase and fucosidase were constructed. The vacuolar signal peptide was replaced by Cel1 signal peptide using NcoI, MunI restriction sites to construct pUC18 35S-Cel1-Xylosidase, pUC18 35S-Cel1-Fucosidase, pBINPLUS 35S-Cel1-Xylosidase, pBINPLUS-35S-Cel1-Fucosidase.

III. Transformation to *E. Coli*

Transformation was performed using Heat Shock. 50 µl of DH5α competent cells were used for transformations and 100 µl of competent cells for ligation. 50 ng of circular DNA was added into E.coli cells which then were thawed on ice for 20 minutes. Heat Shock was performed in 42 °C for 1 minute and back on ice for 5 minutes. 1 ml of LB was added and incubated for 1 hour at 37 °C. Bacterial cells were cultured on LB plates (with appropriate antibiotic added _ e.g., Ampicillin or Kanamycin, dependent on the resistance gene on the construct) 100 µl for transformation and 1000 µl for ligation. Cells were incubated overnight.

IV. Agrobacterium Transformation

Electroporation was carried out in cuvettes with a 1 mm gap distance using competent Agrobacterium tumefaciens (strain LBA 4404). Electroporation conditions were set to 25 µF, 2.5 kV, 200 Ω. Agrobacterium cells were thaw on ice. 1 µl of miniprepped DNA was mixed briefly with 80 µl bacteria and transferred to a pre-chilled cuvette. Following electroporation, 1 ml of sterile LB medium was added and transferred to a test tube. Bacteria were incubated at 28 °C for 3 to 4 hours on a roller drum. Following this, bacteria were plated on selective LB Ampicillin or Kanamycin medium.

V. Transgenic Tobacco plants production

Tobacco plants were grown under sterile conditions to about 4-5 weeks. An Agrobacterium starter was prepared in 25 ml of LB medium with the addition of 50 mg/ml kanamycin. Cells were incubated for 48 h in a shaking incubator at 28 °C to a stationary stage. Starters were then centrifuge for 10 min at 5500 rpm at room temperature. Upper medium was removed, pellet was resuspended in sterile liquid MS medium (4.4 g/L Murashige & Skoog (MS) medium including vitamins from Duchefa (cat# M0222.0050), 30 g/L sucrose from J.T.Baker (cat#4072-05), pH=5.8) to final turbidity of O.D.600 0.5. About 10 ml of the MS containing bacteria were placed on a sterile petri dish. Green leaves of the tobacco plants were cut with sterile tweezers and scalpel and incubated for 5 min with Agrobacterium in the MS suspension. Leaves were then transferred to petri dishes that contained a solid MS (liquid medium with 0.7 % plant agar from Duchefa (cat# P1001.1000)) medium that included 0.8 ml/L IAA and 2 ml/L kinetin). Plates were incubated at 28 °C in the dark for 48 hours. Following two days, the leaves were transferred to petri dishes containing a selective MS medium (0.8 ml/L IAA and 2 ml/L kinetin + 400 mg/L carbenicillin and 100 mg/L kanamycin). Plates were placed at a light room for 3 weeks and media was changed every 10 days. During this period shoots were formed from the leaves. The shoots were transferred to petri dishes containing MS medium with 100 mg/L kanamycin and 400 mg/L carbenicillin at the same light condition. Shoots that produced roots were transferred to the soil, covered with nylon for two days. Plants were then transferred to pots with ground, for further analysis.

EXAMPLE 3

CBD-PrtA expression and activity in tobacco

CBD-PrtA expression in tobacco was assayed by Western blot with anti-CBD antibody.

SDS-PAGE Western Blot

SDS-PAGE analysis was performed using a “mini protein gel system” (Hercules, CA, USA). Western Blot analysis was performed as described before (Ausubel et al 1987). Protein samples were loaded onto a 12.5% SDS PAGE system. After electrophoresis protein were transferred on to a nitrocellulose membrane

(Amersham Biosciences, England) using the "mini trans blot cell" - (Hercules, CA, USA) for 2 hours in a cooled buffer with 10% ethanol and steady current of 150V. After transfer, the membrane was blocked with 4% skim milk for 0.5 hour in R.T. The membranes were exposed to primary antibody overnight at RT and afterwards washed 3 times with TBST. Secondary antibody exposure (alkaline phosphatase (AP) conjugated) was performed for 2 hours followed by additional three washes. Finally, the membrane was washed and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) substrates (Sigma). BCIP/NBT Substrate Solution was prepare by adding 33 ml of 50 mg/ml BCIP Stock Solution and 66 µl of 50 mg/ml NBT Stock Solution to 10 ml of Substrate Buffer (100 mM Tris, 100 mM sodium chloride, and 5 mM MgCl2, pH 9.5. Adjusted with HCl).

Specifically, for Figure 7A - 100 mg of the plant tissue was homogenized in SAB (weight/volume ratio 1:1), the pellet was separated from the soup by centrifugation and the 30 µl of the soup and the pellet were loaded to the SDS PAGE, then we proceeded to the Western blot analysis with anti-CBD antibody. For Figure 7B – 100 mg of the tobacco tissue of the plant expressing CBD-PrtA were homogenized in the 100 µl Buffer 1 (100 mM Tris-HCl pH 7.4, 250 mM NaCl, 10 mM EDTA, Complete protease inhibitor) with 5 ng, and 10 ng of adalimumab. The plant pellet was separated from the soup and washed. The pellet extract was applied on the nitrocellulose membrane and the mAb was detected by anti human IgG-HRP.

Thus as shown, the protein was detected at the pellet of the plant tissue and its size was approximately 55 kDa which corresponds to the size of CBD-PrtA fusion protein (Figure 7A). Figure 7B shows that CBD-PrtA expressed in tobacco and binds human IgG (Figure 7B).

EXAMPLE 4

Xylosidase and Fucosidase are expressed and are active in tobacco plants

I. Fucosidase and Xylosidase activity scanning

Fucosidase and Xylosidase activity were tested in black 96 well plate (Nunc). For each reaction, 0.5 mm^2 fresh tobacco leaf tissue was taken and immediately incubated in 200 µl of 50 mM sodium acetate buffer pH=5.0. 10 µl of 0.15 mM substrate were added and the samples were incubated for 1 hour in 65 ºC. 4-

Methylumbelliferyl β-D-fucopyranoside (Mu-Fuc) (Sigma Aldrich M5510) and 4-Methylumbelliferyl-b-D-xylopyranoside (MU-Xyl) (Sigma Aldrich M 7008) were used as substrates for Fucosidase and xylosidase, respectively. The reaction was terminated by the addition of 21 µl NaOH (f.c. 100 mM). Fluorescence was measured at excitation 360 nm, emulsion 460 nm.

II. Enzymatic activity quantification

Plant tissue from transgenic plant lines expressing Fucosidase and Xylosidase was extracted by grinding leaf in liquid nitrogen, with acetate buffer (50mM Sodium Acetate, 15mM Potassium Meta bi-sulfite, complete (Sigma Aldrich) protease inhibitor cocktail (1 tablet per 100 ml)). Extracts were incubated for 1 hour at RT, centrifuged at 11,300 g for 10 min at 4 °C, soluble fraction was separated from pellet and filtered through 0.2 µm PVDF filter. The soluble fractions were diluted in 50 mM sodium acetate buffer pH=5.0 to concentrations ranging from 0-5.5 µl/well for Xylosidase and 0-70 µl/well for Fucosidase. The ability of the enzymes to hydrolyze 10 µl of Mu-Fuc for Fucosidase and 10 µl of MU-Xyl for Xylosidase was tested and activity calculated using a 4-Methylumbelliferone calibration curve. 4-Methylumbelliferone calibration curve was made from commercially available 4-Methylumbelliferone that was diluted to a final concentration range of 0.01-10 µg/ml. The trend line equation was obtained and fluorescence units (FU) per mass of 4-Methylumbelliferone were calculated.

Calculation of enzyme units per 1 g of plant tissue was calculated using the following equation:

$$\frac{enzyme\ units}{1g\ leaf\ tissue} = \frac{activity\ per\ 1g\ per 1\ \min(FU)}{4-Methylumbelliferone\ 1umol\ flourescence(FU)}$$

Results

The activity of xylosidase and fucosidase was measured in recombinant tobacco plants. A number of tobacco plants with a substantial expression of recombinant xylosidase and fucosidase were detected (Figure 8).

Conditions for Xylose and Fucose residues reduction were determined by treating 4.8 µg of plant-derived adalimumab (PDA) antibody with 1.5 ml of plant extracts expressing β-xylosidase and α-fucosidase. Reactions were carried out in 15

mM PBS buffer pH 7.5 for 2, 3 and 4 hours in RT. Different types of treatment were made: β-xylosidase and α-fucosidase alone (data not shown) and β-xylosidase and α-fucosidase combined together (Figure 13). Each treatment was analyzed with three different sets of antibodies: Anti-Xylose, Anti-Fucose and Anti-Human IgG.

It was observed, that individual treatments with β-xylosidase or α-fucosidase was less efficient (not shown) than a combined treatment with both enzymes. In the combined treatment, best results were obtained after 2 hours (Figure 13, 2h) incubation. Almost all Xylose and Fucose residues were cleared, while the PDA degradation was the lowest (Figure 13 with anti-human IgG). Positive control – not treated PDA (N) shows clear band with both Anti-Xylose and Anti-Fucose. Detection with Anti-Human IgG shows evidence for PDA existence in all treatment.

EXAMPLE 5

Adalimumab is expressed and is active in tobacco plants

I. Adalimumab activity test

Adalimumab activity assay was performed by Harlan Biotech Ltd. Israel. Briefly, activity was tested by antibody neutralization of TNF- α mediated cytotoxicity in L929 fibroblast cell line. Two 96-well tissue culture plates were filled with 100 µl of L929 cells suspension at a density of 3.5 x 10^5 cells/ml and incubated overnight at 37 °C, 5 % CO_2 in a humidified incubator. Following 12 hr incubation, rhTNF-α and Actinomycin D were added to achieve a final concentration of 1 ng/ml rhTNF-α and 1µg/ml Actinomycin D, followed by an additional incubation of 2 hours at 37 °C, 5 % CO2 in a humidified incubator. Then, the first plate (the experiment plate) was incubated with Plant Derived Adalimumab (PDA) at a concentration range of 0 - 2000 ng/ml, and a second plate (control plate) was incubated with commercial Humira at a final concentration range of 0-2000 ng/ml. An MTT solution was added to each well at a final concentration of 0.5 mg/ml. Labeling was carried out 4 hours 37 °C. Following incubation, the MTT solution was removed and 100 µl of Iso-propanol added to each well for no less than 30 minutes. Absorbance signal was measured in a microplate spectrophotometer (Multiscan® FC; Thermo Scientific) at 570-650nm wavelength filters.

Adalimumab ELISA

Plant sample preparation was performed as follows: six leaf discs were sampled

directly into a pre-weighed eppendorf containing grinding buffer (100 mM Tris-HCl pH 8, 25 mM NaCl, 1 mM PMSF, 10 mM EDTA, 1 mM PMBS) by clipping with the 1.5ml Eppendorf lid on a plant leaf, and immediately placed on ice. Care was taken to sample leaves from lower, middle and upper plant sections. The samples were then weighed and ground for 30 seconds using a plastic mortar at 500 RPM. The soluble fraction was extracted by centrifugation for 15 min at 11,000 RPM in 4 °C. Samples were diluted 500 folds before application on Elisa plate. Calibration curve was made from commercial Humira. Adalimumab was serially diluted to achieve a final concentration range of 0-100 ng/ml.

ELISA: untreated 96-well plate were coated with 100µl of 100ng/ml rhTNF-α solution and afterwards thoroughly washed. Samples for calibration curve and test samples were loaded in duplicate and incubated for 1h in 37 °C. The plate was washed 4 times with TBST wash solution and then loaded with 1:50,000 goat anti-Human IgG HRP conjugated, and incubated for 1h in 37 °C. After 4 washes with TBST, 100µl TMB substrate Solution was added. The reaction was stopped after 20 minutes with 100µl H_2SO_4 0.5N. Absorbance signal was measured in microplate reader at 450nm wavelength.

Results

Apoplast expressed adalimimab was purified from 40 g homogenized tobacco leaves on Protein A column. Lines 2-5: 30 µl of the elution fractions were analyzed by SDS PAGE, two bands corresponding to the heavy (55 kDa) and the light chain (25 kDa) of the antibody are seen in the lines 3 and 4 of Figure 9 (RUBISCO promotor and terminator with Cel1 signal peptide (RbCel1)).

Figure 10: Plant derived adalimumab shows in-vitro activity similar to the commercial therapeutics. TNF-α precoated ELISA plates were incubated with commercial therapeutics (gray) and plant derived adalimumab (red), binding of the mAb to the target was detected by using anti-human IgG-HRP.

In a separate experiment, apoplast expressed adalimumab was tested for yield quantification. Plant Derived Adalimumab (PDA) was purified from three different transgenic tobacco plant lines with stable expression of PDA and analyzed by SDS-PAGE Western blot and ELISA. Western blot (Figure 14) showed bands at approximately 55 and 25 kDa, corresponding to adalimumab heavy and light chain,

respectively. ELISA quantification (Figure 15) showed that plants 1, 2 and 3 yielded 4.88, 2.21 and 3.56 mg PDA/kg leaves, respectively. The WB and ELISA results were consistent, with SDS-PAGE bands corresponding in strength to the ELISA quantification.

PDA bioactivity, of neutralization of rhTNF-α, was tested in comparison to bioactivity of commercially available Adalimumab (Humira), in L929 cell line. PDA shows almost the same results as commercial Humira (Figure 16). Downwards trend was observed in PDA.

EXAMPLE 6

In-planta purification of the plant derived mAb by protein A fusion with transmembrane domain (TMD) or a cellulose binding domain (CBD)

Protein A –TMD

The present inventors have also utilized a protein A fusion with membrane anchoring domain in order to attach a mAb to the plasma membrane of the plant cells as a first step of the purification process. The mAb is bound by protein A which is anchored to the plasma membrane by TMD. Thus after the plant is harvested and a pellet is separated from the soup, the mAb is found in the pellet part. -DON'T YOU HAVE TO RUPTURE THE CELLS? WHAT SP WILL THE AB HAVE? No, the procedure is the same as with CBD-ProteinA but instead CBD we use TMD, the detachment of mAb made by pH change. The SP is Cel1 for targeting to the apoplast.

The constructs are shown in Figure 12A.

Schematic representation of the constructs is shown in Figures 12A-C. The transmembrane domain was attached via a linker to protein A (SEQ ID Nos: 30-36). The resultant sequences optimized for protein expression in plant cells are as set forth in SEQ ID NOs: 35-36.

The PrtA-TMD construct was first incorporated into 35S expression cassette in pUC18 plasmid using MunI and SacI restriction sites. Then the full cassette was transferred into pBINPLUS binary plasmid.

Protein A-CBD

In order to obtain a double transgenic plant, expressing both adalimumab and CBD-Protein A, a stable adalimumab expressing transgene (Apoplast expressed) was

transiently transfected with a CBD-Protein A construct. Transient expression was performed as described by Li et al., 2008 Plant Physiol. 147(4):1675-1689. Briefly, one single colony of Agrobacterium was inoculated in 5ml LB with 100µg/ml kanamycin and grown overnight at 28 °C. 1 ml of the overnight culture was used to inoculate 25ml LB (with 100µg/ml kanamycin and 20 µM Acetosyringone). The inoculate was grown overnight at 28 °C to final A600 = 0.4. Infiltration was performed with 5ml syringe.

Plant tissue preparation for *In vitro* binding assay: plant tissue was ground in Binding buffer (20mM Sodium Phosphate pH 7.5) or Grinding buffer (100 mM Tris-HCl pH 7.5, 25 mM NaCl, 1 mM PMSF, 10 mM EDTA, 1 mM PMBS) w/v with 50mg of cellulose. Extracts were incubated for 1h in 4°C for better binding. Pellet separation from soluble fraction was made by centrifugation 30 min at 10,000RPM. Soluble fraction was discarded and kept for further analysis. Pellet was washed with 1 volume binding buffer, followed by centrifugation 15 min at 10000RPM. Both pellet and soluble fractions were resuspended in Sample Aplication Bufer, heated for 10 min in 100°C and analyzed by Western Blot (as described above).

Results

PrtA-TMD

The fusion PrtA-TMD proteins were transiently expressed in mAb expressing tobacco plants. At day 6 the plant tissue was homogenized, the soup (designated by s) was separated from the pellet (p) and both were analyzed by Western blot with anti-human IgG Ab and with anti-protein A Ab. As can be seen at the left side of the figure (Figure 12D), most of the mAb was found in the pellet part of the sample, where the protein A fusion was found (right side of the figure).

PrtA-CBD

In a separate experiment where a Prt-CBD was used, mAb was extracted and purified from plant leaves and analyzed by western blot with anti-Protein A and anti-Human IgG separately. As a positive control, spiking of 5µg of Comercial Humira to a CBD-Protein A exprassing plants extract, was used at the same conditions. It is shown, that soluble fraction was almost clear when extraction was performed with Binding buffer whereas when Grinding buffer was used bands corresponding to heavy and light chains were detected in both commercial Humira control (Com) and in plant derived adalimumab (PDA) samples (Figure 17C) in the pellet mAb (Figure 17B) and CBD

Protein A (Figure 17A) were detected also in both control (Com) and PDA samples also when using Binding buffer, showing that binding of mAb to protein A and CBD to cellulose occurs and Binding buffer is the most efficient one.

EXAMPLE 7

Suppression of Xylose and Fucose glycosylation by RNAi (GMD) and RNA suppression (XylT)

Suppression of Xylose and Fucose glycosylation was performed according to Matsuo, et all, 2014. Briefly, SGDP-mannose-4,6-dehydratase (GMD) RNAi silencing technique was chosen to perform deletion of plant-specific sugar residues in plant N-glycans by repression of GDP-D-mannose 4,6-dehydratase genes. RNAi GMD demolishes α-1,4-Fucose and α-1,3-Fucose residues by interfering in α-1,4-Fucose transferase and α-1,3-Fucose transferase pathway

I. Cloning of GMD RNAi to the pBINPLUS binary vector for tobacco transformation mediated by agrobacterium:

The genes encoding GMD RNAi fragment were inserted in a 3 step cloning into pUC18 plasmid containing under the 35S promoter (Figure 18A). Step 1: GMD antisense encoding DNA was inserted by restriction with NotI and BamHI (Figure 18B) to form the pin structure. Step 2: β-Xylose transferase (XylT) intron encoding DNA from *Arabidopsis* was inserted by restriction with BamHI and MfeI (Figure 18C) to form the loop structure. Step 3: GMD sense encoding DNA was inserted by restriction with MfeI and NcoI (Figure 18D) to complete the pin structure of double stranded RNA. The expression cassette was then cloned into pBINPLUS binary plasmid by HindIII and SacI restriction enzymes to generate pBINPLUS GMD RNAi plasmids (Figure 19A-B).

Plasmids were then transformed into Agrobacterium strains LB4404 or EHA105 and into tobacco plants.

II. Cloning of XylT RNA Suppression construct to the pBINPLUS binary vector for tobacco transformation mediated by agrobacterium:

The Xylosyltransferase RNA suppression fragment encoding DNA was obtained from *Nicotiana tabacum* XylT gene for putative β-(1,2)-xylosyltransferase, exon 3 (Sequence ID: emblAJ627183.1).

For RNA suppression, a of 617 bp DNA fragment from exon 3 of the XylT gene were amplified by PCR and NcoI and NotI restriction sites were added to the 5' and 3'ends, respectively. The fragment was cloned into pUC18 plasmid under the RUBISCO promotor (Figure 20A). The Adalimumab heavy chain (1362 bp) was removed by NcoI and NotI restriction and replaced with the XylT fragment (Figure 20B). The expression cassette was then cloned into pBINPLUS binary plasmid with HindIII restriction enzyme to generate pBINPLUS XylT plasmid (Figures 20C-D). Plasmids than were transformed into agrobacterium strains LB4404 or EHA105 and then to tobacco plants.

RNA silencing and RNAi sequences for silencing expression of GMD and XylT in tobacco (SEQ ID NOs: 37-40).

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

References

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Matsuo K., and T. Matsumura Deletion of fucose residues in plant *N*-glycans by repression of the *GDP-mannose 4,6-dehydratase* gene using virus-induced gene silencing and RNA interference (2011). Plant Biotechnology Journal. 9: 264–281.

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WHAT IS CLAIMED IS:

1. A method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell, the method comprising expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that said at least one glycosidase and the polypeptide-of-interest are co-localized to said subcellular compartment of the plant or plant cell, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

2. A method of producing a polypeptide-of-interest, the method comprising:

(a) expressing in a plant or plant cell transformed to express at least one glycosidase in a subcellular compartment, a nucleic acid sequence encoding the polypeptide-of-interest, such that said at least one glycosidase and the polypeptide-of-interest are co-localized to said subcellular compartment of the plant or plant cell; and subsequently

(b) isolating the polypeptide-of-interest.

3. The method of claim 1 or 2, wherein said plant or plant cell transformed to express at least one glycosidase in said subcellular compartment further comprises reduced level or activity of at least one glycosyl transferase as compared to a plant or plant cell of the same species expressing wild-type levels or exhibiting wild-type activity of said at least one glycosyl transferase.

4. The method of claim 3, wherein said glycosyl transferase comprises Beta-(1-2)-xylosyltransferase and/or Alpha-(1, 3)-fucosyltransferase.

5. The method of any one of claims 1-4, wherein said plant or plant cell transformed to express at least one glycosidase in said subcellular compartment further comprises a nucleic acid sequence encoding a fusion polypeptide comprising a cell wall binding peptide translationally fused to an affinity moiety for binding said polypeptide of interest.

6. An isolated polypeptide comprising a cell wall binding peptide translationally fused to a heterologous affinity moiety.

7. The method of claim 5 or the isolated polypeptide of claim 6, wherein said cell wall binding peptide is a cellulose binding domain (CBD).

8. The method of claim 5 or the isolated polypeptide of any one of claims 6-7, wherein said affinity moiety is for binding an antibody.

9. The method or isolated polypeptide of any one of claims 6-7, wherein said affinity moiety for binding said antibody comprises protein A/G/L.

10. The isolated polypeptide of claim 9 as set forth in SEQ ID NO: 10.

11. An isolated polynucleotide comprising a nucleic acid sequence encoding the polypeptide of any one of claims 6-10.

12. The isolated polynucleotide of claim 11 as set forth in SEQ ID NO: 9.

13. A nucleic acid construct comprising the isolated polynucleotide of any one of claims 11-12 and a cis-acting regulatory element for directing expression of the polypeptide in a plant cell.

14. The nucleic acid construct of claim 13, comprising an additional nucleic acid sequence encoding at least one glycosidase.

15. A transgenic plant or plant cell comprising the polynucleotide of nucleic acid construct of any one of claims 11-13.

16. The transgenic plant or plant cell of claim 15 is transformed to express as least one glycosidase in a subcellular compartment.

17. The transgenic plant of plant cell of any one of claims 15-16, comprises reduced level or activity of at least one glycosyl transferase as compared to a plant or plant cell of the same species expressing wild-type levels or exhibiting wild-type activity of said at least one glycosyl transferase.

18. The transgenic plant of plant cell of any one of claims 15-17, wherein said glycosyl transferase comprises Beta-(1-2)-xylosyltransferase and/or Alpha-(1, 3)-fucosyltransferase.

19. A method of producing a transgenic plant or plant cell, the method comprising expressing in the plant or plant cell at least two glycosidases such that said at least two glycosidases are co-localized to a subcellular compartment of the plant or plant cell.

20. The method of claim 19, wherein said expressing said at least two glycosidases comprises:

(a) expressing a first glycosidase of said at least two glycosidases in said subcellular compartment of a first plant;

(b) expressing a second glycosidase of said at least two glycosidases in said subcellular compartment of a second plant; and

(c) crossing said first plant and said second plant.

21. The method of claim 19, wherein said expressing said at least two glycosidases comprises:

(i) introducing into the plant or plant cell a nucleic acid construct comprising a nucleic acid sequence encoding said at least two glycosidases, wherein each of said at least two glycosidases is translationally fused to a signal peptide for co-localization in said subcellular compartment of the plant or plant cell; or

(ii) introducing into the plant or plant cell a nucleic acid construct system comprising:

a first nucleic acid construct comprising a nucleic acid sequence encoding a first glycosidase;

a second nucleic acid construct comprising a nucleic acid sequence encoding a second glycosidase,

wherein each of said first glycosidase and said second glycosidase is translationally fused to a signal peptide for co-localization in said subcellular compartment of the plant or plant cell.

22. A method of producing a transgenic plant or plant cell, the method comprising expressing in the plant or plant cell at least one glycosidase and an affinity moiety to a polypeptide-of-interest, wherein said affinity moiety is translationally fused to a cell wall binding peptide.

23. A nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding at least one glycosidase;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding an affinity moiety to a polypeptide-of-interest,

wherein said affinity moiety is translationally fused to a cell wall binding peptide.

24. The method of claim 1 or 2, wherein said expressing said nucleic acid sequence encoding the polypeptide-of-interest comprises crossing:

(i) a first transgenic plant transformed to express said at least one glycosidase; and

(ii) a second transgenic plant transformed to express the polypeptide of interest.

25. The method of claim 24, wherein said first plant is transformed to express an affinity moiety translationally fused to a cell wall binding peptide, wherein said affinity moiety is for binding said polypeptide of interest.

26. A nucleic acid construct comprising a nucleic acid sequence encoding at least two glycosidases, wherein each of said at least two glycosidases is translationally fused

to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

27. A nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a first glycosidase of at least two glycosidases;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding a second glycosidase of said at least two glycosidases,

wherein each of said first glycosidase and said second glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

28. The nucleic acid construct or construct system of claim 26 or 27, wherein said signal peptide is a vacuolar signal peptide or an apoplast signal peptide.

29. The nucleic acid construct or construct system of claim 26 or 27, wherein said signal peptide is a vacuolar signal peptide or an apoplast signal peptide fused at an N-terminus of said first glycosidase and said second glycosidase.

30. A transgenic plant or plant cell transformed to express at least two glycosidases in a subcellular compartment in a co-localized manner.

31. A nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest and at least one glycosidase, wherein each of said polypeptide of interest and said at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

32. A nucleic acid construct system comprising:

(i) a first nucleic acid construct comprising a nucleic acid sequence encoding a polypeptide of interest;

(ii) a second nucleic acid construct comprising a nucleic acid sequence encoding and at least one glycosidase,

wherein each of said at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of a plant or plant cell.

33. A nucleic acid construct comprising a nucleic acid sequence encoding a glycosidase translationally fused to a signal peptide for localization in a subcellular compartment of interest.

34. A transgenic plant or plant cell comprising the nucleic acid construct of claim 33.

35. A method of modifying a glycosylation pattern of a polypeptide-of-interest in a plant or plant cell, the method comprising introducing into a plant or plant cell the nucleic acid construct of claim 31 or the nucleic acid construct system of claim 32, thereby modifying the glycosylation pattern of the polypeptide-of-interest in the plant or plant cell.

36. A method of producing a polypeptide-of-interest, the method comprising:

(a) introducing into a plant or plant cell the nucleic acid construct of claim 31 or the nucleic acid construct system of claim 34; and subsequently

(b) isolating the polypeptide-of-interest.

37. A transgenic plant or plant cell recombinantly expressing:

(i) a polypeptide of interest; and

(ii) at least one glycosidase

wherein each of said polypeptide of interest and said at least one glycosidase is translationally fused to a signal peptide for co-localization in a subcellular compartment of the plant or plant cell.

38. A transgenic plant or plant cell comprising the nucleic acid construct or nucleic acid construct system of any one of claims 26, 27, 31 and 32.

39. The method of any one of claims 19, 20, 21 or the nucleic acid construct or construct system of any one of claims 26 and 27 or the transgenic plant or plant cell of claim 34, wherein said at least two glycosidases comprise a fucosidase and a xylosidase.

40. The method of any one of claims 1, 2, 35 and 36 or the nucleic acid construct or construct system of any one of claims 31-34 or the transgenic plant or plant cell of any one of claims 37 and 38, wherein said at least one glycosidase is selected from the group consisting of a fucosidase and a xylosidase.

41. The method, nucleic acid construct, nucleic acid construct system or transgenic plant or plant cell of any one of claims 1-40, wherein said subcellular compartment is selected from the group consisting of a vacuole, an apoplast, an endoplasmic reticulum and golgi.

42. The method, nucleic acid construct, nucleic acid construct system or transgenic plant or plant cell of any one of claims 1-40, wherein said subcellular compartment is a vacuole.

43. The method, nucleic acid construct, nucleic acid construct system or transgenic plant or plant cell of any one of claims 1-42, wherein said plant or plant cell is a tobacco plant or plant cell.

44. The method, nucleic acid construct, nucleic acid construct system or transgenic plant or plant cell of any one of claims 1-42, wherein plant cell is a root cell.

45. The method of claim 21, the nucleic acid construct of any one of claims 26 and 31, the nucleic acid construct system of any one of claims 27 and 33, or the transgenic plant or plant cell of claim 34 or 37, wherein said signal peptide is selected from the group consisting of a vacuolar targeting signal, an endoplasmic targeting signal, an apoplast targeting signal, a mitochondria targeting signal and a plastid targeting signal.

46. The method of claim 1, wherein said plant or plant cell transformed to express at least one glycosidase in said subcellular compartment, is further transformed to express an additional glycosidase in said subcellular compartment.

47. The method of claim 21, the nucleic acid construct of any one of claims 26 and 31, the nucleic acid construct system of any one of claims 27 and 33, or the transgenic plant or plant cell of claim 34 or 37, wherein said signal peptide is translationally fused at a C-terminus of said polypeptide of interest or said glycosidase.

48. An isolated polypeptide produced according to the method of any one of claims 2 and 36.

49. A nucleic acid construct comprising a nucleic acid sequence encoding a glycosidase translationally fused to a signal peptide for localization in a subcellular compartment of interest.

50. A transgenic plant or plant cell comprising the nucleic acid construct of claim 49.

51. The method of any one of claims 1, 2, 35 and 36, the nucleic acid construct or construct system of any one of claims 31-33, and 49 or the transgenic plant of claim 37, wherein said polypeptide-of interest is a human polypeptide.

52. The method of any one of claims 1, 2, 35 and 36, the nucleic acid construct or construct system of any one of claims 31-33, and 49 or the transgenic plant of claim 37, wherein said polypeptide-of interest is a pharmaceutical.

53. The method of any one of claims 1, 2, 35 and 36, the nucleic acid construct or construct system of any one of claims 31-33, and 49 or the transgenic plant of claim 37, wherein said polypeptide-of interest is selected from the group consisting of an antibody, a vaccine, an enzyme, a growth factor, a hormone and a structural protein.

54. The method of any one of claims 1, 2, 35 and 36, the nucleic acid construct or construct system of any one of claims 31-33, and 49 or the transgenic plant of claim 37, wherein said polypeptide-of interest is an antibody or an antibody fragment.

55. The method, nucleic acid construct or construct system or transgenic plant of claim 54, wherein said antibody is bevacizumab or adalimumab.

56. A seed of the transgenic plant of any one of claims 15, 16, 17, 18, 30, 34, 37, 38 and 50.

57. The seed of claim 56 being a hybrid seed.

58. An isolated polypeptide comprising an amino acid sequence of a protein A/G/L tranlationally fused to a heterologous transmembrane domain.

59. The isolated polypeptide of claim 58, wherein said tranlationally fused is via a linker.

60. An isolated polynucleotide comprising a nucleic acid sequence encoding the polypeptide of claim 58 or 59.

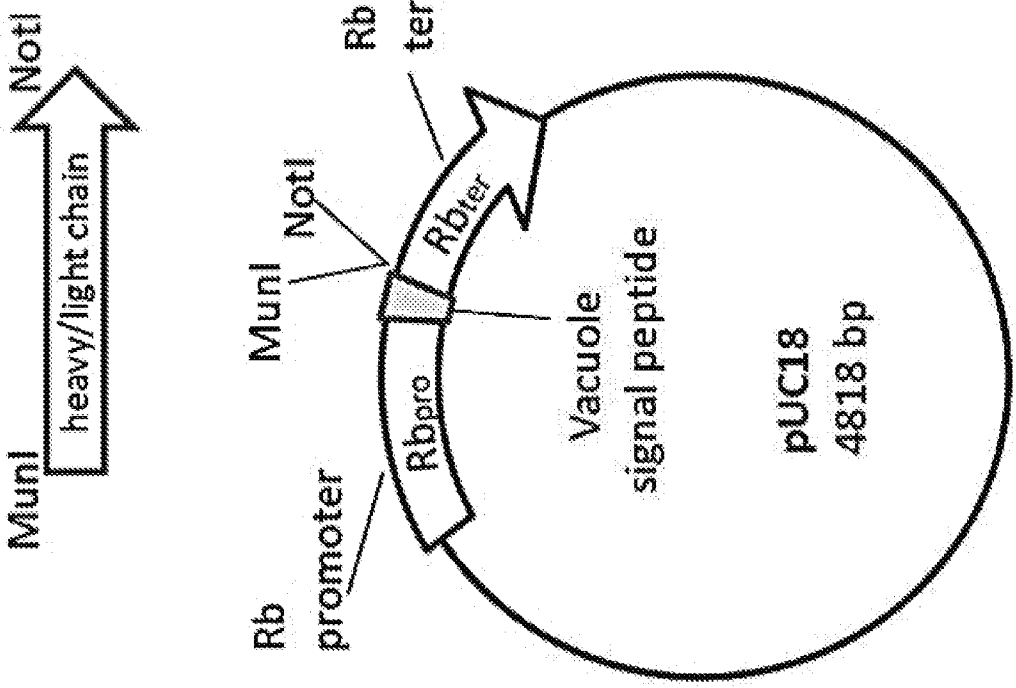

MunI
heavy/light chain
NotI
Rb
promoter
MunI
NotI
Rb
terminator
Rbpro
Rbter
Vacuole
signal peptide
pUC18
4818 bp

FIG. 1A

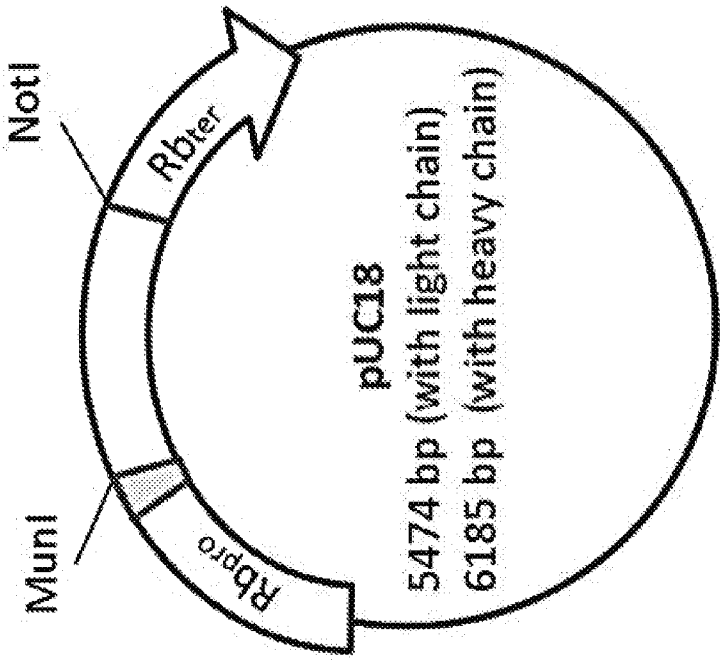

NotI
Rbter
MunI
Rbpro
pUC18
5474 bp (with light chain)
6185 bp (with heavy chain)

FIG. 1B

FIG. 2A

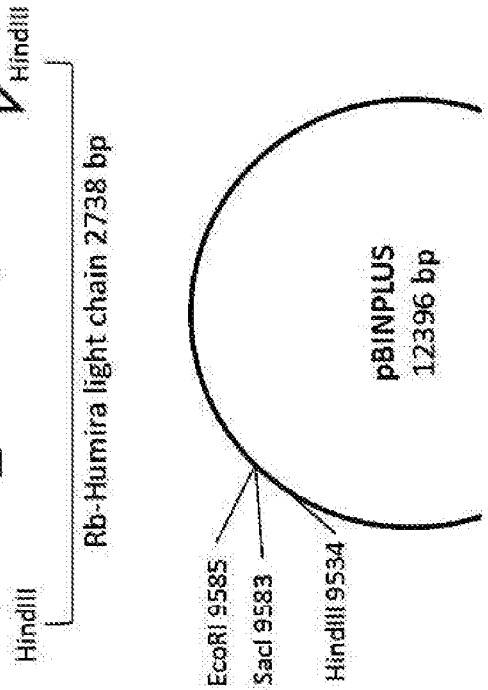


FIG. 2B

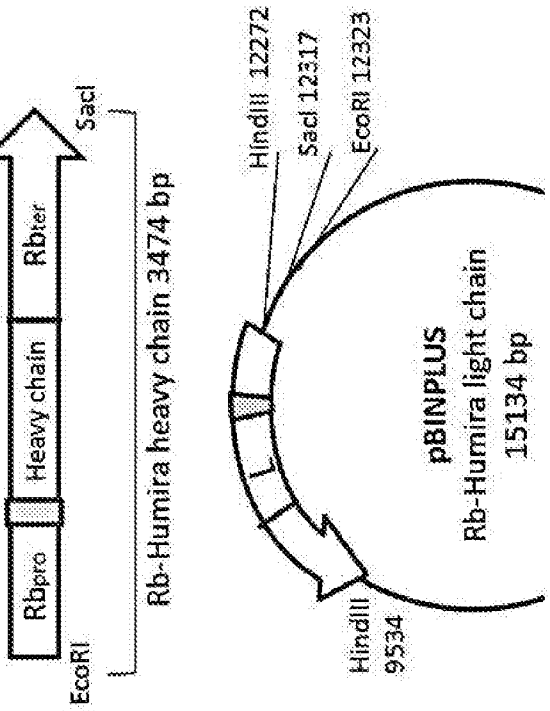


FIG. 2C

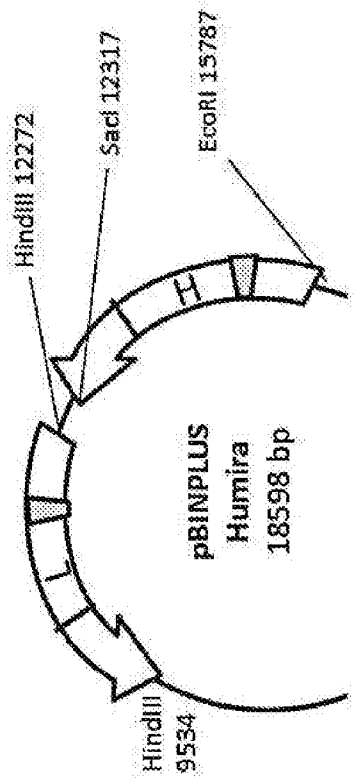


FIG. 3A

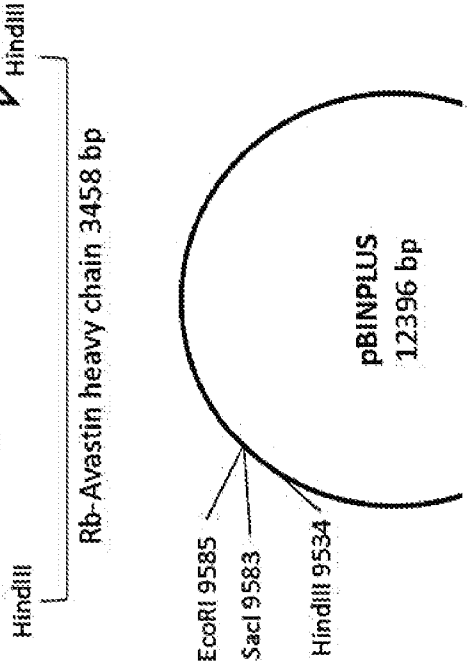


FIG. 3B

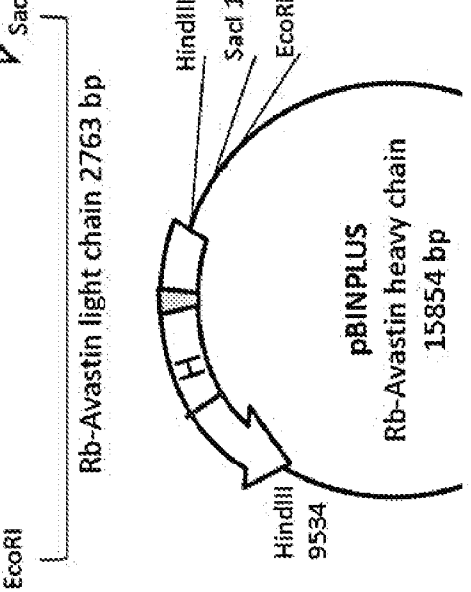


FIG. 3C

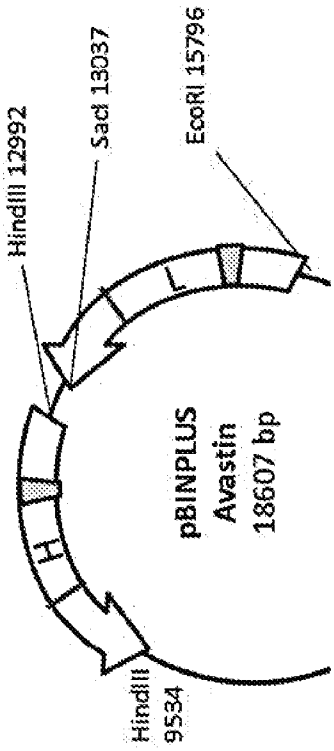
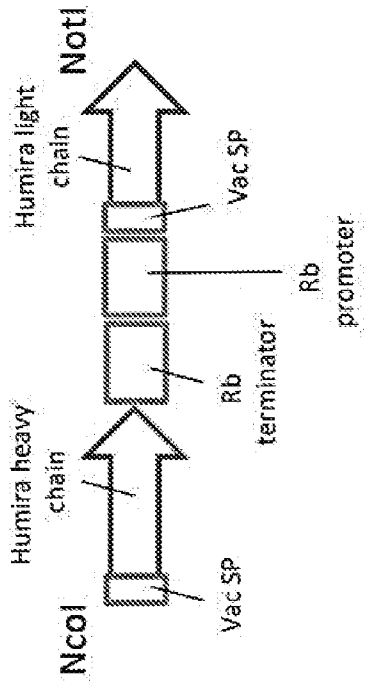


FIG. 4A


NcoI
Vac SP
Humira heavy chain
Rb terminator
Rb promoter
Vac SP
Humira light chain
NotI

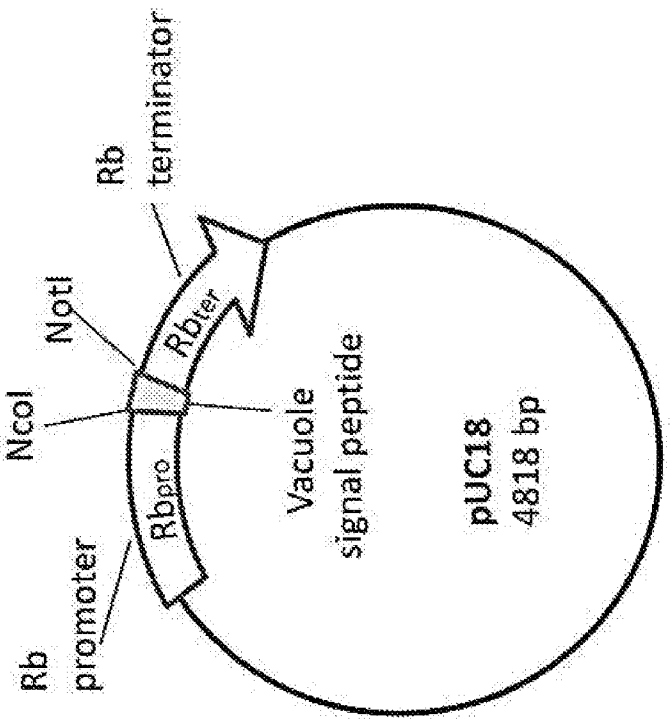

Rb promoter
NcoI
NotI
Rb terminator
Rbpro
Rbter
Vacuole signal peptide
pUC18
4818 bp

FIG. 4B

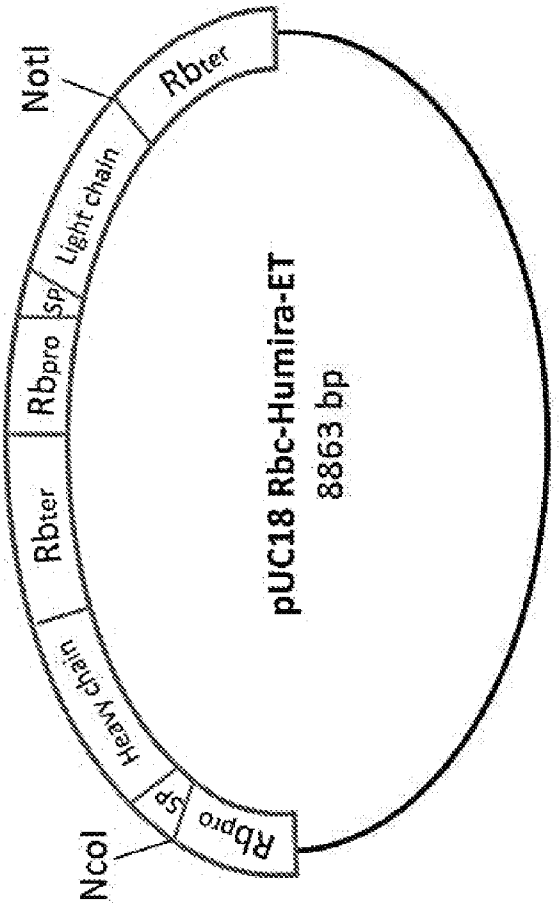
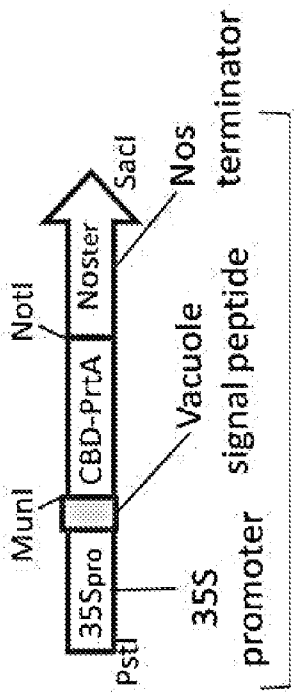

NcoI
Rbpro
SP
Heavy chain
Rbter
Rbpro
SP
Light chain
NotI
Rbter
pUC18 Rbc-Humira-ET
8863 bp

FIG. 5A



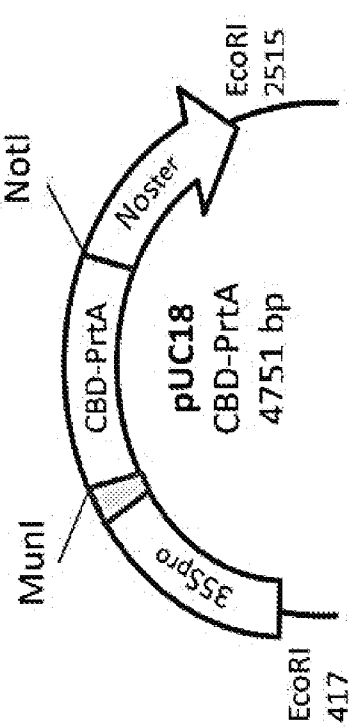
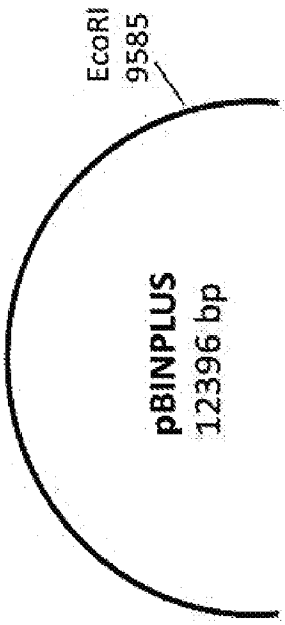
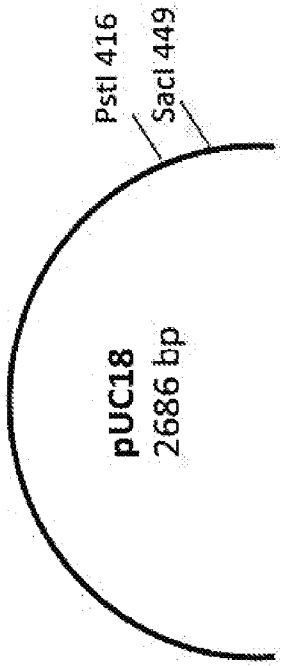


FIG. 5B





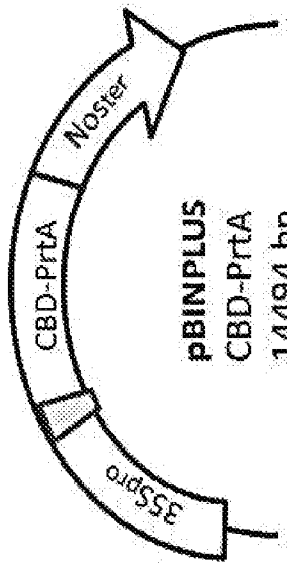
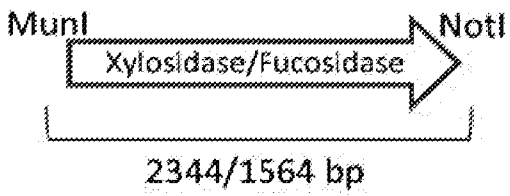


FIG. 5C

FIG. 6A



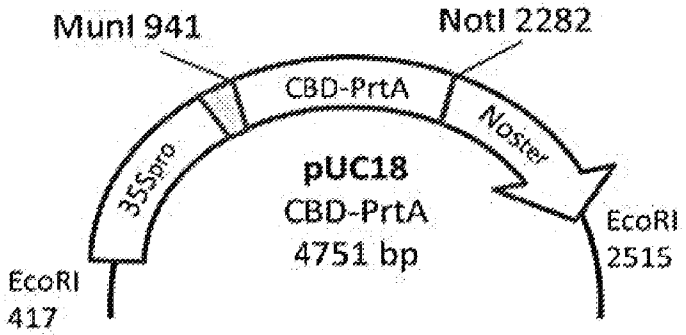


FIG. 6B

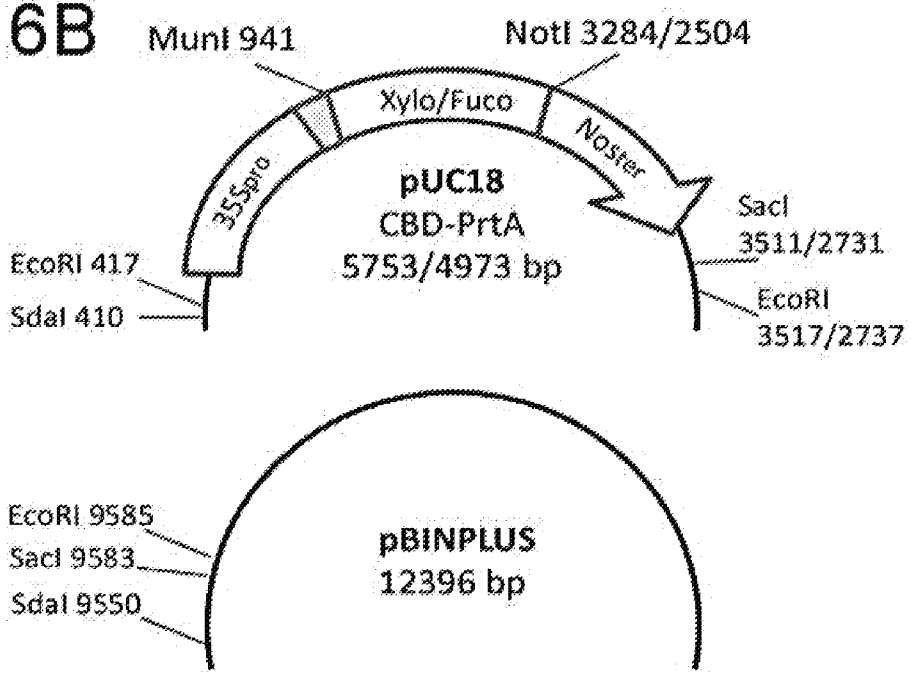


FIG. 6C

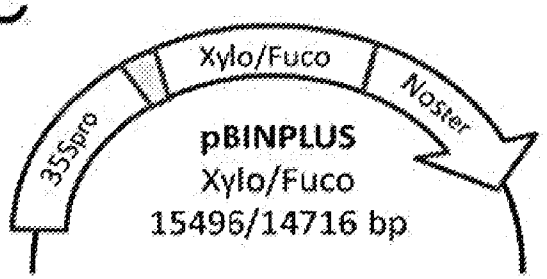


FIG. 7A

1 2 3 4 5 6 7 8 9 10 wt
70 kDa
55 kDa
1. CBD-PrtA 1 sup (s)
2. CBD-PrtA 1 pellet (p)
3. CBD-PrtA 2-s
4. CBD-PrtA 2-p
5. CBD-PrtA 3-s
6. CBD-PrtA 3-p
7. CBD-PrtA 4-s
8. CBD-PrtA 4-p
9. CBD-PrtA 5-s
10. CBD-PrtA 5-p

FIG. 7B

1 2 3
1. CBD-PrtA + 5 ng Humira
2. CBD-PrtA + 10 ng Humira
3. WT + 10 ng Humira

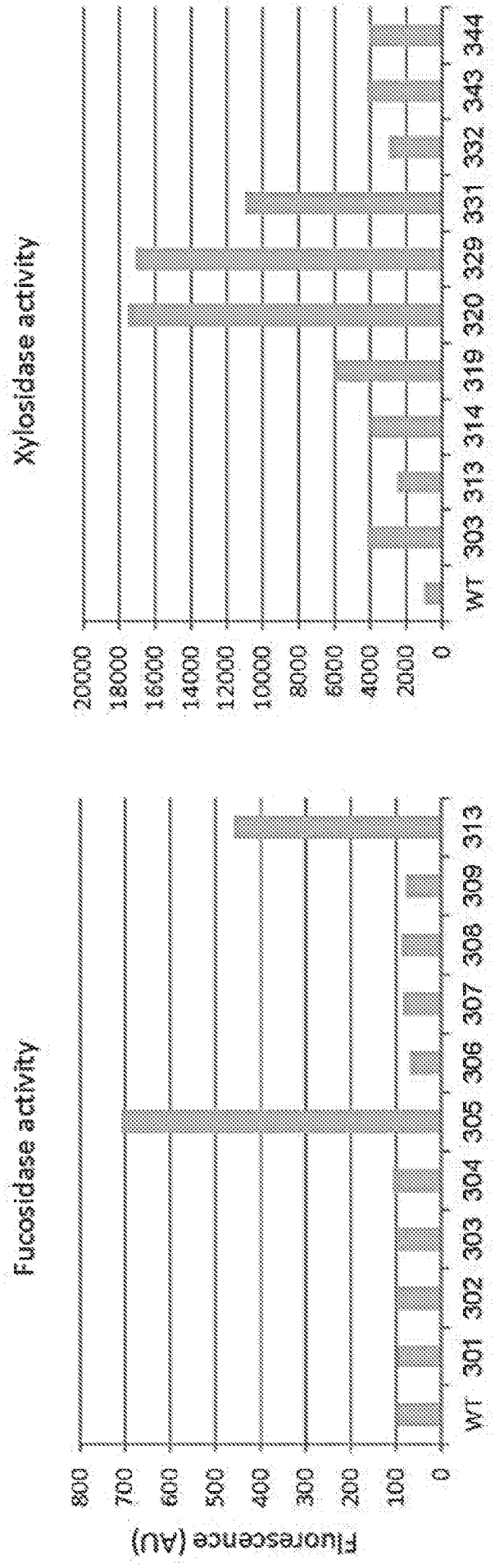

Fucosidase activity
Xylosidase activity
Fluorescence (AU)
800
700
600
500
400
300
200
100
0
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
WT 301 302 303 304 305 306 307 308 309 313
WT 303 313 314 319 320 329 331 332 343 344
Plant name/number

FIG. 8

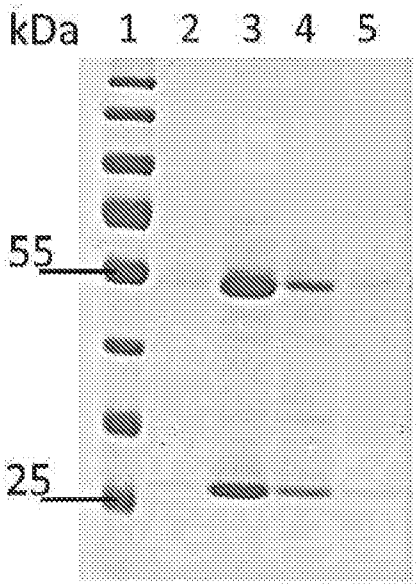

kDa
1
2
3
4
5
55
25

FIG. 9

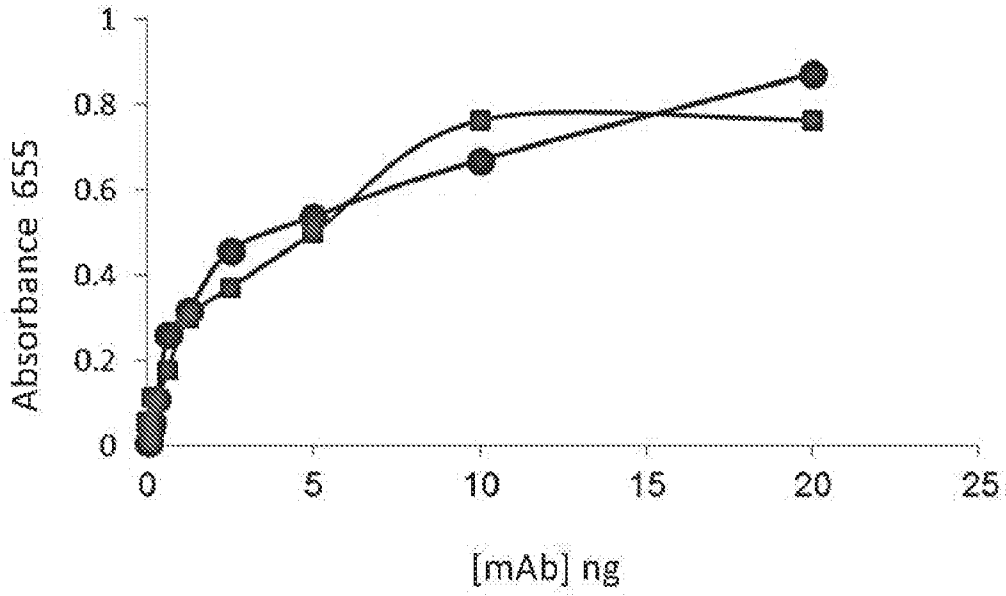

Absorbance 655
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
[mAb] ng

FIG. 10

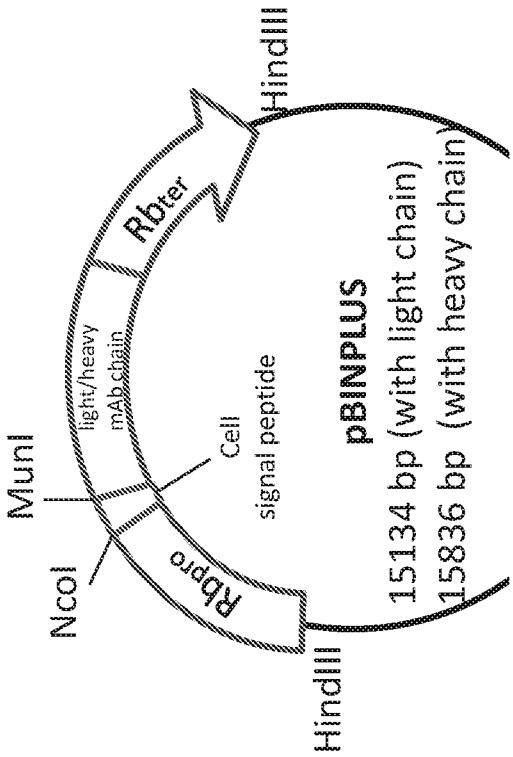


FIG. 11B

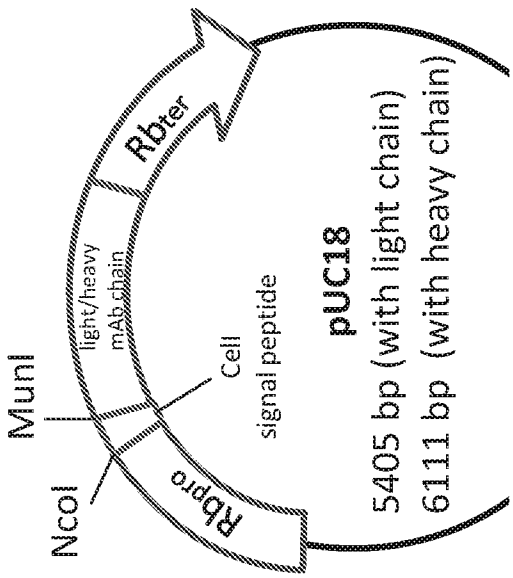


FIG. 11A

FIG. 12A

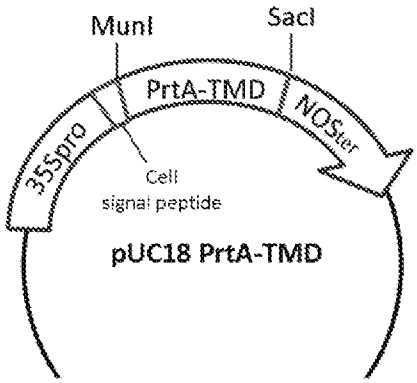


FIG. 12B

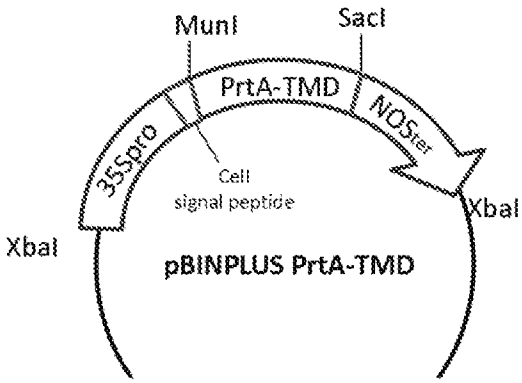
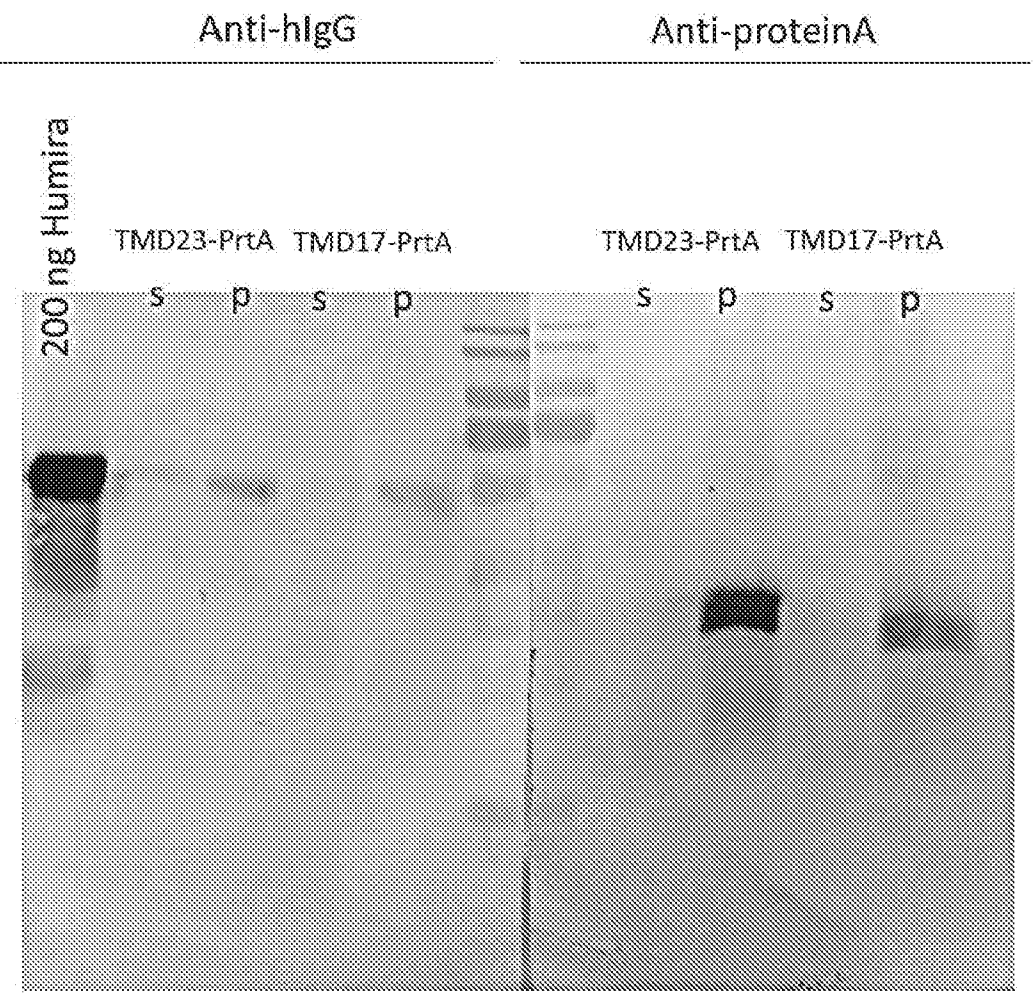


FIG. 12C

FIG. 12D


Anti-hIgG
Anti-proteinA
200 ng Humira
TMD23-PrtA
TMD17-PrtA
TMD23-PrtA
TMD17-PrtA
s
p
s
p
s
p
s
p

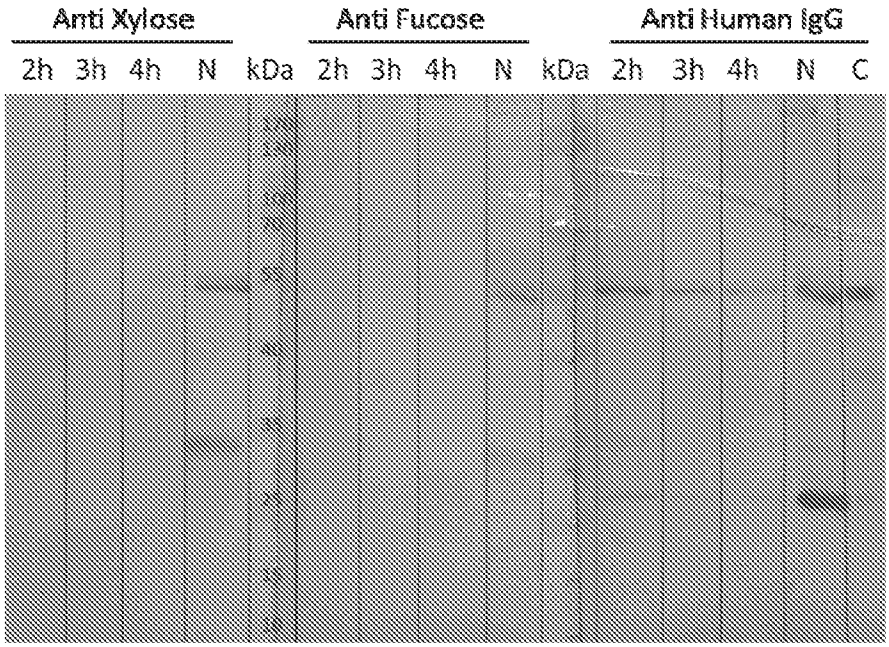

Anti Xylose
Anti Fucose
Anti Human IgG
2h 3h 4h N kDa 2h 3h 4h N kDa 2h 3h 4h N C

FIG. 13

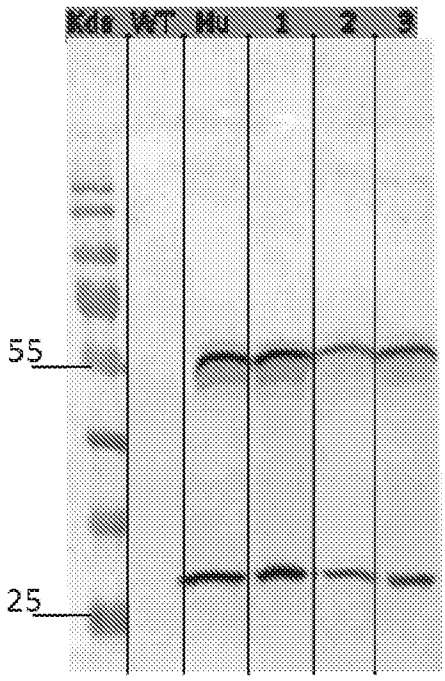

Kda WT Mu 1 2 3
55
25

FIG. 14

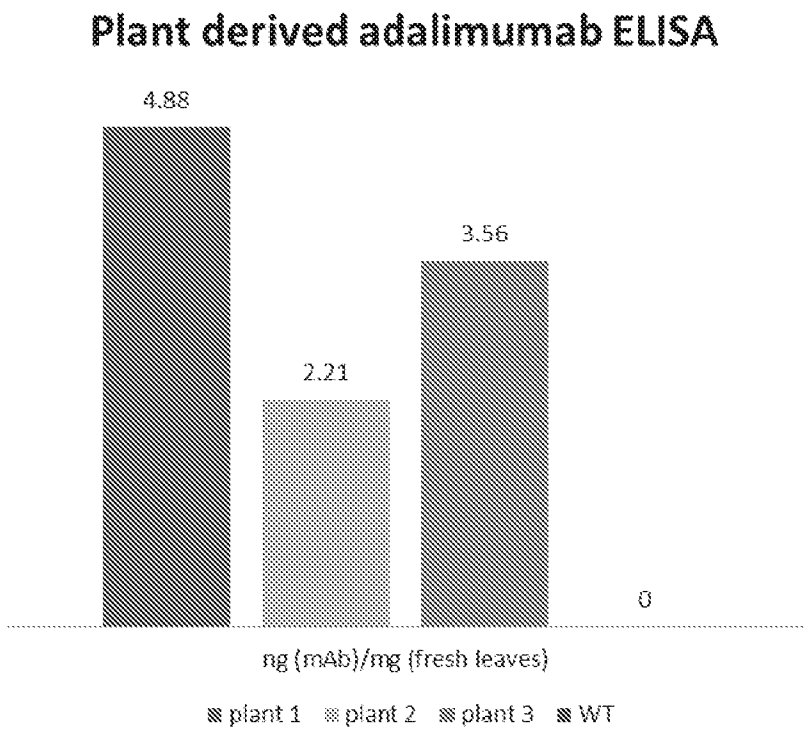

Plant derived adalimumab ELISA
4.88
2.21
3.56
0
ng (mAb)/mg (fresh leaves)
plant 1
plant 2
plant 3
WT

FIG. 15

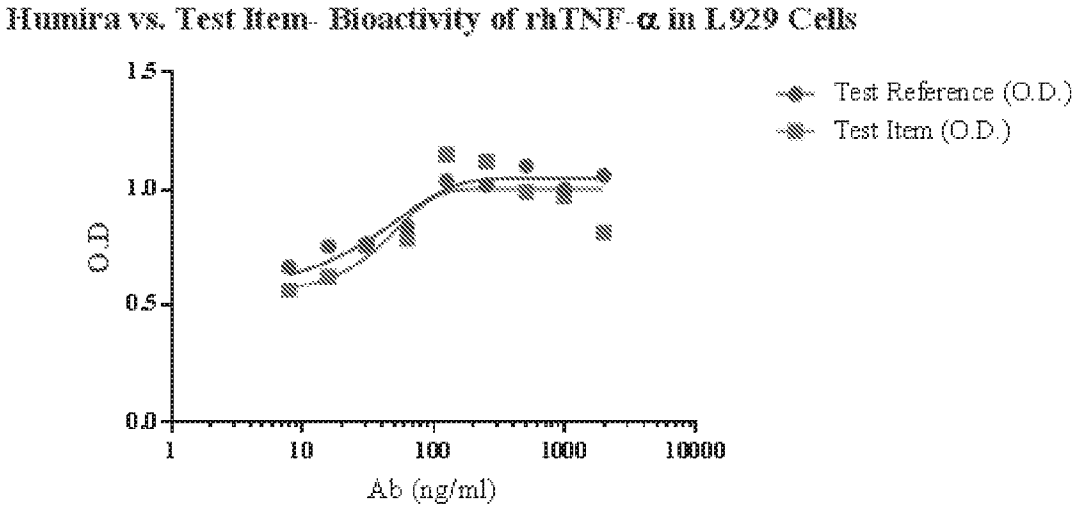

Humira vs. Test Item- Bioactivity of rhTNF-α in L929 Cells
Test Reference (O.D.)
Test Item (O.D.)
O.D
1.5
1.0
0.5
0.0
1
10
100
1000
10000
Ab (ng/ml)

FIG. 16

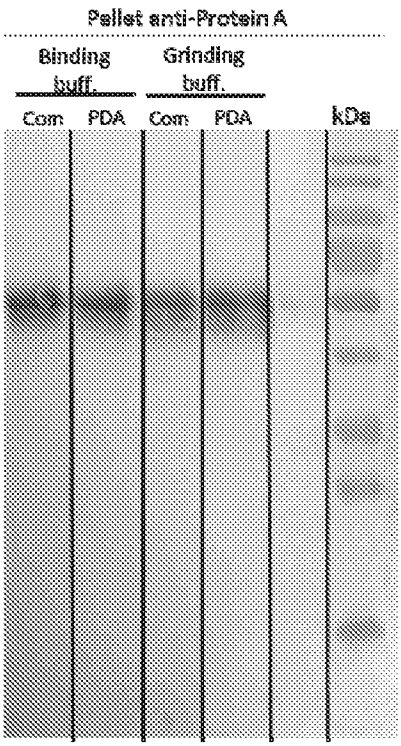


FIG. 17A

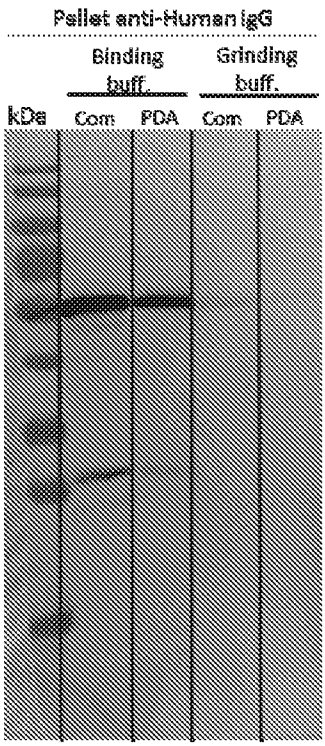


FIG. 17B

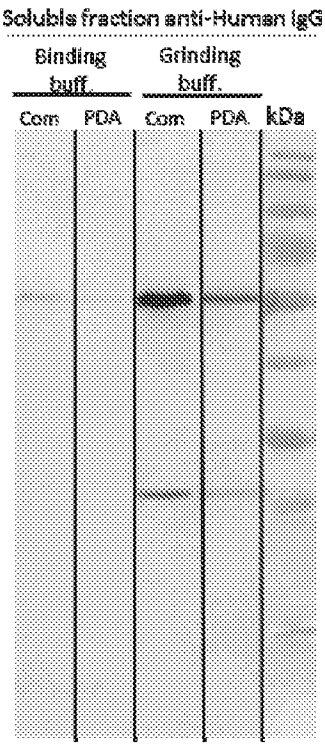


FIG. 17C

FIG. 18A

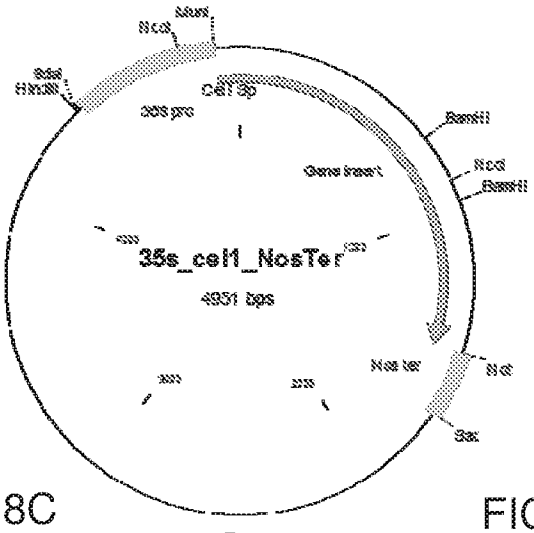


FIG. 18B

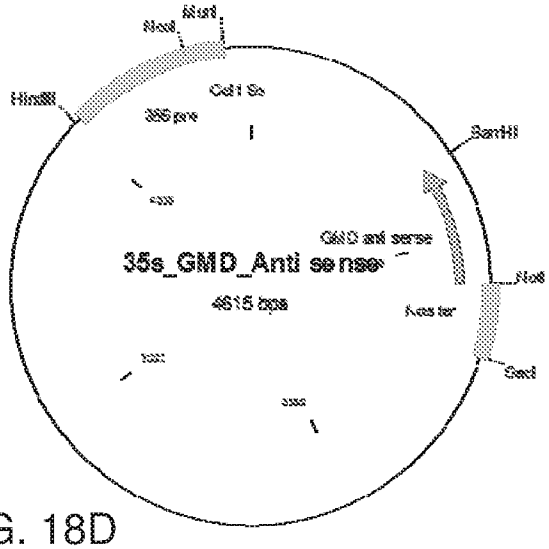


FIG. 18C

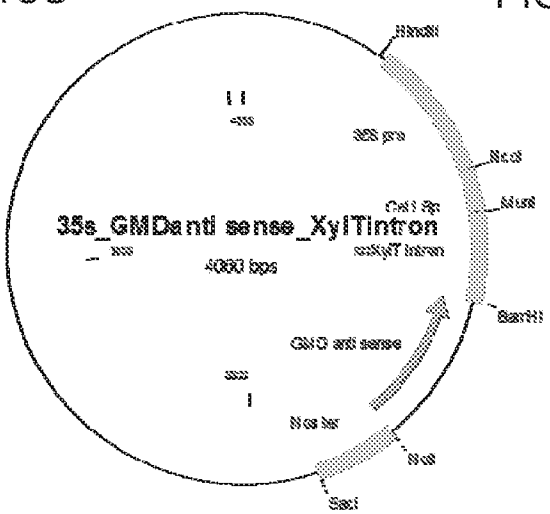


FIG. 18D

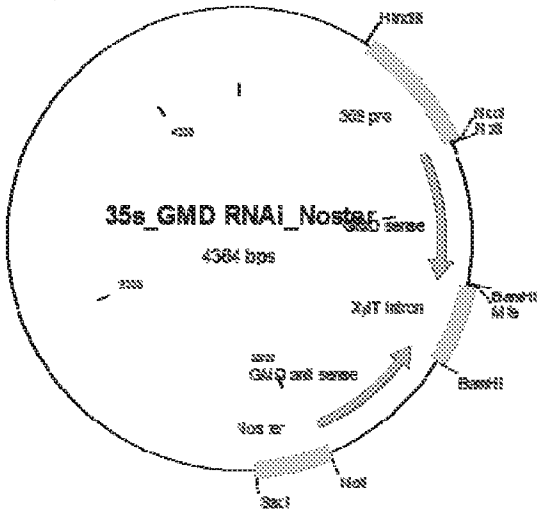


FIG. 19A

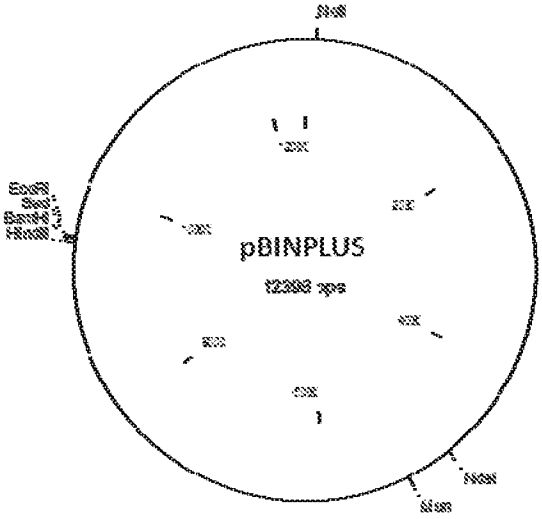


FIG. 19B

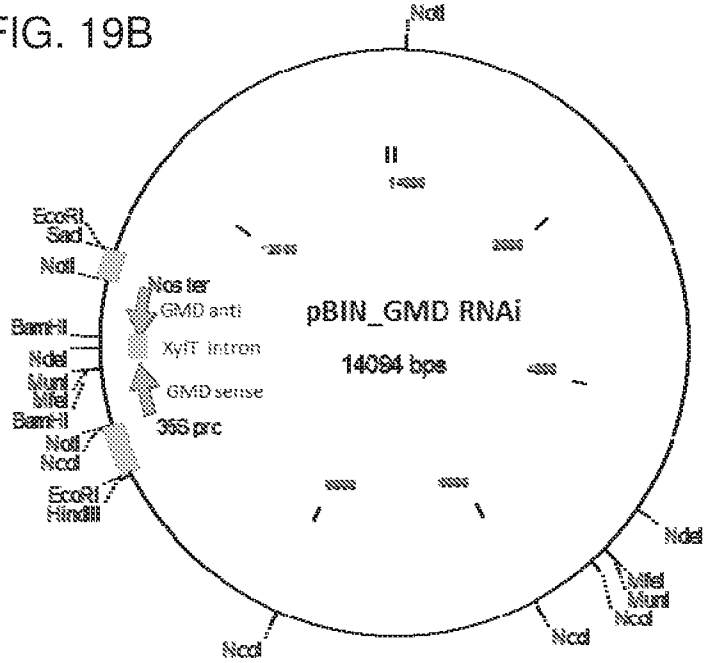


FIG. 20A

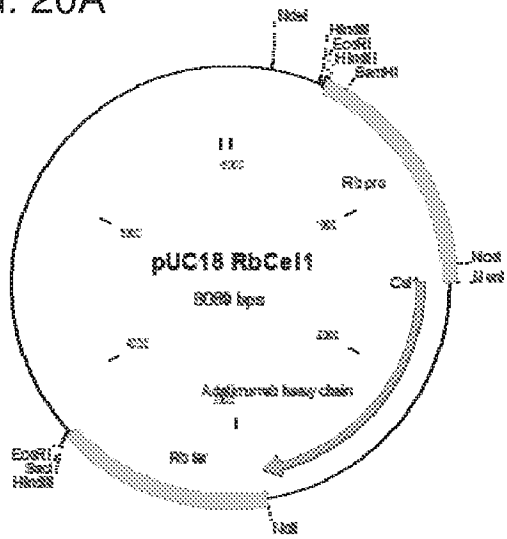


FIG. 20B

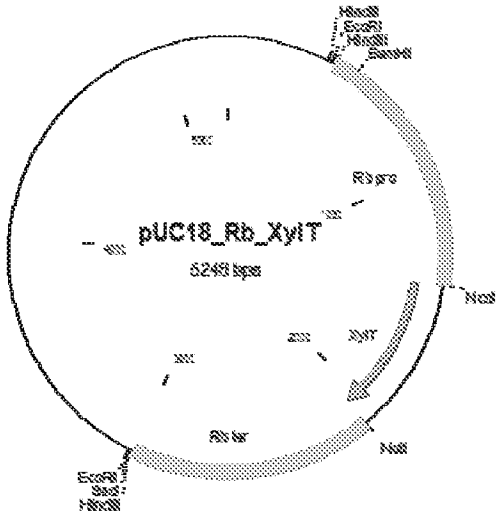


FIG. 20C

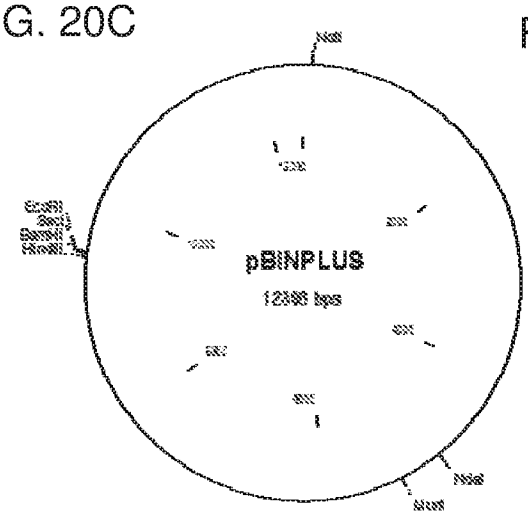


FIG. 20D