Provided are novel β l,2-xylosyltransferase nucleotide sequences and uses thereof.

Abstract: Provided are novel β l,2-xylosyltransferase nucleotide sequences and uses thereof.
Novel nucleotide sequences encoding *Nicotiana* beta-1,2-xylosyltransferase

The following invention relates to novel nucleotide sequences from *Nicotiana* species and cultivars, particularly from *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Petite Havana SRI, encoding β1,2-xylosyltransferase (XyIT) and their use to produce modified *Nicotiana* plants, particularly *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Petite Havana SRI plants, which have a lower level or altered pattern of immunogenic protein-bound N-glycans, particularly a lower level of beta-1,2-xylose residues on the protein-bound N-glycans, than counterpart unmodified *Nicotiana* plants. Such *Nicotiana* plants may be obtained by lowering the expression of the endogenous *Nicotiana* XyIT gene(s), e.g., by modifying the activity of endogenous *Nicotiana* XyIT gene(s), by exchanging the endogenous *Nicotiana* XyIT gene for another allele of the XyIT gene which provides a lower level of beta-1,2-xylose residues on the protein-bound N-glycans, or by any combination thereof.

**Description of related art**

The use of transgenic plants for the production of value-added recombinant proteins, such as antibodies, vaccines, human blood products, hormones, growth regulators and the like, is described to offer many practical, economic and safety advantages compared with more conventional systems such as animal and insect cell cultures, yeast, filamentous fungi and bacteria (reviewed by Stoger *et al.*, 2002; Twyman *et al.*, 2003; Fischer *et al.*, 2004).

Although the protein synthesis pathway is largely the same in plants and animals, there are some differences in posttranslational modifications, particularly with respect to glycan-chain structures. Thus, plant-derived recombinant human proteins tend to have the carbohydrate groups beta(1→2)-xylose and alpha(1→3)-fucose, which are absent in mammals, but lack the terminal galactose and sialic acid residues that are found on many native human glycoproteins (Twyman *et al.*, 2003).

The enzyme that catalyses the transfer of xylose from UDP-xylose to the core beta-linked mannose of protein-bound N-glycans is beta-1,2-xylosyltransferase (XyIT). XyIT is an enzyme
unique to plants and some non-vertebrate animal species, e.g. in *Schistosoma* species (Khoo et al., 1997) and snail (e.g. Mulder et al., 1995) and does not occur in human beings or in other vertebrates.

Tezuka et al. (1992) characterized a XyIT of sycamore (*Acer pseudoplatanus* L.).

Zeng et al. (1997) described the purification of a XyIT from soybean microsomes. Only a part of the soybean XyIT cDNA was isolated (W099/29835 A1).

Strasser et al. (2000) and WOO1/64901 describe the isolation of an *Arabidopsis* XyIT gene, the predicted amino acid sequence of the encoded XyIT protein and its enzymatic activity in vitro and in vivo.

The following database entries identifying experimentally demonstrated and putative XyIT cDNA and gene sequences, parts thereof or homologous sequences, could be identified: AJ627182, AJ627183 (Nicotiana tabacum cv. Xanthi), AM179855 (Solanum tuberosum), AM179856 (Vitis vinifera), AJ891042 (Populus alba x Populus tremula), AY302251 (Medicago sativa), AJ864704 (Saccharum officinarum), AMI79857 (Zea mays), AMI79853 (Hordeum vulgare), AM179854 (Sorghum bicolor), BD434535, AJ277603, AJ272121, AF272852, AX236965 (Arabidopsis thaliana), AJ621918 (Oryza sativa), AR359783, AR359782, AR123000, AR123001 (Soybean), AJ618933 (Physcomitrella patens).

Strasser et al. (2004a) report on two approaches for the modulation of the N-glycosylation pathway in plants: First posttranscriptional gene silencing was used to knock down the expression of beta-1,2-N-acetylglucosaminyltransferase I (GnTI), an enzyme involved in the processing of oligomannosidic residues to hybrid and complex N-glycans in higher eukaryotes, to assess the influence of GnTI expression on the formation of complex N-glycans in *Nicotiana benthamiana*. N-glycan profiling revealed no significant changes of the total N-glycan pattern, indicating that even a minor residual activity of GnTI allows the biosynthesis of complex N-glycans in *Nicotiana benthamiana*. They further report that a similar approach for the knock down of XyIT resulted in a significant reduction of beta-1,2-xylosylated N-glycans. Second, in
order to achieve a complete elimination of beta-1,2-xylose and alpha-1,3-fucose residues from N-glycans, triple knock out *Arabidopsis* plants were generated using insertion mutation lines. These plants exhibit complete deficiency of active beta-1,2-xylosyltransferase and core alpha-i,3-fucosyltransferase, lack immunogenic protein-bound N-glycans and synthesize predominantly humanized structures with terminal beta-N-acetylglucosamine residues (Strasser *et al*, 2004b).

Leafy crops, such as tobacco, are considered to be strong candidates for the commercial production of recombinant proteins (see e.g. Twyman *et al*, 2003).

The aim of the current invention is to provide alternative XyIT cDNA and gene sequences from *Nicotiana* species and cultivars, particularly from *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Petite Havana SR1, which are better suited to modify the expression of XyIT in particular *Nicotiana* species or cultivars.
Summary of the invention

In one aspect of the invention, a method is provided to produce a *Nicotiana* plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of introducing a chimeric gene into plant cells of a *Nicotiana* species or cultivar to generate transgenic plant cells, the chimeric gene comprising operably linked a plant expressible promoter; a transcribable DNA region comprising a first sense DNA region comprising a nucleotide sequence of at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, the nucleotide sequence preferably obtainable from the *Nicotiana* species or cultivar, wherein the at least 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from a nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, the nucleotide sequence preferably obtainable from the *Nicotiana* species or cultivar, wherein the at least 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide; a second antisense DNA region comprising a nucleotide sequence of at least 19 consecutive nucleotides which have at least 95% sequence identity to the complement of the first DNA region; wherein an RNA molecule transcribed from the transcribable DNA region is capable of forming a double stranded RNA region at least between an RNA region transcribed from the first sense DNA region and an RNA region transcribed from the second antisense DNA region; and a DNA region comprising a transcription termination and polyadenylation signal functional in plants; optionally, identifying a transgenic *Nicotiana* plant cell which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than an untransformed *Nicotiana* plant cell; optionally, regenerating the transgenic *Nicotiana* plant cells to obtain transgenic *Nicotiana* plants; and optionally, identifying a transgenic *Nicotiana* plant which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than an untransformed *Nicotiana* plant. The *Nicotiana* species- or cultivar-specific XyIT amino acid or nucleotide may be a *Nicotiana benthamiana*-specific or *Nicotiana tabacum* cv. Petite Havana SR1-specific XyIT amino acid or nucleotide and the *Nicotiana* species or cultivar may preferably be *Nicotiana benthamiana* or *Nicotiana tabacum* cv. Petite Havana SR1, respectively. The nucleotide sequence encoding a *Nicotiana* XyIT protein may comprise a nucleotide sequence encoding the amino acid
sequence of SEQ ID No.: 12 or SEQ ID No.: 14 or the amino acid sequence of SEQ ID No.: 4, 
SEQ ID No.: 6, SEQ ID No.: 8 or SEQ ID No.: 10, and the nucleotide sequence of the Nicotiana 
XyIT gene may comprise the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ 
ID No. 21, or the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 8, SEQ 
ID No.: 10, or SEQ ID No.: 17.

It is another object of the invention to provide a method to produce a Nicotiana plant cell or 
plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising 
the steps of providing one or more double stranded RNA molecules to plant cells or plants of a 
Nicotiana species or cultivar, wherein the double stranded RNA molecules comprise two RNA 
strands, one RNA strand consisting essentially of an RNA nucleotide sequence of 19 out of 20 
to 21 consecutive nucleotides selected from a nucleotide sequence encoding a Nicotiana XyIT 
protein, or the complement thereof, the nucleotide sequence preferably obtainable from the 
Nicotiana species or cultivar, wherein the 19 out of 20 to 21 consecutive nucleotides encode at 
least one Nicotiana species- or cultivar-specific XyIT amino acid, or selected from the 
nucleotide sequence of a Nicotiana XyIT gene or a Nicotiana XyIT cDNA, or the complement 
thereof, the nucleotide sequence preferably obtainable from the Nicotiana species or cultivar, 
wherein the 19 out of 20 to 21 consecutive nucleotides comprise at least one Nicotiana species- 
or cultivar-specific XyIT nucleotide; and identifying a Nicotiana plant cell or plant comprising 
the double stranded RNA molecule or molecules which has a lower level of beta-1,2-xylose 
residues on protein-bound N-glycans than a same Nicotiana plant cell or plant which does not 
comprise the double stranded RNA molecule or molecules. The double stranded RNA may be 
provided to the plant cells or plants by integrating a chimeric gene into the genome of plant 
cells of the Nicotiana species or cultivar to generate transgenic plant cells and, optionally, 
regenerating the plant cells to obtain transgenic plants, the chimeric gene comprising a DNA 
region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide 
sequence encoding a Nicotiana XyIT protein, or the complement thereof, the nucleotide 
sequence preferably obtainable from the Nicotiana species or cultivar, wherein the 19 out of 20 
consecutive nucleotides encode at least one Nicotiana species- or cultivar-specific XyIT amino 
acid, or selected from the nucleotide sequence of a Nicotiana XyIT gene or a Nicotiana XyIT 
cDNA, or the complement thereof, the nucleotide sequence preferably obtainable from the
Nicotiana species or cultivar, wherein the 19 out of 20 consecutive nucleotides comprise at least one Nicotiana species-specific XyIT nucleotide, in antisense and/or sense orientation; operably linked to a plant expressible promoter and a DNA region comprising a transcription termination and polyadenylation signal functional in plants. The Nicotiana species- or cultivar-specific XyIT amino acid or nucleotide may be a Nicotiana benthamiana-specific or Nicotiana tabacum cv. Petite Havana SRI-specific XyIT amino acid or nucleotide and the Nicotiana species or cultivar may preferably be Nicotiana benthamiana or Nicotiana tabacum cv. Petite Havana SRI, respectively. The nucleotide sequence encoding a Nicotiana XyIT protein may comprise a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 12 or SEQ ID No.: 14 or the amino acid sequence of SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8 or SEQ ID No.: 10, and the nucleotide sequence of the Nicotiana XyIT gene may comprise the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No. 21, or the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 8, SEQ ID No.: 10, or SEQ ID No.: 17.

It is yet another object of the invention to provide a method to identify a Nicotiana XyIT DNA fragment, comprising the steps of providing genomic DNA or cDNA obtainable from a Nicotiana species or cultivar; selecting a means from the following group: a DNA fragment comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a probe; a DNA fragment comprising the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a probe; a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 1513 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a probe; a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14 for use as a probe; a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ
ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21 for use as a probe; an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a primer in a PCR reaction; an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a primer in a PCR reaction; an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a primer in a PCR reaction; or an oligonucleotide having the nucleotide sequence of any one of SEQ ID No.: 1, SEQ ID No.: 2, SEQ ID No.: 15 or SEQ ID No.: 16, SEQ ID No.: 19 or SEQ ID No. 20 for use as a primer in a PCR reaction; and utilizing that means to identify a XyIT DNA fragment from the Nicotiana species or cultivar by performing a PCR using the genomic DNA or the cDNA and the primers, or by performing hybridization using the genomic DNA or the cDNA and the probes. The identified fragment may subsequently be isolated and used to obtain a Nicotiana plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans.

The invention also provides a method to identify a Nicotiana XyIT allele correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of providing a population, optionally a mutagenized population, of different plant lines of a Nicotiana species or cultivar; identifying in each plant line of the population a Nicotiana XyIT allele according to the method described above; analyzing the level of beta-1,2-xylose residues on protein-bound N-glycans of each plant line of the population and identifying those plant lines having a lower level of beta-1,2-xylose residues on protein-bound N-glycans than other plant lines; and correlating the low level of beta-1,2-xylose residues on protein-bound N-glycans in a plant line to the presence of a specific Nicotiana XyIT allele. The Nicotiana XyIT allele may be introduced into a Nicotiana plant cell or plant of choice to obtain a Nicotiana plant cell or plant with a low level of beta-1,2-xylose residues on protein-bound N-glycans.
It is yet another object of the invention to provide: an isolated DNA fragment encoding a protein comprising the amino acid sequence of SEQ ID No.: 12, or SEQ ID No.: 14, or any part thereof encoding at least one *Nicotiana benthamiana*-specific XyIT amino acid; an isolated DNA fragment comprising the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No.: 21, or any part thereof comprising at least one *Nicotiana benthamiana*-specific XyIT nucleotide; an isolated DNA fragment encoding a protein comprising the amino acid sequence of SEQ ID No.: 4 or SEQ ID No.: 6, SEQ ID No.: 8, SEQ ID No.: 10, or any part thereof encoding at least one *Nicotiana tabacum* cv. Petite Havana SRL-specific XyIT amino acid; an isolated DNA fragment comprising the nucleotide sequence of SEQ ID No.: 3 or SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, or SEQ ID No.: 17, or any part thereof comprising at least one *Nicotiana tabacum* cv. Petite Havana SRL-specific XyIT nucleotide.

The invention further provides a chimeric gene comprising the following operably linked DNA fragments: a plant expressible promoter; a transcribable DNA region comprising a first DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in antisense orientation; a second DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense orientation, whereby an RNA molecule produced by transcription of the transcribable DNA region is capable of forming a double stranded RNA region by base-pairing at least between an RNA region corresponding to the first DNA region and an RNA region corresponding to the second DNA region; and a DNA region comprising a transcription termination and polyadenylation signal functional in plants. The chimeric gene may also comprise a plant
expressible promoter; a DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein the 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense or antisense orientation; and a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

*Nicotiana* plant cells comprising such chimeric genes and *Nicotiana* plants consisting essentially of such *Nicotiana* plant cells, as well as seed thereof are also provided by the invention.

The invention also relates to the use of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, or any part thereof comprising at least 19 out of 20 consecutive nucleotides encoding at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, to decrease the level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* plant, or the use of a nucleotide sequence comprising the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.:5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17 or SEQ ID No.: 21, or any part thereof comprising at least 19 out of 20 consecutive nucleotides comprising at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, to decrease the level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* plant, to identify a XyIT gene or XyIT cDNA in a *Nicotiana* species or cultivar, to identify an allele of a XyIT gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar, or to introduce an allele of a XyIT gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference
to the following detailed description of different embodiments of the invention, the appended claims and the figures.
Brief description of the Figures

**Figure 1** is a global DNA alignment (based on the standard linear scoring matrix with following parameters: mismatch penalty = 2, open gap penalty = 4 and extend gap penalty = 1) between a cDNA sequence of *Nicotiana tabacum* cv. Xanthi encoding a putative XyIT protein (accession number AJ627182; SEQ ID NO:23) and two different XyIT cDNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRl (SEQ ID NO: 3 and 5). Dots represent nucleotides in the *Nicotiana tabacum* cv. Petite Havana SRl cDNA sequences that are identical to the corresponding nucleotides in the *Nicotiana tabacum* cv. Xanthi cDNA sequence; dashes represent the absence of nucleotides in the *Nicotiana tabacum* cv. Petite Havana SRl cDNA sequences corresponding to nucleotides in the *Nicotiana tabacum* cv. Xanthi cDNA sequence.

**Figure 2** is a global protein alignment (based on the blossom 62 scoring matrix) between the putative XyIT protein encoded by the cDNA sequence from *Nicotiana tabacum* cv. Xanthi (accession number AJ627182; SEQ ID NO:24) and by the two different XyIT cDNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRl (SEQ ID NO: 4 and 6). Dots represent amino acids in the *Nicotiana tabacum* cv. Petite Havana SRl protein sequences that are identical to the corresponding amino acids in the *Nicotiana tabacum* cv. Xanthi protein sequence; dashes represent the absence of amino acids in the *Nicotiana tabacum* cv. Petite Havana SRl protein sequences corresponding to amino acids in the *Nicotiana tabacum* cv. Xanthi protein sequence.

**Figure 3** is a global DNA alignment (based on the standard linear scoring matrix with following parameters: mismatch penalty = 2, open gap penalty = 4 and extend gap penalty = 1) between the genomic DNA sequence from *Nicotiana tabacum* cv. Xanthi encoding a putative XyIT protein (accession number AJ627183; SEQ ID NO:25) and two different XyIT genomic DNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRl (SEQ ID NO: 7 and 9) and two different XyIT genomic DNA sequences isolated from *Nicotiana benthamiana* (SEQ ID NO: 11 and 13). Dots represent nucleotides in the *Nicotiana tabacum* cv. Petite Havana SRl genomic DNA sequences that are identical to the corresponding nucleotides in the
*Nicotiana tabacum* cv. Xanthi genomic DNA sequence; dashes represent the absence of nucleotides in the *Nicotiana tabacum* cv. Petite Havana SRI genomic DNA sequences corresponding to nucleotides in the *Nicotiana tabacum* cv. Xanthi genomic DNA sequence.

Figure 4 is a global protein alignment (based on the blossom 62 scoring matrix) between the putative XyIT protein encoded by the genomic DNA sequence from *Nicotiana tabacum* cv. Xanthi (accession number AJ627183; SEQ ID NO:26) and by the two different XyIT genomic DNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRI (SEQ ID NO:8 and 10) and by the two different XyIT genomic DNA sequences isolated from *Nicotiana benthamiana* (SEQ ID NO: 12 and 14). Dots represent amino acids in the *Nicotiana tabacum* cv. Petite Havana SRI protein sequences that are identical to the corresponding amino acids in the *Nicotiana tabacum* cv. Xanthi protein sequence; dashes represent the absence of amino acids in the *Nicotiana tabacum* cv. Petite Havana SRI protein sequences corresponding to amino acids in the *Nicotiana tabacum* cv. Xanthi protein sequence.
Detailed description of different embodiments of the invention

The current invention is based on the finding that XyIT genes and XyIT cDNAs from *Nicotiana* species and cultivars, particularly *Nicotiana benthamiana* and *Nicotiana tabacum* cv. Petite Havana SRL, are excellent source nucleotide sequences to obtain plants of those *Nicotiana* species and cultivars, particularly *Nicotiana benthamiana* plants and *Nicotiana tabacum* cv. Petite Havana SRL plants, respectively, having a low level of beta-1,2-xylose residues on protein-bound N-glycans, e.g., by modifying the activity of endogenous *Nicotiana* XyIT gene(s), by exchanging an endogenous *Nicotiana* XyIT gene for another allele of the *Nicotiana* XyIT gene which provides a low level of beta-1,2-xylose residues on protein-bound N-glycans, or by any combination thereof.

In one embodiment, the invention is related to a method for obtaining a *Nicotiana* plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans by reducing the expression of the endogenous XyIT gene(s) in the *Nicotiana* plant cell or plant by providing one or more silencing RNA molecules to plant cells or plants of a *Nicotiana* species or cultivar, wherein the silencing RNA molecules comprise a part of a nucleotide sequence encoding a *Nicotiana* XyIT protein, preferably obtained from said *Nicotiana* species or cultivar, wherein said part encodes at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or wherein the silencing RNA molecules comprise a part of a nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, preferably obtained from said *Nicotiana* species or cultivar, wherein said part comprises at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide.

As used herein, "silencing RNA" or "silencing RNA molecule" refers to any RNA molecule, which upon introduction into a plant cell, reduces the expression of a target gene. Such silencing RNA may e.g. be so-called "antisense RNA", whereby the RNA molecule comprises a sequence of at least 20 consecutive nucleotides having 95% sequence identity to the complement of the sequence of the target nucleic acid, preferably the coding sequence of the target gene. However, antisense RNA may also be directed to regulatory sequences of target genes, including the promoter sequences and transcription termination and polyadenylation
signals. Silencing RNA further includes so-called "sense RNA" whereby the RNA molecule comprises a sequence of at least 20 consecutive nucleotides having 95% sequence identity to the sequence of the target nucleic acid. Other silencing RNA may be "unpolyadenylated RNA" comprising at least 20 consecutive nucleotides having 95% sequence identity to the complement of the sequence of the target nucleic acid, such as described in WO01/12824 or US6423885 (both documents herein incorporated by reference). Yet another type of silencing RNA is an RNA molecule as described in WO03/076619 (herein incorporated by reference) comprising at least 20 consecutive nucleotides having 95% sequence identity to the sequence of the target nucleic acid or the complement thereof, and further comprising a largely-double stranded region as described in WO03/076619 (including largely double stranded regions comprising a nuclear localization signal from a viroid of the Potato spindle tuber viroid-type or comprising CUG trinucleotide repeats). Silencing RNA may also be double stranded RNA comprising a sense and antisense strand as herein defined, wherein the sense and antisense strand are capable of base-pairing with each other to form a double stranded RNA region (preferably the said at least 20 consecutive nucleotides of the sense and antisense RNA are complementary to each other). The sense and antisense region may also be present within one RNA molecule such that a hairpin RNA (hpRNA) can be formed when the sense and antisense region form a double stranded RNA region. hpRNA is well-known within the art (see e.g WO99/53050, herein incorporated by reference). The hpRNA may be classified as long hpRNA, having long, sense and antisense regions which can be largely complementary, but need not be entirely complementary (typically larger than about 200 bp, ranging between 200-1000 bp). hpRNA can also be rather small ranging in size from about 30 to about 42 bp, but not much longer than 94 bp (see WO04/073390, herein incorporated by reference). Silencing RNA may also be artificial micro-RNA molecules as described e.g. in WO2005/052170, WO2005/047505 or US 2005/0144667 (all documents incorporated herein by reference).

In another embodiment, the silencing RNA molecules are provided to the plant cell or plant of the Nicotiana species or cultivar by producing a transgenic plant cell or plant of the Nicotiana species or cultivar comprising a chimeric gene capable of producing a silencing RNA molecule, particularly a double stranded RNA ("dsRNA") molecule, wherein the complementary RNA strands of such a dsRNA molecule comprises a part of a nucleotide
sequence encoding a *Nicotiana* XyIT protein, preferably obtained from said *Nicotiana* species or cultivar, wherein said part encodes at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or wherein the complementary RNA strands of such a dsRNA molecule comprises a part of the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, preferably obtained from said *Nicotiana* species or cultivar, wherein said part comprises at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide.

"*Nicotiana*", as used herein, includes all known *Nicotiana* species, such as, but not limited to, *Nicotiana acaulis*, *N. acuminata*, *N. africana*, *N. alata*, *N. amplexicaulis*, *N. arentsi*, *N. attenuata*, *N. benavidesii*, *N. benthamiana*, *N. bigelovii*, *N. bonariensis*, *N. cavicola*, *N. clevelandii*, *N. cordifolia*, *N. corymbosa*, *N. debneyi*, *N. excelsior*, *N. forgetiana*, *N. fragrans*, *N. glauca*, *N. glutinosa*, *N. goodspeedii*, *N. gossei*, *N. hybrid*, *N. ingulba*, *N. kawakamii*, *N. knightiana*, *N. langsdorffii*, *N. linearis*, *N. longiflora*, *N. maritima*, *N. megalosiphon*, *N. miersii*, *N. noctiflora*, *N. nudicaulis*, *N. obtusifolia*, *N. occidentalis*, *N. otophora*, *N. paniculata*, *N. paucijoba*, *N. petunioides*, *N. plumbaginifolia*, *N. quadrivalvis*, *N. raimondii*, *N. repanda*, *N. rosulata*, *N. rotundifolia*, *N. rustica*, *N. setchellii*, *N. simulans*, *N. solanifolia*, *N. spegazzinii*, *N. stocktonii*, *N. suaveolens*, *N. sylvestris*, *N. tabacum*, *N. thyrsiflora*, *N. tomentosa*, *N. tomentosiformis*, *N. trigonophylla*, *N. umbratica*, *N. undulata*, *N. velutina*, *N. wigandioides*, and *Nicotiana x sandera*, and all known *Nicotiana* cultivars, such as, but not limited to, cultivars of *Nicotiana tabacum*, such as cv. Burley2.1, cv. Delgold, cv. Petit Havana, cv. Petit Havana SRI, cv. Samsun, and cv. Xanthi.

*Nicotiana tabacum*, which is common tobacco, is a tetraploid hybrid species, which originated from the diploid species *Nicotiana sylvestris* and *Nicotiana tomentosiformis."

A *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA refers to a nucleotide sequence of a XyIT gene that naturally occurs in a *Nicotiana* species or cultivar or to cDNA corresponding to the mRNA of a XyIT gene that naturally occurs in a *Nicotiana* species or cultivar. Similarly, a *Nicotiana* XyIT protein refers to a protein as it naturally occurs in a *Nicotiana* species or cultivar.
Examples of nucleotide sequences encoding a *Nicotiana* XyIT protein, include those obtained from *Nicotiana benthamiana* encoding the amino acid sequence set forth in SEQ ID No.: 12 or SEQ ID No.: 14, and those obtained from *Nicotiana tabacum* cv. Petite Havana SRI encoding the amino acid sequence set forth in SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8, or SEQ ID No.: 10.

Examples of nucleotide sequences of a *Nicotiana* XyIT gene include those obtained from *Nicotiana benthamiana* comprising the nucleotide sequence set forth in SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No.: 21, and those obtained from *Nicotiana tabacum* cv. Petite Havana SRI comprising the nucleotide sequence set forth in SEQ ID No.: 7 or SEQ ID No.: 9.

Examples of nucleotide sequences of a *Nicotiana* XyIT cDNA, include those obtained from *Nicotiana tabacum* cv. Petite Havana SRI comprising the nucleotide sequence set forth in SEQ ID No.: 3, SEQ ID No.: 5 or SEQ ID No.: 17.

However, it will be immediately clear to the person skilled in the art that the exemplified nucleotide sequences or parts thereof can be used to identify further nucleotide sequences of *Nicotiana* XyIT genes or *Nicotiana* XyIT cDNAs in *Nicotiana* species or cultivars, and that such nucleotide sequences or parts thereof may also be used e.g. to decrease the level of beta-1,2-xylose residues on protein-bound N-glycans in *Nicotiana* plants. The exemplified nucleotide sequences could be used to select:

i) a DNA fragment comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a probe;

ii) a DNA fragment comprising the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a probe;

iii) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 1513 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a probe;
iv) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a probe

v) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a probe;

vi) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a primer in a PCR reaction;

vii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a primer in a PCR reaction;

viii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a primer in a PCR reaction;

or

ix) an oligonucleotide having the nucleotide sequence of any one of SEQ ID No.: 1, SEQ ID No.: 2, SEQ ID No.: 15 or SEQ ID No.: 16, SEQ ID No.: 19 or SEQ ID No.20 for use as a primer in a PCR reaction.

By performing a PCR using genomic DNA or cDNA from *Nicotiana* species or cultivars and the mentioned oligonucleotides as primers or by performing hybridization, preferably under stringent conditions between genomic or cDNA from *Nicotiana* species or cultivars and the mentioned probes, such other *Nicotiana* XyIT genes or *Nicotiana* XyIT cDNAs or fragments thereof can be identified and/or isolated.
"Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C, e.g. for about 10 min (twice). Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

A "Nicotiana species-specific XyIT nucleotide" or a "Nicotiana cultivar-specific XyIT nucleotide", refers to a nucleotide of the nucleotide sequence of a XyIT gene or a XyIT cDNA from a Nicotiana species or cultivar that differs from or is not present in the corresponding nucleotide sequence of the XyIT gene from Nicotiana tabacum cv. Xanthi represented in SEQ ID NO: 25, or of the XyIT cDNA from Nicotiana tabacum cv. Xanthi represented in SEQ ID NO: 23, respectively.

A "Nicotiana species-specific XyIT amino acid" or a "Nicotiana cultivar-specific XyIT amino acid", refers to an amino acid of the amino acid sequence of a XyIT protein encoded by a XyIT gene or encoded by a XyIT cDNA from a Nicotiana species or cultivar that differs from or is not present in the corresponding amino acid sequence of the XyIT protein encoded by the XyIT gene from Nicotiana tabacum cv. Xanthi represented in SEQ ID NO: 26, or encoded by the XyIT cDNA from Nicotiana tabacum cv. Xanthi represented in SEQ ID NO: 24, respectively.

To determine the presence of a Nicotiana species- or cultivar-specific XyIT nucleotide or amino acid in the nucleotide sequence of a XyIT gene or a XyIT cDNA from a Nicotiana species or cultivar or in the amino acid sequence of a XyIT protein encoded by a XyIT gene or encoded by a XyIT cDNA from a Nicotiana species or cultivar, for the purpose of this invention, the XyIT nucleotide or amino acid sequences from the Nicotiana species or cultivar
are compared with the corresponding XyIT nucleotide or amino acid sequences from *Nicotiana tabacum* cv. Xanthi by aligning the sequences using a global alignment procedure (For nucleotide sequences the default scoring matrix used is "standard linear" with mismatch penalty = 2, open gap penalty = 4 and extend gap penalty = 1. For protein sequences the default scoring matrix is "blosum 62"; Henikoff and Henikoff, 1992.) To perform the alignment the Align Plus program (provided by Scientific & Educational Software, USA) may be used.

One example of such a global DNA alignment is the global DNA alignment of the XyIT cDNA sequence from *Nicotiana tabacum* cv. Xanthi represented in SEQ ID NO:23 with the XyIT cDNA sequences from *Nicotiana tabacum* cv. Petite Havana SRI represented in SEQ ID NO:3 and 5, in Figure 1. Examples of *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotides determined based on this global DNA alignment include:

- the nucleotide at position 1041, 1323, 1332, or 1421 of SEQ ID NO:3,

Another example of such a global DNA alignment is the global DNA alignment of the XyIT gene sequence from *Nicotiana tabacum* cv. Xanthi represented in SEQ ID NO:25 with the XyIT gene sequences from *Nicotiana tabacum* cv. Petite Havana SRI represented in SEQ ID NO:7 and 9 and with the XyIT gene sequences from *Nicotiana benthamiana* represented in SEQ ID NO:11 and 13, in Figure 3. Examples of *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotides determined based on this global DNA alignment include:

Examples of *Nicotiana benthamiana*-specific XyIT nucleotides determined based on this global DNA alignment include:


One example of such a global protein alignment is the global protein alignment of the XyIT protein sequence encoded by the XyIT cDNA sequence from *Nicotiana tabacum* cv. Xanthi represented in SEQ ID NO:24 with the XyIT protein sequences encoded by the XyIT cDNA sequences from *Nicotiana tabacum* cv. Petite Havana SRI represented in SEQ ID NO:4 and 6, in Figure 2. Examples of *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT amino acids determined based on this global protein alignment include:

- the amino acid at position 472 or 502 of SEQ ID NO:4,
- the amino acid at position 20, 28, 38, 40, 46, 51, 70, 71, 95, 97, 184, 213, 298, 313, 334, or 497 of SEQ ID NO:6.

Another example of such a global protein alignment is the global protein alignment of the XyIT protein sequences encoded by the XyIT gene sequence from *Nicotiana tabacum* cv. Xanthi represented in SEQ ID NO:26 with the XyIT protein sequences encoded by the XyIT gene sequences from *Nicotiana benthamiana* represented in SEQ ID NO:12 and 14, in Figure 4. Examples of *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT amino acids determined based on this global protein alignment include:

- the amino acid at position 19, 27, 32-38, 44, 46, 52, 57, 76, 77, 101, 103, 190, 219, 304, 319, 340, or 356 of SEQ ID NO:8,
- the amino acid at position 183, 212, 297, 312, 333, or 349 of SEQ ID NO:10.

Examples of *Nicotiana benthamiana*-specific XyIT amino acids determined based on this global protein alignment include:

- the amino acid at position 22, 24, 27, 33, 37, 51, 69, 94, 104, 156, 158, 161, 174, 182, 211, 238, 297, 349, or 414 of SEQ ID NO:12,
the amino acid at position 1, 26, 36, 68, 99, 133, 150, 157, 166, 180, 189, 211, 218, 245, 257, 296, 327, 348, or 392 of SEQ ID NO: 14.

The part of the nucleotide sequence encoding a *Nicotiana* XyIT protein and the part of the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA comprised within the silencing RNA molecule, particularly within one strand of the double stranded RNA molecule, should be at least 19 nucleotides long, but may vary from about 19 nucleotides (nt) up to a length equalling the length (in nucleotides) of the *Nicotiana* XyIT protein-encoding sequence or the *Nicotiana* XyIT gene or cDNA sequence. The total length of the sense or antisense nucleotide sequence may thus be at least 25 nt, or at least about 50 nt, or at least about 100 nt, or at least about 150 nt, or at least about 200 nt, or at least about 500 nt. It is expected that there is no upper limit to the total length of the sense or the antisense nucleotide sequence. However for practical reason (such as e.g. stability of the chimeric genes) it is expected that the length of the sense or antisense nucleotide sequence should not exceed 5000 nt, particularly should not exceed 2500 nt and could be limited to about 1000 nt.

It will be appreciated that the longer the total length of the part of nucleotide sequence encoding a *Nicotiana* XyIT protein or the part of the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA (sense or antisense region), the less stringent the requirements for sequence identity between these regions and the corresponding sequence in the endogenous XyIT gene from the *Nicotiana* species or cultivar it complements are. Preferably, the nucleic acid of interest should have a sequence identity of at least about 75% with the corresponding target sequence, particularly at least about 80%, more particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially be identical to the corresponding part of the target sequence or its complement. However, it is preferred that the nucleic acid of interest always includes a sequence of about 19 consecutive nucleotides, particularly about 25 nt, more particularly about 50 nt, especially about 100 nt, quite especially about 150 nt with 100% sequence identity to the corresponding part of the target XyIT nucleic acid, wherein said about 19 consecutive nucleotides, particularly about 25 nt, more particularly about 50 nt, especially about 100 nt, quite especially about 150 nt, encode
at least one *Nicotiana* species- or cultivar-specific XyIT amino acid or comprise at least one
*Nicotiana* species- or cultivar-specific XyIT nucleotide.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino
acid sequences, expressed as a percentage, refers to the number of positions in the two
optimally aligned sequences which have identical residues (x100) divided by the number of
positions compared. A gap, i.e. a position in an alignment where a residue is present in one
sequence but not in the other, is regarded as a position with non-identical residues. Preferably,
for calculating the sequence identity and designing the corresponding sense or antisense
sequence, the number of gaps should be minimized, particularly for the shorter sense
sequences.

It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference
to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide
sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA
molecules will be clear from the context of the application.

It has been demonstrated that the minimum requirement for silencing a particular target gene is
the presence in the silencing chimeric gene nucleotide sequence of a nucleotide sequence of
about 20-21 consecutive nucleotides long corresponding to the target gene sequence, in which
at least 19 of the 20-21 consecutive nucleotides are identical to the corresponding target gene
sequence. "19 out of 20 consecutive nucleotides" as used herein refers to a nucleotide sequence
of 20 consecutive nucleotides selected from the target gene having one mismatch nucleotide.

For silencing the endogenous XyIT gene from a *Nicotiana* species or cultivar, it is preferred
that the silencing chimeric gene nucleotide sequence comprises at least 19 out of 20-21
consecutive nucleotides from a nucleotide sequence encoding a *Nicotiana* XyIT protein,
wherein said at least 19 out of 20-21 consecutive nucleotides encode at least one *Nicotiana*
species- or cultivar-specific XyIT amino acid, or comprises at least 19 out of 20-21 consecutive
nucleotides from a nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA,
wherein said at least 19 out of 20-21 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide.

As used herein "a *Nicotiana* plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans" is a plant (particularly a *Nicotiana* plant obtained according to the methods of the invention), in which the XyIT activity is decreased or abolished resulting in a lower level of beta-1,2-xylose residues on protein-bound N-glycans than the level of beta-1,2-xylose residues on protein-bound N-glycans in a control *Nicotiana* plant not treated according to the methods of the invention or resulting in the absence of beta-1,2-xylose residues on protein-bound N-glycans. An indication of XyIT activity can be obtained by comparing the level of beta-1,2-xylose residues present on the glycans of proteins from the *Nicotiana* plant obtained according to the methods of the invention with the level of beta-1,2-xylose residues present on the glycans of proteins from a control *Nicotiana* plant not treated according to the methods of the invention. The level of beta-1,2-xylose residues on protein-bound N-glycans of plants can be measured e.g. by Western blot analysis using xylose-specific antibodies as described e.g. by Faye et al. (Analytical Biochemistry, 1993, 209: 104-108) or by mass spectrometry on glycans isolated from the plant's glycoproteins using Matrix-assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS) as described e.g. by Kolarich and Altmann (2000, Anal. Biochem. 285: 64-75) or using Liquid Chromatography Tandem mass spectrometry (LC/MS/MS) as described e.g. by Henriksson et al. (2003, Biochem. J. 375: 61-73).

dsRNA encoding *Nicotiana* XyIT expression reducing chimeric genes according to the invention may comprise an intron, such as a heterologous intron, located e.g. in the spacer sequence between the sense and antisense RNA regions in accordance with the disclosure of WO 99/53050 (incorporated herein by reference).

It has become apparent that double stranded RNA molecules, such as the ones described above, are cleaved in plant cells into small RNA fragments of about 20-21 nucleotides, which serve as guide sequence in the degeneration of the corresponding mRNA (reviewed by Baulcombe, 2004). Thus, in another embodiment, the invention is drawn to a method for producing a
Nicotiana plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of:

a) providing one or more double stranded RNA molecules to cells of a plant of a Nicotiana species or cultivar, wherein the double stranded RNA molecules comprise two RNA strands, one RNA strand consisting essentially of an RNA nucleotide sequence of 20 to 21 consecutive nucleotides selected from a nucleotide sequence encoding a Nicotiana XyIT protein, preferably obtained from said Nicotiana species or cultivar, wherein said 20 to 21 consecutive nucleotides encode at least one Nicotiana species- or cultivar-specific XyIT amino acid, or one RNA strand consisting essentially of an RNA nucleotide sequence of 20 to 21 consecutive nucleotides from a nucleotide sequence of a Nicotiana XyIT gene or a Nicotiana XyIT cDNA, preferably obtained from said Nicotiana species or cultivar, wherein said 20 to 21 consecutive nucleotides comprise at least one Nicotiana species- or cultivar-specific XyIT nucleotide; and

b) identifying a Nicotiana plant comprising these double stranded RNA molecule or molecules which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than a same Nicotiana plant which does not comprise the double stranded RNA molecule or molecules.

The mentioned 20-21 nt long dsRNA sequences are also generated in the course of conventional antisense RNA mediated silencing or sense RNA mediated silencing. Thus, in another embodiment of the invention, a method is provided for producing a Nicotiana plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans, comprising the step of providing to cells of a plant of the Nicotiana species or cultivar a chimeric gene comprising, operably linked, the following DNA fragments

a) a plant expressible promoter;

b) a DNA region comprising at least 20 consecutive nucleotides selected from a nucleotide sequence encoding a Nicotiana XyIT protein, preferably obtained from said Nicotiana species or cultivar, wherein said at least 20 consecutive nucleotides encode at least one Nicotiana species- or cultivar-specific XyIT amino acid, or comprising at least 20 consecutive nucleotides from a nucleotide sequence of a Nicotiana XyIT gene or a Nicotiana XyIT cDNA, preferably obtained from said Nicotiana species or cultivar,
wherein said at least 20 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, in antisense or in sense orientation;

c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

The mentioned antisense or sense nucleotide regions may thus be from about 21 nt to about 5000 nt long, such as 21nt, 40 nt, 50 nt, 100nt, 200 nt, 300nt, 500nt, 1000 nt, or even about 2000 nt or larger in length. Moreover, it is not required for the purpose of the invention that the nucleotide sequence of the used inhibitory XyIT gene molecule or the encoding region of the chimeric gene, is completely identical or complementary to the endogenous *Nicotiana* XyIT gene the expression of which is targeted to be reduced in the *Nicotiana* plant cell. The longer the sequence, the less stringent the requirement for the overall sequence identity is. Thus, the sense or antisense regions may have an overall sequence identity of about 40% or 50% or 60% or 70% or 80% or 90% or 100% to the nucleotide sequence of the endogenous *Nicotiana* gene or the complement thereof. However, as mentioned, antisense or sense regions should preferably comprise a nucleotide sequence of 19-20 consecutive nucleotides having about 100% sequence identity to the nucleotide sequence of the XyIT gene, wherein said 19-20 consecutive nucleotides, encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid or comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide. The stretch of about 100% sequence identity may be about 50, 75 or 100 nt.

The efficiency of the above mentioned chimeric genes which when transcribed yield antisense or sense silencing RNA may be further enhanced by inclusion of DNA elements which result in the expression of aberrant, unpolyadenylated XyIT inhibitory RNA molecules. One such DNA element suitable for that purpose is a DNA region encoding a self-splicing ribozyme, as described in WO 00/01 133. The efficiency may also be enhanced by providing the generated RNA molecules with nuclear localization or retention signals as described in WO 03/076619.

The exemplified XyIT nucleotide sequences from *Nicotiana benthamiana* and from *Nicotiana tabacum* can also be used to identify XyIT alleles in a population of plants of a *Nicotiana* species or cultivar which are correlated with low levels of beta-1,2-xylose residues on protein-
bound N-glycans. Such populations of plants of a *Nicotiana* species or cultivar may be populations which have been previously mutagenized. The identified XyIT alleles may then be introduced into a plant line of a *Nicotiana* species or cultivar of choice using conventional breeding techniques.

Methods to transform *Nicotiana* plants are also well known in the art. *Agrobacterium*-mediated transformation of *Nicotiana* has been described e.g. in Zambryski *et al.* (1983, EMBO J. 2: 2143-2150), De Block *et al.* (1984, EMBO J. 3(8):1681-1689), or Horsch *et al.* (Science (1985) 227: 1229-1231).

The obtained transformed *Nicotiana* plants according to the invention, or the obtained *Nicotiana* plants having a low level of beta-1,2-xylose residues on protein-bound N-glycans wherein the endogenous XyIT gene has been replaced by a XyIT allele, which is correlated with a lower levels of beta-1,2-xylose residues on protein-bound N-glycans than the original XyIT allele, can be used in a conventional breeding scheme to produce more plants with the same characteristics or to introduce the chimeric gene according to the invention in other cultivars of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

Furthermore, it is known that introduction of antisense, sense or doublestranded RNA or the encoding chimeric genes may lead to a distribution of phenotypes, ranging from almost no or very little suppression of the expression of the target gene to a very strong or even a 100% suppression of the expression of the target gene. However, a person skilled in the art will be able to select those plant cells, plants, events or plant lines leading to the desired degree of silencing and desired phenotype.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise
more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region, which is functionally or structurally defined, may comprise additional DNA regions etc.


Throughout the description and Examples, reference is made to the following sequences represented in the sequence listing:

SEQ ID NO: 1: nucleotide sequence of the oligonucleotide XylF4 suitable to amplify a part of a *Nicotiana* XyIT gene or cDNA.
SEQ ID NO: 2: nucleotide sequence of the oligonucleotide XylR4 suitable to amplify a part of a *Nicotiana* XyIT gene or cDNA.
SEQ ID NO: 3: partial cDNA sequence of *Nicotiana tabacum* cv. Petite Havana SRI XyIT gene variant 1.
SEQ ID NO: 4: partial amino acid sequence of *Nicotiana tabacum* cv. Petite Havana SRI XyIT protein variant 1.
SEQ ID NO: 5: partial cDNA sequence of *Nicotiana tabacum* cv. Petite Havana SRI XyIT gene variant 2.
SEQ ID NO: 6: partial amino acid sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT protein variant 2.

SEQ ID NO: 7: partial nucleotide sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT gene variant 1.

SEQ ID NO: 8: partial amino acid sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT protein variant 1.

SEQ ID NO: 9: partial nucleotide sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT gene variant 2.

SEQ ID NO: 10: partial amino acid sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT protein variant 2.

SEQ ID NO: 11: partial nucleotide sequence of *Nicotiana benthamiana* XyIT gene variant 1.

SEQ ID NO: 12: partial amino acid sequence of *Nicotiana benthamiana* XyIT protein variant 1.

SEQ ID NO: 13: partial nucleotide sequence of *Nicotiana benthamiana* XyIT gene variant 2.

SEQ ID NO: 14: partial amino acid sequence of *Nicotiana benthamiana* XyIT protein variant 2.

SEQ ID NO: 15: nucleotide sequence of the oligonucleotide XylF8 suitable to amplify a part of a *Nicotiana tabacum* cv. Petite Havana SRl XyIT gene or cDNA.

SEQ ID NO: 16: nucleotide sequence of the oligonucleotide XylR8 suitable to amplify a part of a *Nicotiana tabacum* cv. Petite Havana SRl XyIT gene or cDNA.

SEQ ID NO: 17: partial cDNA sequence of *Nicotiana tabacum* cv. Petite Havana SRl XyIT gene variant 1.

SEQ ID NO: 18: nucleotide sequence of T-DNA region of vector pTKW20.

SEQ ID NO: 19: nucleotide sequence of the oligonucleotide XylF9 suitable to amplify a part of a *Nicotiana benthamiana* XyIT gene or cDNA.

SEQ ID NO: 20: nucleotide sequence of the oligonucleotide XylR9 suitable to amplify a part of a *Nicotiana benthamiana* XyIT gene or cDNA.
SEQ ID NO: 21: partial sequence of *Nicotiana benthamiana* XyIT gene variant 1.

SEQ ID NO: 23: *Nicotiana tabacum* cv. Xanthi mRNA for putative beta-(1,2)-xylosyltransferase (accession number AJ627182)
SEQ ID NO: 24: putative beta-(1,2)-xylosyltransferase encoded by SEQ ID NO:23

SEQ ID NO: 25: *Nicotiana tabacum* cv. Xanthi xylt gene for putative beta-(1,2)-xylosyltransferase (accession number AJ627183)
SEQ ID NO: 26: putative beta-(1,2)-xylosyltransferase encoded by SEQ ID NO:25
Examples

Example 1: Design of degenerated primers for the isolation of XyIT cDNA and gene sequences from *Nicotiana tabacum* cv. Petite Havana SRI and *Nicotiana benthamiana*

Oligonucleotide sequences to be used as degenerated primers in a PCR amplification of XyIT cDNA and genomic DNA from *Nicotiana tabacum* cv. Petite Havana SRI and *Nicotiana benthamiana* were designed based on exon sequences of a genomic DNA sequence from *Nicotiana tabacum* cv. Xanthi encoding a putative XyIT protein (accession number AJ627183). The forward primer (SEQ ID NO:1) was designed with CACC at its 5′ end for cloning purposes. In this way the following degenerated primers were generated:

XylF4: 5′-CACCTTGGTTTCTCTTCGCTCTCAACTCAATC-3′
       (SEQ ID NO: 1)
XylR4: 5′-TCGATCACAACCTGGAGGATCCGCAA-3′
       (SEQ ID NO: 2)

Example 2: Isolation of XyIT cDNA sequences from *Nicotiana tabacum* cv. Petite Havana SRI

The degenerated primers described in Example 1 were used to isolate XyIT cDNA sequences from *Nicotiana tabacum* cv. Petite Havana SRI:

RNA was extracted from leaves of *Nicotiana tabacum* cv. Petite Havana SRI using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s protocol and used for cDNA synthesis using Superscript™ First-strand synthesis System for RT-PCR (Invitrogen Life Technologies) according to the manufacturer's instructions.

Using the cDNA as template and primer pair XylF4 / XylR4, PCR amplification was performed under the following conditions: 15 sec at 94°C (denaturation) and 3 min at 68°C for 40 cycles (annealing and elongation).
A DNA fragment of about 1500 basepairs was amplified, cloned into a pENTR™/D-TOPO® vector (Invitrogen) and several clones were sequenced (comprising the sequences of SEQ ID NO: 3 - XylTc2Nt - and SEQ ID NO: 5 - XylTc7Nt).

An alignment between a mRNA sequence from *Nicotiana tabacum* cv. Xanthi encoding a putative XyIT protein (accession number AJ627182; SEQ ID NO:23) and the XyIT cDNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRI (SEQ ID NO: 3 and 5) is shown in Figure 1.

An alignment between the putative XyIT protein encoded by the mRNA sequence from *Nicotiana tabacum* cv. Xanthi (accession number AJ627182; SEQ ID NO:24) and by the cDNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRI (SEQ ID NO: 4 and 6) is shown in Figure 2.

**Example 3: Isolation of XyIT gene sequences of *Nicotiana tabacum* cv. Petite Havana SRI and of *Nicotiana benthamiana***

The degenerated primers described in Example 1 were used to isolate XyIT gene sequences from *Nicotiana tabacum* cv. Petite Havana SRI and from *Nicotiana benthamiana*:

DNA was extracted from leaves of *Nicotiana tabacum* cv. Petite Havana SRI and of *Nicotiana benthamiana* based on the protocol described by Bernatzky and Tanksley (1986).

Using the genomic DNA as template and primer pair XylF4 / XylR4, PCR amplification was performed under the following conditions: 15 sec at 94°C (denaturation) and 4 min 30 sec at 68°C for 40 cycles (annealing and elongation).

Using the genomic DNA from *Nicotiana tabacum* cv. Petite Havana SRI as template for the PCR amplification, a DNA fragment of about 3400 basepairs was amplified, cloned into a pENTR™/D-TOPO® vector (Invitrogen) and several clones were sequenced (comprising the sequences of SEQ ID NO: 7 - XylTgINt - and SEQ ID NO: 9 - XylTg3Nt).
The XyIT genomic DNA sequences XyITgINt and XylTg3Nt comprise two putative intron sequences and three putative exon sequences. The location of the intron sequences are:

- For XyITgINt: from the nucleotide at position 679 to the nucleotide at position 1974 and from the nucleotide at position 2125 to the nucleotide at position 2722 of SEQ ID NO: 7, and
- For XylTg3Nt: from the nucleotide at position 658 to the nucleotide at position 1953 and from the nucleotide at position 2104 to the nucleotide at position 2701 of SEQ ID NO: 9.

Using the genomic DNA from *Nicotiana benthamiana* as template for the PCR amplification, a DNA fragment of between about 3300 and about 3600 basepairs was amplified, cloned into a pENTR™/D-TOPO® vector (Invitrogen) and several clones were sequenced (comprising the sequences of SEQ ID NO: 11 - XyITgHNb - and SEQ ID NO: 13 - XylTgL9Nt).

The XyIT genomic DNA sequences XyITgHNb and XylTg19Nt comprise two putative intron sequences and three putative exon sequences. The location of the intron sequences is:

- XyITgHNb from the nucleotide at position 658 to the nucleotide at position 1917 and from the nucleotide at position 2068 to the nucleotide at position 2612 of SEQ ID NO: 11, and
- XylTgL9Nt from the nucleotide at position 649 to the nucleotide at position 2194 and from the nucleotide at position 2345 to the nucleotide at position 2888 of SEQ ID NO: 13.

An alignment between the genomic DNA sequence from *Nicotiana tabacum* cv. Xanthi encoding a putative XyIT protein (accession number AJ627183; SEQ ID NO: 25) and the XyIT genomic DNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRI (SEQ ID NO: 7 and 9) and from *Nicotiana benthamiana* (SEQ ID NO: 11 and 13) is shown in Figure 3.

An alignment between the putative XyIT protein encoded by the genomic DNA sequence from *Nicotiana tabacum* cv. Xanthi (accession number AJ627183; SEQ ID NO: 26) and by the
genomic DNA sequences isolated from *Nicotiana tabacum* cv. Petite Havana SRl (SEQ ID NO:8 and 10) and from *Nicotiana benthamiana* (SEQ ID NO: 12 and 14) is shown in Figure 4.

Example 4: Construction of a T-DNA vector containing a *Nicotiana* XyIT silencing gene

DNA fragments amplified from *Nicotiana* XyIT sequences described in Examples 2 and 3 were used to construct T-DNA vectors comprising a chimeric gene which upon transcription yields an RNA molecule comprising a sense and antisense DNA sequence from the amplified DNA fragment, and which could basepair to form a double stranded RNA molecule. Such chimeric genes can be used to reduce the expression of a XyIT gene in *Nicotiana*, particularly in *Nicotiana tabacum* cv. Petite Havana SRl and *Nicotiana benthamiana*.

4.1. Construction of a T-DNA vector comprising a XyIT silencing gene with a DNA fragment amplified from a XyIT sequence from *Nicotiana tabacum* cv. Petite Havana SRl

Oligonucleotide sequences to be used as non-degenerated primers in a PCR amplification of a XyIT cDNA sequence from *Nicotiana tabacum* cv. Petite Havana SRl were designed based on the cDNA sequence from *Nicotiana tabacum* cv. Petite Havana SRl isolated in Example 2. The forward primer (SEQ ID NO:15) was designed with CACC at its 5' end for cloning purposes. In this way the following non-degenerated primers were generated:

XylF8: 5'-CACCTCTCGCCTTTGGGATATGAAACT -S' (SEQ ID NO: 15)
XylR8: 5'-ACAGCTTTGTGCTGC AGAAACT -3' (SEQ ID NO: 16)

Using the vector comprising a DNA fragment amplified from a XyIT cDNA sequence of *Nicotiana tabacum* cv. Petite Havana SRl as described in Example 2 (SEQ ID NO:3 - XylTc2Nt) as template and primer pair XylF8 / XylR8, a PCR amplification was performed under the following conditions: 15 sec at 94°C (denaturation), 30 sec at 56°C (annealing) and 45 sec at 68°C (elongation) for 25 cycles.

A DNA fragment of about 470 bp (XylTi4Nt; SEQ ID NO: 17) was amplified and cloned into a pENTR™/D-TOPO® vector (Invitrogen) yielding plasmid pKW19. Plasmid pKW19 was

The T-DNA sequence of pTKW20 (SEQ ID NO: 18) thus comprises:

- A chimeric XyIT silencing gene comprising:
  - a fragment including the promoter region of the Cauliflower Mosaic Virus 35S transcript (Odell et al., 1985)
    (SEQ ID NO: 18 from nucleotide 969 to nucleotide 2314)
  - a fragment including a part of the Nicotiana tabacum cv. Petite Havana SRI XyIT cDNA sequence cloned in sense orientation
    (SEQ ID NO: 18 from nucleotide 2365 to nucleotide 2834)
  - a fragment containing the intron of the catase-1 gene from castor bean
    (SEQ ID NO: 18 from nucleotide 2893 to nucleotide 3088)
  - a fragment containing the second intron of the pyruvate orthophosphate dikinase gene from Flaveria trinervia as described by Rosche and Westhoff (1995) in reverse orientation
    (SEQ ID NO: 18 from nucleotide 3130 to nucleotide 3871).
  - a fragment including a part of the Nicotiana tabacum cv. Petite Havana SRI XyIT cDNA sequence cloned in antisense orientation
    (SEQ ID NO: 18 from nucleotide 3957 to nucleotide 4426).
  - a fragment including the 3’ untranslated region of the octopine synthase gene of Agrobacterium tumefaciens as described by De Greve et al. (1982)
    (SEQ ID NO: 18 from nucleotide 4479 to nucleotide 5244).

- A chimeric gene encoding a selectable marker comprising:
  - a fragment including the promoter region of the nopaline synthase gene of Agrobacterium tumefaciens T-DNA
    (SEQ ID NO: 18 from nucleotide 5512 to nucleotide 5744).
  - a fragment including the nptll antibiotic resistance gene
    (SEQ ID NO: 18 from nucleotide 5745 to nucleotide 6690).
  - A fragment including the 3’ untranslated region of the nopaline synthase gene of A. tumefaciens T-DNA.
The T-DNA vector was introduced into Agrobacterium tumefaciens comprising a helper Ti-plasmid.

4.2. Construction of a T-DNA vector comprising a XyIT silencing gene with a DNA fragment amplified from a XyIT sequence from Nicotiana benthamiana

Oligonucleotide sequences to be used as non-degenerated primers in a PCR amplification of a XyIT gene sequence from Nicotiana benthamiana were designed based on the gene sequence from Nicotiana benthamiana isolated in Example 3. The forward primer (SEQ ID NO: 19) was designed with GGCCGGATCCTCG at its 5' end and the reverse primer (SEQ ID NO:20) was designed with GGCCATCGATGGTACC at its 5' end for cloning purposes. In this way the following non-degenerated primers were generated:

XylF9: 5'-GGCCGGATCCTCGAGACACAATTGGAGGAAACATGGAAAGC-S'
(SEQ ID NO: 19)
XylR9: 5'-GGCCATCGATGGTACCGGCCCAGCTCTTTATGGAATCAAA-3'
(SEQ ID NO: 20)

Using the vector comprising a DNA fragment amplified from a XyIT gene sequence of Nicotiana benthamiana as described in Example 3 (SEQ ID NO:11 - XyITg14Nb) as template and primer pair XylF9 / XylR9, a PCR amplification was performed under the following conditions: 15 sec at 94°C (denaturation), 30 sec at 58°C (annealing) and 30 sec at 68°C (elongation) for 25 cycles.

A DNA fragment of about 430 bp (XyITiNb; SEQ ID NO: 21) was amplified and digested with Xhol and Kpnl and with BamHI and Clal, respectively. The Xhol / Kpnl and the BamHI / Clal digested fragments were cloned in pHANNIBAL (Helliwell and Waterhouse, 2003) digested with Xhol / Kpnl and BamHI / Clal yielding pKW28.

Plasmid pKW28 thus comprises a chimeric XyIT silencing gene comprising:
a fragment including the promoter region of the Cauliflower Mosaic Virus 35S transcript (Odell et al., 1985)
(SEQ ID NO:22 from nucleotide 3779 to nucleotide 2434)
a fragment including a part of the *Nicotiana benthamiana* XyIT gene sequence cloned in sense orientation
(SEQ ID NO:22 from nucleotide 2427 to nucleotide 2023).
a fragment including the second intron of the pyruvate orthophosphate dikinase gene from *Flaveria trinervia* as described by Rosche and Westhoff (1995)
(SEQ ID NO:22 from nucleotide 1991 to nucleotide 1250).
a fragment including a part of the *Nicotiana benthamiana* XyIT gene sequence cloned in antisense orientation
(SEQ ID NO:22 from nucleotide 1211 to nucleotide 807).
a fragment including the 3' untranslated region of the octopine synthase gene of *Agrobacterium tumefaciens* as described by De Greve et al. (1982)
(SEQ ID NO:22 from nucleotide 786 to nucleotide 76).

between restriction sites Mscl and Pstl.

Plasmid pKW28 is digested with Mscl and Pstl and the chimeric gene is introduced between the T-DNA borders of a T-DNA vector cut with Pstl and Smal together with a chimeric gene encoding a selectable marker comprising:
a fragment including the promoter region of the nopaline synthase gene of *A. tumefaciens* T-DNA
(SEQ ID NO:22 from nucleotide 3854 to nucleotide 4140).
a fragment including the *bar* phosphinothricin resistance gene (De Block et al., 1987)
(SEQ ID NO:22 from nucleotide 4161 to nucleotide 4712).
a fragment including the 3' untranslated region of the nopaline synthase gene of *A. tumefaciens* T-DNA
(SEQ ID NO:22 from nucleotide 4731 to nucleotide 4991).

to yield pTKW29 (sequence of the T-DNA of pTKW29 is represented in SEQ ID NO: 22).
The vector pTKW29 is derived from pGSC1700 (Cornelissen and Vandewiele, 1989). The vector backbone contains the following genetic elements:

- the plasmid core comprising the origin of replication from the plasmid pBR322 (Bolivar et al., 1977) for replication in *Escherichia coli* (ORI CoIE1) and a restriction fragment comprising the origin of replication from the *Pseudomonas* plasmid pVSl (Itoh et al., 1984) for replication in *Agrobacterium tumefaciens* (ORI pVSl).

- a selectable marker gene conferring resistance to streptomycin and spectinomycin (aadA) for propagation and selection of the plasmid in *Escherichia coli* and *Agrobacterium tumefaciens*.

- a DNA region consisting of a fragment of the neomycin phosphotransferase coding sequence of the *nptI* gene from transposon *Tn903* (Oka et al., 1981).

The T-DNA vector is introduced into *Agrobacterium tumefaciens* comprising a helper Ti-plasmid.

**Example 5:** Analysis of transgenic *Nicotiana* plants harboring a XyIT silencing gene.

Nicotiana plants were transformed using the *Agrobacterium tumefaciens* strains described in Example 4:

5.1. Analysis of transgenic *Nicotiana tabacum* cv. Petite Havana SRI plants harboring a XyIT silencing gene

*Nicotiana tabacum* cv. Petite Havana SRI plants were transformed using the *Agrobacterium tumefaciens* strain described in Example 4.1. according to the protocol as described in Zambryski et al. (1983). Fifty-two transgenic *Nicotiana tabacum* lines, comprising the chimeric genes as described in Example 4.1. were obtained.

Transgenic plant lines were analyzed on molecular level using Southern blot analysis. Similarly, the plant lines are analyzed for XyIT RNA expression using Northern blot analysis.
An indication of XyIT activity can be obtained by comparing the level of beta-1,2-xylose residues present on the glycans of proteins from the transgenic lines with that of untransformed plants. The level of beta-1,2-xylose residues on protein-bound N-glycans of plants can be measured e.g. by Western blot analysis using xylose-specific antibodies as described e.g. by Faye et al. (1993) or by mass spectrometry on glycans isolated from the plant's glycoproteins using Matrix-assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS) as described e.g. by Kolarich and Altmann (2000) or using Liquid Chromatography Tandem mass spectrometry (LC/MS/MS) as described e.g. by Henriksson et al. (2003).

5.2. Analysis of transgenic *Nicotiana benthamiana* plants harboring a XyIT silencing gene

Similarly, *Nicotiana benthamiana* plants were transformed using the *Agrobacterium tumefaciens* strain described in Example 4.2. and the expression of XyIT and the level of beta-1,2-xylose residues present on the glycans of proteins was analyzed as described above.

Fifty four transgenic *Nicotiana benthamiana* lines comprising the chimeric genes described in Example 4.2. were obtained after leaf disk transformation with pTKW29.

To determine the level of beta-1,2-xylose residues present on the glycans of endogenous proteins of these plant lines, soluble leaf proteins of each individual were analyzed by Western blot using a beta-1,2-xylose-specific antibody.

Six samples showed very weak reaction with the antibody and six samples had no detectable reaction with the antibody. For the other samples, the level of reaction with the antibody ranged from weak to wild-type level.

To determine the number of insertions of the chimeric XyIT silencing gene from pTKW29, genome DNA from the plant lines showing very weak or negative reactions to the beta-1,2-xylose-specific antibody was isolated, digested with EcoRI and analyzed by Southern blot using a probe spanning the 35S promoter region and a probe spanning the bar phosphinotricin resistance gene's coding region.
None of the twelve plant lines showed a single insertion. One plant line contained two insertions and was negative for xylose using Western blot analysis.

To test whether these two chimeric XyIT silencing genes inserted independently and to obtain plants which are negative for xylose on Western blot and which contain a single chimeric XyIT silencing gene, progeny resulting from self fertilization of the plant line containing two insertions were sown and twenty five plant lines were analyzed by Western blot analysis.
References


WE CLAIM

1) A method to produce a *Nicotiana* plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of

a) introducing a chimeric gene into plant cells of a *Nicotiana* species or cultivar to generate transgenic plant cells, said chimeric gene comprising the following operably linked DNA fragments:

i) a plant expressible promoter;

ii) a transcribable DNA region comprising

(1) a first sense DNA region comprising a nucleotide sequence of at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said at least 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from a nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said at least 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide;

(2) a second antisense DNA region comprising a nucleotide sequence of at least 19 consecutive nucleotides which have at least 95% sequence identity to the complement of said first DNA region;

wherein an RNA molecule transcribed from said transcribable DNA region is capable of forming a double stranded RNA region at least between an RNA region transcribed from said first sense DNA region and an RNA region transcribed from said second antisense DNA region; and

iii) a DNA region comprising a transcription termination and polyadenylation signal functional in plants;

b) optionally, identifying a transgenic *Nicotiana* plant cell which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than an untransformed *Nicotiana* plant cell;
c) optionally, regenerating said transgenic *Nicotiana* plant cells to obtain transgenic *Nicotiana* plants; and

d) optionally, identifying a transgenic *Nicotiana* plant which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than an untransformed *Nicotiana* plant.

2) The method of claim 1, wherein said *Nicotiana* species-specific XyIT amino acid is a *Nicotiana benthamiana*-specific XyIT amino acid and said *Nicotiana* species is preferably *Nicotiana benthamiana*.

3) The method of claim 1 or 2, wherein said nucleotide sequence encoding a *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 12 or SEQ ID No.: 14.

4) The method of any one of claims 1 to 3, wherein said *Nicotiana* species-specific XyIT nucleotide is a *Nicotiana benthamiana*-specific XyIT nucleotide and said *Nicotiana* species is preferably *Nicotiana benthamiana*.

5) The method of any one of claims 1 to 4, wherein said nucleotide sequence of said *Nicotiana* XyIT gene comprises the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No. 21.

6) The method of claim 1, wherein said *Nicotiana* cultivar-specific XyIT amino acid is a *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT amino acid and said *Nicotiana* cultivar is preferably *Nicotiana tabacum* cv. Petite Havana SRI.

7) The method of claim 1 or 6, wherein said nucleotide sequence encoding said *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8 or SEQ ID No.: 10.
8) The method of any one of claims 1, 6 or 7, wherein said *Nicotiana* cultivar-specific XyIT nucleotide is a *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotide and said *Nicotiana* cultivar is preferably *Nicotiana tabacum* cv. Petite Havana SRI.

9) The method of any one of claims 1 or 6 to 8, wherein said nucleotide sequence of said *Nicotiana* XyIT gene or said *Nicotiana* XyIT cDNA comprises the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 8, SEQ ID No.: 10, or SEQ ID No.: 17.

10) The method of any one of claims 1 to 9, wherein said first and said second DNA region comprise at least 50 consecutive nucleotides.

11) The method of any one of claims 1 to 9, wherein said first and said second DNA region comprise at least 200 consecutive nucleotides.

12) A method to produce a *Nicotiana* plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of:

a) providing one or more double stranded RNA molecules to plant cells or plants of a *Nicotiana* species or cultivar, wherein the double stranded RNA molecules comprise two RNA strands, one RNA strand consisting essentially of an RNA nucleotide sequence of 19 out of 20 to 21 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 to 21 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 to 21 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide;

b) identifying a *Nicotiana* plant cell or plant comprising said double stranded RNA molecule or molecules which has a lower level of beta-1,2-xylose residues on protein-
bound N-glycans than a same *Nicotiana* plant cell or plant which does not comprise said double stranded RNA molecule or molecules.

13) The method of claim 12, wherein said double stranded RNA is provided to said plant cells or plants by integrating a chimeric gene into the genome of plant cells of said *Nicotiana* species or cultivar to generate transgenic plant cells and, optionally, regenerating said plant cells to obtain transgenic plants, said chimeric gene comprising the following operably linked DNA fragments:

a) a plant expressible promoter;

b) a DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in antisense orientation;

c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

14) The method of claim 12, wherein said double stranded RNA is provided to said plant cells or plants by integrating a chimeric gene into the genome of said plant cells to generate transgenic plant cells and, optionally, regenerating said plant cells to obtain transgenic plants, said chimeric gene comprising the following operably linked DNA fragments:

a) a plant expressible promoter;

b) a DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid it complements, or selected from the
nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense orientation;

\[c\] a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

15) The method of claim 12, wherein said double stranded RNA is provided to said plant cells or plants by integrating a chimeric gene into the genome of said plant cells to generate transgenic plant cells and, optionally, regenerating said plant cells to obtain transgenic plants, said chimeric gene comprising the following operably linked DNA fragments:

\[a\] a plant expressible promoter;

\[b\] a transcribable DNA region comprising:

\[i\] a first DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in antisense orientation;

\[ii\] a second DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, said nucleotide sequence preferably obtainable from said *Nicotiana* species or cultivar, wherein said 19 out of 20...
consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense orientation,

whereby an RNA molecule produced by transcription of said transcribable DNA region is capable of forming a double stranded RNA region by base-pairing at least between an RNA region corresponding to said first DNA region and an RNA region corresponding to said second RNA region; and
c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

16) The method of any one of claims 12 to 15, wherein said *Nicotiana* species-specific XyIT amino acid is a *Nicotiana benthamiana*-specific XyIT amino acid and said *Nicotiana* species is preferably *Nicotiana benthamiana*.

17) The method of any one of claims 12 to 16, wherein said nucleotide sequence encoding a *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 12 or SEQ ID No.: 14.

18) The method of any one of claims 12 to 17, wherein said *Nicotiana* species-specific XyIT nucleotide is a *Nicotiana benthamiana*-specific XyIT nucleotide and said *Nicotiana* species is preferably *Nicotiana benthamiana*.

19) The method of any one of claims 12 to 18, wherein said nucleotide sequence of said *Nicotiana* XyIT gene comprises the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No. 21.

20) The method of any one of claims 12 to 15, wherein said *Nicotiana* cultivar-specific XyIT amino acid is a *Nicotiana tabacum* cv. Petite Havana SRl-specfic XyIT amino acid and said *Nicotiana* cultivar is preferably *Nicotiana tabacum* cv. Petite Havana SRl.
21) The method of any one of claims 12 to 15 or 20, wherein said nucleotide sequence encoding said *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8 or SEQ ID No.:10.

22) The method of any one of claims 12 to 15, 20 or 21, wherein said *Nicotiana* cultivar-specific XyIT nucleotide is a *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotide and said *Nicotiana* cultivar is preferably *Nicotiana tabacum* cv. Petite Havana SRI.

23) The method of any one of claims 12 to 15, or 20 to 22, wherein said nucleotide sequence of said *Nicotiana* XyIT gene or said *Nicotiana* XyIT cDNA comprises the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 8, SEQ ID No.:10, or SEQ ID No.: 17.

24) A method to produce a *Nicotiana* plant cell or plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of:

a) identifying a fragment of a XyIT protein encoding DNA sequence obtainable from a first *Nicotiana* species or cultivar, using a means selected from the following group:

i) a DNA fragment comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.:10, SEQ ID No.: 12, or SEQ ID No.:14, for use as a probe;

ii) a DNA fragment comprising the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a probe;

iii) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 1513 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.:6, for use as a probe;

iv) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence
encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14 for use as a probe.

v) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21 for use as a probe;

vi) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a primer in a PCR reaction;

vii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a primer in a PCR reaction;

viii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a primer in a PCR reaction; or

ix) an oligonucleotide having the nucleotide sequence of any one of SEQ ID No.: 1, SEQ ID No.: 2, SEQ ID No.: 15 or SEQ ID No.: 16, SEQ ID No.: 19 or SEQ ID No. 20 for use as a primer in a PCR reaction;

b) providing one or more double stranded RNA molecules to plant cells or plants of said first or a second *Nicotiana* species or cultivar, wherein said double stranded RNA molecules comprise two RNA strands, one RNA strand consisting essentially of an RNA nucleotide sequence of 20 to 21 consecutive nucleotides selected from a nucleotide sequence of said XyIT protein encoding DNA fragment, or the complement thereof, wherein said 20 to 21 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, respectively, or wherein said 20 to 21 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, respectively; and
c) identifying a *Nicotiana* plant cell or plant comprising said double stranded RNA molecule or molecules which has a lower level of beta-1,2-xylose residues on protein-bound N-glycans than a same *Nicotiana* plant cell or plant, which does not comprise said double stranded RNA molecule or molecules.

25) The method of claim 24, wherein provision of said double stranded RNA molecule or molecules is achieved by providing to said plant cells or plants a double stranded RNA molecule or molecules comprising a first nucleotide sequence of at least 19 out of 20 consecutive nucleotides selected from the nucleotide sequence of said XyIT protein encoding DNA fragment, or the complement thereof, wherein said at least 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or wherein said at least 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, and a second nucleotide sequence which is the complement of said first nucleotide sequence.

26) The method of claim 24, wherein said double stranded RNA molecules are provided to said plant cells or plants by integrating a chimeric DNA into the genome of said plant cells to generate transgenic plant cells and, optionally, regenerating said plant cells to obtain transgenic plants, said chimeric DNA comprising the following operably linked DNA fragments:

a) a plant expressible promoter;

b) a transcribable DNA region comprising

i) a first sense DNA region comprising a nucleotide sequence of at least 19 out of 20 consecutive nucleotides selected from the nucleotide sequence of said XyIT protein encoding DNA fragment, or the complement thereof, wherein said at least 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, respectively, or wherein said at least 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, respectively;
ii) a second antisense DNA region comprising a nucleotide sequence of at least 19 consecutive nucleotides which have at least 95% sequence identity to the complement of said first DNA region;

wherein an RNA molecule transcribed from said transcribable region is capable of forming a double stranded RNA region at least between an RNA region transcribed from said first sense DNA region and an RNA region transcribed from said second antisense DNA region; and

c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

27) The method according to any one of claims 1 to 26 further comprising the step of crossing said Nicotiana plant having a low level of beta-1,2-xylose residues on protein-bound N-glycans to another Nicotiana plant to obtain Nicotiana progeny plants having a low level of beta-1,2-xylose residues on protein-bound N-glycans.

28) A method to identify a Nicotiana XyIT DNA fragment, comprising the steps of

a) providing genomic DNA or cDNA obtainable from a Nicotiana species or cultivar;

b) selecting a means from the following group:

i) a DNA fragment comprising a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a probe;

ii) a DNA fragment comprising the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a probe;

iii) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 1513 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.:6, for use as a probe;

iv) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence
encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14 for use as a probe

v) a DNA fragment or oligonucleotide comprising a nucleotide sequence consisting of between 20 to 3574 consecutive nucleotides selected from a nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21 for use as a probe;

vi) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, or SEQ ID No.: 6, for use as a primer in a PCR reaction;

vii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 8, SEQ ID No.: 10, SEQ ID No.: 12, or SEQ ID No.: 14, for use as a primer in a PCR reaction;

viii) an oligonucleotide sequence having a nucleotide sequence comprising between 20 to 200 consecutive nucleotides selected from the nucleotide sequence of any one of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17, or SEQ ID No.: 21, for use as a primer in a PCR reaction; or

ix) an oligonucleotide having the nucleotide sequence of any one of SEQ ID No.: 1, SEQ ID No.: 2, SEQ ID No.: 15 or SEQ ID No.: 16, SEQ ID No.: 19 or SEQ ID No. 20 for use as a primer in a PCR reaction;

c) identifying a XyIT DNA fragment from said Nicotiana species or cultivar by performing a PCR using said genomic DNA or said cDNA and said primers, or by performing hybridization using said genomic DNA or said cDNA and said probes.

29) A method to isolate a Nicotiana XyIT DNA fragment, comprising the steps of

a) identifying said Nicotiana XyIT DNA fragment according to the method of claim 28; and

b) isolating said Nicotiana XyIT DNA fragment.
30) A method to identify a *Nicotiana* XyIT allele correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans comprising the steps of:

(a) providing a population, optionally a mutagenized population, of different plant lines of a *Nicotiana* species or cultivar;

(b) identifying in each plant line of said population a *Nicotiana* XyIT allele according to the method of claim 28;

(c) analyzing the level of beta-1,2-xylose residues on protein-bound N-glycans of each plant line of said population and identifying those plant lines having a lower level of beta-1,2-xylose residues on protein-bound N-glycans than other plant lines;

(d) correlating the low level of beta-1,2-xylose residues on protein-bound N-glycans in a plant line to the presence of a specific *Nicotiana* XyIT allele.

31) A method to obtain a *Nicotiana* plant cell or plant with a low level of beta-1,2-xylose residues on protein-bound N-glycans, comprising the steps of:

a) identifying a *Nicotiana* XyIT allele correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans according to the method of claim 30;

b) introducing said *Nicotiana* XyIT allele into a *Nicotiana* plant line of choice.

32) An isolated DNA fragment encoding a protein comprising the amino acid sequence of SEQ ID No.: 12, or SEQ ID No.: 14, or any part thereof encoding at least one *Nicotiana benthamiana*-specific XyIT amino acid.

33) The isolated DNA fragment of claim 32, comprising the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No.: 21, or any part thereof comprising at least one *Nicotiana benthamiana*-specific XyIT nucleotide.

34) An isolated DNA fragment encoding a protein comprising the amino acid sequence of SEQ ID No.: 4 or SEQ ID No.: 6, SEQ ID No.: 8, SEQ ID No.: 10, or any part thereof encoding at least one *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT amino acid.
35) The isolated DNA fragment of claim 34, comprising the nucleotide sequence of SEQ ID No.: 3 or SEQ ID No.: 5, SEQ ID No.: 7, SEQ ID No.: 9, or SEQ ID No.: 17, or any part thereof comprising at least one *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotide.

36) An isolated DNA fragment obtainable by the method of claim 28, encoding at least one *Nicotiana* species- or *Nicotiana* cultivar-specific XyIT amino acid.

37) The isolated DNA fragment of claim 36, comprising at least one *Nicotiana* species- or *Nicotiana* cultivar-specific XyIT nucleotide.

38) A chimeric gene comprising the following operably linked DNA fragments:
   a) a plant expressible promoter;
   b) a transcribable DNA region comprising
      i) a first DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in antisense orientation;
      ii) a second DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense orientation,
   whereby an RNA molecule produced by transcription of said transcribable DNA region is capable of forming a double stranded RNA region by base-pairing at least between
an RNA region corresponding to said first DNA region and an RNA region corresponding to said second RNA region; and
c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

39) A chimeric gene comprising the following operably linked DNA fragments
   a) a plant expressible promoter;
   b) a DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in sense orientation; and
c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

40) A chimeric gene comprising the following operably linked DNA fragments
   a) a plant expressible promoter;
   b) a DNA region comprising at least 19 out of 20 consecutive nucleotides selected from a nucleotide sequence encoding a *Nicotiana* XyIT protein, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides encode at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, or selected from the nucleotide sequence of a *Nicotiana* XyIT gene or a *Nicotiana* XyIT cDNA, or the complement thereof, wherein said 19 out of 20 consecutive nucleotides comprise at least one *Nicotiana* species-specific XyIT nucleotide, in antisense orientation; and
c) a DNA region comprising a transcription termination and polyadenylation signal functional in plants.

41) The chimeric gene according to any one of claims 38 to 40, wherein said *Nicotiana* species-specific XyIT amino acid is a *Nicotiana benthamiana*-specific XyIT amino acid.
42) The chimeric gene according to any one of claims 38 to 41, wherein said nucleotide sequence encoding a *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 12 or SEQ ID No.: 14.

43) The chimeric gene according to any one of claims 38 to 42, wherein said *Nicotiana* species-specific XyIT nucleotide is a *Nicotiana benthamiana*-specific XyIT nucleotide.

44) The chimeric gene according to any one of claims 38 to 43, wherein said nucleotide sequence of said *Nicotiana* XyIT gene comprises the nucleotide sequence of SEQ ID No.: 11, SEQ ID No.: 13, or SEQ ID No. 21.

45) The chimeric gene according to any one of claims 38 to 40, wherein said *Nicotiana* cultivar-specific XyIT amino acid is a *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT amino acid.

46) The chimeric gene according to any one of claims 38 to 40 or 45, wherein said nucleotide sequence encoding said *Nicotiana* XyIT protein comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID No.: 4, SEQ ID No.: 6, SEQ ID No.: 8 or SEQ ID No.: 10.

47) The chimeric gene according to any one of claims 38 to 40, 45 or 46, wherein said *Nicotiana* cultivar-specific XyIT nucleotide is a *Nicotiana tabacum* cv. Petite Havana SRI-specific XyIT nucleotide.

48) The chimeric gene according to any one of claims 38 to 40, or 45 to 47, wherein said nucleotide sequence of said *Nicotiana* XyIT gene or said *Nicotiana* XyIT cDNA comprises the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.: 5, SEQ ID No.: 8, SEQ ID No.: 10, or SEQ ID No.: 17.

49) A *Nicotiana* plant cell comprising the chimeric gene of any one of claims 38 to 48.
50) A *Nicotiana* plant consisting essentially of the *Nicotiana* plant cells of claim 49.

51) A *Nicotiana* plant cell or plant obtained by the method of claim 31.

52) A seed of a *Nicotiana* plant according to claim 50 or claim 51.

53) Use of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.:10, SEQ ID No.: 12, or SEQ ID No.:14, or any part thereof comprising at least 19 out of 20 consecutive nucleotides encoding at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, to decrease the level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* plant.

54) Use of a nucleotide sequence comprising the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.:5, SEQ ID No.: 7, SEQ ID No.:9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17 or SEQ ID No.: 21, or any part thereof comprising at least 19 out of 20 consecutive nucleotides comprising at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, to decrease the level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* plant.

55) Use of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.:10, SEQ ID No.: 12, or SEQ ID No.:14, or any part thereof encoding at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, to identify a XyIT gene or XyIT cDNA in a *Nicotiana* species or cultivar.

56) Use of a nucleotide sequence comprising the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.:5, SEQ ID No.: 7, SEQ ID No.:9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17 or SEQ ID No.: 21, or any part thereof comprising at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, to identify a XyIT gene or XyIT cDNA in a *Nicotiana* species or cultivar.
57) Use of a nucleotide sequence encoding a protein comprising the amino acid sequence of
SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.:10, SEQ ID No.: 12, or SEQ ID No.: 14, or any part thereof encoding at least one *Nicotiana* species- or cultivar-specific XyII amino acid, to identify an allele of a *XyIT* gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar.

58) Use of a nucleotide sequence comprising the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.:5, SEQ ID No.: 7, SEQ ID No.:9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17 or SEQ ID No.: 21, or any part thereof comprising at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, to identify an allele of a *XyIT* gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar.

59) Use of a nucleotide sequence encoding a protein comprising the amino acid sequence of
SEQ ID No.: 4, SEQ ID No.:6, SEQ ID No.: 8, SEQ ID No.:10, SEQ ID No.: 12, or SEQ ID No.: 14, or any part thereof encoding at least one *Nicotiana* species- or cultivar-specific XyIT amino acid, to introduce an allele of a *XyIT* gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar.

60) Use of a nucleotide sequence comprising the nucleotide sequence of SEQ ID No.: 3, SEQ ID No.:5, SEQ ID No.: 7, SEQ ID No.:9, SEQ ID No.: 11, SEQ ID No.: 13, SEQ ID No.: 17 or SEQ ID No.: 21, or any part thereof comprising at least one *Nicotiana* species- or cultivar-specific XyIT nucleotide, to introduce an allele of a *XyIT* gene correlated with a low level of beta-1,2-xylose residues on protein-bound N-glycans in a *Nicotiana* species or cultivar.
### Figure 1 (continued)

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Figure 3

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| xylTg3Nt   | 2656 | ..........t.t............................t.g..................gg......................c..... |
| xylTg14Nb  | 2568 | ..........t.t............................t.t............................t...........c... |
| xylTg19Nb  | 2846 | ..........t.t............................t.t............................t...........c... |

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| xylTg14Nb  | 2646 | ..........c...tt...............................c...tt...............................c...tt |
| xylTg19Nb  | 2922 | ..........c...tt...............................c...tt...............................c...tt |

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| xylTg3Nt   | 2815 | ..........a..............a..................a.............................a..................a |
| xylTg14Nb  | 2726 | ..........a..............a..................a.............................a..................a |
| xylTg19Nb  | 3002 | ..........a..............a..................a.............................a..................a |

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| xylTg3Nt   | 2895 | ..........c..............c..................c.............................c..................c |
| xylTg14Nb  | 2806 | ..........c..............c..................c.............................c..................c |
| xylTg19Nb  | 3082 | ..........c..............c..................c.............................c..................c |

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 A01H5/00 C12Q1/68 C12N9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of Box C

* Special categories of cited documents
*A* document defining the general state of the art which is not considered to be of particular relevance
*E* earlier document but published on or after the international filing date
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another document and/or special reason (as specified)
*O* document referring to an oral disclosure, use, exhibition or other means
*P* document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 15 June 2007

Date of mailing of the international search report: 27/06/2007

Name and mailing address of the ISA:
European Patent Office, P B 5818 Patentlaan 2 NL-2280 HV RIJSWijk Tel (+31-70) 340-2040, Tx 31 651 epp nl, Fax (+31-70) 340-3016

Authorized officer: Bilang, Jürg
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