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(54) **Titre : PROCÉDES DE FABRICATION DE PAROIS DE CELLULES VÉGÉTALES COMPRENANT DE LA CHITINE**
(54) **Title: METHODS FOR MANUFACTURING PLANT CELL WALLS COMPRISING CHITIN**

(57) **Abrégé/Abstract:**

Methods and means are provided for the modification of the reactivity of plant secondary cell walls, particularly in cotton cell walls found in cotton fibers. This can be conveniently achieved by expressing a chimeric gene encoding a *Saprolegnia monoica* chitin synthase in cotton plants.

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(54) Title: METHODS FOR MANUFACTURING PLANT CELL WALLS COMPRISING CHITIN

(57) Abstract: Methods and means are provided for the modification of the reactivity of plant secondary cell walls, particularly in cotton cell walls found in cotton fibers. This can be conveniently achieved by expressing a chimeric gene encoding a Saprolegnia monoica chitin synthase in cotton plants.



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Methods for manufacturing plant cell walls comprising chitin**Field of the invention**

The present invention relates to the modification of the reactivity of plant cell walls such as secondary plant cell walls. In particular, the present invention provides cotton fibers comprising positively charged polysaccharides such as chitin and chitosan. These cotton fibers have a modified reactivity which can be exploited for dyeing the fibers with fiber-reactive dyes. In addition, the modified reactivity can be applied to improve the reactivity of the fibers with reactants such as flame retardants, water, oil and soil repellents, softeners, antistatic agents, fluorescent whitening agents and the like.

Introduction to the invention

Mankind has been using natural fibers, including cellulose containing natural fibers from plants, such as cotton and linen, for several thousand years to produce many different kinds of textiles. With increasing demand and improved fiber quality, a global textile industry based on cotton developed rapidly. Today, cotton contributes approximately 45 percent to the world's clothing, and many of the best fashion houses use textiles made from high quality cotton. Cotton fiber consists of cellulose, a natural polymer composed of many molecules of the sugar glucose. Its unique structure is ideally suited for textile production. Each fiber is basically a hollow tube a few centimetres in length that, when spun and woven, provides the very special characteristic "feel" of cotton. Natural cellulose containing fibers, however, do not possess the chemical versatility of synthetic fibers, due to the relative inert nature of the cellulose consisting of β -1-4 linked glucose monomers. This relatively inert nature is apparent during the dyeing process of cotton fibers and fabrics. Direct dyes and fiber-reactive dyes are two types of anionic dyes used to color cotton. Cotton itself develops an anionic charge in water, so that without special treatment, the uptake of dye by the fiber or fabric is quite elaborate. Direct dyes create a relatively weak hydrogen bond with the cellulose polymer forming a semi-permanent attachment because it does not withstand well washing. Fiber-reactive dyes are molecules that combine chromophores with a reactive group that forms strong covalent bonds with the fiber via reaction with hydroxyl groups. The covalent bonds provide a good resistance of the dyed fiber against laundering. During the dyeing process, large amounts of electrolytes are needed to shield the anionic dyes from the anionic fiber charges. Unreacted hydrolyzed dyes (up to 40%) need to be removed by a washing step,

generating large volumes of wastewater, also containing the above mentioned electrolytes.

Providing the cellulose fiber with a positive electric charge, e.g. by incorporation of positively charged chemical compounds, could therefore improve the dyeability of natural cellulose fibers, as well as improve any chemical reaction of the modified cellulose fiber with negatively charged chemical compounds. It would also make the use of acidic dyes possible.

Several publications have described the coating of chitosan oligomers onto cellulose fibers to make chitosan/cellulose blends, yarns or fabrics. Chitosan is a positively charged polymer of glucosamine, which can be obtained by deacetylation of chitin, e.g. by alkaline treatments. Chitin itself is a polymer of β -1-4 linked N-acetylglucosamine (GlcNAc).

Liu et al. (*Carbohydrate Polymers* 44(2003) 233-238) described a chemical method for surface coating cotton fibers with chitosan. With this chitosan coating, the cotton fiber surface became physiologically and biologically active because of the chemical reactivity of the amino group leading to a higher potential for subsequent chemical modification of the fibers. Based on the physiological function of chitosan in inhibiting e.g. dermatophytes, many functional clothes, fabrics and fibers employ cellulose-chitosan blend fibers, cellulose fiber-chitosan conjugates and fabrics coated with chitosan-containing resins.

WO 00/09729 describes the expression of chitin synthase and chitin deacetylase genes in plants to alter the cell wall for industrial uses and improved disease resistance. Specifically cited uses are: to provide a single plant source of cellulose, chitin and chitosan, to increase tensile strength and to increase brittle snap. Specifically suggested chitin synthase genes are derived from fungal organisms. Experimental data are neither provided on the production of chitin or chitosan in plants, nor on the incorporation thereof in plant cell walls. WO2006/136351 showed that the strategy as proposed in WO00/09729 does not lead to the functional incorporation of chitin into the plant cell wall. Instead WO 2006/136351 discloses that chitin is effectively produced in the secondary cell wall of cotton fibers only when the fungal chitin synthase (from *Neurospora crassa*) is actively relocated to the Golgi apparatus. The latter is achieved by operable fusion of this fungal chitin synthase with a signal anchor sequence specific for the Golgi apparatus, and by expressing the resulting chimeric gene in plants. Although, chitin could be

efficiently produced in cotton plant cell walls it was also observed that the transgenic plants remained smaller presumably due to toxicity of the expression of the chimeric chitin synthase in cotton outside of the cotton fibers.

Thus there remains a need for alternative methods to produce plant cell walls such as secondary cell walls which comprise positively charged polysaccharides. In particular a need exists for providing methods to produce fibers which can be directly harvested from plants and which contain positively charged chemical groups and/or group which are more reactive than hydroxyl groups of cellulose, which can be used directly without the need for further chemical treatment to introduce such chemical groups and wherein the transgenic plants do not show a growth retardation. These and other problems are solved as described hereinafter in the different embodiments, examples and claims.

Summary of the invention

The invention shows that the expression of a chitin synthase derived from *Saprolegnia monoica*, which is not operably linked with a Golgi targeting signal, in plant cells leads to the efficient incorporation of N-acetylglucosamine polymers in the plant cell wall, in particular the plant secondary cell wall. The invention described herein in the different embodiments, examples, and claims provides *Saprolegnia* chitin synthases and chimeric genes thereof comprised in expression cassettes which can be used to modify the plant cell walls with positively charged polysaccharides. Accordingly, the invention provides in a first object an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide comprising an MIT domain, wherein said nucleotide sequence is at least 97% identical to the full-length sequence of SEQ ID NO: 1, or a full-length complementary sequence thereof. In another embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to the full-length sequence of SEQ ID NO: 2 and comprises an MIT domain. In yet another embodiment the invention provides a chimeric gene comprising the following operably linked DNA regions a) a plant-expressible promoter, b) a nucleotide sequence which is at least 97% identical to the full-length sequence of SEQ ID NO: 1 and which encodes a chitin synthase polypeptide comprising an MIT domain, or a complementary sequence thereof, or a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to the full-length sequence of SEQ ID NO: 2 and comprises an MIT domain, and c) a transcription termination and polyadenylation region. In yet another embodiment the chimeric gene comprises a constitutive promoter. In yet another embodiment the chimeric gene comprises a fiber-selective promoter. In yet another embodiment the chimeric gene comprises an expansin promoter.

In yet another embodiment the invention provides a chimeric gene as defined in any of the previous embodiments. In yet another embodiment the invention provides a plant consisting essentially of a plant cell comprising a chimeric gene as defined in any of the previous embodiments. In a particular embodiment the plant is a cotton plant. In yet another embodiment the invention provides fibers
 5 comprising the chimeric gene as defined in any of the previous embodiments. In yet another embodiment the invention provides a transgenic seed comprising a chimeric gene as defined in any of the previous embodiments. In yet another embodiment the invention provides a method for the manufacture of a plant cell wall comprising positively charged polysaccharides, said method comprises expressing a chimeric gene according to any of the previous embodiments in a plant and isolating said plant cell wall. In a
 10 particular embodiment said method provides a cotton plant cell wall. In yet another particular embodiment said method provides a cotton plant cell wall which is present in a cotton fiber.

Figure

Figure 1:

Fig. 1a shows untransformed mature trichomes (*Arabidopsis thaliana*) which are stained with a
 15 rhodamine-conjugated chitin-binding probe while Fig. 1b shows recombinant mature trichomes (*Arabidopsis thaliana*) comprising the SmCHS2 chimeric gene stained with a rhodamine-conjugated chitin-binding probe. A clear reaction with the chitin binding probe is observed since the recombinant trichomes (Fig. 1b) are stained more intensively than trichomes derived from control plants (Fig. 1a). Chitin is witnessed by the presence of red dots in the recombinant trichomes (Fig. 1b) while the red
 20 dots are absent in the untransformed trichomes (Fig. 1a).

Detailed description of different embodiments of the inventions

The current invention hinges on the remarkable finding that expression of the *Saprolegnia monoica* gene coding for a chitin synthase in plant cells leads to the efficient incorporation of N-acetylglucosamine (GlcNAc) polymers into plant cell walls. The *Saprolegnia monoica* gene coding
 25 for a chitin synthase does not require to be operably linked (or fused) with a Golgi targeting localisation signal as was required with the *Neurospora crassa* chitin synthase in WO2006/136351. In the present invention it was observed that chitin (i.e. a polymer of N-acetylglucosamine) was made in the cell wall, in particular the secondary cell wall, of the transgenic plants. The *Saprolegnia monoica*

gene coding for a chitin synthase can be used e. g. for expression in cotton plants for the generation of more reactive cotton fibers.

Thus the invention provides in a first embodiment an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide wherein said nucleotide sequence is at least 97% identical to SEQ ID NO: 1 or a complementary sequence thereof. SEQ ID NO: 1 depicts the nucleotide sequence of the *Saprolegnia monoica* chitin synthase 2 (SmCHS2).

In another embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to SEQ ID NO: 2. SEQ ID NO: 2 depicts the amino acid sequence of the *Saprolegnia monoica* chitin synthase 2 (SmCHS2). In a specific embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 85%, at least 90%, at least 95% or at least 98% or even at least 99% identical to SEQ ID NO: 2. In yet another embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide wherein said nucleotide sequence is at least 90% identical to SEQ ID NO: 3 or a complementary sequence thereof. In a specific embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide wherein said nucleotide sequence is at least 95%, at least 98 or even at least 99% identical to SEQ ID NO: 3 or a complementary sequence thereof. SEQ ID NO: 3 is the *Saprolegnia monoica* chitin synthase 1 (SmCHS1). In yet another embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to SEQ ID NO: 4. In a specific embodiment the invention provides an isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 85%, at least 90% identical, at least 95%, at least 98% or even at least 99% identical to SEQ ID NO: 4. SEQ ID NO: 4 is the amino acid sequence of the *Saprolegnia monoica* chitin synthase 1.

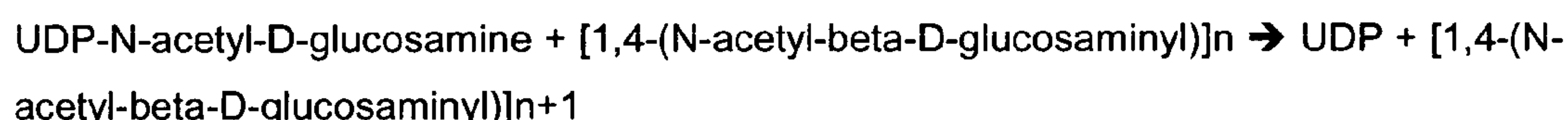
Nucleic acids can be DNA or RNA, single- or double-stranded. Nucleic acids can be synthesized chemically or produced by biological expression in vitro or even in vivo.

Nucleic acids can be chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. Suppliers of

RNA synthesis reagents are Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, USA), Pierce Chemical (part of Perbio Science, Rockford, IL, USA), Glen Research (Sterling, VA, USA), ChemGenes (Ashland, MA, USA), and Cruachem (Glasgow, UK).

In connection with the chimeric gene of the present disclosure, DNA includes cDNA and genomic DNA.

In enzymology, a chitin synthase (EC 2.4.1.16) is an enzyme that catalyzes the chemical reaction:



Thus, the two substrates of this enzyme are UDP-N-acetyl-D-glucosamine and $[[[1,4\text{-(N-acetyl-beta-D-glucosaminyl)}]_n]]$, whereas its two products are UDP and $[[[1,4\text{-(N-acetyl-beta-D-glucosaminyl)}]_{n+1}]]$. This enzyme belongs to the family of glycosyltransferases, specifically the hexosyltransferases. The systematic name of this enzyme class is UDP-N-acetyl-D-glucosamine:chitin 4-beta-N-acetylglucosaminyl-transferase. Other names in common use include chitin-UDP N-acetylglucosaminyltransferase, chitin-uridine diphosphate acetylglucosaminyltransferase, chitin synthetase, chitin synthase and trans-N-acetylglucosaminosylase. This enzyme participates in aminosugars metabolism. Thus chitin is a polymer of beta-1,4-linked N-acetyl-D-glucosamine (GlcNAc).

The chitin synthases 1 and 2 (respectively depicted in SEQ ID NO: 3 and 1) are derived from *Saprolegnia monoica*. The genus *Saprolegnia* belongs to the family of Oomycetes. Based on their general morphology and lifestyles, this group was traditionally classified as fungi. However, a cladistic classification based on modern insights, supports a relatively close relationship with the photosynthetic organisms such as brown algae and diatoms, within the eukaryotic group - the heterokonts. The sequences of the chitin synthases 1 and 2 derived from *S. monoica* comprise a conserved aminoterminal domain of approximately 70 amino acids, also known as a MIT domain. A MIT domain is a conserved domain comprising approximately 70 amino acids, with alternating conserved hydrophobic and polar areas and a predicted helical secondary structure first identified in proteins involved in microtubule binding and in intracellular transport. Such a domain is designated as a MIT domain (contained within Microtubule Interacting and Trafficking molecules). The MIT domain is also found in sorting nexins, the nuclear thiol protease PalBH, the AAA protein spastin and archaeobacterial proteins with similar

domain architecture, vacuolar sorting proteins and others. The precise molecular function of the MIT domain is unclear. Without intending to limit the invention to a particular mode of action, it is thought that the MIT domain present in the chitin synthases of *S. monoica* is responsible for the efficient synthesis of chitin in the cell walls of transgenic plants. It was previously shown in WO2006/136351 that chitin synthases of fungal origin (such as a *Neurospora crassa* chitin synthase) had to be adapted by means of operable coupling of the fungal chitin synthase with a Golgi localisation signal, to obtain for efficient synthesis of chitin in the cell wall of transgenic plants. Since a MIT domain is present in a few other chitin synthases, in particular in some chitin synthases derived from oomycetes, the invention provides the use of chitin synthases naturally comprising an MIT domain for the manufacture of chitin in the plant secondary cell wall. Such chitin synthases naturally comprising an MIT domain include those of *Saprolegnia parasitica* having the amino acid sequence depicted in SEQ ID NO: 22 and SEQ ID NO: 24 or encoded by the nucleic acid sequences depicted in SEQ ID NO: 21 and SEQ ID NO: 23.

In another embodiment the invention provides the operable coupling of a MIT domain with a chitin synthase, such as a fungal chitin synthase, not comprising an MIT domain, e. g. a non-oomycete chitin synthase to generate a chimeric chitin synthase for efficient production of chitin in the plant secondary cell wall. In other words, disclosed is a chimeric gene comprising a DNA region coding for a chitin synthase polypeptide not comprising an MIT domain operably linked to a DNA region encoding an MIT domain such that, upon expression, a fusion protein is made, a plant expressible promoter and a transcription termination and polyadenylation region. Also disclosed is a method for the manufacture of a plant cell wall comprising positively charged polysaccharides comprising a) expressing a chimeric gene comprising a DNA region coding for a chitin synthase polypeptide not comprising an MIT domain operably linked to a DNA region encoding an MIT domain such that, upon expression, a fusion protein is made, a plant expressible promoter and a transcription termination and polyadenylation region; and b) isolating the plant cell wall obtained in a).

MIT domains have been assigned the Pfam family number PF04212. Currently, 495 proteins are known which are known or predicted to comprise an MIT domain. The sequences of these proteins can be retrieved e. g. from the UniProt server (<http://www.uniprot.org/>) under the following UniProt accession numbers which are followed by the position of the (predicted) MIT domain within the sequence.

C5L7B3/6-74; C5K7I8/6-74; B9PJ69/9-77; B6K9M2/9-77; B9QA65/9-77; A7ASR9 /7-74;
 B6AJD9/4-72; Q5CFS7/1-69; Q5CSB4/3-71; Q4UC87/4-82; B3L9J0/6-74; A5K3I1/6-74;
 Q8IKQ5/6-74; Q4Z291/6-74; Q4X5E3/6-74; Q7RRP8/6-74; A8IAJ1/7-74; Q6ETH5/5-73;
 B8AI60/5-73; B9SG62/5-73; B9HVV7/5-73; B9HL02/5-73; B9NGD1/5-73; Q3EDG2/1-41;
 Q9SGD4/1-41; Q8LKV4/5-73; B6TLN7/5-73; B8A2I4/5-73; B6T3Y2/5-73; A2WKH8/5-73;
 A2ZP36/5-73; B8A2W9/5-73; A2WKI0/5-73; Q5ZEN9/5-73; Q8LAK9/5-73; Q9ZNT0/5-73;
 Q9SEA8/5-73; Q1W2L1/5-73; B9HQW8/5-73; B9H1R8/5-73; A5BIG1/5-73; A7R0D5/5-
 73; A9SGM2/5-73; A9TBU2/5-73; A9P2N1/5-73; B9SCR4/5-73; A4S3E8/8-75;
 C1ECR7/11-78; C1NA06/11-78; C1E2F1/103-169; B6K5C2/6-74; Q09803/6-74;
 A8PSV3/1-33; Q5KC30/6-74; Q4PDZ4/6-74; A8N0F3/6-74; B0DXQ0/6-74; A7F3H9/6-74;
 Q7S0H4/6-74; Q2GQ74/6-74; B2AFE6/6-74; C5FLK6/6-74; C5JDP2/6-74; C5GXE6/6-
 74; C0NGS1/6-74; C0SHS5/6-74; C1H9G7/6-74; C1GCX1/6-74; C4JW95/6-74;
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 B5VTV5/1-34; C5DUT4/6-74; Q6CVM8/6-74; C5DBA6/6-74; A7TH89/6-74; Q758U9/6-
 74; C4Y9U8/7-75; Q5AGH7/7-75; Q5AG40/7-75; B9WHM5/7-75; A5E2L0/55-123;
 C5MHK4/5-73; A5DQ68/6-74; A3LVF1/7-75; Q6BPY2/7-75; A9V5Z2/5-73; Q57V58/5-74;
 Q4E658/5-74; Q4D9C2/5-74; Q4FXF2/5-74; A4I4W4/5-74; A4HHP9/5-74; Q54PT2/6-74;
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 Q4SKA0/1-69; Q2HJB1/5-74; Q793F9/5-74; Q8VEJ9/5-74; Q6IRG3/5-74; Q3TDX2/5-74;
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 Q4S8A8/285-352; A4IG43/277-345; A4IID6/279-347; Q4V7Q6/279-347; Q5ZJH6/280-

348; B4DFS6/190-258; B4DRQ7/280-348; B2RXK3/280-348; B4DDG2/163-231;
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 C4Q8U0/273-338; B0E7C2/4-72; C4LYN8/4-72; A3DP09/6-74; B8D2W2/9-77;
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 A8XST3/5-70; Q54CX7/246-316; Q55GN8/5-73; A4QZC1/8-77; A2D8M7/10-80;
 Q54RJ5/10-79; C4LYH7/2-70; Q8I8X1/16-84; Q54CX7/120-188; Q0UZT0/375-444;
 C4JEZ5/18-85; Q1E904/19-86; C5FCB8/23-90; C1GDJ4/63-130; C0SAK1/19-86;
 C1HDA2/19-86; C5JPG7/78-145; C5G855/19-86; A6RHR0/1-68; C0NMG2/1-68;
 A2QKF2/239-307; B6H2K6/100-168; Q0CFY4/216-284; B0XW44/210-278;
 A1DG63/193-261; Q4X270/211-279; A1CSH7/266-334; B8NC13/82-150; Q2TZ95/233-
 301; Q5B0X3/178-246; B8M0Z2/44-113; B6Q8T3/44-113; A7EZ31/1-69; A6S2Q3/293-
 361; Q7S0P7/402-470; B2ADU1/368-424; Q2GTY0/352-420; C1E4H7/3-68; C1MPZ7/3-
 69; C3ZZL4/6-70; B7P5B6/8-73; Q9R1S8/6-71; Q499T5/6-71; Q9Y6W3/6-71;
 B2RAM2/6-71; Q7Z479/6-71; A0FKG7/6-71; B0FGU2/6-71; B4F6P1/6-71; C0H9M1/6-
 71; Q4S467/6-71; B3RQK1/8-73; A7RRP7/7-70; Q54JG4/13-81; Q55GN8/176-244;
 C4LYH7/369-438; Q8I8X1/383-452; B0EV39/371-440; A9V3C1/6-74; A7URZ9/6-72;
 B0X0P8/1-69; Q16FL8/1-69; Q0IFG1/1-69; B4KNS6/15-76; B4MS07/21-82; B3M8E0/5-
 71; B4IZ41/4-72; B4KXT1/5-71; B4MFW3/3-71; B4MLA7/3-71; Q29EY7/4-71;
 B3NG09/3-71; B4PH91/3-71; Q9VZK1/3-71; Q7YU93/3-71; B4QQ18/3-62; B4HTX3/3-
 71; B3RKL7/450-515; Q7T332/322-390; A9JT54/322-390; A4IIB7/268-336;
 Q28CB9/261-329; Q63ZH0/269-337; Q0P3R2/270-338; A7SVK8/274-341; Q20821/252-
 320; A8Y3X4/266-339; Q148E7/275-343; B3KU31/268-336; B2RDR2/268-336;
 Q9NRS6/268-336; B0CM75 /268-336; Q4V896/269-337; Q91WE1/268-336;
 Q9CW22/63-131; Q6NU31/257-325; KS6C1/239-307; B1APS8/227-295; B3KVM4/239-
 307; B4DRK0/58-126; Q5R5U6/239-307; KS6C1/238-306; B8JLT3/232-300;
 Q4RR16/253-353; B4NG93/233-301; B3MTR0/236-304; B3NZ90/246-314;
 Q9VEA9/242-310; B4QUH5/242-310; B4IB83/242-310; B4PL88/246-314; B4GLM4/255-

323; Q293U2/255-323; B4JI50/203-271; B4K7H6/227-295; B4M0W2/211-279; Q7PYF1/252-320; B0X4C2/216-284; Q16ZH6/224-292; Q4RLU8/6-73; Q8R2S1/49-117; B4DSP6/49-117; Q5RA67/49-117; Q9Y6S9/49-117; Q32PG2/49-117; A9RII8/29-104; B8B1Z6/49-124; Q658G8/49-124; B4FVB5/49-124; Q944N4/90-165; A8MRR2/55-130; Q0WMJ4/55-130; O64630/55-130; B9RDF4/52-127; B9IC21/13-88; A7PY13/56-131; A5BB69/56-131; A5BIC4/76-120; A9V1R3/29-100; B3S0V7/15-93; C3XZ15/1-79; C3ZJS8/19-88; B7PXE3/98-174; B4NBP4/237-313; B4M0H8/223-299; B4K799/217-293; B4JII0/234-310; B4G437/239-315; B4QSF0/232-308; B3M301/235-311; B4HGG6/232-308; B3P8A3/232-308; Q298L4/239-315; B4PL32/232-308; Q8I0P1/232-308; Q16IA7/179-255; Q7QBW0/230-306; B0X289/2-60; Q4TCF6/6-91; Q4TCD9/16-91; Q6NW58/83-158; Q6AZT2/110-185; Q05AS3/113-188; Q9QYY8/117-193; Q9UBP0/119-195; A2VDN5/117-193; Q719N1/116-192; Q5ZK92/116-192; B2RYN7/117-192; C3XW83/29-96; B7Q7T2/7-83; A7RQX2/15-88; Q4RRG4/9-85; C0H9Y4/20-96; Q0P3W0/20-96; Q4SG05/11-87; A8WFZ3/15-90; B3DLA2/21-97; Q8R1X6/19-95; Q3TVW1/19-95; A0JNJ3/19-95; Q4R7V2/19-95; Q8N0X7/19-95; B3KMI3/19-95; A8K6Q9/19-95; A2Q8K6/7-77; Q00204/6-77; B8MY71/5-77; Q9Y6Z8/5-77; Q0CUT6/6-77; A1CRW2/6-77; Q1DXZ5/5-72; B6QNP8/12-76; B0DXY2/11-78; Q7QGN5/7-67; B0WDD1/4-67; Q179Z1/4-67; Q179Z0/4-67; B7P5B6/89-157; Q5DBL1/86-142; A8P1B5/37-127; Q9R1S8/86-154; Q7Z479/86-154; B2RAM2/86-154; Q9Y6W3/86-154; A0FKG7/86-154; B0FGU2/86-154; Q499T5/86-154; C0H9M1/86-154; B4F6P1/86-154; Q4S467/86-149; Q6NVT4/42-110; C1BMZ4/11-74; A8PMC5/4-73; Q5D999/6-77; Q5D9R4/6-77; C4Q8N9/35-106; A7RL55/2-70; Q5I0J5/12-79; Q8VDV8/12-79; Q8WV92/12-79; B8ZZL5/12-79; A8YXZ4/12-79; Q6DJ62/7-75; C3XWE6/1-69; B0S8J9/9-77; B5XBB0/7-75; Q4S0N8/9-77; Q54KQ7/168-232; B3RXW5/5-72; A8PZJ1/282-350; C3Z907/423-490; Q4V7Q6/374-442; Q3U3Q1/375-444; B2RXB9/375-444; B4DFT0/386-455; B4DRQ7/375-444; B4DDG2/258-327; B4DFS6/285-354; B4DQN3/258-327; B2RXK3/375-444; A4IG43/372-441; Q5ZJH6/375-444; Q7QEQ2/417-485; B0XAE0/287-363; B3M073/404-479; B4HKF5/399-474; B3P1R4/399-474; B4PUL9/399-474; B4QX75/399-474; B4JYT0/404-479; Q86P98/399-474; Q9VHF6/399-474; Q8T0L6/399-464; Q29BD1/399-474; B4NAJ4/403-478; B4LW06/400-475; B4K4Y3/397-472. Further MIT domains include those disclosed in Ciccarelli et al. (2003; Genomics 81: 437-41) or Patel et al. (2002; Nature Genetics 31: 347-8) or in the pdb (protein data bank) files (or related publications): 1wfd (Suetake et al.; Solution structure of mouse MIT domain); 1wr0 (Takasu et al. (2005). *Biochem*

Biophys Res Commun, **334**, 460-465.), 1yxr (Scott et al. (2005) *Proc Natl Acad Sci U S A.* 102:13813-13818), 2cpt (Suetake et al.; Solution structure of MIT domain from human skd1), 2dl1 (Suetake et al.; Solution structure of MIT domain from human spartin), 2jq9 and 2jqh (Stuchell-Brereton et al. (2007). *Nature*, **449**, 740-744.), 2k3w (C.Kieffer et al. (2008). *Dev Cell*, **15**, 62-73.), 2v6x, 2v6y (T.Obita et al. (2007). *Nature*, **449**, 735-739.), 2w2u (Samson et al. (2008). *Science*, **322**, 1710-1713.), 2zam, 2zan, 2zao (all Inoue et al. (2008). *Traffic*, **9**, 2180-2189.) or 3eab (Yang et al. (2008). *Nat Struct Biol*, **15**, 1278-1286.).

In the chitin synthases identified herein, the MIT domain is located in an amino acid stretch from position 103 to 171 in SEQ ID NO: 4 (CHS1), corresponding to SEQ ID NO: 19, and from position 141 to 209 in SEQ ID NO: 2 (CHS2), corresponding to SEQ ID NO: 20.

Further example MIT domains show at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99% or even 100% sequence identity to the MIT domains present in CHS1 (SEQ ID NO: 4), corresponding to SEQ ID NO: 20, and CHS2 (SEQ ID NO: 2), corresponding to SEQ ID NO: 19, or any one of the MIT domains present in the UniProt sequences or in the references listed above. All MIT domains disclosed herein have the biological function of the MIT domains present in CHS1 (SEQ ID NO: 4) and CHS2 (SEQ ID NO: 2). Said biological function can e. g. be evaluated by checking the location of a fusion protein comprising said MIT domain in a plant cell as outlined in the examples.

As used herein, the term "% sequence identity" refers to the percentage of identical nucleotides between two segments of a window of optimally aligned DNA. Optimal alignment of sequences for aligning a comparison window are well-known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman (Waterman, M. S., Chapman & Hall. London, 1995), the homology alignment algorithm of Needleman and Wunsch (1970), the search for similarity method of Pearson and Lipman (1988), and preferably by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG (Registered Trade Mark), Wisconsin Package (Registered Trade Mark from Accelrys Inc., San Diego, Calif.). An "identity fraction" for aligned segments of a test

sequence and a reference sequence is the number of identical components that are shared by the two aligned sequences divided by the total number of components in the reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction times 100. The comparison of one or more DNA sequences may be to a full-length DNA sequence or a portion thereof, or to a longer DNA sequence.

An MIT domain may e. g. be operably linked with any of the following chitin synthases (Chitin UDP- acetyl-glucosaminyl transferases) which can be found in the different databases including amino acid sequences with the following identifiers (accession numbers): CHS1_AJECA (P30576) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) from *Ajellomyces capsulata* (*Histoplasma capsulatum*); CHS1_AJEDE (P30579) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) from *Ajellomyces dermatitidis* (*Blastomyces dermatitidis*); CHS1_ASPNG (P30581) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) from *Aspergillus niger*; CHS1_BOTCI (P49603) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) from *Botrytis cinerea* (Noble rot fungus) (*Botryotinia fuckeliana*); CHS1_CANAL (P23316) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1). from *Candida albicans* (Yeast); CHS1_CRYNV (O13356) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-IV chitin synthase 1). {GENE: Name=CHS1} - *Cryptococcus neoformans* var. *grubii* (*Filobasidiella neoformans* var. *grubii*); CHS1_EMENI (P30583) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) (Fragment). {GENE: Name=chs1} - *Emericella nidulans* (*Aspergillus nidulans*); CHS1_EXODE (P30600) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-II chitin synthase 1). {GENE: Name=CHS1} - *Exophiala dermatitidis* (*Wangiella dermatitidis*); CHS1_EXOJE (P30585) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) (Fragment). {GENE: Name=CHS1} - *Exophiala jeanselmei*; CHS1_NEUCR (P29070) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-III chitin synthase 3). {GENE: Name=chs-1; ORFNames=B11H24.170, NCU03611.1} - *Neurospora crassa*; CHS1_PHAEX (P30590); Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1)

(Fragment). {GENE: Name=CHS1} - *Phaeococcomyces exophialae*; CHS1_PHYBL (P87073) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-II chitin synthase 1). {GENE: Name=chs1} - *Phycomyces blakesleeanus*; CHS1_RHIAT (P30592) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-I chitin synthase 1) (Fragment). {GENE: Name=CHS1} - *Rhinocladia atrovirens*; CHS1_RHIOL (P30594) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1). {GENE: Name=CHS1} - *Rhizopus oligosporus*; CHS1_RHIRA (Q12632) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Class-II chitin synthase 1). {GENE: Name=CHS1} - *Rhizomucor racemosus* (*Mucor circinelloides* f. *lusitanicus*); CHS1_SCHCO (P30596); Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Fragment). {GENE: Name=CHS1} - *Schizophyllum commune* (Bracket fungus); CHS1_SCHPO (P30597) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1). {GENE: Name=chs1; ORFNames=SPAC13G6.12c, SPAC24B11.01c} - *Schizosaccharomyces pombe* (Fission yeast); CHS1_TUBUN (P55003) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Fragment). {GENE: Name=CHS1} - *Tuber uncinatum* (Burgundy truffle); CHS1_USTMA (P30598) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Fragment). {GENE: Name=CHS1} - *Ustilago maydis* (Smut fungus); CHS1_XYLBA (P30603) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1) (Fragment). {GENE: Name=CHS1} - *Xylohypha bantiana*; CHS1_YEAST (P08004) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1). {GENE: Name=CHS1; *Saccharomyces cerevisiae* (Baker's yeast); CHS2_AJECA (P30577) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-III chitin synthase 2) *Ajellomyces capsulata* (*Histoplasma capsulatum*); CHS2_AJEDE (P30580) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-II chitin synthase 2) {GENE: Name=CHS2} - *Ajellomyces dermatitidis* (*Blastomyces dermatitidis*) CHS2_ASPNG (P30582); Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-II chitin synthase 2) (Fragment). {GENE: Name=chs2} - *Aspergillus niger*; CHS2_CANAL (P30572) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2). {GENE: Name=CHS2} - *Candida albicans* (Yeast); CHS2_EXODE (P30601) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-I chitin synthase 2). {GENE: Name=CHS2} -

Exophiala dermatitidis (Wangiella dermatitidis); CHS2_EXOJE (P30586) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Fragment). {GENE: Name=CHS2} - Exophiala jeanselmei; CHS2_NEUCR (P30589); Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2). {GENE: Name=chs-2; ORFNames=NCU05239.1} - Neurospora crassa; CHS2_PARBR (Q92444) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-II chitin synthase 2). {GENE: Name=CHS2} - Paracoccidioides brasiliensis; CHS2_PHAEX (P30591); Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-II chitin synthase 2) (Fragment). {GENE: Name=CHS2} - Phaeococcomyces exophialae; CHS2_RHIAT (P30593) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-III chitin synthase 2) (Fragment). {GENE: Name=CHS2} - Rhinocladiella atrovirens; CHS2_RHIOL (P30595) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2). {GENE: Name=CHS2} - Rhizopus oligosporus; CHS2_SCHPO (O74756) Chitin synthase-like protein 2. {GENE: Name=chs2; ORFNames=SPBC1709.01, SPBC1734.17} - Schizosaccharomyces pombe (Fission yeast); CHS2_USTMA (P30599) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Fragment). {GENE: Name=CHS2} - Ustilago maydis (Smut fungus); CHS2_XYLBA (P30604) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2) (Class-II chitin synthase 2) (Fragment). {GENE: Name=CHS2} - Xylohypha bantiana; CHS2_YEAST (P14180); Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 2). {GENE: Name=CHS2; OrderedLocusNames=YBR038W; ORFNames=YBR0407} - Saccharomyces cerevisiae (Baker's yeast); CHS3_AJECA (P30578) Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3) (Class-II chitin synthase 3) (Fragment). {GENE: Name=CHS3} - Ajellomyces capsulata (Histoplasma capsulatum); CHS3_CANAL (P30573) Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3) (Class-IV chitin synthase 3). {GENE: Name=CHS3} - Candida albicans (Yeast); CHS3_EXODE (P30602) Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3) (Class-III chitin synthase 3). {GENE: Name=CHS3} - Exophiala dermatitidis (Wangiella dermatitidis); CHS3_EXOJE (P30587); Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3) (Class-III chitin synthase 3) (Fragment). {GENE: Name=CHS3} - Exophiala jeanselmei; CHS3_NEUCR (P30588) Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3). {GENE: Name=chs-3; ORFNames=G65A3.040} - Neurospora crassa; CHS3_YEAST

(P29465) Chitin synthase 3 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 3) (Class-IV chitin synthase 3). {GENE: Name=CHS3; Synonyms=CAL1, CSD2, DIT101, KIT2; Ordered Locus Names=YBR023C; ORFNames=YBR0305} - *Saccharomyces cerevisiae* (Baker's yeast); CHS4_MAGGR (O13353); Chitin synthase 4 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 4) (Class-IV chitin synthase 4). {GENE: Name=CHS4} - *Magnaporthe grisea* (Rice blast fungus) (*Pyricularia grisea*); CHS4_NEUCR (Q01285) Chitin synthase 4 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 4) (Class-IV chitin synthase 4). {GENE: Name=chs-4; ORFNames=NCU09324.1} - *Neurospora crassa*; CHS5_USTMA (O13394) Chitin synthase 5 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 5) (Class-IV chitin synthase 5). {GENE: Name=CHS5} - *Ustilago maydis* (Smut fungus); CHS6_USTMA (O13395) Chitin synthase 6 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 6) (Class-V chitin synthase 6). {GENE: Name=CHS6} - *Ustilago maydis* (Smut fungus); CHSA_AMPQU (Q12564); Chitin synthase A (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase A) (Class-I chitin synthase A). {GENE: Name=CHSA} - *Ampelomyces quisqualis*; CHSA_EMENI (P30584) Chitin synthase A (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase A) (Class-II chitin synthase A). {GENE: Name=chsA; Synonyms=chs2} - *Emericella nidulans* (*Aspergillus nidulans*); CHSB_EMENI (Q00757) Chitin synthase B (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase B) (Class-III chitin synthase B). {GENE: Name=chsB} - *Emericella nidulans* (*Aspergillus nidulans*); CHSC_ASPFU (Q92197) Chitin synthase C (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase C) (Class-III chitin synthase C). {GENE: Name=chsC} - *Aspergillus fumigatus* (*Sartorya fumigata*); CHSD_ASPFU (P78746) Chitin synthase D (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase D) (Class-VI chitin synthase D). {GENE: Name=chsD} - *Aspergillus fumigatus* (*Sartorya fumigata*); CHSD_EMENI (P78611) Chitin synthase D (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase D) (Class-V chitin synthase D). {GENE: Name=chsD; Synonyms=chsE} - *Emericella nidulans* (*Aspergillus nidulans*); CHSG_ASPFU (P54267); Chitin synthase G (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase G) (Class-III chitin synthase G). {GENE: Name=chsG} - *Aspergillus fumigatus* (*Sartorya fumigata*); CHSX_USTMA (Q99126) Chitin synthase 1 (EC 2.4.1.16) (Chitin-UDP acetyl-glucosaminyl transferase 1). {GENE: Name=CHS1} - *Ustilago maydis* (Smut fungus); or CHSY_USTMA (Q99127) Chitin synthase 2 (EC 2.4.1.16) (Chitin-UDP acetyl-

glucosaminyl transferase 2). {GENE: Name=CHS2} - *Ustilago maydis* (Smut fungus).

Operably linking can result in the MIT domain being present at the N-terminus, at the C-terminus or within a chitin synthase sequence as long as this position does essentially not impair the chitin synthase activity of the resulting protein. "Essentially not impair" in this regard means that the chitin synthase is still sufficiently active to provide for a detectable amount of positively charged polysaccharides in a plant cell wall, when expressed in said plant cell. This means that the chitin synthase has at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90% or at least 100% of the chitin synthase activity of either CHS1 or CHS2. Methods of determining chitin synthase activity are known in the art and also disclosed in the present application.

In a particular embodiment the invention also provides variant sequences of SmCHS1 and SmCHS2 which may be obtained by induced generation of variation *in vitro* or *in vivo*. Several methods for *in vitro* induced generation of variant nucleotide sequences are available in the art including but not limited to DNA shuffling or directed evolution techniques as described in US patent 5605793, US patent 5,811,238 and US patent 5,830,721. Methods for *in vivo* induced generation of variant nucleotide sequence are also well known in the art and may include exposure of *Saprolegnia monoica* to mutagens such as ionizing radiation, EMS, MMS or the like, followed by isolation of the chitin synthase 1 or 2 encoding nucleic acids, e.g. as elsewhere described herein. In another particular embodiment the SmCHS1 and SmCHS2, in particular the nucleic acids encoding them, can be modified by adapting the codon usage for the specific expression in plants. Such methods for codon-modification (or codon-adaptation which is an equivalent wording) for the expression in specific plants are well known in the art. SEQ ID NO: 18 depicts a variant SmCHS2 nucleotide sequence which is a codon-modified variant optimized for expression in cotton. SEQ ID NO: 18 encodes for the chitin synthase polypeptide depicted in SEQ ID NO: 2.

In another embodiment the invention provides a chimeric gene comprising the following operably linked DNA regions: 1) a plant-expressible promoter, 2) a DNA region coding for a chitin synthase polypeptide wherein the nucleotide sequence of the DNA region is at least 97% identical to SEQ ID NO: 1, or wherein nucleotide sequence of the DNA

region is at least 90% identical to SEQ ID NO: 3, or wherein the nucleotide sequence of the DNA region comprises a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to SEQ ID NO: 2 or wherein the nucleotide sequence of the DNA region comprises a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 90% identical to SEQ ID NO: 4 and 3) a transcription termination and polyadenylation region.

The chimeric genes according to the invention comprise a plant-expressible promoter. As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S, the subterranean clover virus promoter No 4 or No 7, or T-DNA gene promoters and the like.

A plant-expressible promoter that controls initiation and maintenance of transcription preferentially in fiber cells is a promoter that drives transcription of the operably linked DNA region to a higher level in fiber cells and the underlying epidermis cells than in other cells or tissues of the plant. A higher level can be for example a transcription of at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, at least 100-fold or at least 500-fold more in fiber cells and optionally the underlying epidermis cells than in other cells or tissues of the plant. Such definition also applies to the term "fiber selective promoter". In other words, the term includes a promoter that drives at least 2-fold, at least 5-fold, or at least 10-fold more transcription in fiber cells and optionally the underlying epidermis cells than in other cells or tissues of the plant. Such promoters include the promoter from cotton from a fiber-specific β - tubulin gene (as described in WO0210377), the promoter from cotton from a fiber-specific actin gene (as described in WO0210413), the promoter from a fiber specific lipid transfer protein gene from cotton (as described in US5792933), a promoter from an expansin gene from cotton (WO9830698) or a promoter from a chitinase gene in cotton (US2003106097) or the promoters of the fiber specific genes described in US6259003 or US6166294 or the

promoters derived from the E6 family as disclosed in US6096950. In a particular embodiment the plant-expressible promoter is a constitutive promoter. As discussed in example 3, last 6 lines, expressing the chitin synthase of the invention does not cause differences in plant growth as compared to untransformed plants.

In a particular embodiment the invention provides a plant cell comprising a chimeric gene herein described before.

The chimeric gene may be introduced into a plant cell by methods well-known in the art. "Introducing" in connection with the present application relate to the placing of genetic information in a plant cell or plant by artificial means. This can be effected by any method known in the art for introducing RNA or DNA into plant cells, tissues, protoplasts or whole plants.

A number of methods are available to transfer DNA into plant cells or plants. Agrobacterium-mediated transformation of cotton has been described e.g. in US patent 5,004,863, in US patent 6,483,013 or in WO2000/71733.

Plants or plant cells may also be transformed by particle bombardment: Particles of gold or tungsten are coated with DNA and then shot into young plant cells or plant embryos. This method also allows transformation of plant plastids. Cotton transformation by particle bombardment is reported e.g. in WO 92/15675.

Viral transformation (transduction) may be used for transient or stable expression of a gene, depending on the nature of the virus genome. The desired genetic material is packaged into a suitable plant virus and the modified virus is allowed to infect the plant or plant cell. The progeny of the infected plants is virus free and also free of the inserted gene. In an exemplary viral transformation protocol, DNA such as DNA comprising the chimeric gene described herein as well as a helper virus, can be introduced into whole plants by mechanical inoculation with DNA comprising the chimeric gene described herein alone or with virions containing the said DNA. The selection of the best suited transformation protocol including the manner and parameters of transformation, timing of transformation, etc. is well within the knowledge of persons of ordinary skill in the art. Suitable methods are described or further detailed e. g. in WO 90/12107, WO 03/052108 or WO 2005/098004.

In another particular embodiment the invention provides a plant consisting essentially of plant cells comprising a chimeric gene herein described before. In a particular embodiment the plant is a transgenic cotton plant.

Apart from the methods for introducing the chimeric gene described above, said chimeric gene may also be introgressed into a plant. "Introgressing" means the integration of a gene in a plant's genome by natural means, i. e. by crossing a plant comprising the chimeric gene described herein with a plant not comprising said chimeric gene. The offspring can be selected for those comprising the chimeric gene.

The plant may be any fiber producing plant such as cotton. Other examples include other fiber producing plants such as hemp, jute, flax and woody plants, including but not limited to *Pinus spp.*, *Populus spp.*, *Picea spp.*, *Eucalyptus spp.* etc.

In another particular embodiment the invention provides a transgenic seed comprising a chimeric gene as herein described before.

In yet another embodiment the invention provides a method for the manufacture of a plant cell wall comprising positively charged polysaccharides, said method comprises 1) expressing a chimeric gene as outlined herein before in a plant or plant cell and 2) isolating said plant cell wall. Said isolating is from the plant obtained after carrying out step 1).

In a specific embodiment said method for manufacturing (or production which is an equivalent wording) produces positively charged polysaccharides in a plant secondary cell wall. In another particular embodiment said method for manufacturing produces chitin in a plant secondary cell wall. In another particular embodiment said method for manufacturing produces chitosan in a plant secondary cell wall, for example due to the presence and expression of a further chimeric gene comprising a chitin deacetylase as described further below. In a particular embodiment said plant cell wall, or said plant secondary cell wall is a cotton plant cell wall or a cotton secondary plant cell wall. In a particular embodiment said cotton plant cell wall, or said cotton secondary cell wall is present in a cotton fiber.

The invention further provides plant cell walls including the cell walls obtained from plant cells using the methods according to the invention, and fibers comprising said cell walls.

Such plant cell walls comprise positively charged polysaccharides, such as N-acetylglucosamine polymers or chitin, embedded into the cellulose. These plant cell walls may be further modified, e.g. partly or completely deacetylated such that oligomers comprising glucosamine residues are obtained. The amino-group of the resulting glucosamines is chemically more reactive than the aminoacetyl group of N-acetylglucosamine or the hydroxyl group of cellulose. In a particular embodiment plant cell walls, in particular plant secondary cell walls, comprising chitin can be further modified by means of a chemical deacetylation step. In another particular embodiment the method for manufacturing positively charged polysaccharides in a plant cell wall can be carried out by expressing two chimeric genes in a plant or plant cell, one chimeric gene being a chitin synthase of the invention together with a chimeric chitin deacetylase. A chitin deacetylase in enzymology, is an enzyme (EC 3.5.1.41) that catalyzes the chemical reaction:



Thus, the two substrates of this enzyme are chitin and H_2O , whereas its two products are chitosan and acetate. This enzyme belongs to the family of hydrolases, those acting on carbon-nitrogen bonds other than peptide bonds, specifically in linear amides. The systematic name of this enzyme class is chitin amidohydrolase.

In a particular embodiment the plant cell wall obtained according to the invention, particularly those which have been subjected to a deacetylation step, for example in vivo as described above or after harvest of the cell wall, can be further chemically modified. Products containing such plant cell walls, such as fibers, yarns or fabrics have qualities resembling those of the cellulose-chitosan blends described in the art, including improved dyeability, improved inhibition of e.g. dermatophytes, controlled drug release etc.

In a specific embodiment, the invention provides cotton fibers obtained from or which can be obtained from cotton plants according to the methods of the invention. In other words, cotton fibers are provided from cotton plants comprising in the genome, such as the nuclear genome, of their cells a chimeric gene comprising a plant-expressible promoter operably linked to a DNA region coding for a chitin synthase 1 or 2 according to the invention or a combination of two chimeric genes coding for the chitin synthase genes 1 and 2 according to the invention. Particular embodiments of DNA coding

regions or promoters comprised in the chimeric genes transferred into cotton plants are as described elsewhere in this document.

The cotton fibers according to the invention can be distinguished from naturally occurring cotton fibers, i.e. cotton fibers obtained from an isogenic line which does not comprise a chimeric gene according to the invention, by the capacity of such fibers for increased staining with anionic dyes (including e.g. Congo Red), by the capacity of such fibers for increased staining with amine-reactive dyes (including e.g. tetrafluorophenyl ester). The cotton fibers according to the invention also have the capacity of binding of Wheat germ agglutinin which binds chitin. The cotton fibers according to the invention can also be distinguished from naturally occurring cotton fibers by direct detection of the N-acetylglucosamine and GlcNAc polymers, optionally after treatment of the fiber cell wall material with chitinase. The cotton fibers according to the invention may also be distinguished by their increased nitrogen content.

Cotton fibers according to the invention can also be distinguished from the chitosan coated fibers of the prior art, in that the positively charged polymers are evenly distributed in the secondary plant cell walls making up the fibers as opposed to the surface coated chitosan fibers of the prior art. Accordingly, in microscopical sections of cotton fibers, stained e.g. with WGA or with congo red or with tetrafluorophenyl as described hereinafter, the dyes will be distributed evenly throughout the cell walls making up the cotton fibers, whereas in chitosan-coated fibers, the staining will be concentrated at the coat of chitosan located as a sheet at the surface of the treated fibers.

The increased staining of the plant cell wall material according to the invention, by anionic dyes such as congored can be quantified e.g. by dyeing a uniform amount of material under standard conditions, spreading out the material over a standardized area (such as a well in a multiwell plate) digitalizing a picture of the area for the gray scale of the colored layer of material. The less gray, the more stained the plant cell wall material is. In this way, cotton fibers and cell wall material according to the invention showed an increase of at least about 5% in staining by congo-red compared to control cell wall material or fibers from isogenic plant lines without a chitin synthase encoding gene. In a particular embodiment the plant cell material obtained according to the invention can be stained with commercial dyes including cotton reactive dyes (e.g. Reactive Red 120,

Levafix Blue CA), acid dyes (Acid Orange 7, Acid Blue 281) and wool reactive dyes (e.g. Reactive Red 116, Realan Amber EHF). In an example, the cotton fibers and the cell wall material according to the invention show an increase of at least about 5 %, at least about 10%, at least 20%, at least 30%, at least 40%, at least 50% at least 60, at least 70%, at least 80%, at least 90% or even at least 100% such as 2-fold, 5-fold or 10-fold in staining as compared to said fibers and material not comprising a chitin synthase encoding gene.

The capacity of the novel cotton fibers to specifically bind wheat germ agglutinin (detectable by the coupled fluorophoric group) is a clear distinguishing feature of the provided novel cotton fibers over the naturally occurring cotton fibers. Except for a very low background fluorescence, naturally occurring cotton fibers do not stain/fluoresce when treated with WGA –alexa fluor 488 or 555. The fluorescence of cotton fibers increases at least 5 times when chitin polymers are present. Accordingly, the invention provides cotton fibers which are capable of specifically binding wheat germ agglutinin, or WGA coupled to a fluorophore, such as WGA Alexa 488 or WGA Alexa 555 or which, when treated with WGA Alexa 488 or WGA Alexa 555 provide a bright fluorescence under UV light. This fluorescence is not restricted to the surface of the cotton fiber but is distributed throughout the cell wall of the fiber cells.

Cotton fibers according to the invention can also be distinguished from only chitosan coated fibers by applying the detection method disclosed in WO2010/015423 and checking for the presence of the chimeric gene of the invention in the fibers.

Wherever the methods of the invention are directed to introduction of a chimeric gene in a plant cell, it will be clear that such methods can also be applied in cases whereby the plant cell is incorporated into a mature plant. E.g. transgenic cells may be regenerated into transgenic plants according to established methods.

Methods to transform plants cells and plants are well known in the art. Methods to transform cotton plants are also well known in the art. Agrobacterium-mediated transformation of cotton has been described e.g. in US patent 5,004,863 or in US patent 6,483,013 and cotton transformation by particle bombardment is reported e.g. in WO 92/15675.

The chimeric genes may be introduced by transformation in cotton plants from which embryogenic callus can be derived, such as Coker 312, Coker310, Coker 5Acala SJ-5, GSC25110, FiberMax 819 , Siokra 1-3, T25, GSA75, Acala SJ2, Acala SJ4, Acala SJ5, Acala SJ-C1, Acala B1644, Acala B1654-26, Acala B1654-43, Acala B3991, Acala GC356, Acala GC510, Acala GAM1, Acala C1, Acala Royale, Acala Maxxa, Acala Prema, Acala B638, Acala B1810, Acala B2724, Acala B4894, Acala B5002, non Acala "picker" Siokra, "stripper" variety FC2017, Coker 315, STONEVILLE 506, STONEVILLE 825, DP50, DP61, DP90, DP77, DES119, McN235, HBX87, HBX191, HBX107, FC 3027, CHEMBRED A1, CHEMBRED A2, CHEMBRED A3, CHEMBRED A4, CHEMBRED B1, CHEMBRED B2, CHEMBRED B3, CHEMBRED C1, CHEMBRED C2, CHEMBRED C3, CHEMBRED C4, PAYMASTER 145, HS26, HS46, SICALA, PIMA S6 and ORO BLANCO PIMA, Fibermax® FM5013, FM5015, FM5017, FM989, FM832, FM966 and FM958, FM989, FM958, FM832, FM991, FM819, FM800, FM960, FM966, FM981, FM5035, FM5044, FM5045, FM5013, FM5015, FM5017 or FM5024 and plants with genotypes derived thereof.

"Cotton" as used herein includes *Gossypium hirsutum*, *Gossypium barbadense*, *Gossypium arboreum* and *Gossypium herbaceum* or progeny from crosses of such species with other species or crosses between such species.

The methods and means of the current invention may also be employed for other plant species such as hemp, jute, flax and woody plants, including but not limited to *Pinus spp.*, *Populus spp.*, *Picea spp.*, *Eucalyptus spp.* etc.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene according to the invention in other varieties 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.

The chimeric gene of the invention is advantageously combined in plants with other genes which encode proteins or RNAs that confer useful agronomic properties to such plants. Among the genes which encode proteins or RNAs that confer useful agronomic

properties on the transformed plants, mention can be made of the DNA sequences encoding proteins which confer tolerance to one or more herbicides, and others which confer tolerance to certain insects or those which confer tolerance to certain diseases. Such genes are in described for example in published PCT Patent Applications WO 91/02071 and WO95/06128.

Among the DNA sequences encoding proteins which confer tolerance to certain herbicides on the transformed plant cells and plants, mention can be made of a bar or PAT gene or the *Streptomyces coelicolor* gene described in WO2009/152359 which confers tolerance to glufosinate herbicides, a gene encoding a suitable EPSPS which confers tolerance to herbicides having EPSPS as a target, such as glyphosate and its salts (US 4,535,060, US 4,769,061, US 5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,310,667, US 5,312,910, US 5,627,061, US 5,633,435, US 6,566,587 and WO 97/04103), a gene encoding glyphosate oxydoreductase (US 5,463,175), or a gene encoding the HPPD enzyme for tolerance to HPPD inhibitor herbicides such as isoxazoles (WO 96/38567).

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.

The following non-limiting Examples describe the methods for altering plant cell walls. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

The transformed plant cells and plants obtained by the methods described herein may be further used in breeding procedures well known in the art, such as crossing, selfing, and backcrossing. Breeding programs may involve crossing to generate an F1 (first filial) generation, followed by several generations of selfing (generating F2, F3, etc.). The breeding program may also involve backcrossing (BC) steps, whereby the offspring is backcrossed to one of the parental lines, termed the recurrent parent.

The transgenic plant cells and plants obtained by the methods disclosed herein may also be further used in subsequent transformation procedures, e. g. to introduce a further chimeric gene.

The plants comprising the chimeric gene disclosed herein may further be treated with cotton herbicides such as Diuron, Fluometuron, MSMA, Oxyfluorfen, Prometryn, Trifluralin, Carfentrazone, Clethodim, Fluazifop-butyl, Glyphosate, Norflurazon, Pendimethalin, Pyriithiobac-sodium, Trifloxysulfuron, Tepraloxymdim, Glufosinate, Flumioxazin, Thidiazuron; cotton insecticides such as Acephate, Aldicarb, Chlorpyrifos, Cypermethrin, Deltamethrin, Abamectin, Acetamiprid, Emamectin Benzoate, Imidacloprid, Indoxacarb, Lambda-Cyhalothrin, Spinosad, Thiodicarb, Gamma-Cyhalothrin, Spiromesifen, Pyridalyl, Flonicamid, Flubendiamide, Triflumuron, Rynaxypyr, Beta-Cyfluthrin, Spirotetramat, Clothianidin, Thiamethoxam, Thiacloprid, Dinetofuran, Flubendiamide, Cyazypyr, Spinosad, Spinotoram, gamma Cyhalothrin, 4-[[[(6-Chlorpyridin-3-yl)methyl](2,2-difluorethyl)amino]furan-2(5H)-on, Thiodicarb, Avermectin, Flonicamid, Pyridalyl, Spiromesifen, Sulfoxaflor; and cotton fungicides such as Azoxystrobin, Bixafen, Boscalid, Carbendazim, Chlorothalonil, Copper, Cyproconazole, Difenconazole, Dimoxystrobin, Epoxiconazole, Fenamidone, Fluazinam, Fluopyram, Fluoxastrobin, Fluxapyroxad, Iprodione, Isopyrazam, Isotianil, Mancozeb, Maneb, Metominostrobin, Penthiopyrad, Picoxystrobin, Propineb, Prothioconazole, Pyraclostrobin, Quintozene, Tebuconazole, Tetraconazole, Thiophanate-methyl, Trifloxystrobin.

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 *Saprolegnia monoica* chitin synthase 2 gene

SEQ ID No: 2: amino acid sequence of the *Saprolegnia monoica* chitin synthase 2 gene
 SEQ ID No: 3: nucleotide sequence of the *Saprolegnia monoica* chitin synthase 1 gene
 SEQ ID No: 4: amino acid sequence of the *Saprolegnia monoica* chitin synthase 1 gene
 SEQ ID NOs: 5-17 are synthetic primers for which the sequences are depicted in Table 1.

SEQ ID NO: 18: modified nucleotide sequence of *Saprolegnia monoica* chitin synthase 2 (codon optimized for expression in cotton)

SEQ ID No: 19: amino acid sequence of the MIT domain of *Saprolegnia monoica* chitin synthase 1

SEQ ID NO: 20: amino acid sequence of the MIT domain of *Saprolegnia monoica* chitin synthase 2

SEQ ID NO: 21: nucleotide sequence of a *Saprolegnia parasitica* chitin synthase gene

SEQ ID NO: 22: amino acid sequence encoded by the *Saprolegnia parasitica* chitin synthase gene of SEQ ID NO: 21

SEQ ID NO: 23: nucleotide sequence of a *Saprolegnia parasitica* chitin synthase gene

SEQ ID NO: 24: amino acid sequence encoded by the *Saprolegnia parasitica* chitin synthase gene of SEQ ID NO: 23

EXAMPLES

1. Cloning of chitin synthase genes from *Saprolegnia monoica*

The oomycete *Saprolegnia monoica* Pringsheim 53-967 Dick was obtained from the "Centraal Bureau voor Schimmel Culturen" (CBS, Baarn, The Netherlands) and maintained on Potato Dextrose Agar in 90-mm Petri dishes. The mycelium used for the experiments was grown in the liquid medium of Machlis (Machlis L. (1953) Am. J. Bot. 40, 449-460) for 3-5 days at 25°C in the dark.

Total RNA was extracted from 100 mg of 3-days old *S. monoica* mycelium, using the RNeasy Plant Mini Kit (Qiagen), coupled with the on-column DNaseI digestion. Reverse transcription was carried out with 4.5 µg total RNA using the Superscript III First Strand cDNA Synthesis kit (Invitrogen) following the manufacturer's instructions. 1 µl of the synthesized cDNA was used in the PCR reactions using the primers CHS2Fwd and CHS2Rev which were designed based on the sequence deposited under the accession number U19946 (this sequence is also disclosed in Mort-Bontemps M. *et al* (1997) *Microbiology* 143, 2009-2020 wherein a chitin synthase 2 (CHS2) sequence is depicted in Figure 3 of said reference), for the amplification of the CHS2 sequence. The RNA

ligase-mediated RACE kit (RLM-RACE, Ambion) was used to isolate and determine the sequence of the 3' end of CHS2. The 3' end of CHS2 was obtained with the primers CHS2Fwd1 and CHS2Fwd2. The complete full-length CHS2 coding sequence (depicted in SEQ ID NO: 1) was amplified from *S. monoica* mycelium cDNA using the primers CHS2Fwd and CHS2FLRev. This resulting sequence was cloned into pENTR-D-TOPO (Invitrogen) using the Gateway technology according to the manufacturer's instructions. The construct was transformed into One Shot TOP10 chemically competent cells (Invitrogen) and the nucleotide sequence was determined (MWG, Germany). It was observed that the disclosed coding sequence of the *S. monoica* CHS2 (accession number U19946) is only 96% identical to the isolated CHS2 sequence depicted in SEQ ID NO: 1. The differences in the nucleotide sequence are due to a number of small deletions in the coding sequence of the chitin synthase 2 disclosed with accession number U19946. As a consequence the translation of the coding sequence depicted in SEQ ID NO: 1 is only 79% identical to the translated coding sequence of accession number U19946. The latter is caused by small deletions in the nucleotide sequence of U19946, which leads to frame shifts with respect to the translation of SEQ ID NO: 1, upon translation of the coding sequence of U19946. It was apparent that SEQ ID NO: 2 comprises the conserved Microtubule Interacting and Trafficking molecule domain (MIT domain) which is absent in the chitin synthase protein translated from the accession U19946.

The sequences were edited using the software BioEdit (Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis – <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Alignment was performed using ClustalW (Larkin MA *et al* (2007) Bioinformatics 23, 2947-2948). SmCHS1 and SmCHS2 full-length sequences were used to perform a BLASTp search against non redundant protein database from the National Centre for Biotechnology (NCBI, <http://www.ncbi.nlm.nih.gov>).

Table 1 depicts the sequence of the primers:

CHS2Fwd, SEQ ID NO: 5	CACCATGAGTGACCAGCTCGACCTCGCGGC
CHS2Rev, SEQ ID NO: 6	TGCTCTCTGCACGGGCAACCACAACCCGAC
CHS1Fwd, SEQ ID NO: 7	AATGAGGACGAGAACGAGCTCCGGTCTG
CHS1Rev, SEQ ID NO: 8	AGCTTGTAAGGACGACTTGGTTGGC

CHS1Rev1, SEQ ID NO: 9	TCTCCTTGGTCATTTGCAGCGAGTG TTC
CHS1Rev2, SEQ ID NO: 10	CAGAACGTTGTTGCAAACCTTGCGGAGT
CHSFwd1, SEQ ID NO: 11	TCCGGTCGACACTCCGCAAGGTTTGCAA
CHS1Fwd2, SEQ ID NO: 12	ACTACACGGTCCTCCTCGATGTTGGGAC
CHS2Fwd1, SEQ ID NO: 13	TGTCGGTGGCTTGATTGTCTTTGC
CHS2Fwd2, SEQ ID NO: 14	TTTGGCTCTACGTTGTGACGGACT
CHS1FLFwd, SEQ ID NO: 15	CACCATGCCGCCCAAGCGACCGACGACCGA
CHS1FLRev, SEQ ID NO: 16	CTAGCGCATGCGGTTGTACGGCGCTTGG
CHS2FLRev, SEQ ID NO: 17	TTAGACTTGTTGGTAGGCGCCGCCGCGG

Two primers (CHS1Fwd and CHS1Rev) were designed based on the partial sequence of the *S. monoica* chitin synthase 1 (CHS1), corresponding to the accession number U42304, for the amplification of a conserved region of CHS1. The RNA ligase-mediated RACE kit (RLM-RACE, Ambion) was used to amplify the 5' and 3' ends of CHS1. The following sets of nested reverse primers were used to obtain the 5' end of CHS1: CHS1Rev1 and CHS1Rev2. The nested forward primers CHSFwd1 and CHS1Fwd2 were used to isolate the 3' end. Full-length CHS1 was amplified from *S. monoica* mycelium cDNA to confirm the complete sequence using the primers CHS1FLFwd and CHS1FLRev for the amplification of the coding sequence of the CHS1 gene. The full-length gene was cloned into pENTR-D-TOPO (Invitrogen) using the Gateway technology according to the manufacturer's instructions. The construct was transformed into One Shot TOP10 chemically competent cells (Invitrogen) and the nucleotide sequence was determined by sequencing (MWG, Germany). All PCR reactions were carried out using Phusion High-Fidelity DNA Polymerase (Finnzymes), according to the manufacturer's instructions. The coding region of the chitin synthase 1 nucleotide sequence is depicted in SEQ ID NO: 3. The amino acid sequence of the chitin synthase 1 is depicted in SEQ ID NO: 4.

2. Construction of chimeric plant-expressible genes encoding a *Saprolegnia monoica* chitin synthase 2 (CHS2) protein

Using standard recombinant DNA techniques, a plant expressible *Saprolegnia monoica* CHS2 chimeric gene was constructed containing the following operably linked DNA fragments:

- a 35S promoter region from CaMV

- a DNA fragment coding for an untranslated leader sequence (5'Cab22L)
- a DNA fragment coding for CHS2 of *Saprolegnia monoica* (depicted in SEQ ID NO: 1)
- a transcription termination and polyadenylation signal from the octopine synthase gene

Briefly, this construct was generated by PCR-cloning the CHS2 gene into the Gateway entry vector pENTR/D/TOPO, using the directional TOPO cloning kit (Invitrogen), according to the manufacturer's instructions. The PCR reaction was carried out using Phusion DNA polymerase (Finnzymes). The resulting entry vector was recombined into the destination vector pEarleyGate 103 using the Gateway LR Clonase Enzyme Mix (Invitrogen) (Earley KW *et al* (2006) *Plant J.* 45: 616-629). All constructs were sequence verified by sequence analysis. The selection marker for plant transformation was the *bar* gene providing resistance to phosphinotricin. The recombinant binary vector pEarleyGate 103 comprising the *SmCHS2* gene was used to transform the *Agrobacterium* strain C58C1 (Voinnet O. *et al* (2003) *Plant J.* 33: 949-956) which was used to transform *Arabidopsis thaliana* by means of the floral dip method (Clough SJ and Bent AF (1998) *Plant J.* 16: 735-743). The transformed *A. thaliana* plants comprising the chimeric *Saprolegnia monoica* chitin synthase 2 did not differ in growth compared to the untransformed *A. thaliana* plants. This is in contrast with the reduced growth of the transgenic *A. thaliana* plants comprising the *Neurospora crassa* chitin synthase operably linked with a Golgi localisation signal (as disclosed in WO2006/136351, see example 9).

3. Histochemical analysis of the trichomes of recombinant *Arabidopsis thaliana*

Trichomes (plant hairs) in *Arabidopsis thaliana* are large non-secreting epidermal cells with a characteristic three-dimensional structure. Because trichomes are easily accessible to a combination of genetic, cell biological and molecular methods we used mature trichomes derived from recombinant *A. thaliana* plants produced in example 2 for the analysis of the plant cell walls of *SmCHS2*-transformed *A. thaliana* plants. During our recent investigations we observed that the cell wall analysis of hairy root cultures (see WO2006/136351, example 2) leads to similar results as the cell wall analysis of mature trichomes (the latter are also less cumbersome to establish and easier to isolate than hairy root cultures).

Trichomes were histochemically stained to visualize different compounds of the cells, and analyzed microscopically.

N-acetylglucosamine can be detected e. g. after immunological reaction with IgM monoclonal antibodies to N-acetylglucosamine (BIODESIGN). Chitin can be detected by for example using Wheat Germ Agglutinin-Alexa Fluor 555 or alternatively by using a rhodamine-conjugated chitin-binding probe. The latter is a recombinant fusion protein that binds specifically to chitin. The fusion protein comprises a small (5 kDa) chitin-binding domain (CBD) derived from the C-terminal region of chitinase A1 of *Bacillus circulans* WL-12 fused to the C-terminus of maltose-binding protein (43kDa) from *E. coli*. The fusion protein is labeled using tetramethylrhodamine isothiocyanate (TRITC) following standard methods. We purchased the rhodamine-conjugated chitin-binding probe from New England BioLabs (catalogue number P 5210).

The histochemically stained trichomes were examined by means of fluorescence microscopy, using an Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with Apotome (Zeiss) to allow optical sections. Axio Vision 4.2 (Zeiss) was used for image processing.

Calcofluor staining of plant cell walls can be carried out with the following protocol. Calcofluor White (or Fluorescent Brightener 28) is a colourless organic compound that fluoresces in a clear bluish color under ultraviolet radiation ($\lambda_{\text{max}} = 350 \text{ nm}$). The specimen (i.e. the trichome) to be stained is immersed for 15 to 30 minutes in culture medium or PBS (buffer solution) comprising Fluorescent Brightener 28 at 50 $\mu\text{g/mL}$ final concentration. The specimen is then washed with medium or buffer, and samples are examined using a microscope equipped for fluorescence microscopy using Zeiss filter set 18. Cell walls fluoresce in a clear bluish color. Recombinant mature trichomes comprising the SmCHS2 chimeric gene are immunohistochemically stained for the presence of N-acetylglucosamine and subsequently stained with Calcofluor to visualize the cell walls. As can be observed from the superposition of the optical sections, the presence of N-acetylglucosamine is exclusively detected in the cell walls of recombinant trichomes.

Method for Wheat Germ Agglutinin-Alexa Fluor 555 staining or staining with the rhodamine-conjugated chitin-binding probe:

Mature leaves comprising trichomes were isolated after about three weeks from *in vitro* generated transgenic *Arabidopsis* plants (i.e. recombinant plants comprising SmCHS2 according to example 2) and also from control *Arabidopsis* plants (i.e. non-transformed

plants). The leaves were first fixed in a fixative (10% formalin, 0.25% glutaraldehyde in PBS) for 1 hour. The sample was vacuum infiltrated and the fixative was refreshed and incubated with the leaves for another 3 hours. Finally, after fixation the leaves could be further processed or stored at 4°C for some days. In a next step fixated leaves were washed (for 60 minutes) with PBS followed by washing with PBT for 60 minutes (i.e. PBS + 0.1% Tween20). The leaves were subsequently incubated with wheat germ agglutinin-Alexa Fluor 555 (Alexa fluor 555 TFP ester is available as a kit from the company Molecular Probes) in PBT (3mg/ml) for 4 to 6 hours. Or alternatively the leaves were incubated with the rhodamine-chitin-binding-probe for 16 hours. After the incubation the fluorochrome or the rhodamine-chitin-binding-probe was washed away (twice with PBT and once with PBS).

The stained recombinant trichomes were examined at the edges of the leaves using fluorescence microscopy e.g. with Zeiss filter 38. It was observed that the cell wall material from recombinant trichomes reproducibly stained more intense than cell wall material from control plants (see Figure 1).

4. Fiber specific expression of a chitin synthase in cotton

Transgenic cotton plants comprising a chimeric chitin synthase 2 gene as outlined in example 2, under control of the F286 fiber-selective promoter (which is disclosed in US2003/106097) as well as transgenic plants comprising two chimeric genes, i. e. said chimeric chitin synthase 2 gene under control of the F286 promoter and a glutamine:fructose-6-phosphate amidotransferase (gfa) gene under control of said F286 promoter, or said chimeric chitin synthase 2 gene under the control of the E6 promoter and a gfa gene under control of the E6 promoter, have been? generated using the transformation method as described in US6483013. Fibers from these transgenic cotton plants are isolated and used to produce yarns and fabrics with improved reactivity, such as improved dyeability. Fibers isolated from cotton bolls of transgenic plants have an increased amount of N-acetylglucosamine polymers which are evenly distributed throughout the cell wall.

5. Cotton fibers with increased reactivity

Transgenic cotton plants comprising a chimeric SmCHS2 coding region operably linked to a fiber-specific promoter have been generated as described in Example 4. Mature cotton fibers are harvested from these plants and can be stained with Congo Red or can

be reacted with WGA-Alexa fluor 555. In addition, the resulting mature cotton fibers can be stained with commercial dyes including cotton reactive dyes (e.g. Reactive Red 120, Levafix Blue CA), acid dyes (Acid Orange 7, Acid Blue 281) and wool reactive dyes (e.g. Reactive Red 116, Realan Amber EHF).

WGA-Alexa 555 staining

Cotton fibers do not need to be dehydrated or permeabilized. Instead, lipids and waxes are removed by treating the fibers for 3 times 10 minutes in a chloroform: methanol mixture (1:1), follow by twice a treatment of 10 minutes in acetone and twice 5 minutes in ether. The fibers are allowed to air dry.

Fibers can be stained with WGA-Alexa555, WGA-Alexa488 or WGA-tetramethylrhodamine.

The fibers are placed in blocking solution (150 mM NaCl, 10 mM sodiumphosphate buffer pH 7.4; 0.1% Tween 20 and 1% bovine serum albumin) and are incubated for one hour. Thereafter, the buffer is replaced by the same buffer containing WGA-fluorochrome and incubated for 4 hrs. The WGA-fluorochrome solution is replaced by blocking solution, washed 10 minutes, followed by 3 times 10 min washing with blocking solution without BSA, and 2 times 5 min washing with blocking solution without BSA and without Tween. The stained fibers are mounted on a microscope slide and evaluated by means of fluorescence microscopy (Axioplan 2 (Zeiss, Jena, Germany) using Filterset 38 (excitation: BP470/40; emission: BP525/50) for Alexa fluor 488 conjugate or Filterset 20 (excitation: BP546/12; emission: BP575-640) for Alexa fluor 555 or tetramethylrhodamine conjugate. Whereas no specific fluorescence can be detected in cotton fibers from non-transgenic cotton plants, a bright fluorescence is detectable in cotton fibers from transgenic cotton plants comprising a chimeric SmCHS2 gene. Virtual microscopic sections of the cotton fibers show that the WGA-fluor555 is evenly distributed throughout the secondary cell wall of the cotton fiber cells.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 75749-74 Seq 15-07-12 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

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<211> 910

<212> PRT

<213> Saprolegnia monoica

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Trp	Thr	Met	His	Tyr	Val	Lys	Asp	Ala	Ile	Ala	Arg	Thr	Asp	Val	Pro	515	520	525	
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Ala	Phe	Asn	Gly	Ile	Arg	Leu	Leu	Gly	Cys	Ile	Gly	Tyr	Leu	Leu	Tyr	835	840	845	
Tyr	Ala	Arg	Gln	Phe	Leu	Leu	Phe	Asn	Thr	Leu	Ser	Ala	Thr	Gly	Val	850	855	860	
Leu	His	Lys	Arg	His	Glu	Ala	Arg	Lys	His	Lys	Lys	Ala	Glu	Glu	Asp	865	870	875	880
Pro	Asp	Pro	Ile	Asp	Phe	Glu	Met	Gly	Thr	Phe	Gln	Asn	Asp	Leu	Pro	885	890	895	
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 <210> 17
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 <220>
 <223> CHS2FLRev

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 <210> 18
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 synthase 2 (codon optimized for expression in cotton)

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 cagagattac caccctcta tacacaagag agtctggagt ttggtgggac ttatgctact 180
 ggatctcctg tcggagctga agctgaagga tcctatagtc aagtcccggg gtggaaggat 240

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<212> PRT

<213> Artificial Sequence

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<210> 20
 <211> 69
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> MIT domain from Saprolegnia monoica chitin synthase 1 gene

<400> 20
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<210> 21
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 <212> DNA
 <213> Saprolegnia parasitica

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 35 40 45
 Ser Ser Tyr Glu Tyr Asp Tyr Glu Tyr Asn Met Met Pro Met Met Gln
 50 55 60
 Ala Pro Pro Lys Ser Gln Pro Thr Phe Leu Ser Asn Ile Ala Pro Ile
 65 70 75 80
 Ser Ala Lys Glu Ala Ser Met Lys Gly Ser Asn Ala Met Gln Leu Leu
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CLAIMS:

1. An isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide comprising an MIT domain, wherein said nucleotide sequence is at least 97% identical to the full-length sequence of SEQ ID NO: 1, or a full-length
5 complementary sequence thereof.
2. An isolated nucleic acid molecule comprising a nucleotide sequence which encodes a chitin synthase polypeptide which is at least 80% identical to the full-length sequence of SEQ ID NO: 2 and comprises an MIT domain.
3. A chimeric gene comprising the following operably linked DNA regions:
10 a) a plant-expressible promoter,
 b) a DNA region comprising the nucleic acid molecule according to claim 1 or 2, and
 c) a transcription termination and polyadenylation region.
4. The chimeric gene according to claim 3 wherein said promoter is a constitutive
15 promoter.
5. The chimeric gene according to claim 3 wherein said promoter is a fiber-selective promoter.
6. A plant cell comprising the chimeric gene as defined in any one of claims 3 to 5.
7. Fibers comprising the chimeric gene as defined in any one of claims 3 to 5.
- 20 8. A method for the manufacture of a plant cell wall comprising positively charged polysaccharides, said method comprising
 a) expressing the chimeric gene according to any one of claims 3 to 5 in a plant, and
 b) isolating said plant cell wall.

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9. The method according to claim 8 wherein said plant cell wall is a cotton plant cell wall.

10. The method according to claim 9 wherein said cotton plant cell wall is present in a cotton fiber.

Figure 1a



Figure 1b

