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**(54) Title**: METHOD FOR PRODUCING TRANSGENIC PLANTS HAVING INCREASED RESISTANCE TO PATHOGENS

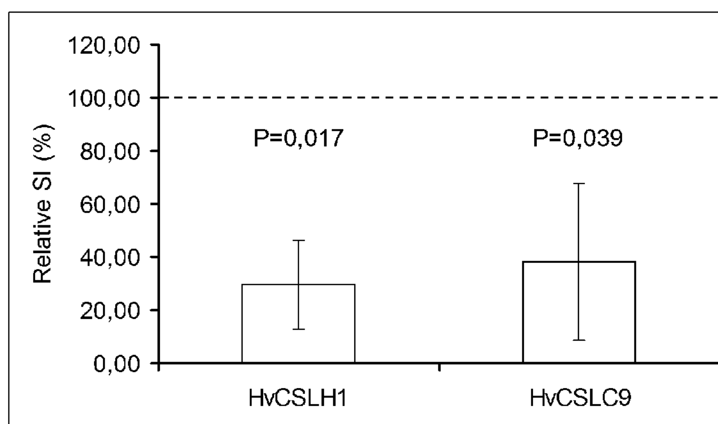


Figure 3

**(57) Abstract**: A method for producing a transgenic plant cell, a transgenic plant or a transgenic part thereof having increased resistance to pathogens is provided. The method comprises a step of reducing the content and/or activity of a cellulose synthase-like protein, preferably by RNA interference.

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METHOD FOR PRODUCING TRANSGENIC PLANTS HAVING INCREASED RESISTANCE TO PATHOGENS

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**FIELD OF THE INVENTION**

The present invention relates to a method of producing a transgenic plant cell, a transgenic plant or a transgenic part thereof having an increased resistance to pathogens, wherein the content and/or activity of a cellulose synthase-like protein is reduced, preferably by RNA interference.

**BACKGROUND OF THE INVENTION**

Plant diseases, which are caused by various pathogens such as viruses, bacteria and fungi, may lead to significant crop losses of cultivated plants, resulting in economic consequences and in threatening human food supply. For example, infestation of cereals with *Blumeria graminis*, the pathogen that causes powdery mildew, may cause yield losses of up to 30%.

Since the last century, chemical fungicides have been utilised for controlling fungal diseases. A different approach is to examine the natural pathogen defence of plants against different pathogens and to use the same specifically for the production of pathogen resistant plants by gene technological manipulation, e.g. by means of introducing external resistance genes or by means of manipulating of the endogenous gene expression of the plants.

Resistance is the ability of a plant to inhibit or at least limit any infestation or population of a pest. The plants have a certain degree of natural resistance which is imparted by the formation of specific defence substances, such as isoprenoids, flavonoids, enzymes and reactive oxygen species.

Therefore, one approach for producing pathogen resistant plants is the (over)expression of a transgene in said plants, resulting in the formation of specific defence substances. For example, chitinase (WO 92/17591) and pathogenesis-related genes (WO 92/20800) as well as genes for various oxidizing enzymes, such as glucose oxidase (WO 95/21924) and oxalate oxidase (WO 99/04013), have already been overexpressed in plants, thus creating plants having increased fungal resistance.

Conversely, it could be shown that some of the plant genes help a fungus to enter the plant. Thus, an alternative approach for producing transgenic plants having increased fungal resistance is to inhibit the expression of said plant genes which code for example for a polyphenoloxidase (WO 02/061101), NADPH oxidase (WO 2004/009820) and the Mlo gene (WO 00/01722) in transgenic plants.

Another alternative for causing resistance to pathogenic fungi is to introduce gene constructs into plants which inhibit the expression and/or activity of fungal genes that are essential for the proliferation and/or development of fungi (US 2007/0061918).

Nevertheless, there is still a need to identify further genes which code for polypeptides involved in pathogen resistance and to develop methods for producing transgenic plants with increased pathogen resistance by using these genes.

**OBJECT AND SUMMARY OF THE INVENTION**

It is thus an object of the present invention to identify genes which are involved in the pathogen resistance of plants.

It is a further object of the present invention to provide a method for producing transgenic plants with increased pathogen resistance, preferably resistance to fungal pathogens such as *Blumeria graminis*, *Septoria tritici* and/or *Puccinia triticina*.

These and further objects of the invention, as will become apparent from the description, are attained by the subject-matter of the independent claims.

Some of the preferred embodiments of the present invention form the subject-matter of the dependent claims.

The present inventors have found that the reduction of the content of cellulose synthase-like proteins by RNA interference leads to an enhanced resistance of barley cells to *Blumeria graminis*.

Together with the cellulose synthases (CesA)which catalyze the synthesis of cellulose the cellulose synthase-like (CSL) proteins which catalyze the synthesis of non-cellulosic polysaccharides make up the cellulose synthase superfamily the members of which are involved in plant cell wall synthesis. The cellulose synthase-like proteins are currently subdivided into nine families designated *CSLA* to *CSLH* and *CSLJ* (Dwivany et al. (2009) Molecular Plant 2(5): 1025-1039). Proteins of the CSLA, CSLC, CSLF and CSLH families are able to make the β-linked backbones for various non-cellulosic polysaccharides found in primary walls (Dwivany et al. (2009) Molecular Plant 2(5): 1025-1039). For example, it has been proposed that CSLC proteins are involved in the synthesis of the xyloglucan backbone which is composed

of (1,4)-β-D-glucosyl residues to which α-D-xylosyl residues and other sugars are attached (Cocuron et al. (2007) Proc. Natl. Acad. Sci. USA 104: 8550-8555). However, while a potential role of cellulose synthases in mediating pathogen resistance has been discussed (Cano-Delgado *et al.* (2003) The Plant Journal 34(3): 351-362; Hernandez-Blanco *et al.* (2007) Plant Cell 19: 890-903) it was not known that the cellulose synthase-like proteins are also involved in plant defense against pathogens.

Accordingly, the present invention provides a method of producing a transgenic plant cell, a transgenic plant or a transgenic part thereof having an increased resistance to pathogens compared to a control plant cell, plant or plant part, wherein in the transgenic plant cell, transgenic plant or transgenic part thereof the content and/or activity of at least one cellulose synthase-like protein is reduced in comparison to the control plant cell, plant or plant part.

Preferably, the cellulose synthase-like protein is encoded by a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1 to 5 or a fragment of any of these sequences;
b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5.

Preferably, the method comprises the steps of

(a) introducing into a plant cell a vector which comprises:

(i) a promoter functional in plant cells,

(ii) operatively linked thereto at least one nucleic acid sequence as defined above, preferably selected from the nucleic acid sequences according to any of SEQ ID Nos. 6, 7, 9 and 10 or a fragment of any of these sequences,

(iii) optionally a spacer sequence,

(iv) the nucleic acid sequence being reverse-complementary to the nucleic acid sequence mentioned in (ii);

(v) optionally, a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operatively linked thereto a nucleic acid sequence which is complementary to a nucleic acid as defined above, preferably selected from the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and

(iii) optionally, a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operatively linked thereto a nucleic acid sequence which is identical to a nucleic acid as defined above, preferably the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

(iii) optionally, a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operably linked thereto a nucleic acid sequence encoding a precursor micro RNA sequence comprising a micro RNA sequence which targets the nucleic acid sequence as defined above, preferably the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and

(b) optionally, regenerating a transgenic plant from the transformed cell.

In a preferred embodiment the transgenic plant is a wheat plant and the nucleic acid used in the recombinant nucleic acid molecule is selected from the sequence according to SEQ ID Nos. 7 and 10, more preferably the recombinant nucleic acid molecule comprises a sequence selected from SEQ ID Nos. 8 and 11.

In an alternative preferred embodiment the transgenic plant is a barley plant and the nucleic acid used in the recombinant nucleic acid molecule is selected from the sequences according to SEQ ID Nos. 6 and 9.

Preferably, the promoter is a tissue-specific and/or a pathogen-inducible promoter.

In another preferred embodiment, the method further comprises reducing the content and/or activity of at least one other protein which mediates pathogen susceptibility and/or increasing the content and/or activity of at least one protein which mediates pathogen resistance.

In another embodiment the method further comprises the step of crossing the transgenic plant produced by the above method with another plant in which the content and/or the activity of the cellulose synthase-like protein as defined herein is not reduced and selecting transgenic progeny in which the content and/or the activity of the cellulose synthase-like protein as defined herein is reduced.

In a preferred embodiment the method is for producing true breeding plants and comprises inbreeding the transgenic progeny of the above crossing and repeating this inbreeding step until a true breeding plant is obtained.

In another embodiment the present invention relates to a method of producing mutant plants, plant cells or plant parts having an increased resistance to pathogens compared to control plants, plant cells or plant parts, comprising the steps of:
(a) mutagenizing plant material;
(b) identifying plant material having at least one point mutation in a nucleic acid sequence comprising the nucleic acid sequence according to any of SEQ ID Nos.1-5.

Preferably, the transgenic or mutant plant has an increased resistance to a fungal pathogen, more preferably to *Blumeria graminis*, *Septoria tritici* and/or *Puccinia triticina*.

Also preferably, the transgenic or mutant plant is a monocotyledonous plant. More preferably it is a wheat or barley plant.

In another embodiment the present invention relates to an expression construct comprising at least one nucleic acid sequence selected from the group consisting of:
(a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
(d) a nucleic acid sequence which is reverse-complementary to a nucleic acid sequence of any of (a) to (c),
operatively linked to a promoter functional in plant cells.

In a preferred embodiment the expression construct comprises:
(a) a promoter functional in plant cells,
(b) operatively linked thereto at least one nucleic acid sequence as defined above, preferably at least one nucleic acid sequence selected from the group consisting of SEQ ID Nos. 6, 7, 9 and 10 and fragments of these sequences;
(c) optionally a spacer sequence,
(d) the nucleic acid sequence being reverse-complementary to the nucleic acid sequence mentioned in b); and
(e) optionally, a termination sequence;

or:
(a) a promoter functional in plant cells;
(b) operatively linked thereto at least one nucleic acid sequence which is complementary to a nucleic acid sequence as defined above, preferably the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
(c) optionally, a termination sequence;
or:
(a) a promoter functional in plant cells;
(b) operatively linked thereto at least one nucleic acid sequence which is identical to a nucleic acid sequence as defined above, preferably the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(c) optionally, a termination sequence;
or:
(a) a promoter functional in plant cells;
(b) operably linked thereto nucleic acid sequence encoding a precursor micro RNA sequence comprising a micro RNA sequence which targets the nucleic acid sequence as defined above, preferably the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences.

In another embodiment the invention relates to a vector comprising an expression construct as defined above.

In another embodiment the invention relates to a transgenic plant or plant cell with an increased resistance to pathogens, produced by the method of the present invention or containing an expression construct or a vector of the present invention.

In another embodiment the invention relates to the use of the transgenic or mutant plant or parts thereof as feed or as fodder material.

The present invention also relates to transgenic or mutant seed produced from the transgenic or mutant plant and to flour produced from said transgenic or mutant seed, wherein the presence of the transgene or the mutation which reduces the content and/or the activity of a cellulose synthase-like protein as defined herein can be detected in said flour.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1: Flow diagram for the high-throughput production of RNAi constructs.
I, PCR amplification of cDNA fragments of interest;
IIa, Ligation of the PCR fragments in the intermediate vector pIPKTA38 in the presence of the restriction endonuclease, *Swa* I, which inhibits the re-ligation of the vector;
IIb, Re-cutting all re-ligated vector molecules;
III, Recombination of the cloned cDNA fragments in the RNAi vector pIPKTA30 by means of LR clonase.

Figure 2: Flow diagram showing how the effect of the RNAi constructs on plant resistance to *Blumeria graminis* is tested.

Figure 3: Effect of the RNAi constructs on barley resistance to *Blumeria graminis* f.sp. *hordei*.
The results represent the mean of five independent experiments as shown in Table 10. SI: susceptibility index

Figure 4: Plasmid map of vector TaCSLH1 RNAi

Figure 5: Plasmid map of vector TaCSLC9 RNAi

## DETAILED DESCRIPTION OF THE INVENTION

The present invention as illustratively described in the following may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein.

The present invention will be described with respect to particular embodiments but the invention is not limited thereto but only by the claims.

Where the term “comprising” is used in the present description and claims, it does not exclude other elements. For the purposes of the present invention, the term “consisting of” is considered to be a preferred embodiment of the term “comprising". If hereinafter a group is defined to comprise at least a certain number of embodiments, this is also to be understood to disclose a group which preferably consists only of these embodiments.

For the purposes of the present invention, the term “obtained” is considered to be a preferred embodiment of the term “obtainable”. If hereinafter e.g. a plant is defined to be obtainable by a specific method, this is also to be understood to disclose a plant which is obtained by this method.

Where an indefinite or definite article is used when referring to a singular noun, e.g. “a”, “an” or “the”, this includes a plural of that noun unless something else is specifically stated.

The term "transgenic" means that a plant cell, plant or plant part has been altered using recombinant DNA technology to contain a nucleic acid sequence which would otherwise not be present in said plant cell, plant, or plant part or which would be expressed to a considerably lower extent. Within the present invention, the transgenic

plant cell, plant or plant part contains a nucleic acid sequence selected from the group consisting of

(a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

(b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

(c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and

(d) a nucleic acid sequence which is reverse-complementary to a nucleic acid sequence of any of (a) to (c),

which is not present at the natural locus of this sequence in the genome of the control plant and/or which has been linked to sequences to which the nucleic acid sequence is not linked in the genome of the control plant and/or which is present in another 5' to 3' orientation compared to the orientation of this sequence in the natural locus of the control plant. Natural locus means the location on a specific chromosome, preferably the location between certain genes, more preferably the same sequence background as in the original plant which is transformed.

Preferably, the nucleic acid sequence is introduced by means of a vector. Also preferably, the nucleic acid sequence is stably integrated into the genome of the transgenic plant. In particular, the transgenic plant cell, plant or plant part of the present invention contains a nucleic acid sequence which reduces the content and/or activity of a cellulose synthase-like protein compared to a control plant cell, plant or plant part. In addition to the nucleic acid sequence which reduces the content and/or activity of a cellulose synthase-like protein, the transgenic plant cell, plant or plant part may contain one or more other transgenic nucleic acid sequences, for example nucleic acid sequences conferring resistance to biotic or abiotic stress and/or altering the chemical composition of the transgenic plant cell, plant or plant part. The term

"transgenic" does not refer to plants having alterations in the genome which are the result of naturally occurring events, such as spontaneous mutations or of induced mutagenesis followed by breeding and selection.

The term "mutant" means that a plant cell, plant or plant part has been altered by mutagenesis so that a nucleic acid sequence selected from the group consisting of

(a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

(b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

(c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;

contains at least one point mutation, i.e. at least one nucleotide substitution, deletion and/or addition, in comparison to a control plant, plant cell or part thereof which has been used as a starting material in the mutagenesis and which has not been mutagenized. Preferably, the mutant plant contains at least one nucleotide substitution in the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences.

The transgenic or mutant plant of the present invention may be a monocotyledonous or a dicotyledonous plant.

Examples of monocotyledonous plants are plants belonging to the genera Avena (oat), Triticum (wheat), Secale (rye), Hordeum (barley), Oryza (rice), Panicum, Pennisetum, Setaria, Sorghum (millet), Zea (maize), and the like.

Dicotyledonous useful plants comprise, *inter alia*, cotton, legumes, like leguminous plants and in particular alfalfa, soy bean, rape, tomato, sugar beet, potato, ornamental

plants, and trees. Further useful plants can comprise fruit (in particular apples, pears, cherries, grapes, citrus, pineapple, and bananas), pumpkin, cucumber, wine, oil palms, tea shrubs, cacao trees, and coffee shrubs, tobacco, sisal, as well as, with medicinal plants, rauwolfia and digitalis.

Particularly preferred are the cereals wheat, rye, oat, barley, rice, maize and millet, sugar beet, rape, soy, tomato, potato, cotton and tobacco. Further useful plants can be taken from US 6,137,030.

More preferably the transgenic or mutant plants are oat, barley, rye, wheat or rice plants and most preferably the transgenic or mutant plants are barley or wheat plants.

Within the meaning of the present invention the term "transgenic plant" includes the transgenic progeny of the transgenic plant and the term "mutant plant" also includes the mutant progeny of the mutant plant. The transgenic or mutant progeny of the transgenic or mutant plant may be the result of a cross of the transgenic or mutant plant with another transgenic or mutant plant of the present invention, respectively, or it may be the result of a cross with a wild-type plant or a transgenic plant having a transgene other than the transgene of the present invention. In particular, the term "transgenic plant" also comprises true breeding transgenic plants which are obtained by repeated inbreeding steps as described below.

Plant parts include, but are not limited to, stems, roots, ovules, stamens, leaves, embryos, meristematic regions, callus tissue, gametophytes, sporophytes, pollen, microspores, and the like.

The term "cell" or "plant cell" as used herein refers to a single cell, and also includes a population of cells. The population may be a pure population comprising one cell type. Likewise, the population may comprise more than one cell type. A plant cell

within the meaning of the invention may be isolated (e.g., in suspension culture) or comprised in a plant tissue, plant organ or plant at any developmental stage

According to the present invention, “pathogen resistance” means reducing or attenuating disease symptoms of a plant as a result of attack by a pathogen, preferably by a fungus. While said symptoms can be manifold, they preferably comprise such symptoms directly or indirectly leading to impairment of plant quality, yield quantity, or suitability for use as feed or food, or impeding sowing, cultivation, harvest, or processing of the crop. Furthermore, "resistance" also means that pests and/or a pathogen and preferably a fungus and especially preferably the fungi described below display reduced growth in a plant and reduced or absent propagation. The term "resistance" also includes a so-called transient resistance, i.e. the transgenic or mutant plants or plant cells of the present invention have an increased resistance to pests and/or pathogens or fungi, compared to the corresponding control plants, only for a limited period of time.

According to the present invention, the term “increased pathogen resistance” is understood to denote that the transgenic or mutant plants or plant cells of the present invention are infected less severely and/or less frequently by plant pathogens.

In one embodiment the reduced frequency and the reduced extent of pathogen infection, respectively, on the transgenic plants or plant cells according to the present invention is determined as compared to the corresponding control plant. According to the present invention, an increase in resistance means that an infection of the plant by the pathogen occurs less frequently or less severely by at least 5%, preferably by at least 20%, also preferably by at least 50%, 60% or 70%, especially preferably by at least 80%, 90% or 100%, also especially preferably by the factor 5, particularly preferably by at least the factor 10, also particularly preferably by at least the factor 50, and more preferably by at least the factor 100, and most preferably by at least the factor 1000, as compared to the control plant.

Alternatively, the pathogen resistance may be described by reference to a relative susceptibility index (SI) which compares the susceptibility of a plant of the present invention to a pathogen with the susceptibility of a control plant to said pathogen. The susceptibility of the control plant is set to 100%. The relative susceptibility index of the plants of the present invention is less than 80%, preferably less than 70 or 60%, more preferably less than 50% and most preferably less than 40%.

When used in connection with transgenic plants, the term "control plant", "control plant cell" or "control plant part" refers to a plant cell, an explant, seed, plant component, plant tissue, plant organ, or whole plant used to compare against a transgenic plant, plant cell or plant part which has been modified by the method of the present invention for the purpose of identifying an enhanced phenotype or a desirable trait in the transgenic plant. A "control plant" may in some cases be a transgenic plant line that comprises an empty vector or marker gene, but does not contain the recombinant polynucleotide of interest that is present in the transgenic plant being evaluated, i.e. the nucleic acid sequence reducing the content and/or the activity of the cellulose synthase-like protein. A control plant may be a plant of the same line or variety as the transgenic plant being tested, or it may be another line or variety, such as a plant known to have a specific phenotype, characteristic, or known genotype. Another suitable control plant is a genetically unaltered or non-transgenic plant of the parental line used to generate the transgenic plant of the present invention, i.e. the wild-type plant.

When used in connection with mutant plants, the terms "control plant", "control plant cell" or "control plant part" refers to a plant cell, an explant, seed, plant component, plant tissue, plant organ, or whole plant which has been used as starting material for the mutagenization and which does not contain the at least one point mutation of the mutant plant, plant cell or plant part.

The infection of test plants with pathogens such as fungi in order to examine potential resistance phenomena is a method well-known to those skilled in the art. The test plants used must be responsive to the pathogen used, i.e. they must be able to serve as a host plant for said pathogen, and the pathogen attack must be detectable by simple means. Preferred test plants are wheat or barley plants, which are, for example, inoculated with the powdery mildew fungus *Blumeria graminis*. "Inoculating" denotes contacting the plant with the fungus the plant is to be infected with, or with infectious parts thereof, under conditions in which the fungus may enter a wild type plant.

The fungal infestation of the plant may then be evaluated by means of a suitable evaluation procedure. The visual inspection, in which the formed fungal structures are detected in the plant and quantified, is particularly suitable. In order to identify successfully transformed cells in transient experiments, a reporter gene, such as the beta-glucuronidase (GUS) gene from *E. coli*, a fluorescence gene, such as the green fluorescence protein (GFP) gene from *Aequorea victoria*, the luciferase gene from *Photinus pyralis* or the beta-galactosidase (lacZ) gene from *E. coli*, the expression of which in the plant cells may be proven by simple methods, is co-transformed in a suitable vector with the vector mediating the inhibition of the expression of the cellulose synthase-like protein. Optionally, the formed fungal structures may be stained by methods well-known to those skilled in the art in order to improve the determination thereof, e.g. by staining with coomassie or trypan blue. Then, the number of infected plants transformed with the nucleic acid molecule to be tested is compared to the number of infected wild-type or control plants and the degree of pathogen resistance is calculated. Alternatively, fungal resistance may be scored by determining the symptoms of fungal infection on the infected plant, for example by eye, and calculating the diseased leaf area, The diseased leaf area is the percentage of the leaf area showing symptoms of fungal infection, such as fungal pycnidia or fungal colonies. The diseased leaf area of infected plants transformed with the vector mediating the inhibition of the expression of the cellulose synthase-like protein or of

the mutant plants with at least one point mutation within the nucleic acid sequence coding for the cellulose synthase-like protein is lower than the diseased leaf area of infected control plants.

According to the present invention, the term “plant pathogens” includes viral, bacterial, fungal and other pathogens. Preferably, the term “plant pathogens” comprises fungal pathogens.

According to the present invention, the term “plant pathogens” includes biotrophic, hemibiotrophic and necrotrophic pathogens. Preferably, the plant pathogen is a biotrophic pathogen, more preferably a biotrophic fungal pathogen.

The biotrophic phytopathogenic fungi, such as many rusts, depend for their nutrition on the metabolism of living cells of the plants. This type of fungi belong to the group of biotrophic fungi, like other rust fungi, powdery mildew fungi or oomycete pathogens like the genus Phytophthora or Peronopora. The necrotrophic phytopathogenic fungi depend for their nutrition on dead cells of the plants, e.g. species from the genus Fusarium, Rhizoctonia or Mycospaerella. Soybean rust has occupied an intermediate position, since it penetrates the epidermis directly, whereupon the penetrated cell becomes necrotic. After the penetration, the fungus changes over to an obligatory-biotrophic lifestyle. The subgroup of the biotrophic fungal pathogens which follows essentially such an infection strategy is hemibiotrophic.

Table 1: Diseases caused by biotrophic phytopathogenic fungi

| Disease | Pathogen |
|---|---|
| Black rust | *Puccinia triticina* |
| Leaf rust | *Puccinia recondita* |
| Yellow rust | *Puccinia striiformis* |
| Powdery mildew | *Erysiphe graminis / Blumeria graminis* |
| Rust (common corn) | *Puccinia sorghi* |
| Rust (Southern corn) | *Puccinia polysora* |
| Tobacco leaf spot | *Cercospora nicotianae* |
| Rust (soybean) | *Phakopsora pachyrhizi, P. meibomiae* |
| Rust (tropical corn) | *Physopella pallescens, P. zeae = Angiopsora zeae* |

Table 2: Diseases caused by necrotrophic and/or hemibiotrophic fungi and Oomycetes

| Disease | Pathogen |
|---|---|
| Plume blotch | *Septoria (Stagonospora) nodorum* |
| Leaf blotch | *Septoria tritici* |
| Ear fusarioses | *Fusarium spp.* |
| Eyespot | *Pseudocercosporella herpotrichoides* |
| Smut | *Ustilago spp.* |
| Late blight | *Phytophthora infestans* |
| Bunt | *Tilletia caries* |
| Take-all | *Gaeumannomyces graminis* |
| Anthrocnose leaf blight<br><br>Anthracnose stalk rot | *Colletotrichum graminicola (teleomorph: Glomerella graminicola Politis); Glomerella tucumanensis (anamorph: Glomerella falcatum Went)* |
| Aspergillus ear and kernel rot | *Aspergillus flavus* |

| | |
|---|---|
| Banded leaf and sheath spot ("Wurzeltöter") | *Rhizoctonia solani Kuhn = Rhizoctonia microsclerotia J. Matz (telomorph: Thanatephorus cucumeris)* |
| Black bundle disease | *Acremonium strictum W. Gams = alosporium acremonium Auct. non Corda* |
| Black kernel rot | *Lasiodiplodia theobromae = Botryodiplodia theobromae* |
| Borde blanco | *Marasmiellus sp.* |
| Brown spot (black spot, stalk rot) | *Physoderma maydis* |
| Cephalosporium kernel rot | *Acremonium strictum = Cephalosporium acremonium* |
| Charcoal rot | *Macrophomina phaseolina* |
| Corticium ear rot | *Thanatephorus cucumeris = Corticium sasakii* |
| Curvularia leaf spot | *Curvularia clavata, C. eragrostidis, = C. maculans (teleomorph: Cochliobolus eragrostidis), Curvularia inaequalis, C. intermedia (teleomorph: Cochliobolus intermedius), Curvularia lunata (teleomorph: Cochliobolus lunatus), Curvularia pallescens (teleomorph: Cochliobolus pallescens), Curvularia senegalensis, C. tuberculata (teleomorph: Cochliobolus tuberculatus)* |
| Didymella leaf spot | *Didymella exitalis* |
| Diplodia ear and stalk rot | *Diplodia frumenti (teleomorph: Botryosphaeria festucae)* |
| Diplodia ear and stalk rot, seed rot and seedling blight | *Diplodia maydis = Stenocarpella maydis* |

| | |
|---|---|
| Diplodia leaf spot or streak | *Stenocarpella macrospora = Diplodia macrospora* |
| Brown stripe downy mildew | *Sclerophthora rayssiae var. zeae* |
| Crazy top downy mildew | *Sclerophthora macrospora = Sclerospora macrospora* |
| Green ear downy mildew (graminicola downy mildew) | *Sclerospora graminicola* |
| Dry ear rot (cob, kernel and stalk rot) | *Nigrospora oryzae (teleomorph: Khuskia oryzae)* |
| Ear rots (minor) | *Alternaria alternata = A. tenuis, Aspergillus glaucus, A. niger, Aspergillus spp., Botrytis cinerea (teleomorph: Botryotinia fuckeliana), Cunninghamella sp., Curvularia pallescens, Doratomyces stemonitis = Cephalotrichum stemonitis, Fusarium culmorum, Gonatobotrys simplex, Pithomyces maydicus, Rhizopus microsporus Tiegh., R. stolonifer = R. nigricans, Scopulariopsis brumptii* |
| Ergot (horse's tooth) | *Claviceps gigantea (anamorph: Sphacelia sp.)* |
| Eyespot | *Aureobasidium zeae = Kabatiella zeae* |
| Fusarium ear and stalk rot | *Fusarium subglutinans = F. moniliforme var.subglutinans* |
| Fusarium kernel, root and stalk rot, | *Fusarium moniliforme* |

| | |
|---|---|
| seed rot and seedling blight | *(teleomorph: Gibberella fujikuroi)* |
| Fusarium stalk rot,<br>seedling root rot | *Fusarium avenaceum*<br>*(teleomorph: Gibberella avenacea)* |
| Gibberella ear and stalk rot | *Gibberella zeae*<br>*(anamorph: Fusarium graminearum)* |
| Gray ear rot | *Botryosphaeria zeae = Physalospora zeae*<br>*(anamorph: Macrophoma zeae)* |
| Gray leaf spot<br>(Cercospora leaf spot) | *Cercospora sorghi = C. sorghi var. maydis, C. zeae-maydis* |
| Helminthosporium root rot | *Exserohilum pedicellatum = Helminthosporium pedicellatum (teleomorph: Setosphaeria pedicellata)* |
| Hormodendrum ear rot<br>(Cladosporium rot) | *Cladosporium cladosporioides = Hormodendrum cladosporioides, C. herbarum (teleomorph: Mycosphaerella tassiana)* |
| Leaf spots, minor | *Alternaria alternata,*<br>*Ascochyta maydis, A. tritici,*<br>*A. zeicola, Bipolaris victoriae = Helminthosporium victoriae*<br>*(teleomorph: Cochliobolus victoriae), C. sativus (anamorph: Bipolaris sorokiniana = H. sorokinianum = H. sativum), Epicoccum nigrum,*<br>*Exserohilum prolatum = Drechslera prolata (teleomorph: Setosphaeria prolata)*<br>*Graphium penicillioides,*<br>*Leptosphaeria maydis, Leptothyrium zeae, Ophiosphaerella herpotricha, (anamorph: Scolecosporiella sp.),*<br>*Paraphaeosphaeria michotii, Phoma sp.,* |

| | |
|---|---|
| | *Septoria zeae, S. zeicola, S. zeina* |
| Northern corn leaf blight (white blast, crown stalk rot, stripe) | *Setosphaeria turcica (anamorph: Exserohilum turcicum = Helminthosporium turcicum)* |
| Northern corn leaf spot Helminthosporium ear rot (race 1) | *Cochliobolus carbonum (anamorph: Bipolaris zeicola = Helminthosporium carbonum)* |
| Penicillium ear rot (blue eye, blue mold) | *Penicillium spp., P. chrysogenum, P. expansum, P. oxalicum* |
| Phaeocytostroma stalk and root rot | *Phaeocytostroma ambiguum, = Phaeocytosporella zeae* |
| Phaeosphaeria leaf spot | *Phaeosphaeria maydis = Sphaerulina maydis* |
| Physalospora ear rot (Botryosphaeria ear rot) | *Botryosphaeria festucae = Physalospora zeicola (anamorph: Diplodia frumenti)* |
| Purple leaf sheath | *Hemiparasitic bacteria and fungi* |
| Pyrenochaeta stalk and root rot | *Phoma terrestris = Pyrenochaeta terrestris* |
| Pythium root rot | *Pythium spp., P. arrhenomanes, P. graminicola* |
| Pythium stalk rot | *Pythium aphanidermatum = P. butleri L.* |
| Red kernel disease (ear mold, leaf and seed rot) | *Epicoccum nigrum* |
| Rhizoctonia ear rot (sclerotial rot) | *Rhizoctonia zeae (teleomorph: Waitea circinata)* |
| Rhizoctonia root and stalk rot | *Rhizoctonia solani, Rhizoctonia zeae* |
| Root rots (minor) | *Alternaria alternata, Cercospora sorghi, Dictochaeta fertilis, Fusarium acuminatum (teleomorph: Gibberella acuminata), F. equiseti (teleomorph: G. intricans), F. oxysporum, F. pallidoroseum, F. poae, F.* |

| | |
|---|---|
| | *roseum, G. cyanogena, (anamorph: F. sulphureum), Microdochium bolleyi, Mucor sp., Periconia circinata, Phytophthora cactorum, P. drechsleri, P. nicotianae var. parasitica, Rhizopus arrhizus* |
| Rostratum leaf spot (Helminthosporium leaf disease, ear and stalk rot) | *Setosphaeria rostrata, (anamorph: xserohilum rostratum = Helminthosporium rostratum)* |
| Java downy mildew | *Peronosclerospora maydis = Sclerospora maydis* |
| Philippine downy mildew | *Peronosclerospora philippinensis = Sclerospora philippinensis* |
| Sorghum downy mildew | *Peronosclerospora sorghi = Sclerospora sorghi* |
| Spontaneum downy mildew | *Peronosclerospora spontanea = Sclerospora spontanea* |
| Sugarcane downy mildew | *Peronosclerospora sacchari = Sclerospora sacchari* |
| Sclerotium ear rot (southern blight) | *Sclerotium rolfsii Sacc. (teleomorph: Athelia rolfsii)* |
| Seed rot-seedling blight | *Bipolaris sorokiniana, B. zeicola = Helminthosporium carbonum, Diplodia maydis, Exserohilum pedicillatum, Exserohilum turcicum = Helminthosporium turcicum, Fusarium avenaceum, F. culmorum, F. moniliforme, Gibberella zeae (anamorph: F. graminearum), Macrophomina phaseolina, Penicillium spp., Phomopsis sp., Pythium spp., Rhizoctonia solani, R. zeae, Sclerotium rolfsii, Spicaria sp.* |

| | |
|---|---|
| Selenophoma leaf spot | *Selenophoma sp.* |
| Sheath rot | *Gaeumannomyces graminis* |
| Shuck rot | *Myrothecium gramineum* |
| Silage mold | *Monascus purpureus, M ruber* |
| Smut, common | *Ustilago zeae = U. maydis* |
| Smut, false | *Ustilaginoidea virens* |
| Smut, head | *Sphacelotheca reiliana = Sporisorium holcisorghi* |
| Southern corn leaf blight and stalk rot | *Cochliobolus heterostrophus (anamorph: Bipolaris maydis = Helminthosporium maydis)* |
| Southern leaf spot | *Stenocarpella macrospora = Diplodia macrospora* |
| Stalk rots (minor) | *Cercospora sorghi, Fusarium episphaeria, F. merismoides, F. oxysporum Schlechtend, F. poae, F. roseum, F. solani (teleomorph: Nectria haematococca), F. tricinctum, Mariannaea elegans, Mucor sp., Rhopographus zeae, Spicaria sp.* |
| Storage rots | *Aspergillus spp., Penicillium spp. und weitere Pilze* |
| Tar spot | *Phyllachora maydis* |
| Trichoderma ear rot and root rot | *Trichoderma viride = T. lignorum teleomorph: Hypocrea sp.* |
| White ear rot, root and stalk rot | *Stenocarpella maydis = Diplodia zeae* |

| | |
|---|---|
| Yellow leaf blight | *Ascochyta ischaemi, Phyllosticta maydis (teleomorph: Mycosphaerella zeae-maydis)* |
| Zonate leaf spot | *Gloeocercospora sorghi* |

Preferably, fungal pathogens or fungal-like pathogens (like for example *Chromista*) are from the group comprising Plasmodiophoramycetes, Oomycetes, Ascomycetes, Chytridiomycetes, Zygomycetes, Basidiomycetes, and Deuteromycetes (Fungi imperfecti). The fungal pathogens listed in Tables 1 and 2 as well as the diseases associated therewith are to be mentioned in an exemplary, yet not limiting manner.

Particularly preferred are:

- Plasmodiophoromycetes like *Plasmodiophora brassicae* (clubroot of crucifers), *Spongospora subterranea* (powdery scab of potato tubers), *Polymyxa graminis* (root disease of cereals and grasses),

- Oomycetes like *Bremia lactucae* (downy mildew of lettuce), *Peronospora* (downy mildew) of snapdragon (*P. antirrhini*), onion (*P. destructor*), spinach (*P. effusa*), soy bean (*P. manchurica*), tobacco ("blue mold" , *P. tabacina*) alfalfa and clover (*P. trifolium*), *Pseudoperonospora humuli* (downy mildew of hop), *Plasmopara* (downy mildew) of grapes (*P. viticola*) and sun flower (*P. halstedii*), *Sclerophthora macrospora* (downy mildew of cereals and grasses), *Pythium* (seed rot, seedling damping-off, and root rot and all types of plants, for example black root disease of beet caused by *P. debaryanum*), *Phytophthora infestans* (potato light blight, tomato late blight, etc.), *Albugo spec.* (white rust on cruciferous plants)

- Ascomycetes like *Microdochium nivale* (snow mold of rye and wheat), *Fusarium graminearum*, *Fusarium culmorum* (head blight, in particular of wheat), *Fusarium oxysporum* (fusarium wilt of tomato), *Blumeria graminis*

(powdery mildew of barley (f. sp. hordei) and wheat (f. sp. tritici)), *Erysiphe pisi* (pea mildew), *Nectria galligena* (Nectria canker of fruit trees), *Unicnula necator* (grapevine powdery mildew), *Pseudopeziza tracheiphila* (grapevine red fire disease), *Claviceps purpurea* (ergot on, for example, rye and grasses), *Gaeumannomyces graminis* (black leg disease of wheat, rye and, *inter alia*, grasses), *Magnaporthe grisea* (rice blast disease), *Pyrenophora graminea* (leaf stripe disease of barley), *Pyrenophora teres* (net blotch disease of barley), *Pyrenophora tritici-repentis* (tan spot disease) *Septoria tritici* (leaf spot of wheat), *Venturia inaequalis* (apple scab disease), *Sclerotinia sclerotium* (white mold, stem canker of rape), *Pseudopeziza medicaginis* (leaf spot diseases of lucerne, white and red clover).

- Basidiomycetes like *Typhula incarnata* (typhula snow mold of barley, rye, and wheat), *Ustilago maydis* (corn smut), *Ustilago nuda* (loose smut of barley), *Ustilago tritici* (loose smut of wheat and spelt), *Ustilago avenae* (loose smut of oat), *Rhizoctonia solani* (taproot lesions of potatoes), *Sphacelotheca spp.* (head smut of sorghum), *Melampsora lini* (rust of flax), *Puccinia graminis* (stem rust of wheat, barley, rye, oat), *Puccinia recondita* (brown rust of wheat), *Puccinia triticina* (wheat leaf rust), *Puccinia dispersa* (brown rust of rye), *Puccinia hordei* (brown rust of barley), *Puccinia coronata* (crown rust of oat), *Puccinia striiformis* (yellow rust of wheat, barley, rye, and various grasses), *Uromyces appendiculatus* (bean rust), *Phakopsora pachyrhizi* (Asian soybean rust), *Sclerotium rolfsii* (root and stem rots of many plants).

- Deuteromycetes (Fungi imperfecti) like *Septoria nodorum* (glume blotch) of wheat (*Septoria tritici*), *Pseudocercosporella herpotrichoides* (stem break disease in wheat, barley, rye), *Rynchosporium secalis* (scald disease in rye and barley), *Alternaria solani* (early blight of potato and tomato), *Phoma betae* (black rot of beet), *Cercospora beticola* (Cercospora leaf spot of beet),

*Alternaria brassicae* (dark leaf spot of rape, cabbage and other cruciferous plants), *Verticillium dahliae* (Verticillium wilt and stalk rot of rape), *Colletotrichum lindemuthianum* (bean anthracnose), *Phoma lingam – phoma* stem canker (black leg disease of cabbage; crown and stem canker of rape), *Botrytis cinerea* (gray mold diseases of grapevine, strawberry, tomato, hop, etc.).

Likewise preferred are: *Phytophthora infestans* (late blight of tomato, root and foot rot of tomato, etc.), *Microdochium nivale* (formerly Fusarium nivale; snow mold of rye and wheat), *Fusarium graminearum*, *Fusarium culmorum* (head blight of wheat), *Fusarium oxysporum* (Fusarium wilt of tomato), *Blumeria graminis* (powdery mildew of barley (f. sp. hordei) and wheat (f. sp. tritici)), *Puccinia triticina* (wheat leaf rust), *Magnaporthe grisea* (rice blast disease), *Sclerotinia sclerotium* (white mold, stem canker of rape), *Septoria nodorum* and *Septoria tritici* (glume blotch of wheat), *Alternaria brassicae* (dark leaf spot of rape, cabbage and other cruciferous plants), *Phakopsora pachyrhizi* (Asian soybean rust), *Phoma lingam* (phoma stem canker, black leg disease of cabbage; crown and stem canker of rape).

The pathogens listed in Table 3 as well as the diseases associated therewith are to be mentioned as bacterial pathogens in an exemplary, yet not limiting manner.

Table 3: Bacterial diseases

| **Disease** | **Pathogen** |
|---|---|
| Bacterial leaf blight and stalk rot | *Pseudomonas avenae subsp. avenae* |
| Bacterial leaf spot | *Xanthomonas campestris* pv. holcicola |
| Bacterial stalk rot | *Enterobacter dissolvens* = *Erwinia dissolvens* |
| Bacterial stalk and top rot | *Erwinia carotovora* subsp. carotovora, *Erwinia chrysanthemi* pv. zeae |
| Bacterial stripe | *Pseudomonas andropogonis* |
| Chocolate spot | *Pseudomonas syringae* pv. coronafaciens |
| Goss's bacterial wilt and blight (leaf freckles and wilt) | *Clavibacter michiganensis* subsp. nebraskensis = *Corynebacterium michiganense* pv.andnebraskense |
| Holcus spot | *Pseudomonas syringae* pv. syringae |
| Purple leaf sheath | Hemiparasitic bacteria |
| Seed rot-seedling blight | *Bacillus subtilis* |
| Stewart's disease (bacterial wilt) | *Pantoea stewartii* = *Erwinia stewartii* |
| Corn stunt (achapparramiento, maize stunt, Mesa Central or Rio Grande maize stunt) | *Spiroplasma kunkelii* |

Particularly preferably, the transgenic plants produced according to the present invention are resistant to the following pathogenic bacteria:

*Corynebacterium sepedonicum* (bacterial ring rot of potato), *Erwinia carotovora* (black leg rot of potato), *Erwinia amylovora* (fire blight of pear, apple, quince), *Streptomyces scabies* (common scab of potato), *Pseudomonas syringae* pv. tabaci (wild fire disease of tobacco), *Pseudomonas syringae* pv. phaseolicola (halo blight disease of dwarf bean), *Pseudomonas syringae* pv. tomato ("bacterial speck" of tomato), *Xanthomonas campestris* pv. malvacearum (angular leaf spot of cotton), and *Xanthomonas campestris* pv. oryzae (bacterial blight of rice and other grasses).

The term "viral pathogens" includes all plant viruses, like for example tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, maize dwarf mosaic virus, etc.

The pathogens listed in Table 4 as well as the diseases associated therewith are to be mentioned as viral pathogens in an exemplary, yet not limiting manner.

Table 4: Viral diseases

| **Disease** | **Pathogen** |
|---|---|
| American wheat striate (wheat striate mosaic) | American wheat striate mosaic virus (AWSMV) |
| Barley stripe mosaic | Barley stripe mosaic virus (BSMV) |
| Barley yellow dwarf | Barley yellow dwarf virus (BYDV) |
| Brome mosaic | Brome mosaic virus (BMV) |
| Cereal chlorotic mottle | Cereal chlorotic mottle virus (CCMV) |
| Corn chlorotic vein banding (Braizilian maize mosaic) | Corn chlorotic vein banding virus (CCVBV) |
| Corn lethal necrosis | Virus complex from Maize chlorotic mottle virus (MCMV) and Maize dwarf mosaic virus (MDMV) |

| | |
|---|---|
| | A or B or Wheat streak mosaic virus(WSMV) |
| Cucumber mosaic | Cucumber mosaic virus (CMV) |
| Cynodon chlorotic streak | Cynodon chlorotic streak virus (CCSV) |
| Johnsongrass mosaic | Johnsongrass mosaic virus (JGMV) |
| Maize bushy stunt | Mycoplasma-like organism (MLO) associated |
| Maize chlorotic dwarf | Maize chlorotic dwarf virus (MCDV) |
| Maize chlorotic mottle | Maize chlorotic mottle virus (MCMV) |
| Maize dwarf mosaic | Maize dwarf mosaic virus (MDMV) strains A, D, E and F |
| Maize leaf fleck | Maize leaf fleck virus (MLFV) |
| Maize line | Maize line virus (MLV) |
| Maize mosaic (corn leaf stripe, enanismo rayado) | Maize mosaic virus (MMV) |
| Maize mottle and chlorotic stunt | Maize mottle and chlorotic stunt virus |
| Maize pellucid ringspot | Maize pellucid ringspot virus (MPRV) |
| Maize raya gruesa | Maize raya gruesa virus (MRGV) |
| maize rayado fino (fine striping disease) | Maize rayado fino virus (MRFV) |
| Maize red leaf and red stripe | Mollicute |
| Maize red stripe | Maize red stripe virus (MRSV) |
| Maize ring mottle | Maize ring mottle virus (MRMV) |
| Maize rio IV | Maize rio cuarto virus (MRCV) |
| Maize rough dwarf (nanismo ruvido) | Maize rough dwarf virus (MRDV) (Cereal tillering disease virus) |
| Maize sterile stunt | Maize sterile stunt virus |

| | |
|---|---|
| | (strains of barley yellow striate virus) |
| Maize streak | Maize streak virus (MSV) |
| Maize stripe (maize chlorotic stripe, maize hoja blanca) | Maize stripe virus |
| Maize stunting | Maize stunting virus |
| Maize tassel abortion | Maize tassel abortion virus (MTAV) |
| Maize vein enation | Maize vein enation virus (MVEV) |
| Maize wallaby ear | Maize wallaby ear virus (MWEV) |
| Maize white leaf | Maize white leaf virus |
| Maize white line mosaic | Maize white line mosaic virus (MWLMV) |
| Millet red leaf | Millet red leaf virus (MRLV) |
| Northern cereal mosaic | Northern cereal mosaic virus (NCMV) |
| Oat pseudorosette (zakuklivanie) | Oat pseudorosette virus |
| Oat sterile dwarf | Oat sterile dwarf virus (OSDV) |
| Rice black-streaked dwarf | Rice black-streaked dwarf virus (RBSDV) |
| Rice stripe | Rice stripe virus (RSV) |
| Sorghum mosaic | Sorghum mosaic virus (SrMV) (also: sugarcane mosaic virus (SCMV) strains H, I and M) |
| Sugarcane Fiji disease | Sugarcane Fiji disease virus (FDV) |
| Sugarcane mosaic | Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B) |
| Wheat spot mosaic | Wheat spot mosaic virus (WSMV) |

The plants and plant cells according to the present invention can also be resistant to animal pests like insects and nematodes. Insects, like for example beetles,

caterpillars, lice, or mites are to be mentioned in an exemplary, yet not limiting manner.

Preferably, the plants according to the present invention are resistant to insects of the species of Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera. Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc. Insects of the following species are particularly preferred: Coleoptera and Lepidoptera, like, for example, the European corn borer (ECB), *Diabrotica barberi* (Northern corn rootworm), *Diabrotica undecimpunctata* (Southern corn rootworm), *Diabrotica virgifera* (Western corn rootworm), *Agrotis ipsilon* (black cutworm), *Crymodes devastator* (glassy cutworm), *Feltia ducens* (dingy cutworm), *Agrotis gladiaria* (claybacked cutworm), Melanotus spp., *Aeolus mellillus* (wireworm), *Aeolus mancus* (wheat wireworm), *Horistonotus uhlerii* (sand wireworm), *Sphenophorus maidis* (maize billbug), *Sphenophorus zeae* (timothy billbug), *Sphenophorus parvulus* (bluegrass billbug), *Sphenophorus callosus* (southern corn billbug), *Phyllogphaga* spp.(white grubs), *Anuraphis maidiradicis* (corn root aphid), *Delia platura* (seedcorn maggot), *Colaspis brunnea* (grape colaspis), *Stenolophus lecontei* (seedcorn beetle), and *Clivinia impressifrons* (lender seedcorn beetle).

Furthermore, there are to be mentioned: the cereal leaf beetle (*Oulema melanopus*), the frit fly (*Oscinella frit*), wireworms (*Agrotis lineatus*), and aphids (like for example the bird cherry-oat aphid *Rhopalosiphum padi,* the grain aphid *Sitobion avenae*).

The pathogens listed in Table 5 as well as the diseases associated therewith are to be mentioned as nematode pests in an exemplary, yet not limiting manner.

Table 5: Parasitic nematodes

| **Damage** | **Pathogenic nematode** |
|---|---|
| Awl | *Dolichodorus spp., D. heterocephalus* |
| Bulb and stem nematode, beet eelworm ("Bulb and stem"; Europe) | *Ditylenchus dipsaci* |
| Burrowing | *Radopholus similis* |
| Cereal cyst nematode ("Cyst") | *Heterodera avenae, H. zeae, Punctodera chalcoensis* |
| Dagger | *Xiphinema spp., X. americanum, X. mediterraneum* |
| False root-knot | *Nacobbus dorsalis* |
| Lance, Columbia | *Hoplolaimus columbus* |
| Lance | *Hoplolaimus spp., H. galeatus* |
| Lesion | *Pratylenchus spp., P. brachyurus, P. crenatus, P. hexincisus, P. neglectus, P. penetrans, P. scribneri, P. thornei, P. zeae* |
| Needle | *Longidorus spp., L. breviannulatus* |
| Ring | *Criconemella spp., C. ornata* |
| Root-knot nematode | *Meloidogyne spp., M. chitwoodi, M. incognita, M. javanica* |
| Spiral | *Helicotylenchus spp.* |
| Sting | *Belonolaimus spp., B. longicaudatus* |
| Stubby-root | *Paratrichodorus spp., P. christiei, P. minor, Quinisulcius acutus, Trichodorus spp.* |

| Stunt | *Tylenchorhynchus dubius* |
|---|---|

Particularly preferably, the transgenic plants produced according to the present invention are resistant to *Globodera rostochiensis* and *G. pallida* (cyst nematodes of potato, tomato, and other solanaceae), *Heterodera schachtii* (beet cyst nematodes of sugar and fodder beets, rape, cabbage, etc.), *Heterodera avenae* (cereal cyst nematode of oat and other types of cereal), *Ditylenchus dipsaci* (bulb and stem nematode, beet eelworm of rye, oat, maize, clover, tobacco, beet), *Anguina tritici* (wheat seed gall nematode), seed galls of wheat (spelt, rye), *Meloidogyne hapla* (root-knot nematode of carrot, cucumber, lettuce, tomato, potato, sugar beet, lucerne).

In individual species of particular agricultural importance, the plants according to the present invention are preferably resistant to the following pathogens:

In barley, the plants are resistant to the fungal, bacterial, and viral pathogens *Puccinia hordei* (barley stem rust), *Blumeria (Erysiphe) graminis* f. sp. hordei (barley powdery mildew), *Rhynchosporium secalis* (barley scald), barley yellow dwarf virus (BYDV), and the pathogenic insects / nematodes *Ostrinia nubilalis* (European corn borer); *Agrotis ipsilon* (black cutworm); *Schizaphis graminum* (greenbug); *Blissus leucopterus* (chinch bug); *Acrosternum hilare* (green stink bug); *Euschistus servus* (brown stink bug); *Deliaplatura* (seedcorn maggot); *Mayetiola destructor* (Hessian fly); *Petrobia latens* (brown wheat mite).

In soy bean, the plants are resistant to the fungal, bacterial, or viral pathogens *Phytophthora megasperma* fsp. glycinea, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Diaporthe phaseolorum* var. sojae (*Phomopsis sojae*), *Diaporthe phaseolorum* var. caulivora, *Sclerotium rolfsii*, *Cercospora kikuchii*, *Cercospora sojina*, *Peronospora manshurica*, *Colletotrichum dematium* (*Colletotrichum truncatum*), *Corynespora cassiicola*, *Septoria glycines*,

*Phyllosticta sojicola*, *Alternaria alternata*, *Pseudomonas syringae* p.v. glycinea, *Xanthomonas campestris* p.v. phaseoli, *Microsphaera diffussa*, *Fusarium semitectum*, *Phialophora gregata*, soy bean mosaic virus, *Glomerella glycines*, tobacco ring spot virus, tobacco streak virus, *Phakopsora pachyrhizi*, *Pythium aphanidermatum*, *Pythium ultimum*, *Pythium debaryanum*, tomato spotted wilt virus, *Heterodera glycines*, *Fusarium solani* and the pathogenic insects / nematodes *Pseudoplusia includens* (soybean looper); *Anticarsia gemmatalis* (velvetbean caterpillar); *Plathypena scabra* (green cloverworm); *Ostrinia nubilalis* (European corn borer); *Agrotis ipsilon* (black cutworm); *Spodoptera exigua* (beet armyworm); *Heliothis virescens* (cotton budworm); *Helicoverpa zea* (cotton bollworm); *Epilachna varivestis* (Mexican bean beetle); *Myzus persicae* (green peach aphid); *Empoasca fabae* (potato leaf hopper); *Acrosternum hilare* (green stink bug); *Melanoplus femurrubrum* (redlegged grasshopper); *Melanoplus differentialis* (differential grasshopper); *Hylemya platura* (seedcom maggot); *Sericothrips variabilis* (soybean thrips); *Thrips tabaci* (onion thrips); *Tetranychus turkestani* (strawberry spider mite); *Tetranychus urticae* (twospotted spider mite).

In canola, the plants are resistant to the fungal, bacterial, or viral pathogens *Albugo candida*, *Alternaria brassicae*, *Leptosphaeria maculans*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Mycosphaerella brassiccola*, *Pythium ultimum*, *Peronospora parasitica*, *Fusarium roseum* and *Alternaria alternata*.

In alfalfa, the plants are resistant to the fungal, bacterial, or viral pathogens *Clavibacter michiganensis* subsp. insidiosum, *Pythium ultimum*, *Pythium irregulare*, *Pythium splendens*, *Pythium debaryanum*, *Pythium aphanidermatum*, *Phytophthora megasperma*, *Peronospora trifoliorum*, *Phoma medicaginis* var. medicaginis, *Cercospora medicaginis*, *Pseudopeziza medicaginis*, *Leptotrochila medicaginis*, *Fusarium*, *Xanthomonas campestris* p.v. alfalfae, *Aphanomyces euteiches*, *Stemphylium herbarum*, *Stemphylium alfalfae*.

In wheat, the plants are resistant to the fungal, bacterial, or viral pathogens *Pseudomonas syringae* p.v. atrofaciens, *Urocystis agropyri*, *Xanthomonas campestris* p.v. translucens, *Pseudomonas syringae* p.v. syringae, *Alternaria alternata*, *Cladosporium herbarum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium culmorum*, *Ustilago tritici*, *Ascochyta tritici*, *Cephalosporium gramineum*, *Collotetrichum graminicola*, *Blumeria (Erysiphe) graminis* f. sp. tritici, *Puccinia graminis* f. sp. tritici, *Puccinia recondita* f. sp. tritici, *Puccinia striiformis*, *Puccinia triticina*, *Pyrenophora tritici-repentis*, *Septoria nodorum*, *Septoria tritici*, *Septoria avenae*, *Pseudocercosporella herpotrichoides*, *Rhizoctonia solani*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. tritici, *Pythium aphanidermatum*, *Pythium arrhenomanes*, *Pythium ultimum*, *Bipolaris sorokiniana*, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American Wheat Striate Virus, *Claviceps purpurea*, *Tilletia tritici*, *Tilletia laevis*, *Ustilago tritici*, *Tilletia indica*, *Pythium gramicola*, High Plains Virus, European wheat striate virus and to the pathogenic insects / nematodes *Pseudaletia unipunctata* (army worm); *Spodoptera frugiperda* (fall armyworm); *Elasmopalpus lignosellus* (lesser cornstalk borer); *Agrotis orthogonia* (western cutworm); *Elasmopalpus Zignosellus* (lesser cornstalk borer); *Oulema melanopus* (cereal leaf beetle); *Hypera punctata* (clover leaf weevil); *Diabrotica undecimpunctata howardi* (southern corn rootworm); Russian wheat aphid; *Schizaphis graminum* (greenbug); *Macrosiphum avenae* (English grain aphid); *Melanoplus femurrubrum* (redlegged grasshopper); *Melanoplus differentialis* (differential grasshopper); *Melanoplus sanguinipes* (migratory grasshopper); *Mayetiola destructor* (Hessian fly); *Sitodiplosis mosellana* (wheat midge); *Meromyza americana* (wheat stem maggot); *Hylemya coarctata* (wheat bulb fly); *Frankliniella fusca* (tobacco thrips); *Cephus cinctus* (wheat stem sawfly); *Aceria tulipae* (wheat curl mite).

In sun flower, the plants are resistant to the fungal, bacterial, or viral pathogens *Plasmophora halstedii*, *Sclerotinia sclerotiorum*, Aster Yellows, *Septoria helianthi*, *Phomopsis helianthi*, *Alternaria helianthi*, *Alternaria zinniae*, *Botrytis cinerea*, *Phoma macdonaldii*, *Macrophomina phaseolina*, *Erysiphe cichoracearum*, *Rhizopus oryzae*, *Rhizopus arrhizus*, *Rhizopus stolonifer*, *Puccinia helianthi*, *Verticillium dahliae*, *Erwinia carotovorum* p.v. Carotovora, *Cephalosporium acremonium*, *Phytophthora cryptogea*, *Albugo tragopogonis* and to the pathogenic insects/nematodes *Suleima helianthana* (sunflower bud moth); *Homoeosoma electellum* (sunflower moth); *Zygogramma exclamationis* (sunflower beetle); *Bothyrus gibbosus* (carrot beetle); *Neolasioptera murtfeldtiana* (sunflower seed midge).

In maize, the plants are resistant to the fungal, bacterial, or viral pathogens *Fusarium moniliforme* var. subglutinans, *Erwinia stewartii*, *Fusarium moniliforme*, *Gibberella zeae* (*Fusarium graminearum*), *Stenocarpella maydi* (*Diplodia maydis*), *Pythium irregulare*, *Pythium debaryanum*, *Pythium graminicola*, *Pythium splendens*, *Pythium ultimum*, *Pythium aphanidermatum*, *Aspergillus flavus*, *Bipolaris maydis* 0, T (*Cochliobolus heterostrophus*), *Helminthosporium carbonum* I, II & III (*Cochliobolus carbonum*), *Exserohilum turcicum* I, II & III, *Helminthosporium pedicellatum*, *Physoderma maydis*, *Phyllosticta maydis*, *Kabatiella maydis*, *Cercospora sorghi*, *Ustilago maydis*, *Puccinia sorghi*, *Puccinia polysora*, *Macrophomina phaseolina*, *Penicillium oxalicum*, *Nigrospora oryzae*, *Cladosporium herbarum, Curvularia lunata*, *Curvularia inaequalis*, *Curvularia pallescens*, *Clavibacter michiganense* subsp. nebraskense, *Trichoderma viride*, Maize Dwarf Mosaic Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, *Claviceps sorghi*, *Pseudonomas avenae*, *Erwinia chrysanthemi* p.v. Zea, *Erwinia corotovora*, *Cornstunt spiroplasma*, *Diplodia macrospora*, *Sclerophthora macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinesis*, *Peronosclerospora maydis*, *Peronosclerospora sacchari*, *Spacelotheca reiliana*, *Physopella zeae*, *Cephalosporium maydis*, *Cephalosporium acremonium*, Maize

Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus (MSV, Maisstrichel-Virus), Maize Stripe Virus, Maize Rough Dwarf Virus, and the pathogenic insects / nematodes *Ostrinia nubilalis* (European corn borer); *Agrotis ipsilon* (black cutworm); *Helicoverpa zea* (corn earworm); *Spodoptera frugiperda*. (fall armyworm); *Diatraea grandiosella* (southwestern corn borer); *Elasmopalpus lignosellus* (lesser cornstalk borer); *Diatraea saccharalis* (surgarcane borer); *Diabrotica virgifera* (western corn rootworm); *Diabrotica longicornis barberi* (northern corn rootworm); *Diabrotica undecimpunctata howardi* (southern corn rootworm); *Melanotus spp*. (wireworms); *Cyclocephala borealis* (northern masked chafer; white grub); *Cyclocephala immaculata* (southern masked chafer; white grub); *Popillia japonica* (Japanese beetle); *Chaetocnema pulicaria* (corn flea beetle); *Sphenophorus maidis* (maize billbug); *Rhopalosiphum maidis* (corn leaf aphid); *Anuraphis maidiradicis* (corn root aphid); *Blissus leucopterus leucopterus* (chinch bug); *Melanoplus femurrubrum* (redlegged grasshopper); *Melanoplus sanguinipes* (migratory grasshopper); *Hylemva platura* (seedcom maggot); *Agromyza parvicornis* (corn blot leafminer); *Anaphothrips obscurus* (grass thrips); *Solenopsis milesta* (thief ant); *Tetranychus urticae* (twospotted spider mite).

In sorghum, the plants are resistant to the fungal, bacterial, or viral pathogens *Exserohilum turcicum*, *Colletotrichum graminicola* (*Glomerella graminicola*), *Cercospora sorghi*, *Gloeocercospora sorghi*, *Ascochyta sorghina*, *Pseudomonas syringae* p.v. syringae, *Xanthomonas campestris* p.v. holcicola, *Pseudomonas andropogonis*, *Puccinia purpurea*, *Macrophomina phaseolina*, *Perconia circinata*, *Fusarium moniliforme*, *Alternaria alternata*, *Bipolaris sorghicola*, *Helminthosporium sorghicola*, *Curvularia lunata*, *Phoma insidiosa*, *Pseudomonas avenae* (*Pseudomonas alboprecipitans*), *Ramulispora sorghi*, *Ramulispora sorghicola*, *Phyllachara sacchari*, *Sporisorium reilianum* (*Sphacelotheca reiliana*), *Sphacelotheca cruenta*, *Sporisorium sorghi*, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, *Claviceps sorghi*, *Rhizoctonia solani*, *Acremonium strictum*,

*Sclerophthona macrospora*, *Peronosclerospora sorghi*, *Peronosclerospora philippinensis*, *Sclerospora graminicola*, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium arrhenomanes*, *Pythium graminicola* and to the pathogenic insects / nematodes *Chilo partellus* (sorghum borer); *Spodoptera frugiperda* (fall armyworm); *Helicoverpa zea* (corn earworm); *Elasmopalpus lignosellus* (lesser cornstalk borer); *Feltia subterranea* (granulate cutworm); *Phyllophaga crinita* (white grub); *Eleodes, Conoderus* und *Aeolus* spp. (wireworm); *Oulema melanopus* (cereal leaf beetle); *Chaetocnema pulicaria* (corn flea beetle); *Sphenophorus maidis* (maize billbug); *Rhopalosiphum maidis* (corn leaf aphid); *Siphaflava* (yellow sugarcane aphid); *Blissus leucopterus leucopterus* (chinch bug); *Contarinia sorghicola* (sorghum midge); *Tetranychus cinnabarinus* (carmine spider mite); *Tetranychus urticae* (two-spotted spider mite).

In cotton, the plants are resistant to the pathogenic insects/nematodes: *Heliothis virescens* (cotton budworm); *Helicoverpa zea* (cotton bollworm); *Spodoptera exigua* (beet armyworm); *Pectinophora gossypiella* (pink bollworm); *Anthonomus grandis grandis* (boll weevil); *Aphis gossypii* (cotton aphid); *Pseudatomoscelis seriatus* (cotton fleahopper); *Trialeurodes abutilonea* (bandedwinged whitefly); *Lygus lineolaris* (tarnished plant bug); *Melanoplus femurrubrum* (redlegged grasshopper); *Melanoplus differentialis* (differential grasshopper); *Thrips tabaci* (onion thrips); *Franklinkiella fusca* (tobacco thrips); *Tetranychus cinnabarinus* (carmine spider mite); *Tetranychus urticae* (two-spotted spider mite);

In rice, the plants are resistant to the pathogenic insects/nematodes *Diatraea saccharalis* (sugarcane borer); *Spodoptera frugiperda* (fall armyworm); *Helicoverpa zea* (corn earworm); *Colaspis brunnea* (grape colaspis); *Lissorhoptrus oryzophilus* (rice water weevil); *Sitophilus oryzae* (rice weevil); *Nephotettix nigropictus* (rice leafhopper); *Blissus Ieucopterus leucopterus* (chinch bug); *Acrosternum hilare* (green stink bug).

In rape, the plants are resistant to the pathogenic insects/nematodes *Brevicoryne brassicae* (cabbage aphid); *Phyllotreta cruciferae* (Flea beetle); *Mamestra configurata* (Bertha armyworm); *Plutella xylostella* (Diamond-back moth); *Delia ssp.* (Root maggots)..

Particularly preferably, the term “plant pathogen” comprises pathogens selected from the group consisting of *Blumeria graminis f. sp. hordei, tritici*, *avenae*, *secalis*, *lycopersici*, *vitis*, *cucumis*, *cucurbitae*, *pisi*, *pruni, solani*, *rosae*, *fragariae*, *rhododendri*, *mali,* and *nicotianae* as well as *Septoria tritici* and *Puccinia triticina.*

Within the meaning of the present invention a "cellulose synthase-like protein" is an enzyme which is involved in the synthesis of the backbone of non-cellulosic β-linked polysaccharides of the plant cell wall. Preferably, the cellulose synthase-like protein of the present invention belongs to the cellulose synthase-like C or H family. More preferably, the cellulose synthase-like protein is CSLC9 or CSLH1. Most preferably, the cellulose synthase-like protein is encoded by a nucleic acid sequence selected from the group consisting of:

(a) a nucleic acid sequence comprising the nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(b) a nucleic acid sequence comprising a sequence which is at least 80% identical to the sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
(c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5.

Members of the cellulose synthase-like C family possess β-1,4 glucan synthase activity (Cocuron et al. (2007) Proc. Natl. Acad. Sci. USA 104(20): 8550-8555). For a barley cellulose synthase like protein CSLH1 it has been shown that it mediates the synthesis of (1,3; 1,4)-β-D-glucan (Doblin et al. (2009) Proc. Natl. Acad. Sci. USA 106(14): 5996-6001). Hence, the sequences according to SEQ ID Nos. 1, 2 and 3

preferably encode a cellulose synthase like protein of the CSLH family which mediates the synthesis of (1,3; 1,4)-β-D-glucan and the sequence according to SEQ ID Nos. 4 and 5 preferably encode a cellulose synthase like protein of the CSLC family which mediates the synthesis of β-1,4-glucan.

The content of a protein within a plant cell is usually determined by the expression level of the protein. Hence, in most cases the terms "content" and "expression" may be used interchangeably. The content of a protein within a cell can be influenced on the level of transcription and/or the level of translation. Typically, the content is reduced on the RNA level, e.g. by RNA interference as described herein.

The person skilled in the art knows that the activity of a protein is not only influenced by the expression level, but also by other mechanisms such as post-translational modifications such as phosphorylations and acetylations. The present invention also encompasses methods of influencing the activity of the cellulose synthase-like proteins which do not affect the content of these proteins.

The person skilled in the art knows methods for reducing the content and/or the activity of a protein.

The expression of the nucleic acid sequence coding for the cellulose synthase-like protein may be substantially inhibited in transgenic plants for example by "silencing". For silencing, a nucleic acid sequence which is substantially identical to the nucleic acid sequence coding for the cellulose synthase-like protein and/or which is substantially complementary thereto is transferred to the plant. To ensure that the plants are transgenic for the introduced nucleic acids, the nucleic acid to be transferred is normally introduced to the plant by a vector, such as a plasmid, which is able to stably replicate in the plant cell or to integrate the introduced nucleic acid into the plant genome.

In this context, those skilled in the art refer to a nucleic acid sequence complementary to a nucleic acid sequence as antisense nucleic acid, wherein the same is typically antisense RNA. Use of antisense RNA leads to suppression of the corresponding endogenous gene. A nucleic acid sequence identical to a nucleic acid sequence is also referred to as sense nucleic acid. The use of sense RNA may also lead to suppression of the corresponding endogenous gene, by means of a process called "co-suppression".

If, in the scope of the present invention, sense sequences are mentioned, it is referred to those sequences, which correspond to the coding strand of a nucleic acid sequence coding for the cellulose synthase-like protein, or which comprise parts thereof. Such sequences do not have to be 100 % identical to the sequence coding for the cellulose synthase-like protein. It should be sufficient if said sequences are at least 80 %, 82 %, 84 %, 86 %, 88 %, particularly preferably at least 90 % and most preferably at least 95 %, 96 %, 97 %, 98 % or 99% identical. In case of such degrees of identity, the sequences are regarded, according to the invention, as homologous to each other or comprising a homology. The deviations to the nucleic acid coding for the cellulose synthase-like protein or parts thereof may originate from deletion, substitution and/or insertion of one or more nucleotides. The skilled person surely knows that with decreasing identity, it becomes more likely that multiple nucleic acids are suppressed in a silencing manner. Sequences having such a low degree of identity or homology that the expression of genes other than those coding for cellulose synthase-like proteins of the transgenic plant is suppressed, are not specific enough for the method of the present invention, and are not suitable, since they may interfere with the metabolism of the plant. However, sequences suppressing the expression of more than one cellulose synthase-like protein, such as sequences suppressing the expression of CSLs from the same or different CSL families, may be used in the method of the present invention, unless they do not suppress the expression of genes other than those coding for cellulose synthase-like proteins.

If antisense sequences are mentioned, those sequences of the invention are referred to which correspond to the codogenous DNA strand of the genes coding for the cellulose synthase-like protein. These sequences are composed of the nucleotides which are complementary to the corresponding nucleotides in the sense sequence. Said sequences are preferably complementary to at least 80 %, 82 %, 84 %, 86 %, 88 %, particularly preferably to at least 90 %, 92%, 94% and most preferably to at least 95 %, 96 %, 97 %, 98 % or 99% to the sequence coding for the cellulose synthase-like protein the expression of which is to be inhibited, i.e. not all nucleotides in the antisense sequence have to be complementary to the nucleotides in the sense sequence, but only 80% or more nucleotides. As mentioned above, it is sufficient if the antisense sequences are able to hybridize specifically with the mRNA of the corresponding gene coding for the cellulose synthase-like protein, but not with the mRNA of genes other than CSLs of the transgenic plant. However, if a double-stranded RNA is to be produced, the antisense sequence should be 100% reverse-complementary to the sense sequence to ensure optimal base-pairing. Within the meaning of the present invention the terms "complementary" and "reverse complementary" are used synonymously.

In one embodiment of the present invention, the at least one nucleic acid sequence is present in antisense orientation, so that upon transcription of said sequence in plant cells a RNA molecule is created, the sequence of which being complementary to the nucleic acid coding for the cellulose synthase-like protein. By hybridizing the antisense sequence with the nucleic acid sequence coding for the cellulose synthase-like protein *in vivo*, the expression of the nucleic acid sequence coding for the cellulose synthase-like protein may be suppressed in plant cells, whereby the plant becomes pathogen resistant. Hence, in this case the expression construct or vector comprises a promoter functional in plant cells; operatively linked thereto a nucleic acid sequence which is complementary to a nucleic acid coding for the cellulose synthase-like protein; and, optionally, a termination sequence. Preferably, the nucleic

acid sequence is complementary to a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1 to 5 or a fragment of any of these sequences.

In another preferred embodiment of the present invention, the at least one nucleic acid sequence is present in sense orientation, so that upon transcription of said sequence in plant cells a RNA molecule is created, the sequence of which being identical to the nucleic acid sequence coding for the cellulose synthase-like protein. Those skilled in the art know that, upon expression of such sense sequences in plants, antisense RNAs (asRNAs) may be formed which may cause silencing of both, the transgene, i.e. the sense sequence which was introduced and the endogenous, corresponding gene (co-suppression). By co-suppressing the nucleic acid sequence coding for the cellulose synthase-like protein *in vivo*, the expression of the nucleic acid sequence coding for the cellulose synthase-like protein may be suppressed in plant cells, whereby the plant becomes pathogen resistant. Hence, in this case the expression construct or vector comprises a promoter functional in plant cells; operatively linked thereto a nucleic acid sequence which is identical to a nucleic acid sequence coding for a cellulose synthase-like protein; and, optionally, a termination sequence. Preferably, the nucleic acid sequence is identical to a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1 to 5 or a fragment of any of these sequences.

In another embodiment of the method of the present invention, expression constructs or vectors are used for introducing the nucleic acids in the plant cells which comprise in 5' - 3'-orientation a promotor functional in plant cells, operatively linked thereto a DNA sequence coding for a ribozyme which specifically recognizes the nucleic acid sequence coding for a cellulose synthase-like protein, and optionally a termination sequence. Those skilled in the art know how ribozymes which exhibit an endonuclease activity directed against a certain nucleic acid sequence, for example a mRNA, may be produced. In the scope of the invention, the term "ribozyme" also refers to those RNA sequences which comprise next to the actual ribozyme leading

sequences which are complementary to the nucleic acid sequence coding for the cellulose synthase-like protein or parts thereof, and thus direct the mRNA-specific ribozyme even more target-orientedly to the mRNA substrate of the ribozyme.

In another preferred embodiment of the above-mentioned method, the expression construct or vector comprises a promoter which is functional in plant cells, operatively linked thereto at least one nucleic acid sequence which after transcription acts as a leading sequence, another nucleic acid sequence coding for ribonuclease P, and optionally a termination sequence. Upon transcription of such vectors, RNA molecules are formed in the cell having a leading sequence (the antisense sequence), which directs the RNAse P to the mRNA of the cellulose synthase-like protein, thereby causing the cleavage of the mRNA by RNAse P (US Patent No. 5,168,053). Preferably, the leading sequence comprises 10 to 15 nucleotides which are complementary to the mRNA of the cellulose synthase-like protein and a 3'-NCCA nucleotide sequence, wherein N preferably is a purine. The transcripts of the external leading sequence bind to the target mRNA by the formation of base pairs, thus enabling cleavage of the mRNA by RNAse P at the nucleotide 5' from the paired region. Such cleaved mRNA cannot be translated into a functional protein.

The skilled person knows diverse techniques for suppressing or inhibiting the expression of an endogenous gene by small double-stranded RNA molecules, so-called small interfering RNAs, or siRNAs.

Thereby, the double-stranded RNA molecule confers the specific degradation of the corresponding nucleic acid sequence, i.e. the nucleic acid sequence, from which the double-stranded RNA sequence has been derived. By enzymatic cleavage, e.g. by the dicer enzyme complex, RNA fragments having a length of 19 - 25 nucleotides, the so-called siRNAs, are produced from double-stranded RNA substrates. Such double-stranded RNA substrates (dicer substrates) must have a length of at least 25 bp. However, those skilled in the art know that also substantially longer double-stranded

RNA molecules are suitable substrates. Due to the very high sequence identity of both siRNAs, the siRNAs are commonly present as double-stranded RNA. The siRNAs may inhibit or prevent gene expression in many different ways:

a) transcription (transcriptional gene silencing or TGS)
b) degradation of the mRNA (post-transcriptional gene silencing or PTGS)
c) translation.

Said method for suppressing the expression of an endogenous nucleic acid sequence by sequence-specific double-stranded RNA is known to those skilled in the art as RNA interference or RNAi (Zamore *et al.* (2000) Cell 101: 25-33; Tang *et al.* (2003) Genes Dev. 17: 49-63; Smith *et al.* (2000) Nature 407: 319-320).

The use of RNAi constructs according to the invention is based on the above-mentioned mechanisms for inhibiting gene expression of a nucleic acid sequence coding for a cellulose synthase-like protein. Thereby, the corresponding polypeptide(s) cannot be formed.

Those skilled in the art know that various strategies may be chosen to make double-stranded RNA available as dicer substrate in the cell, and thus to trigger a specific RNAi effect.

In a preferred embodiment of the above-mentioned method, the expression construct or vector comprises a promoter which is functional in plant cells, operatively linked thereto at least one nucleic acid sequence coding for the cellulose synthase-like protein, preferably the nucleic acid sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences, wherein said sequence has reverse-complementary regions, and a termination sequence. The skilled person knows constructs in which a nucleic acid sequence has reverse-complementary regions, so that after transcription of such a construct and self-hybridization within the nucleic acid sequence with the mentioned reverse-complementary regions, double-stranded

RNA is being formed, which is a substrate for the dicer enzyme complex, for example. Accordingly, siRNA molecules are formed, which lead to the degradation of the corresponding nucleic acid. The above-mentioned reverse-complementary nucleic acid sequences are also referred to as inverted repeats.

In another preferred embodiment, the expression construct or vector comprises a promoter which is functional in plant cells, operatively linked thereto is the at least one nucleic acid sequence coding for the cellulose synthase-like protein, preferably the nucleic acid sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences, a "short hairpin" structure-generating nucleic acid and the nucleic acid sequence which is reverse-complementary to the at least one nucleic acid sequence coding for the cellulose synthase-like protein, preferably the nucleic acid sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences, and optionally a termination sequence.

By refolding the "short hairpin" structure, the at least one nucleic acid sequence and the nucleic acid sequence reverse-complementary thereto may hybridize and form double-stranded RNA. Suitable constructs and double-stranded RNA molecules are known to those skilled in the art for example as "short hairpin" RNAs or shRNAs. Such constructs may be led by a U6 promoter or a CaMV35S promoter (Tuschl (2002) Nat. Biotechnol. 20: 446-448; Paul *et al.* (2002) Nat. Biotechnol. 20: 505-508; Paddison *et al.* (2002) Genes Dev. 16(8): 948-958; Brummelkamp *et al.* (2002) Science 296: 550-553).

In a preferred embodiment of the method of the present invention using the RNAi methodology, the expression construct or vector comprises a promoter which is functional in plant cells, operatively linked thereto at least one nucleic acid sequence coding for the cellulose synthase-like protein, preferably the nucleic acid sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences , optionally a spacer sequence, the nucleic acid sequence reverse-complementary to the at least one

nucleic acid sequence coding for the cellulose synthase-like protein, preferably the nucleic acid sequence of any of SEQ ID Nos. 1-5 or a fragment of any of these sequences, and optionally a termination sequence.

In an especially preferred embodiment, the expression construct or vector comprises a RNAi construct, wherein the at least one nucleic acid sequence coding for the cellulose synthase-like protein comprises a sequence selected from the group consisting of SEQ ID NOs: 1-7, 9 and 10 or parts thereof, and a sequence reverse-complementary thereto. Most preferably, the at least one nucleic acid sequence is selected from the group consisting of SEQ ID NOs. 6, 7, 9 and 10. Even more preferably, the recombinant nucleic acid molecule comprises a sequence according to SEQ ID No. 8 or 11, in particular if it is to be transferred to wheat plants.

If a spacer sequence is used in the RNAi construct, the spacer sequence can be any sequence which is not complementary to another sequence in the construct. The spacer sequence can be derived from both exons and introns. The spacer sequence may also be a part of the sense or antisense sequence which is not reverse-complementary to the antisense or sense sequence, respectively. For example, the sense sequence may be extended by a certain number of nucleotides which are located 5' or 3' of the sense sequence in its natural sequence context, whereas the antisense sequence is not extended by the corresponding complementary nucleotides.

In another embodiment, the spacer sequence is an intron which provides splice donor and splice acceptor sequences, such as the rga1 intron from wheat. If vectors containing an intron as a spacer sequence are stably introduced in plant cells, first a pre-mRNA is formed upon transcription of said vectors which consists of a first exon comprising the at least one nucleic acid sequence of the present invention, an intron and a second exon comprising the nucleic acid sequence reverse-complementary to the at least one nucleic acid sequence. Since the intron is removed by the splicing procedure, a continuous RNA molecule is formed having regions which are

complementary to each other, and thus being a substrate for specific enzyme complexes, such as the dicer enzyme complex. Those skilled in the art know that the position of the antisense (3' - 5') and sense (5' - 3') sequences may be interchanged in the vector.

The spacer sequence typically comprises 20 to 500 nucleotides, preferably 40 to 400 nucleotides, more preferably 60 to 300 nucleotides and most preferably 100 to 200 nucleotides.

In an embodiment of the method of the invention, the recombinant nucleic acid molecule comprises a RNAi construct, optionally comprising a spacer sequence between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-7, 9 and 10, preferably selected from the group consisting of SEQ ID NOs: 6, 7, 9 and 10 or parts thereof, and the sequence reverse-complementary thereto.

In another embodiment of the present invention, the RNAi construct comprises two promoters of which one regulates the expression of the sense sequence and the other one regulates the expression of the antisense sequence. The two promoters may be the same or different promoters. The sense and the antisense nucleic acid molecules may also be expressed under the control of a bidirectional promoter.

Those skilled in the art know that for RNAi and/or PTGS the sense and antisense RNAs used for forming double-stranded RNA molecules may be of different sizes (Tuschl (2002) Nature Biotechnol. 20: 446-448).

In another embodiment the content of the CSL protein is reduced by microRNA. MicroRNAs (miRNAs) have emerged as evolutionarily conserved, RNA-based regulators of gene expression in plants and animals. MiRNAs with a length of 21 to

25 nucleotides arise from larger precursors with a stem loop structure that are transcribed from non-protein-coding genes. MiRNA targets a specific mRNA to suppress gene expression at post-transcriptional (i.e. degrades mRNA) or translational levels (i.e. inhibits protein synthesis) (Bartel (2004) Cell 116: 281-297).

A miRNA precursor (pre-miRNA) can be engineered in such a way that endogenous miRNA encoded by pre-miRNA is replaced by a miRNA to target a gene-of-interest. A native plant microRNA precursor can be engineered as described in Schwab et al. (2006) Plant Cell 18(5): 1121-1133, to produce artificial miRNA which specifically down-regulates target gene expression. A further method for the design of functional microRNAs is the tool WMD3 as described by Ossowski et al (2008) Plant J. 53(4): 674-690. The tool is available in the internet with the following URL http://wmd3.weigelworld.org/cgi-bin/webapp.cgi).

Hence, the present inventions further provides a method of producing a transgenic plant, plant cell or plant part having an increased resistance to pathogens compared to the control plant comprising the steps of:
(a) transforming a plant cell with a miRNA precursor construct, and
(b) generating from the plant cell the transgenic plant,wherein said construct contains a promoter that drives expression in a plant cell operably linked to a nucleotide sequence encoding a precursor micro RNA sequence comprising a micro RNA sequence which targets the cellulose synthase-like protein.

The use of engineered micro RNA precursors and micro-RNA for modulating the expression of a gene is well known and described e.g. in US 2004/0268441. The use of engineered micro-RNA precursors and micro-RNA for modulating the expression of a gene can be combined with other methods of genetic engineering well known to the person skilled in the art.

In another embodiment of the present invention synthetic double-stranded siRNAs which typically have a length of 19 - 21 nucleotides are used for inhibiting the expression of a nucleic acid coding for the cellulose synthase-like protein. Such synthetic siRNAs may be introduced in the corresponding plant cell or plant by biolistic transformation techniques. Such synthetic siRNA molecules may activate the PTGS system in plants, and trigger an RNAi effect (Hamilton and Baulcombe (1999) Science 286: 950-2).

The target sequence for siRNA inhibition as well as the siRNA sequence motif may be selected according to the rules and regulations known to those skilled in the art, for example according to Elbashir *et al.* (2001) Nature 411: 494-8. If the target sequence for the siRNA-mediated inhibition lies within the coding regions of the gene, or within the mRNA, those skilled in the art know, for example, that the target sequence for a siRNA-mediated inhibition may typically be at least 70 nucleotides downstream from the start codon in 5' - 3' direction and at least 50 nucleotides upstream from the stop codon.

The sequence region may then be searched for the sequence motif AA (N19), wherein N may be each nucleotide. Said sequence motif typically comprises the AA dinucleotide, followed by 19 nucleotides, and preferably two additional uridine or thymidine residues. In general, the thymidine residues may be replaced by uridine residues in the siRNA sequence.

Further, those skilled in the art know the rules and regulations, established by Reynolds *et al.* ((2004) Nature Biotechnol. 22:326-30):

1. a guanine/cytosine content of 30 - 50 %
2. at least three adenine or uracil groups at positions 15 to 19 of the sense strand
3. no intermolecular "hairpin" structures
4. one adenine residue at position 19 of the sense strand

5. one adenine residue at position 3 of the sense strand
6. one uracil residue at position 10 of the sense strand
7. no guanine or cytosine residue at position 19 of the sense strand
8. no guanine residue at position 13 of the sense strand.

These eight criteria may be rated as follows:

(i) 1 point each for criteria 1, 3, 4, 5 and 6

(ii) 1 point each for each adenine or uridine residue at position 15 to 19, at least 3 corresponding bases (criterion 2)

(iii) minus 1 point each for not fulfilling the criteria 7 - 8.

According to Reynolds *et al.*, only those siRNAs should be considered which have at least 6 points. Such siRNAs which fulfil the above-mentioned criteria may be checked by appropriate search programs, e.g. BLAST, whether there are any, if possible no or only little, homologies to other nucleic acid sequences of the plant.

The skilled person also knows other methods for reducing the content and/or activity of a protein, such as a cellulose synthase-like protein. For example, a nucleic acid sequence for reducing the content and/or the activity of a protein may be integrated into the natural locus of the sequence by targeted homologous recombination. Such methods are for example described in WO 00/46386 A3, WO 01/89283A1, WO 02/077246 A2 and WO 2007/135022 A1. A method for introducing a targeting sequence differing from the target sequence by 0.1 to 10% by homeologous recombination is described for example in WO 2006/134496 A2.

To cleave DNA sequences within the genomic DNA for introducing a nucleic acid sequence for reducing the content and/or the activity of a protein different enzymes such as meganucleases (WO 2009/114321 A2), zink finger nucleases (WO 2009/042164 A1), transcription activator-like effector nucleases (WO 2011/072246 A2) and chimeric nucleases which comprise a DNA binding

domain targeting the nuclease to a specific sequence within the genome (WO 2009/130695 A2) may be used. Such sequence-specific nucleases may also be used to cut the sequence of interest, thereby introducing one or more mutations into said sequence.

Within the scope of the present invention, the method for producing mutant plants, plant cells or plant parts having an increased resistance to pathogens is preferably the TILLING (Targeting Induced Local Lesions IN Genomes) method. In a first step of this method, plant material is mutagenized to introduce at least one mutation into the genome of the plant material. This mutagenesis may be chemical mutagenesis, for example with ethyl methane sulfonate (EMS), mutagenesis by irradiation such as ionizing irradiation or mutagenesis by using sequence-specific nucleases. Single base mutations or point mutations lead to the formation of heteroduplexes which are then cleaved by single strand nucleases such as *Cel*I at the 3' side of the mutation. The precise position of the mutation within the nucleic acid sequence according to any of SEQ ID NOs. 1-5 can then be determined by denaturing gel electrophoresis or the LICOR gel based system (see, e.g., McCallum *et al.* (2000) Plant Physiol. 123(2): 439-442; Uauy *et al.* (2009) BMC Plant Biol. 9:115). If necessary, the mutant plants can then be screened for their resistance to pathogens.

The expression level of the nucleic acid coding for the cellulose synthase-like protein may be determined in the control plants as well as in the transgenic plants, for example, by RT-PCR analysis or Northern Blot analysis with specific primers or probes. A person skilled in the art knows how to select said probes or primers in order to examine the expression of said nucleic acid. Preferably, the expression of the nucleic acid coding for the cellulose synthase-like protein is statistically significantly reduced by at least 80 %, particularly preferably by at least 90 %, also particularly preferably by at least 95 %, and most preferably by at least 98 % or 99 %.

The activity of the cellulose synthase-like proteins may be determined by isolating the cell walls of control and transgenic plants or plant cells and analyzing the carbohydrate composition using suitable methods such as high-performance anion exchange chromatography (HPAEC), MALDI-linear ion trap mass spectrometry and NMR spectroscopy (as described in Cocuron et al. (2007) Proc. Natl. Acad. Sci. USA 104(20): 8550-8555 and Doblin et al. (2009) Proc. Natl. Acad. Sci. USA 106(14): 5996-6001). The inhibition of CSLC9 may lead to a reduction in β-1,4 glucan content and the inhibition of CSLH1 may lead to a reduction in (1,3; 1,4)-β-glucan content. The β-1,4 glucan content may be reduced by inhibition of CSLC9 expression by at least 5%, preferably at least 10%, more preferably at least 15% or 20%, even more preferably by at least 25 or 30% and most preferably by at least 35% or 40%. The (1,3; 1,4)-β-glucan content may be reduced by inhibition of CSLH1 expression by at least 5%, preferably at least 10%, more preferably at least 15% or 20%, even more preferably by at least 25 or 30% and most preferably by at least 35% or 40%.

The activity of the cellulose synthase-like protein may also be reduced by an antibody specific for said protein. The production of monoclonal, polyclonal, or recombinant antibodies specific for a cellulose synthase-like protein follows standard protocols (Guide to Protein Purification, Meth. Enzymol. 182, pp. 663-679 (1990), M. P. Deutscher, ed.). The expression of antibodies is also known from the literature (Fiedler et al. (1997) Immunotechnology 3: 205-216; Maynard and Georgiou (2000) Annu. Rev. Biomed. Eng. 2: 339-76).

In another embodiment, aptamers can be used to reduce CSL activity. Usually, aptamers are overexpressed from vectors and the design and selection of aptamers is well known to the person skilled in the art (Famulok et al. (1999) Curr Top Microbiol Immunol. 243: 123-36).

Preferably, in the method, the expression construct or the vector of the present invention a nucleic acid sequence is used which is selected from the group consisting of:

a) a nucleic acid sequence comprising the sequence according to SEQ ID No. 1-7, 9 and 10, preferably selected from the group consisting of SEQ ID NOs: 6, 7, 9 and 10 or a fragment of any of these sequences;
b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to SEQ ID No. 1-7, 9 and 10, preferably selected from the group consisting of SEQ ID NOs: 6, 7, 9 and 10, or a fragment of any of these sequences; and
c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to SEQ ID No. 1-7, 9 and 10, preferably selected from the group consisting of SEQ ID NOs: 6, 7, 9 and 10, or a fragment of any of these sequences.

If the method, the expression construct or the vector is used in a barley plant, the nucleic acid sequence is preferably selected from the group consisting of:

a) a nucleic acid sequence comprising the sequence according to SEQ ID No. 1, 2, 4, 6 or 9, preferably selected from the group consisting of SEQ ID NOs: 6 and 9, or a fragment of any of these sequences;
b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to SEQ ID No. 1, 2, 4, 6 or 9, preferably selected from the group consisting of SEQ ID NOs: 6 and 9, or a fragment of any of these sequences; and
c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to SEQ ID No. 1, 2, 4, 6 or 9, preferably selected from the group consisting of SEQ ID NOs: 6 and 9, or a fragment of any of these sequences.

If the method, the expression construct or the vector is used in a wheat plant, the nucleic acid sequence is preferably selected from the group consisting of:

a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 3, 5, 7 and 10, preferably the sequence according to SEQ ID No. 7 or 10, or a fragment of any of these sequences;
b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 3, 5, 7 and 10, preferably the sequence according to SEQ ID No. 7 or 10, or a fragment of any of these sequences; and
c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 3, 5, 7 and 10, preferably the sequence according to SEQ ID No. 7 or 10,or a fragment of any of these sequences.

A "fragment" of the nucleic acid sequence according to any of SEQ ID Nos. 1-7, 9 and 10 is understood to refer to a smaller part of any of these nucleic acid sequences which consists of a contiguous nucleotide sequence found in any of SEQ ID Nos. 1-7, 9 and 10 and which is able to reduce the content and/or activity of the cellulose synthase-like protein when used in a suitable expression system, but not or not considerably of other proteins the expression of which should not be reduced.

The fragment of SEQ ID No. 1 has a length of at least 500, 600, 700, 800 or 900 nucleotides, preferably of at least 1000, 1200, 1400 or 1600 nucleotides, more preferably of at least 1800, 2000, 2200, 2400, 2600 or 2800 nucleotides and most preferably of 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600 or 3700 nucleotides.

The fragment of SEQ ID No. 2 has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500 or 1600 nucleotides.

The fragment of SEQ ID No. 3 has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800 or 900 nucleotides, more preferably of at least 1000, 1100 or 1200, nucleotides and most preferably of 1400 nucleotides.

The fragment of SEQ ID No. 4 has a length of at least 50, 100, 150, 200 or 250 nucleotides, preferably of at least 300, 350 or 400 nucleotides, more preferably of at least 450, 500 or 550, nucleotides and most preferably of 600 or 650 nucleotides.

The fragment of SEQ ID No. 5 has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500, 1600, 1700 or 1800 nucleotides.

The fragment of any of SEQ ID Nos. 6, 7, 9 and 10 has a length of at least 25, 50, 75 or 100 nucleotides, preferably of at least 150, 200 or 250 nucleotides, more preferably of at least 300, 325, 350 or 375 nucleotides and most preferably of 400, 425 or 450 nucleotides.

A preferred fragment of the sequence according to SEQ ID No. 1 or 2 is the sequence according to SEQ ID No. 6, a preferred fragment of the sequence according to SEQ ID No. 3 is the sequence according to SEQ ID No. 7, a preferred fragment of the sequence according to SEQ ID No. 4 is the sequence according to SEQ ID No. 9 and a preferred fragment of the sequence according to SEQ ID No. 5 is the sequence according to SEQ ID No. 10.

The present invention further relates to the use of nucleic acid sequences which are at least 80 %, preferably at least 82, 84 or 86% identical, more preferably 88, 89 or 90% identical, even more preferably 91, 92, 93, 94 or 95% identical and most preferably 96, 97, 98 or 99% identical to the complete sequence according to any of

SEQ ID Nos. 1-7, 9 and 10 or a fragment thereof and which are able to reduce the content and/or activity of the cellulose synthase-like protein, but not or not considerably of other proteins the expression of which should not be reduced.

Within the meaning of the present invention, "sequence identity" denotes the degree of conformity with regard to the 5' - 3' sequence within a nucleic acid molecule in comparison to another nucleic acid molecule. The sequence identity may be determined using a series of programs, which are based on various algorithms, such as BLASTN, ScanProsite, the laser gene software, etc. As an alternative, the BLAST program package of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) may be used with the default parameters. Here, in addition, the program Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) using the "dirtydata"-algorithm for sequence comparisons was employed.

The sequence identity refers to the degree of the sequence identity over a length of 200 or 300 nucleotides, preferably 350 or 380 nucleotides, more preferably 400 or 450 nucleotides and most preferably the whole length of the nucleic acid sequence according to any of the sequences according to 2, 4, 6, 7, 9 and 10. The sequence identity refers to the degree of the sequence identity over a length of 500 or 700 nucleotides, preferably 800 or 900 nucleotides, more preferably 1000, 1100 or 1200 nucleotides, even more preferably 1300 or 1400 nucleotides and most preferably the whole length of the nucleic acid sequence according to any of the sequences according to 1, 3 and 5. If two sequences having a different length are compared, the percent sequence identity is calculated on the basis of the longer sequence.

If the sequence identity is to be determined with respect to a fragment of the sequence according to SEQ ID No. 1, the fragment has a length of at least 500, 600, 700, 800 or 900 nucleotides, preferably of at least 1000, 1200, 1400 or 1600 nucleotides, more preferably of at least 1800, 2000, 2200, 2400, 2600 or 2800

nucleotides and most preferably of 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600 or 3700 nucleotides.

If the sequence identity is to be determined with respect to a fragment of the sequence according to SEQ ID No. 2, the fragment has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500 or 1600 nucleotides.

If the sequence identity is to be determined with respect to a fragment of the sequence according to SEQ ID No. 3, the fragment has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800 or 900 nucleotides, more preferably of at least 1000, 1100 or 1200, nucleotides and most preferably of 1400 nucleotides.

If the sequence identity is to be determined with respect to a fragment of the sequence according to SEQ ID No. 4, the fragment has a length of at least 50, 100, 150, 200 or 250 nucleotides, preferably of at least 300, 350 or 400 nucleotides, more preferably of at least 450, 500 or 550, nucleotides and most preferably of 600 or 650 nucleotides.

If the sequence identity is to be determined with respect to a fragment of the sequence according to SEQ ID No. 5, the fragment has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500, 1600, 1700 or 1800 nucleotides.

If the sequence identity is to be determined with respect to a fragment of the sequence according to any of SEQ ID Nos. 6, 7, 9 and 10, the fragment has a length of at least 25, 50, 75 or 100 nucleotides, preferably of at least 150, 200 or 250

nucleotides, more preferably of at least 300, 325, 350 or 375 nucleotides and most preferably of 400, 425 or 450 nucleotides.

The present invention further relates to the use of nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-7, 9 and 10 or a fragment of any of these sequences and which are able to reduce the content and/or activity of the cellulose synthase-like protein when used in a suitable expression system, but not or not considerably of other proteins the expression of which should not be reduced.

In the context of the present invention the term "hybridizing under stringent conditions" means that the hybridization is implemented *in vitro* under conditions which are stringent enough to ensure a specific hybridization. Stringent *in vitro* hybridization conditions are known to those skilled in the art and may be taken from the literature (e.g. Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY). The term "specific hybridization" means that under stringent conditions a molecule preferably binds to a certain nucleic acid sequence, i.e. the target sequence, if the same is part of a complex mixture of, e.g. DNA or RNA molecules, but does not, or at least very rarely, bind to other sequences.

Stringent conditions depend on the circumstances. Longer sequences hybridize specifically at higher temperatures. In general, stringent conditions are chosen such that the hybridization temperature is about 5 °C below the melting point ($T_m$) of the specific sequence at a defined ionic strength and at a defined pH value. $T_m$ is the temperature (at a defined pH value, a defined ionic strength and a defined nucleic acid concentration), at which 50% of the molecules complementary to the target sequence hybridize to the target sequence in the state of equilibrium. Typically, stringent conditions are conditions, where the salt concentration has a sodium ion concentration (or concentration of a different salt) of at least about 0.01 to 1.0 M at a

pH value between 7.0 and 8.3, and the temperature is at least 30 °C for small molecules (i.e. 10 to 50 nucleotides, for example). In addition, stringent conditions may include the addition of substances, such as, e. g., formamide, which destabilise the hybrids. At hybridization under stringent conditions, as used herein, normally nucleotide sequences which are at least 60 % homologous to each other hybridize to each other. Preferably, said stringent conditions are chosen such that sequences which are about 65 %, preferably at least about 70 %, and especially preferably at least about 75 % or higher homologous to each other, normally remain hybridized to each other. A preferred but non-limiting example of stringent hybridization conditions is hybridizations in 6 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by one or more washing steps in 0.2 x SSC, 0.1 % SDS at 50 to 65 °C. The temperature depends on the type of the nucleic acid and is between 42 °C and 58 °C in an aqueous buffer having a concentration of 0.1 to 5 x SSC (pH value 7.2).

If an organic solvent, e.g. 50 % formamide, is present in the above-mentioned buffer, the temperature is about 42 °C under standard conditions. Preferably, the hybridisation conditions for DNA:DNA hybrids are, for example, 0.1 x SSC and 20 °C to 45 °C, preferably 30 °C to 45 °C. Preferably, the hybridisation conditions for DNA:RNA hybrids are, for example, 0.1 x SSC and 30 °C to 55 °C, preferably between 45 °C and 55 °C. The above-mentioned hybridization temperatures are determined, for example, for a nucleic acid which is 100 base pairs long and has a G/C content of 50 % in the absence of formamide. Those skilled in the art know how to determine the required hybridization conditions using text books, such as those mentioned above, or the following textbooks, Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), Hames and Higgins (publ.) 1985, Nucleic Acids Hybridization: A Practical Approach, IRL Press at Oxford University Press, Oxford; Brown (publ.) 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

Typical hybridization and washing buffers for example have the following composition:

| | |
|---|---|
| *Pre-hybridization solution*: | 0.5 % SDS |
| | 5x SSC |
| | 50 mM $NaPO_4$, pH 6.8 |
| | 0.1 % sodium pyrophosphate |
| | 5x Denhardt's solution |
| | 100 µg/mL salmon sperm DNA |
| *Hybridization solution:* | pre-hybridization solution |
| | $1x10^6$ cpm/mL probe (5 - 10 min 95 °C) |
| *20x SSC:* | 3 M NaCl |
| | 0.3 M sodium citrate |
| | ad pH 7 with HCl |
| *50x Denhardt's reagent:* | 5 g Ficoll |
| | 5 g polyvinylpyrrolidone |
| | 5 g bovine serum albumin |
| | ad 500 mL aqua destillata |

A typical procedure for hybridization is as follows:

*Optional:* wash blot 30 min in 1x SSC/ 0.1 % SDS at 65 °C

*Pre-hybridization:* at least 2 h at 50 - 55 °C

| | | | |
|---|---|---|---|
| *Hybridization:* | over night at 55 - 60 °C | | |
| *Washing:* | 05 min | 2x SSC/ 0.1 % SDS | hybridization temp. |
| | 30 min | 2x SSC/ 0.1 % SDS | hybridization temp. |
| | 30 min | 1x SSC/ 0.1 % SDS | hybridization temp. |
| | 45 min | 0.2x SSC/ 0.1 % SDS | 65 °C |
| | 5 min | 0.1x SSC | room temperature |

Those skilled in the art know that the given solutions and the presented protocol may be modified or have to be modified, depending on the application.

The nucleic acid sequence hybridizing to a fragment of the sequence according to SEQ ID No.1 under stringent conditions has a length of at least 500, 600, 700, 800 or 900 nucleotides, preferably of at least 1000, 1200, 1400 or 1600 nucleotides, more preferably of at least 1800, 2000, 2200, 2400, 2600 or 2800 nucleotides and most preferably of 2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600 or 3700 nucleotides.

The nucleic acid sequence hybridizing to a fragment of the sequence according to SEQ ID No.2 under stringent conditions has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500 or 1600 nucleotides.

The nucleic acid sequence hybridizing to a fragment of the sequence according to SEQ ID No.3 under stringent conditions has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800 or 900 nucleotides, more preferably of at least 1000, 1100 or 1200, nucleotides and most preferably of 1400 nucleotides.

The nucleic acid sequence hybridizing to a fragment of the sequence according to SEQ ID No.4 under stringent conditions has a length of at least 50, 100, 150, 200 or

250 nucleotides, preferably of at least 300, 350 or 400 nucleotides, more preferably of at least 450, 500 or 550, nucleotides and most preferably of 600 or 650 nucleotides.

The nucleic acid sequence hybridizing to a fragment of the sequence according to SEQ ID No.5 under stringent conditions has a length of at least 200, 300, 400, 500 or 600 nucleotides, preferably of at least 700, 800, 900 or 1000 nucleotides, more preferably of at least 1100, 1200, 1300 or 1400 nucleotides and most preferably of 1500, 1600, 1700 or 1800 nucleotides.

The nucleic acid sequence hybridizing to a fragment of the sequence according to any of SEQ ID Nos.6, 7, 9 and 10 under stringent conditions has a length of at least 25, 50, 75 or 100 nucleotides, preferably of at least 150, 200 or 250 nucleotides, more preferably of at least 300, 325, 350 or 375 nucleotides and most preferably of 400, 425 or 450 nucleotides.

The expression of proteins other than the cellulose synthase-like proteins, preferably the cellulose synthase-like protein according to any of SEQ ID Nos. 1-7, 9 and 10, is reduced by less than 10% or 8%, preferably by less than 7, 6 or 5%, more preferably by less than 4, 3 or 2% and most preferably by less than 1%.

In order to produce RNAi constructs, the corresponding sense or antisense nucleic acid sequences for example may be inserted into an appropriate vector by restriction digestion and subsequent ligation.

Alternatively, the corresponding sense or antisense nucleic acid sequences for example may be inserted into the vector by homologous recombination, such as by the GATEWAY® system (Invitrogen) or the BD Creator™ system (BD Biosciences Clontech Co.).

Within the scope of the present invention, the term “expression construct” means a nucleic acid molecule which contains all elements which are necessary for the expression of a nucleic acid sequence, i.e. the nucleic acid sequence to be expressed under the control of a suitable promoter and optionally further regulatory sequences such as termination sequences. An expression cassette of the present invention may be part of an expression vector which is transferred into a plant cell or may be integrated into the chromosome of a transgenic plant after transformation.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked and may be used herein interchangeably with the term "recombinant nucleic acid molecule". One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. A vector can be a binary vector or a T-DNA that comprises aleft border and a right border and that may include a gene of interest in between. The term "expression vector" means a vector capable of directing expression of a particular nucleotide sequence in an appropriate host cell. An expression vector comprises a regulatory nucleic acid element operably linked to a nucleic acid of interest, which is - optionally - operably linked to a termination signal and/or other regulatory element.

The term "promoter" as used herein refers to a DNA sequence which, when ligated to a nucleotide sequence of interest, is capable of controlling the transcription of the nucleotide sequence of interest into mRNA. A promoter is typically, though not necessarily, located 5' (e.g., upstream) of a nucleotide sequence of interest (e.g., proximal to the transcriptional start site of a structural gene) whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription.

The promoter used in the present invention may be a constitutive promoter, an inducible promoter or a tissue-specific promoter.

Constitutive promoters, include the 35S CaMV promoter (Franck et al. (1980) Cell 21: 285-294), the ubiquitin promoter (Binet *et al.* (1991) Plant Science 79: 87-94), the Nos promoter (An G. et al. (1990) The Plant Cell 3: 225-233), the MAS promoter (Velten et al. (1984) EMBO J. 3: 2723-230), the maize H3 histone promoter (Lepetit et al. (1992) MoI Gen. Genet 231: 276-285), the ALS promoter (WO 96/30530), the 19S CaMV promoter (US 5,352,605), the super-promoter (US 5,955,646), the figwort mosaic virus promoter (US 6,051 ,753), the Rubisco small subunit promoter (US 4,962,028) and the actin promoter (McElroy *et al.* (1990) Plant Cell 2: 163-171).

In another embodiment, the promoter is a regulated promoter. A "regulated promoter" refers to a promoter that directs gene expression not constitutively, but in a temporally and/or spatially restricted manner, and includes both tissue-specific and inducible promoters. Different promoters may direct the expression of a polynucleotide or regulatory element in different tissues or cell types, or at different stages of development, or in response to different environmental conditions.

Wound-, light- or pathogen-induced promoters and other development-dependent promoters or control sequences may also be used (Xu *et al.* (1993) Plant Mol. Biol. 22: 573-588; Logemann *et al.* (1989) Plant Cell 1: 151-158; Stockhaus *et al.* (1989) Plant Cell 1: 805-813; Puente *et al.* (1996) EMBO J. 15: 3732-3734; Gough *et al.* (1995) Mol. Gen. Genet. 247: 323-337). A summary of useable control sequences may be found, for example, in Zuo *et al.* (2000) Curr. Opin. Biotech. 11: 146-151.

A "tissue-specific promoter" or "tissue-preferred promoter" refers to a regulated promoter that is not expressed in all plant cells but only in one or more cell types in specific organs (such as leaves or seeds), specific tissues (such as embryo or cotyledon), or specific cell types (such as leaf parenchyma or seed storage cells).

Suitable tissue-specific promoters include, e.g., epidermis-specific promoters, such as the GSTA1 promoter (Altpeter *et al.* (2005) Plant Mol Biol. 57: 271-83), or promoters of photosynthetically active tissues, such as the ST-LS1 promoter (Stockhaus *et al.* (1987) Proc. Natl. Acad. Sci. USA 84: 7943-7947; Stockhaus *et al.* (1989) EMBO J. 8: 2445-2451). The promoters of phosphoenolpyruvate-carboxylase from corn (Hudspeth *et al.* (1989) Plant Mol. Biol. 12:579) or of fructose-1,6-bisphosphatase from potato (WO 98/18940), which impart leaf-specific expression, are also considered as tissue-specific promoters. Further preferred promoters are those which are in particular active in fruits. Examples of these are the promoter of a polygalacturonase gene, e. g. from tomato, which mediates expression during the ripening process of tomato fruits (Nicholass *et al.* (1995) Plant Mol. Biol. 28: 423-435), the promoter of an ACC oxidase, e.g. from apples, which mediates ripening and fruit specificity in transgenic tomatoes (Atkinson *et al.* (1998) Plant Mol. Biol. 38: 449-460), or the 2A11 promoter from tomato (van Haaren *et al.* (1991) Plant Mol. Biol. 17: 615-630). Further, the chemically inducible Tet repressor system (Gatz *et al.* (1991) Mol. Gen. Genet. 227: 229-237) may also be used.

Other suitable promoters may be taken from the literature, e.g. Ward (1993, Plant Mol. Biol. 22: 361-366). The same applies to inducible and cell- or tissue-specific promoters, such as meristem-specific promoters which have also been described in the literature and which are suitable within the scope of the present invention as well.

Particularly suitable promoters for the method of the present invention are pathogen-inducible promoters, and especially those, which are induced by pathogenic fungi and not by useful fungi (e.g. mycorrhiza in the soil, such as the GER4 promoter (WO 2006/128882).

Further promoters which are inducible by fungi include promoters such as the GAFP-2 promoter (Sa *et al.* (2003) Plant Cell Rep. 22: 79-84), which, e.g., is

induced by the fungus *Trichoderma viride*, or the PAL promoter which is induced by inoculation with *Pyricularia oryzae* (Wang *et al.* (2004) Plant Cell Rep. 22: 513-518).

Also particularly suitable in the method of the present invention are promoters which are active on the site of pathogen entry, such as epidermis-specific promoters. Suitable epidermis-specific promoters include, but are not limited to, the GSTA1 promoter (Accession number X56012), the GLP4 promoter (Wei *et al.* (1998) Plant Mol. Biol. 36: 101), the GLP2a promoter (Accession number AJ237942), the Prx7 promoter (Kristensen *et al.* (2001) Mol. Plant Pathol. 2(6): 311), the GerA promoter (Wu *et al.* (2000) Plant Phys Biochem. 38: 685), the OsROC1 promoter (Accession number AP004656), the RTBV promoter (Kloeti *et al.* (1999) PMB 40: 249); the chitinase ChtC2 promoter (Ancillo *et al.* (2003) Planta 217(4): 566), the AtProT3 promoter (Grallath *et al.* (2005) Plant Physiol. 137(1): 117) and the SHN promoters from Arabidopsis (Aaron *et al.* (2004) Plant Cell 16(9): 2463).

Furthermore, those skilled in the art are able to isolate further suitable promoters by means of routine procedures.

The skilled person knows that the use of inducible promoters allows for the production of plants and plant cells which only transiently express the sequences of the present invention, and thus silence transiently. Such transient expression allows for the production of plants which show only transiently increased pathogen resistance. Such transiently increased resistance may be desired, if, for example, there is an acute risk of fungal contamination, and therefore the plants only have to be resistant to the fungus for a certain period of time. Further situations, in which transient resistance is desirable, are known to those skilled in the art. The skilled person also knows that transient expression and thus transient silencing and transient resistance may be achieved using vectors which do not replicate stably in plant cells and which carry the respective sequences for silencing of fungal genes.

In a preferred embodiment of the method of the invention, the actin promoter from *Oryza sativa* is used to express a nucleic acid sequence of the present invention.

The vectors which are used in the method of the present invention may further comprise regulatory elements in addition to the nucleic acid sequence to be transferred. Which specific regulatory elements must be included in said vectors depends on the procedure which is to be used for said vectors. Those skilled in the art, who are familiar with the various methods for producing transgenic plants in which the expression of a protein is inhibited know which regulatory elements and also other elements said vectors must include.

Typically, the regulatory elements which are contained in the vectors ensure the transcription and, if desired, the translation in the plant cell.

The term "transcription regulatory element" as used herein refers to a polynucleotide that is capable of regulating the transcription of an operably linked polynucleotide. It includes, but is not limited to, promoters, enhancers, introns, 5' UTRs, and 3' UTRs.

With respect to nucleic acid sequences or DNA sections in vectors or expression constructs the terms "operably linked" and "operatively linked" mean that nucleic acid sequences are linked to each other such that the function of one nucleic acid sequence is influenced by the other nucleic acid sequence. For example, if a nucleic acid sequence is operably linked to a promoter, its expression is influenced by said promoter.

So-called termination sequences are sequences which ensure that the transcription or the translation is terminated properly. If the introduced nucleic acids are to be translated, said nucleic acids are typically stop codons and corresponding regulatory

sequences; if the introduced nucleic acids are only to be transcribed, said nucleic acids are normally poly-A sequences.

The vectors of the present invention may for example also comprise enhancer elements as regulatory elements, resistance genes, replication signals and further DNA regions which allow for a propagation of the vectors in bacteria, such as *E.coli*. Regulatory elements also comprise sequences which lead to a stabilization of the vectors in the host cells. In particular, such regulatory elements comprise sequences which enable a stable integration of said vector in the host genome of the plant or autonomous replication of said vector in the plant cells. Such regulatory elements are known to those skilled in the art.

A number of well-known techniques are available for introducing DNA into a plant host cell, and those skilled in the art may easily determine the suitable technique for each case. Said techniques comprise the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation means, viral infection by using viral vectors (EP 0 067 553; US 4,407,956, WO 95/34668; WO 93/03161), the fusion of protoplasts, polyethylene glycol-induced DNA uptake, liposome-mediated transformation (US 4,536,475), incubation of dry embryos in DNA-comprising solution, microinjection, the direct gene transfer of isolated DNA in protoplasts, the electroporation of DNA, the introduction of DNA by the biolistic procedure, as well as other possibilities. Thereby, stable as well as transient transformants may be produced.

For injection and electroporation of DNA in plant cells, the used plasmids do not need to fulfil special requirements per se. The same applies to direct gene transfer. Simple plasmids, such as pUC derivatives, may be used. If, however, whole plants are to be regenerated from cells which were transformed in such a manner, the presence of a selectable marker gene may become necessary. Those skilled in the art know all commonly used selection markers, and thus there is no difficulty to select a

suitable marker. Common selection markers create resistance in the transformed plant cells to a biocide or antibiotic, such as kanamycin, G418, bleomycin, hygromycin, methotrexate, glyphosate, streptomycin, sulfonyl urea, gentamycin or phosphinotricin and the like or may confer tolerance to D-amino acids such as D-alanine. However, it is also possible to select transformed cells by PCR, i.e. without the use of selection markers.

Depending on the introduction method of the desired genes into the plant cell, further DNA sequences may become necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, at least the right border, or very often both, the right and the left border of the T-DNA contained in the Ti and Ri plasmid needs to be linked to the genes to be inserted.

If agrobacteria are used for the transformation, the DNA to be inserted needs to be cloned into special plasmids, i.e. either into an intermediate vector or into a binary vector. The intermediate vectors may be integrated into the Ti or Ri plasmid of the agrobacteria by means of homologous recombination due to sequences which are homologous to sequences in the T-DNA, which contains the vir region required for the transfer of the T-DNA. Intermediate vectors are not able to replicate in agrobacteria. By means of a helper plasmid, the intermediate vector may be transferred to *Agrobacterium tumefaciens* (conjugation). Binary vectors are able to replicate in both *E. coli* and in agrobacteria. Said vectors contain a selection marker gene and a linker or polylinker located between the right and left T-DNA border region. The vector may be transformed directly into the agrobacteria (Holsters *et al.* (1978) Molecular and General Genetics 163: 181-187). The agrobacterium, serving as host cell, is to contain a plasmid which includes a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. In addition, T-DNA may be present. The agrobacterium, transformed in such a manner, is used for the transformation of plant cells.

For the transfer of the DNA into the plant cell, plant explants may be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. From the infected plant material (e.g. leaf cuttings, stem sections, roots, but also protoplasts or suspension-cultivated plant cells) whole plants may be regenerated in a suitable medium which may contain antibiotics, biocides or D-amino acids for the selection of transformed cells, if a selection marker was used in the transformation. The regeneration of the plants is performed according to standard regeneration procedures using well-known culture media. The plants or plant cells obtained this way may then be examined for the presence of the introduced DNA.

Other possibilities for introducing foreign DNA using the biolistic method or by protoplast transformation are well-known to those skilled in the art (see L. Willmitzer (1993) *Transgenic Plants in: Biotechnology, A Multi-Volume Comprehensive Treatise* (publisher: H.J. Rehm *et al.*), volume 2, 627 - 659, VCH Weinheim, Germany).

Monocotyledonous plants or the cells thereof may also be transformed using vectors which are based on agrobacteria (see e.g. Chan *et al.* (1993) Plant Mol. Biol. 22: 491-506). Alternative systems for the transformation of monocotyledonous plants or the cells thereof are transformation by the biolistic approach (Wan and Lemaux (1994) Plant Physiol. 104: 37-48; Vasil *et al.* (1993) Bio/Technology 11: 1553-1558; Ritala *et al.* (1994) Plant Mol. Biol. 24: 317-325; Spencer *et al.* (1990) Theor. Appl. Genet. 79: 625-631), the protoplast transformation, the electroporation of partially permeabilized cells, and the insertion of DNA by means of glass fibres.

The vectors described herein can be directly transformed into the plastid genome. Plastid expression, in which genes are inserted by homologous recombination into the several thousand copies of the circular plastid genome present in each plant cell, takes advantage of the enormous copy number over nuclear-expressed genes to permit high expression levels. In one embodiment, the nucleotides are inserted into a

plastid targeting vector and transformed into the plastid genome of a desired plant host. Plants homoplasmic for plastid genomes containing the nucleotide sequences are obtained, and are preferentially capable of high expression of the nucleotides.

Plastid transformation technology is for example extensively described in U.S. Pat. NOs. 5,451,513; 5,545,817; 5,545,818, and 5,877,462 in WO 95/16783 and WO 97/32977, and in McBride et al. (1994) Proc. Natl. Acad. Sci. USA 91: 7301 - 7305.

The method of producing a transgenic plant cell, a transgenic plant or a transgenic part thereof may further comprise the step of selecting a plant cell, a plant or part thereof having an increased resistance to pathogens. As discussed above, the increased pathogen resistance can be determined in comparison with a corresponding control plant cell or plant. For example, both the transgenic plant and a control plant can be infected with a pathogen as described in the Examples section below and the diseased leaf area can be scored. In the transgenic plants the diseased leaf area is less than in the control plants.

The transformed cells grow within the plant in the usual manner (see also McCormick *et al.* (1986) Plant Cell Reports 5: 81-84). The resulting plants may be cultivated in the usual manner, and may be crossed with plants which have the same transformed genes or other genes. The hybrid individuals resulting therefrom have the respective phenotypical properties.

According to common procedures, transgenic lines which are homozygous for the introduced nucleic acid molecules may also be identified and examined with respect to pathogen resistance compared to the pathogen resistance of hemizygous lines. Hence, the method of the present invention may further comprise the step of crossing the transgenic plant produced by the method of the present invention with another plant in which the content and/or the activity of the cellulose synthase-like protein is

not reduced and selecting transgenic progeny in which the content and/or the activity of the cellulose synthase-like protein is reduced. The other plant in which the content and/or the activity of the cellulose synthase-like protein is not reduced is preferably from the same species as the transgenic plant and may be a wild-type plant, i.e. a plant which does not contain any transgenic nucleic acid sequence, or it may be a transgenic plant which contains a transgenic nucleic acid sequence other than the nucleic acid sequences disclosed herein, e.g. a transgenic nucleic acid sequence coding for another protein involved in pathogen resistance or a protein conferring resistance to abiotic stress. The other plant is preferably an elite variety which is characterized by at least one favourable agronomic property which is stably present in said elite variety. Methods for determining whether the content and/or activity of the cellulose synthase-like protein is reduced are discussed above. An "elite variety" within the meaning of the present invention is a variety which is adapted to specific environmental conditions and/or which displays at least one superior characteristic such as an increased yield compared to non-elite varieties.

The transgenic progeny of the above crossing step can be further crossed with each other to produce true breeding lines. For this purpose the transgenic progeny of the above cross in which the content and/or the activity of the cellulose synthase-like protein is decreased is inbred, i.e. crossed among each other, and the transgenic progeny of this crossing step is selected and again inbred. This inbreeding step is repeated until a true breeding line is established, for example at least five times, six times or seven times. A "true breeding plant" or "inbred plant" is a plant for a particular trait which upon self-pollination or crossing with another true breeding or inbred plant for the same trait produces only offspring which is identical to the parent with respect to the at least one trait, in the present case the transgene which decreases the content of the RNA of the cellulose synthase-like protein. Hence, true breeding plants are homozygous for the specific trait.

The true breeding lines can then be used in hybrid breeding yielding F1 hybrids which can be marketed. This method is particularly suitable for example for maize and rice plants.

Alternatively, the true breeding lines can be further inbred in a linebreeding process. This method is particularly suitable for example for wheat and barley plants.

Of course, plant cells which contain the recombinant nucleic acid molecules of the present invention may also be further cultivated as plant cells (including protoplasts, calli, suspension cultures and the like).

The method of the present invention may additionally comprise the reduction of the content and/or the activity of at least one, for example two or three, other plant proteins which mediate pathogen susceptibility. Suitable genes include the Mlo gene (WO 00/01722), the Bax inhibitor-1 gene (Eichmann et al. (2010) Mol. Plant Microbe Interact. 23(9): 1217-1227) and the Pmr genes (Vogel and Somerville (2000) Proc. Natl. Acad. Sci. USA 97(4): 1897-1902).

Within the present invention, the method of producing a transgenic plant cell, a transgenic plant or a transgenic plant part having an increased resistance to pathogens is equivalent with a method of increasing pathogen resistance in a transgenic plant cell, transgenic plant or transgenic part thereof. Hence, the method of increasing pathogen resistance in a transgenic plant cell, a transgenic plant or transgenic part thereof comprises the same steps as the method of producing a transgenic plant cell, a transgenic plant or a transgenic plant part thereof having an increased resistance to pathogens as discussed herein.

The transgenic or mutant plants of the present invention or parts thereof can be used as fodder plants or for producing feed. Fodder is intended to mean any agricultural foodstuff which is specifically used to feed domesticated animals such as cattle,

goats, sheep and horses. It includes hay, straw, silage and also sprouted grains and legumes. The person skilled in the art knows that it may be necessary to treat the transgenic plants of the present invention to make them suitable for use as fodder. The term feed is intended to mean a dry feed which can be blended from various raw materials and additives such as soybean shred or barley shred in a feed mill.

The transgenic or mutant seed of the transgenic or mutant plants of the present invention can be used to prepare flour, in particular if the transgenic or mutant plants are monocotyledonous plants such as barley or wheat. The flour prepared from the transgenic seed of the present invention can be distinguished from the flour prepared from other plants by the presence of the transgenic nucleic acid sequence, the expression construct or the vector of the present invention. For example, if the transgenic nucleic acid sequence is expressed under the control of a promoter which is not endogenous to the transgenic plant, the presence of the promoter can be detected in the flour prepared from the transgenic seed. Alternatively or additionally, if an antisense sequence is part of the transgene mediating the reduction of the content and/or the activity of the cellulose synthase-like protein, the presence of this antisense sequence can be detected in the flour prepared from the transgenic seed. The flour prepared from the mutant seed of the present invention can be distinguished from the flour prepared from other plants by the presence of the at least one point mutation within the nucleic acid sequence defined herein.

The identification of cellulose synthase-like proteins as proteins involved in pathogen resistance and the use thereof for producing transgenic plants with increased pathogen resistance will be described in the following. The following examples shall not limit the scope of the present invention. The content of all literature references, patent applications, patent specifications and patent publications, which are cited in this patent application, is incorporated herein by reference.

## EXAMPLES

### 1) A practical protocol for construction of a RNAi library

A schematic overview of the steps for RNAi library construction is shown in Figure 1.

#### 1.1. Entry vector (pIPKTA38) preparation

The pIPKTA38 plasmid (Douchkov et al. (2005) Mol. Plant Microbe Interact. 18(8): 755-761) with a kanamycvin resistance gene was used as Gateway Entry vector.

Bacteria which contained the plasmid were grown in LB medium containing 50 µg/mL kanamycin. Plasmid DNA was prepared with the *Jetstar* midi DNA kit (Genomed).

As a control the plasmid was digested with the restriction enzyme *Apa*I, yielding bands of 1257 bp and 1054 bp. Then the DNA concentration was measured and adjusted to 150 ng/µL.

#### 1.2. Destination vector (pIPKTA30) preparation

The pIPKTA30 plasmid (Douchkov et al. (2005) Mol. Plant Microbe Interact. 18(8): 755-761) was used as the RNAi vector. It contains an ampicillin resistance gene, a *ccdB* negative selection marker gene which requires the propagation of the plasmid in DB3.1 cells and a chloramphenicol resistance gene.

Plasmid DNA was prepared with the *Jetstar* midi DNA kit (Genomed). The plasmid preparations were digested as a control with *Eco*RI (correct bands - 687, 1007, 2641,

and 2857 bp) or *Sal* I (601, 1589, and 5002 bp). The DNA concentration was measured and adjusted to 150 ng/µL.

1.3. PCR amplification of the cDNA clones

"Master mixes" for 20-µL PCR reactions with two specific EST-primers
Specific primers (SEQ ID Nos. 16 and 17 for CSLH1 and SEQ ID Nos. 18 and 19 for CSLC9) were designed to amplify ~500 bp fragments from the EST clone. *Tm* of the primers is ~65°C.

A "PCR master mix" (see Table 6) was prepared of which 11,0 µL were dispensed to each well of a 96-well PCR plate. 4.25 µL of each EST-specific primer and 0,5 µL EST DNA as template were added to each well.

Table 6: "PCR master mix" content (11 µL "Master mix" + 4.25 µL of each specific primer + 0,5 µL template per reaction):

| Component | For 96 samples | For 2 plates | For 4 plates |
|---|---|---|---|
| Thermal Ace buffer (10x) | 200 µL | 400 µL | 800 µL |
| dNTPs (50x, 10 mM each) | 50 µL | 100 µL | 200 µL |
| Thermal Ace DNA Polymerase (2U/µL) | 25 µL | 50 µL | 100 µL |
| Water | 825 µL | 1650 µL | 3300 µL |
| | Dispense 11,0 µL PCR master mix per well | | |
| Target-specific sense primer 1 (1 µM) | 4,25 µL per well | | |

| Target-specific antisense primer 2 (1 µM) | 4,25 µL per well |
|---|---|
| Template (EST clone DNA) | 0,5 µL per well |

PCR cycle conditions

| | | |
|---|---|---|
| *95 °C* | *3 min* | |
| *95 °C* | *30 sec* | *30 cycles* |
| *65 °C* | *30 sec* | |
| *74 °C* | *60 sec* | |
| *74 °C* | *10 min* | |
| *4 °C* | *Pause* | |

Purification of the PCR product

30 µL $H_2O$ were added to each PCR reaction to obtain 50-µL volumes followed by purification using the Qiagen *MinElute UF 96-well* kit. The PCR product was eluted with 20 µL $H_2O$ (according to Qiagen's *MinElute* protocol). As a control, 2 µL each of the purified PCR product were separated by agarose gel electrophoresis.

1.4. Cloning of the PCR products

A ligation master mix was prepared (see Table 7), 6 µL each of this ligation mix were added to each well and 4 µL of the purified PCR product were added.

The samples were incubated for 1 hour at 25 °C and the reaction was then stopped by heating up to 65 °C for 10 minutes. 5 µL *Swa* I master mix (see Table 8) was added to each well, followed by incubation at 25 °C for 1 hour. Next, the ligation samples were transformed into competent bacteria and suitable clones were isolated after miniprep and control digestion using *Eco*RI.

Table 7: Ligation Master mix for 10-µL reactions (6 µL master mix + 4 µL PCR product per reaction)

| Components | For 96 samples | For 2 plates | For 4 plates |
|---|---|---|---|
| $H_2O$ | 100 µL | 200 µL | 400 µL |
| pIPKTA38 (150 ng/µL) | 100 µL | 200 µL | 400 µL |
| Ligation buffer (10x) | 100 µL | 200 µL | 400 µL |
| 50% PEG 4000 | 100 µL | 200 µL | 400 µL |
| NaCl (0,5 M) | 100 µL | 200 µL | 400 µL |
| *Swa*I (10 U/µL) (New England Biolabs) | 50 µL | 100 µL | 200 µL |
| T4 DNA ligase (5 U/µL) (Fermentas) | 50 µL | 100 µL | 200 µL |
| PCR product (purified) | 4 µL per reaction | | |

Table 8: *Swa*I Master mix (add 5 µL per reaction)

| Components | For 96 samples | For 2 plates | For 4 plates |
|---|---|---|---|
| *Swa*I buffer (10x) | 50 µL | 100 µL | 200 µL |
| NaCl (0,5 M) | 100 µL | 200 µL | 400 µL |
| $H_2O$ | 300 µL | 600 µL | 1200 µL |
| *Swa*I (10 U/µL) | 50 µL | 100 µL | 200 µL |

1.5. LR Reaction

Master mixes for 6 µL LR reactions

Table 9: LR-master mix (5 µL master mix + 1 µL pIPKTA38::EST DNA per reaction)

| Component | For 96 samples | For 2 plates | For 4 plates |
|---|---|---|---|
| LR Buffer (5x) | 120 µL | 240 µL | 480 µL |
| pIPKTA30 (150 ng/µL) | 100 µL | 200 µL | 400 µL |
| $H_20$ | 200 µL | 400 µL | 800 µL |
| LR Clonase Mix (Invitrogen) | 80 µL | 160 µL | 320 µL |
| | Dispense 5 µL Master mix per well | | |
| pIPKTA38::EST DNA | 1 µL per reaction | | |

Incubation at room temperature over night (or at least 6 h).

## 2. Trans*Gen* test and TIGS protocol

Figure 2 shows a schematic drawing of the test procedure for the RNAi constructs.

2.1. Preparation of the plant material

Barley was cultivated in IPK cereal soil for 7 days without fertilization in a Sanyo phyto-cultivator, at constantly 20 °C, 60 - 70 % relative humidity and a 16 h light-cycle. Primary leaves (about 7 cm) were cut off and were arranged in parallel on a phytoagar Petri dish (adaxial side up). Thereby, magnetic stirrers were put onto the leaves such that they repel each other.

2.2. Coating of gold particles with DNA or RNA

7 µL (= 7 µg) DNA (plasmid) of the gene to be tested and 7 µl of the vector pUbiGUS containing the GUS reporter gene were used per bombardment. A Bio-Rad Hepta-Adaptor (7 macro carrier slides) was used.

Per bombardment, 87.5 µL 1 M $Ca(NO_3)_2$ pH 10 were added drop wise to 87.5 µL coating suspension (gold particles, 25 mg/mL in 50 % of glycerol; storage at 4 °C) while vortexing. The particle suspension was left for 10 min at room temperature and was occasionally tipped. The suspension was centrifuged (15 sec, 14000 rpm) and the supernatant was removed with a pipette and discarded. The pellet was washed with 1 ml ethanol, and the ethanol was removed with a pipette. Then, the pellet was resuspended in 30 µL ethanol (absolute).

2.3. Coating of macro carrier

Tensile disks and macro carriers were placed in ethanol (absolute) for 30 sec, subsequently dried at room temperature, and placed in the macro carrier holder using a pipette. The tube containing the coating suspension (DNA/particle mixture) was placed in an ultrasonic bath for 10 sec, and then the coating suspension was mixed with a pipette. 3 µL of the coating suspension were applied to each macro carrier and the suspension was left to dry for 2 to 5 min.

2.4. Biolistic transformation

Leaves and macro carrier holder with the treated macro carriers as well as grids (Hepta Stop Screen) were placed in the chamber for biolistic transformation. Vacuum was applied for biolistic transformation, wherein the bombardment was made at a pressure of 27.5 mm Hg.

2.5. Incubation and inoculation of the leaves with mildew

Bombarded leaves were first incubated for 4 h in slightly opened Petri dishes. Then, the leaves were transferred in large, square Petri dishes containing 1 % w/v phytoagar with 20 ppm of benzimidazole. Thereby, leaves of all preparations were mixed. For inoculation, open Petri dishes were put in dishes with nylon nets (100 µm mesh width) stretched thereover. The leaves were inoculated with mildew (about 200 conidia/mm$^2$). For inoculation, conidia as fresh as possible were used, i.e. either from older plants, which were shaken 24 h - 48 h prior to inoculation, or from fresh plants, which had been inoculated seven days before. The dishes were then placed in the incubation chamber.

2.6. GUS staining (for staining the transformed cells)

40 h after inoculation, the leaves were collected, the leaf tips were cut off and the resulting leaves were transferred to Greiner tubes containing 10 mL of X-glucose solution (100 mM sodium phosphate , pH 7,0; 10 mM sodium EDTA; 1,4 mM K-hexacyanoferrate(II); 1,4 mM K-hexacyanoferrate(III); 0,1% Triton X-100; 20% methanol and 1 mg / ml X-Gluc). The tubes were placed in a suction bottle and vacuum was applied thereto 2 - 3 times. The infiltration is complete when the leaves become transparent and start to sink. The X-glucose solution was refilled to 14 mL and the tubes were sealed. The tubes were incubated over night at 37 °C in the incubator.

2.7. TCA destaining

The leaves were placed in destaining solution (7.5 % TCA, 50 % methanol) for 5 min. Then the leaves were washed with distilled water. Then, the leaves were carefully removed from the tube and were placed onto an object slide with their adaxial side facing upwards.

200µl of distilled water were added to each object slide and the cover glass was carefully applied. The GUS-staining and the fungal structures were then analyzed in the microscope.

## 3. Experiment for primary data acquisition

The effect of the RNAi constructs on plant resistance to the fungal pathogen *Blumeria graminis* was tested in transient experiments.

The barley plants used (*Hordeum vulgare*, cultivar, '*Golden promise*') were cultivated in soil without fertilisation in a phyto-cultivator (20 °C, 70 % rel. humidity). On the day of bombardment, plants were 7 days old. The primary leaves were cut off, placed on 0.5 % phytoagar with 200 ppm benzimidazole and bombarded with 2.2 mg of gold particles, which were coated with a mixture of 7 µg reporter gene vector (pUbiGUS) and 7 µg of a control vector PIPKTA30 or of an RNAi construct. The leaves were stored in closed Petri dishes at 20 °C until inoculation.

Three days after bombardment, the leaves were transferred to 1 % phytoagar with 2 % benzimidazole. A nylon net (mesh width of 200 µm) was stretched over the leaves, and they were inoculated with a conidia density of about 200 conidia/mm$^2$. The conidia (from the pathogen *Blumeria graminis hordei*) originated from barley plants (cultivar ‚*Golden Promise*'), which had been inoculated 6 - 7 days before. Until GUS staining, the leaves were stored in closed Petri dishes with holes for ventilation at 20 °C at a north-facing window.

About 45 h after inoculation, GUS staining was performed. Said staining was stopped after 24 h by incubation in 7.5 % trichloroacetic acid, 50 % v/v methanol, and the leaves were bleached.

Each experiment contained 3 parallel bombardments to 7 leaf sections each of the negative control (empty vector pIPKTA30N). Further, each experiment contained 2 parallel bombardments of the positive control pIPKTA36, which causes resistance by inhibiting the *Mlo* gene of barley. Data per experiment are based on the comparison of the effect of the test constructs with the average value of the 3 negative controls of the respective experiment.

Table 10 shows the relative susceptibility index (Rel. SI) of barley cells transiently transformed with an RNAi construct inhibiting the expression of cellulose synthase-like protein H1 and C9, respectively. The susceptibility index relative to the empty vector control was determined in five independent transformation experiments. As the cells transformed with the RNAi construct have a susceptibility index of less than 100% compared to the control cells transformed with empty vector (pIPKTA30N), the RNAi construct suppresses putative susceptibility genes in barley.

Table 10

| Gene | Exp.1 | Exp.2 | Exp.3 | Exp.4 | Exp.5 | Mean | SD |
|---|---|---|---|---|---|---|---|
| HvCSLH1 | 34,87 | 14,84 | 10,60 | 35,07 | 52,15 | 29,50 | 16,92 |
| HvCSLC9 | 18,70 | 31,77 | 13,47 | 38,81 | 87,76 | 38,10 | 29,54 |

Figure 3 graphically illustrates the results shown in Table 10.

## 4. Cloning of overexpression vector constructs for stable stransformation

Based on the HvCSLC9 and HvCSLH1 sequences from barley the corresponding sequences in wheat were identified using BLAST search (Altschul, S. F., *et al.* (1990) Journal of Molecular Biology 215: 403–410) (TaCSLH1: SEQ ID No. 3 and TaCSLC9: SEQ ID No. 5). Fragments of these sequences are used to select a specific target sequence of 200 – 500 bp length (TaCSLH1: SEQ ID No. 7 and TaCSLC9: SEQ ID No. 10). This target sequence is used to generate an antisense:rga1-

intron:sense hairpin RNAi repeat cassettes (TaCSLH1: SEQ ID NO. 8 and TaCSLC9: SEQ ID No. 11).

The hairpin RNAi repeat cassettes are generated by DNA synthesis in a way that an AttB1 recombination site (Gateway system, Invitrogen, Life Technologies, Carlsbad, California, USA) and an OsAct1D promoter is located 5' of the inserted gene fragment and a cauliflower mosaic virus 35S terminator as well as an AttB2 recombination site is located 3' of the inserted CSLC9 or CSLH1 hairpin RNAi repeat. Via a BP recombination reaction the gene is inserted into a GATEWAY pENTRY vector (Gateway system, Invitrogen, Life Technologies, Carlsbad, California, USA).

To obtain the binary plant transformation vector, a LR reaction (Gateway system, Invitrogen, Life Technologies, Carlsbad, California, USA) is performed according to manufacturer's protocol by using the above described pENTRY vector containing the CSLC9 or CSLH1 RNAi fragment.

As target a binary pDEST vector is used which is composed of: an adenyltransferase cassette conferring spectinomycin resistance for bacterial selection, a pVS1 origin for replication in Agrobacteria, a pBR322 origin of replication for stable maintenance in *E. coli* and a gene coding for D-amino acid oxidase (GenBank U60066) as D-aminoacid tolerance marker under control of a ZmUbi-promoter as D-aminoacid tolerance marker between the right and left border. The recombination reaction is transformed into *E. coli* (DH5alpha), mini-prepped and screened by specific restriction digestions. A positive clone from each vector construct is sequenced and submitted to wheat transformation. (TaCSLH1: Figure 4 and TaCSLC9: Figure 5, sequences: TaCSLH1: SEQ ID No. 12 and TaCSLC9: SEQ ID No. 13).

## 5. **Wheat transformation**

### 5.1. Plant Material and Surface Sterilisation

A comprehensive discussion about wheat transformation methods and a protocol for the *Agrobacterium*-mediated transformation of wheat can be found in Jones et al. (2005) Plant Methods 1: 5.

Immature embryos (IEs) from *Triticum aestivum* (variety 'Bobwhite') are used as explant for *Agrobacterium*-mediated transformation.
Donor plants are grown at 18–20°C day and 14–16°C night temperatures under a 16 h photoperiod (500 – 1000 µmolm-2s-1 photosynthetically active radiation (PAR)) with relative air humidity of 50–70% for approximately 8 to 11 weeks.

The optimal harvesting time is 12-20 days post-anthesis. For transformation IEs should be 0.8 – 1.5 mm in length and translucent in appearance. Donor plants used for harvesting should be at peak vigour to ensure optimal transformation and regeneration frequencies.

Immature seeds are surface sterilized by rinsing them 30-60 sec. in 70% (v/v) aqueous ethanol followed by 15 minutes 10% (v/v) Domestos bleach solution (Lever) gentle shaking. Then the immature seeds are rinsed 3-4 times with sterile distilled water and transferred to a sterile Petri dish, avoiding extreme dehydration. Immature seeds are ready for use.

### 5.2. *Agrobacterium* culture

*Agrobacterium* cultures containing the vector harbouring a selectable marker (SM) cassette and the gene(s) of interests (GOI) described above are grown for 24-72 hours in a 28°C incubator on LB agar plates with appropriate selection.

To obtain a liquid *Agrobacterium* culture one colony is picked from a 1-3 days old plate and re-suspended in liquid medium (5 g mannitol, 1 g L-glutamic acid, 250 mg $KH_2PO_4$, 100 mg NaCl, 100 mg $MgSO_4$ x 7H2O, 5 g tryptone, 2.5 g yeast extract, pH 7.0, add after autoclave 1 µg Biotin incl. appropriate antibiotics). Liquid culture is grown at 28°C for ~16h to reach an $OD_{600}$ ~1. The *Agrobacterium* culture is centrifuged at 4.500 g for 10 minutes and resuspended in 4 ml inoculation medium (1/10 MS complete, 30g maltose, 100mg MES; adjusted to pH 5.8 and add after autoclave 0.01% Pluronic, 200µM acetosyringone to an $OD_{600}$ of ~1. The *Agrobacterium* inoculation medium is ready to use.

5.3. Isolation of immature embryos (IEs)

The IEs are isolated from the immature seed followed by removing and discarding the embryo axis. The IEs are directly transferred in the *Agrobacterium* inoculation culture.

5.4. Co-culture

Following isolation of immature embryos (IEs), the tube is vortexed at full speed for 10 seconds and IEs are allowed to settle in the solution for 30 - 60 minutes.

The *Agrobacterium* solution is removed and the IEs are placed on sterile Whatman filter paper #1 (4-5 pieces) to blot excess *Agrobacterium* solution. The top filter paper containing the IEs are transferred onto a plate containing approx. 20 ml of solidified co-culture media (1/10 MS,complete, 30g maltose , 0.69g proline, 100mg MES , 10g Agar , adjust to pH 5.8, add after autoclave, 4mg 2,4-D, 200µM acetosyringone, 100mg ascorbic acid). The plates are sealed with parafilm and incubated for 2-3 days at 24°C in the dark.

5.5. Callus Induction

Following co-culture, the explants are placed with the embryo axis facing down on recovery media (MS full complete, 30g maltose, 0.69g proline, 20mg thiamine, 1g casein hydrolysate, 100mg myo-inositol , 5µM $CuSO_4$ ; 2.4g NH4NO3, 1.95g MES, 8g agar (Plant TC), adjust to pH 5.8 and add after autoclave 2mg 2,4-D, 200mg timentin, 100mg ascorbic acid) for 4 weeks at 24°C in the dark. The calli are transferred to fresh recovery medium after two weeks.

5.6. Shoot Regeneration, Rooting and Selection

Calli are transferred to shoot regeneration medium (MS full complete, 30g maltose, 20mg thiamine, 100mg myo-inositol, 750mg glutamine, 5µM $CuSO_4$, 1.95g MES; 8g agar (Plant TC), adjust to pH 5.8 and add after autoclave, 0.5mg TDZ, 200mg timentin, 11mM D-alanine) and are cultivated under light conditions at 21-25°C for 3-4 weeks.

After shoot induction the explants are transferred to rooting media (½ MS complete, 30g sucrose, 7g agar and adjust to pH 5.8, add after autoclave, 0.5mg NAA, 200mg timentin, 11mM D-alanine) in 100x20 plates and are cultivated for 4-5 weeks at 21-25°C under light conditions..

Putative transgenic shoots that develop roots are planted out into a nursery soil mix consisting of peat and sand (1:1) and maintained at 22-24°C with elevated humidity (>70%) After two weeks, plants are removed from the humidity chamber and are further cultivated under greenhouse conditions.

## 6. Wheat Septoria screening assay

Transgenic plants are grown in the greenhouse at 19°C and 60-80% humidity. After 11 days plants are inoculated with *Septoria tritici* spores (1,3x$10^6$ Spores/ml in 0.1% Tween20 solution). Plants are incubated for 4 days at 19°C and 80-90% humidity under long day conditions (16h light). Plants are then grown for approx. 3 weeks at 19°C and 60-80% humidity under long day conditions.

The diseased leaf area is scored by eye by trained personal. The percentage of the leaf area showing fungal pycnidia or strong yellowing/browning is considered as diseased leaf area. Per experiment the diseased leaf area of 16 transgenic plants (and 16 WT plants as control) is scored. For analysis the average of the diseased leaf area of the non-transgenic mother plant is set to 100% to calculate the relative diseased leaf area of the transgenic lines.

The knock-down of cellulose synthase-like proteins will lead to enhanced resistance of wheat against Septoria

## 7. Wheat rust screening assay

Transgenic plants are grown in the phytochamber at 22°C and 75% humidity (16/8 h light/dark rhythm) for 2 weeks. The 2 weeks old plants are inoculated with wheat brown rust (*Puccinia triticina*) spores. Generally plants are inoculated with a 0.2% (w/v) spore suspension in HFE (Hydrofluoroether). Plants are incubated for 24 h in darkness under 100% humidity and 24°C. After the dark phase, plants are grown at 23°C, 75% humidity and a 16/8 hours light/dark rhythm.

Diseased leaf area is scored by eye by trained personal. The percentage of the leaf area showing fungal colonies or strong yellowing/browning is considered as diseased leaf area. Per experiment the diseased leaf area of 16 transgenic plants (and 16 WT

plants as control) is scored. For the analysis the average of the diseased leaf area of the non-transgenic mother plant is set to 100% to calculate the relative diseased leaf area of the transgenic lines.

The knock-down of cellulose synthase-like proteins will lead to enhanced resistance of wheat against rust fungi

## 8. Powdery mildew screening assay

Transgenic plants are grown in the phytochamber at 22°C and 75% humidity (16/8 h light/dark rhythm) for 2 weeks. The 2 weeks old plants are inoculated with spores of the powdery mildew fungus (*Blumeria graminis* f.sp. tritci). Generally inoculations with powdery mildew are performed with dry spores using an inoculation tower to a density of approx. 10 spores/mm². Plants are incubated for 7 days at 20°C, 75% humidity and a 16/8 hours light/dark rhythm.

Diseased leaf area is scored by eye by trained personal. The percentage of the leaf area showing white fungal colonies is considered as diseased leaf area. Per experiment the diseased leaf area of 16 transgenic plants (and 16 WT plants as control) is scored. For analysis the average of the diseased leaf area of the non-transgenic mother plant is set to 100% to calculate the relative diseased leaf area of the transgenic lines.

Knockdown of cellulose synthase-like proteins will lead to enhanced resistance of wheat to powdery mildew fungus.

## Claims

1. Method of producing a transgenic plant cell, a transgenic plant or a transgenic part thereof having an increased resistance to pathogens compared to a control plant cell, plant or plant part, wherein in the transgenic plant cell, the transgenic plant or the transgenic part thereof the content and/or activity of at least one cellulose synthase-like protein is reduced in comparison to the control plant cell, plant or plant part.

2. Method of claim 1, wherein the cellulose synthase-like protein is encoded by a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1 to 5 or a fragment of any of these sequences;
b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5.

3. Method of claim 1 or 2, comprising the steps of

(a) introducing into a plant cell a vector which comprises:

(i) a promoter functional in plant cells,

(ii) operatively linked thereto at least one nucleic acid sequence as defined in claim 2;

(iii) optionally a spacer sequence,

(iv) the nucleic acid sequence being reverse-complementary to the nucleic acid sequence of ii);

(v) a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operatively linked thereto a nucleic acid sequence which is complementary to a nucleic acid as defined in claim 2; and

(iii) optionally, a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operatively linked thereto a nucleic acid sequence which is identical to a nucleic acid as defined in claim 2;

(iii) optionally, a termination sequence;

or which comprises:

(i) a promoter functional in plant cells;

(ii) operably linked thereto a nucleic acid sequence encoding a precursor micro RNA sequence comprising a micro RNA sequence which targets the nucleic acid sequence as defined in claim 2;

and

(b) optionally, regenerating a transgenic plant from the transformed cell.

4. Method of claim 3, wherein the nucleic acid sequence in (ii) or (iv) is selected from the nucleic acid sequences according to any of SEQ ID Nos. 6, 7, 9 and 10 or a fragment of any of these sequences.

5. Method of claim 3 or 4, wherein the transgenic plant cell or plant is a wheat plant cell or plant and the nucleic acid sequence in (ii) or (iv) is selected from the group consisting of SEQ ID No. 7 and 10.

6. Method of claim 5, wherein the vector comprises:

(i) a promoter functional in plant cells;

(ii) a nucleic acid sequence selected from the group consisting of SEQ ID Nos. 8 and 11;

(iii) optionally, a termination sequence.

7. Method of claim 3 or 4, wherein the transgenic plant cell or plant is a barley plant cell or plant and the nucleic acid sequence in (ii) or (iv) is selected from the group consisting of SEQ ID No. 6 and 9.

8. Method of any of claims 3 to 7, wherein the promoter is a tissue-specific and/or a pathogen-inducible promoter.

9. Method of any of the preceding claims, further comprising reducing the content and/or activity of at least one other plant protein mediating pathogen susceptibility and/or increasing the content and/or activity of at least one protein which mediates pathogen resistance.

10. Method of any of the preceding claims, further comprising the step of crossing the transgenic plant produced by the method of any of the preceding claims with another plant in which the content and/or the activity of a cellulose synthase-like protein is not reduced and selecting transgenic progeny in which the content and/or the activity of a cellulose synthase-like protein is reduced.

11. Method of producing mutant plants, plant cells or plant parts having an increased resistance to pathogens compared to control plants, plant cells or plant parts, comprising the steps of:

(a) mutagenizing plant material;

(b) identifying plant material having at least one point mutation in a nucleic acid sequence comprising the nucleic acid sequence according to any of SEQ ID Nos.1-5.

12. Method of any of the preceding claims, wherein the transgenic or mutant plant has an increased resistance to a fungal pathogen.

13. Method of claim 12, wherein the transgenic or mutant plant has an increased resistance to *Blumeria graminis*, *Septoria tritici* and/or *Puccinia triticina*.

14. Method of any of the preceding claims, wherein the transgenic or mutant plant is a monocotyledonous plant.

15. Method of claim 14, wherein the transgenic or mutant plant is a wheat or a barley plant.

16. Expression construct comprising at least one nucleic acid sequence selected from the group consisting of:

(a) a nucleic acid sequence comprising the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(b) a nucleic acid sequence comprising a sequence which is at least 80 % identical to the sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences;
(c) a nucleic acid sequence hybridizing under stringent conditions with a nucleic acid sequence according to any of SEQ ID Nos. 1-5 or a fragment of any of these sequences; and
(d) a nucleic acid sequence which is reverse-complementary to a nucleic acid sequence of any of (a) to (c),

operatively linked to a promoter functional in plant cells.

17. Expression construct of claim 16, comprising:

(a) a promoter functional in plant cells,
(b) operatively linked thereto at least one nucleic acid sequence as defined in claim 16;
(c) optionally a spacer sequence,
(d) the nucleic acid sequence being reverse-complementary to the nucleic acid sequence mentioned in b); and

(e) optionally, a termination sequence

or:

(a) a promoter functional in plant cells;

(b) operatively linked thereto a nucleic acid sequence which is complementary to a nucleic acid as defined in claim 16; and

(c) optionally, a termination sequence;

or:

(a) a promoter functional in plant cells;

(b) operatively linked thereto a nucleic acid sequence which is identical to a nucleic acid as defined in claim 16;

(c) optionally, a termination sequence;

or:

(a) a promoter functional in plant cells;

(b) operably linked thereto a nucleic acid sequence encoding a precursor micro RNA sequence comprising a micro RNA sequence which targets the nucleic acid sequence as defined in claim 16;

(c) optionally, regenerating a transgenic plant from the transformed cell.

18. Expression construct of claim 16 or 17, comprising:

(a) a promoter functional in plant cells,

(b) operatively linked thereto at least one nucleic acid sequence selected from the group consisting of SEQ ID Nos. 6, 7, 9 and 10 and fragments of these sequences;

(c) optionally a spacer sequence,

(d) the nucleic acid sequence being complementary to the nucleic acid sequence mentioned in b); and

(e) optionally, a termination sequence.

19. Vector comprising the expression construct of any of claims 16 to 18.

20. Transgenic or mutant plant or plant cell with an increased resistance to pathogens, produced according to the method of any of claims 1 to 15 or containing an expression construct of any of claims 16 to 18 or containing a vector of claim 19.

21. Use of the transgenic or mutant plant of claim 20 or parts thereof as fodder or to produce feed material.

22. Transgenic or mutant seed produced from the transgenic plant of claim 20.

23. Flour produced from the transgenic or mutant seed of claim 22, wherein the presence of the transgene or the mutation which reduces the content and/or the activity of a a cellulose synthase-like protein can be detected in said flour.

24. Method for producing true breeding plants comprising inbreeding the transgenic progeny of the crossing step of claim 10 and repeating this inbreeding step until a true breeding plant is obtained.

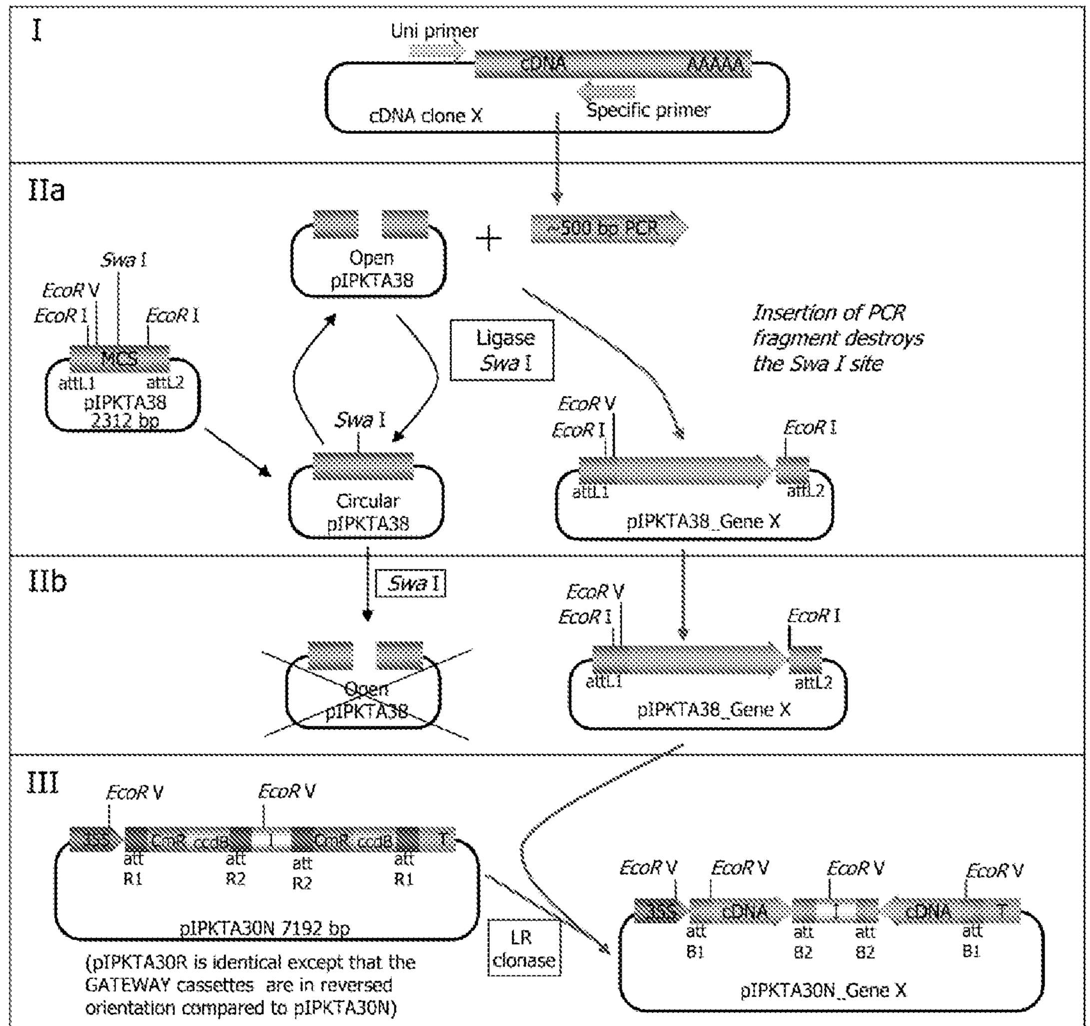


Figure 1

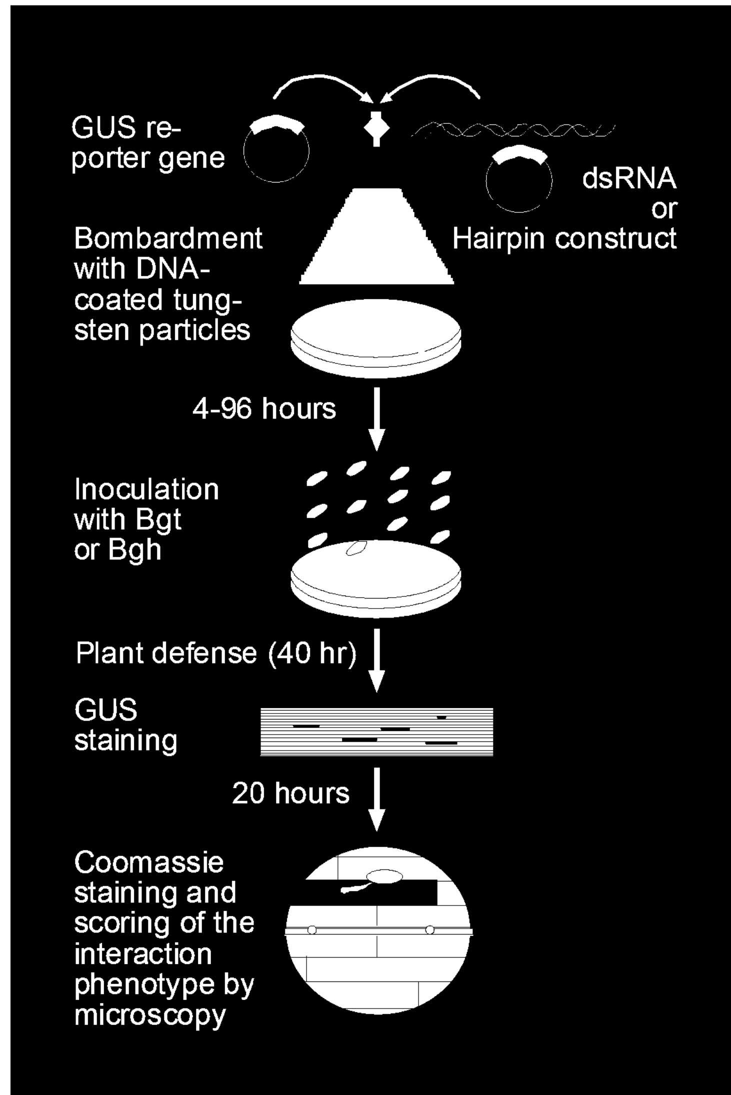


Figure 2

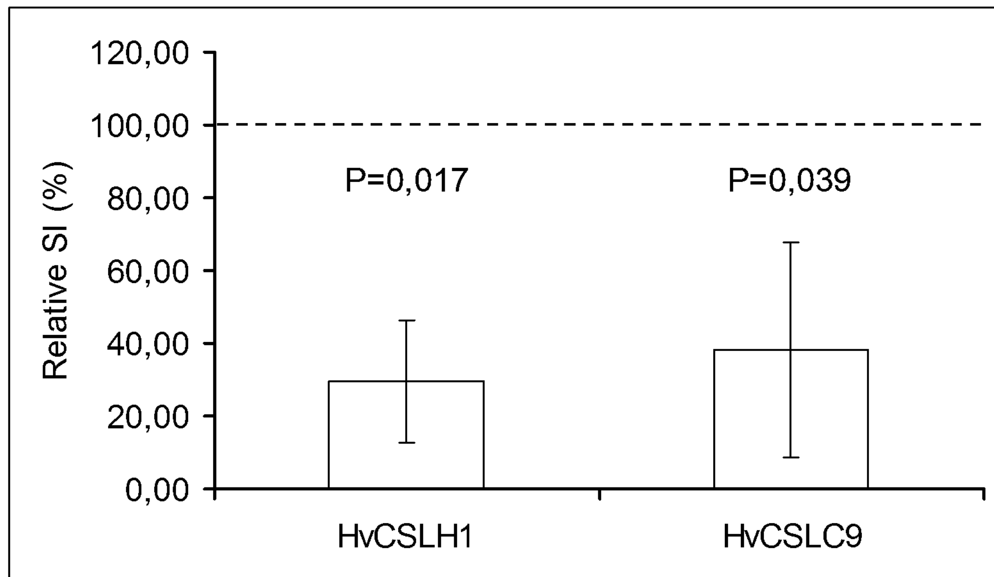


Figure 3

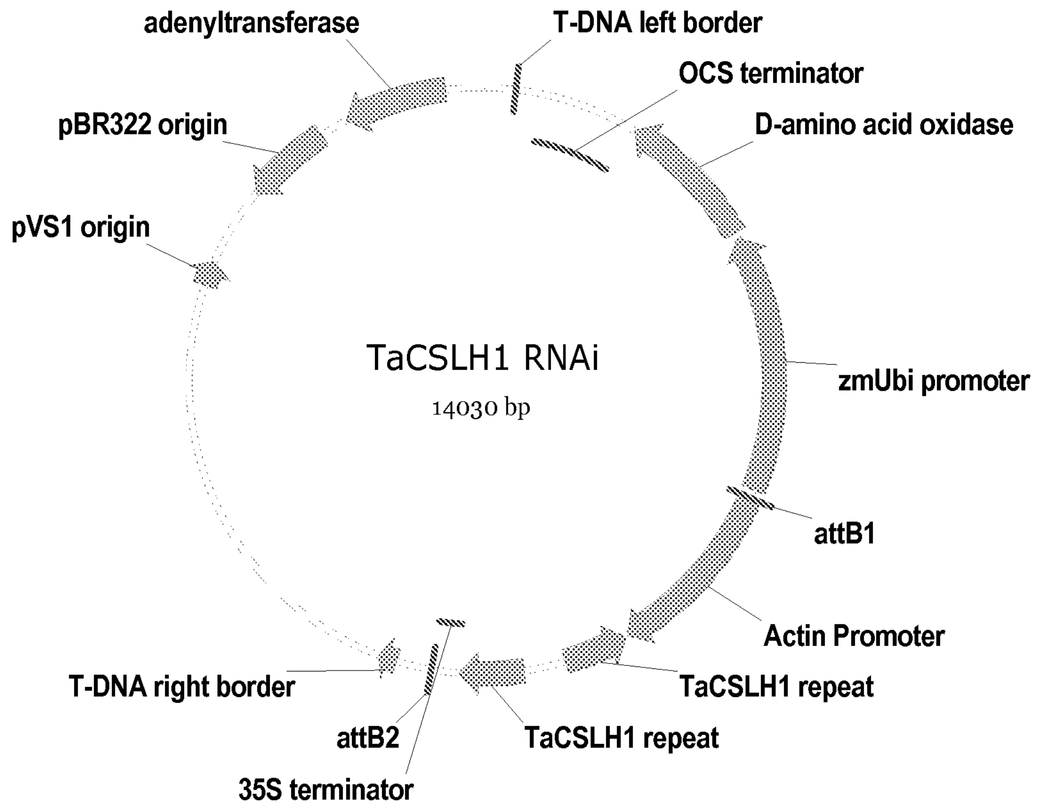


Figure 4

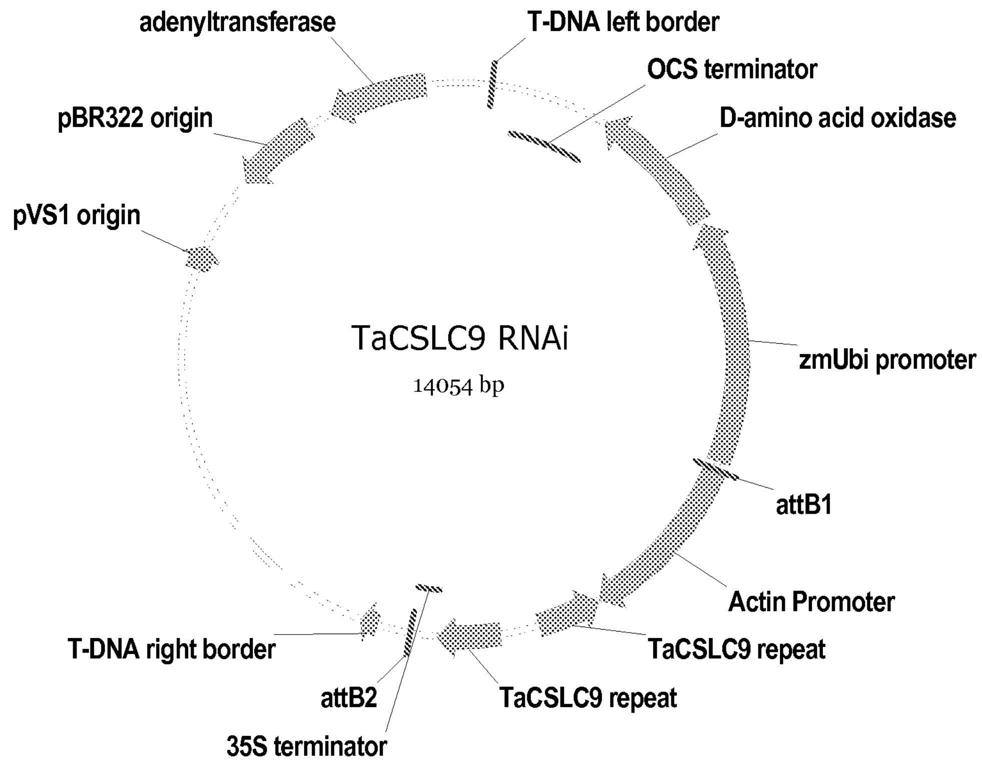


Figure 5

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052999

**A. CLASSIFICATION OF SUBJECT MATTER**

See extra sheet

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)

IPC: C12N, A01H

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 practicable, search terms used)

DATABASE: CNABS, CPRSABS, CNMED, CJFD, CNKI, DWPI, SIPOABS, ISI WEB OF KNOWLEDGE, CA, EMBASE, MEDLINE, PUBMED, GENBANK+EMBL+DDBJ+PDB
SEARCH TERMS: plant or plants or wheat or barley, pathogen, pathogens, pathogenic, germ, germs, bacteria, bacterium, fungal, fungi, fungus, blumeria graminis, septoria tritici, puccinia triticina, puccinia triticinia, cellulose synthase-like, CSL, ??CSLA??, ??CSLB??, ??CSLC??, ??CSLD??, ??CSLE??, ??CSLF??, ??CSLG??, ??CSLH??, RNAi, siRNA, shRNA, miRNA, microRNA, dsRNA; sequence search on SEQ ID NO: 1, 6

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | CN1871352A (ARBORGEN INC),<br>29 November 2006 (29.11.2006), see claims 12-48, SEQ ID NOs: 4, 5, 8, 9, 11, 12, 14, 16-18, 25-29, and pages 12-20 of the description | 1,9,10,12,14,15,20-24(all partial) |
| Y | see claims 12-48, SEQ ID NOs: 4, 5, 8, 9, 11, 12, 14, 16-18, 25-29, and pages 12-20 of the description | 2,3,8,11,16,17,19(all partial) |
| A | see the whole document | 4,7,13,18(all partial) |

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* 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 application or patent 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 citation or other 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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 September 2012 (25.09.2012) | **18 Oct. 2012 (18.10.2012)** |

Name and mailing address of the ISA/CN
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Facsimile No. 86-10-62019451

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# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052999

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | DOBLIN, M.S. et al. Hordeum vulgare subsp. vulgare cellulose synthase-like protein H1 (CSLH1) gene, complete cds. GenBank Database, 23 April 2009 (23.04.2009), Accession No. FJ459581, see the sequence | 2,3,8,11,16,17,19(all partial) |
| A | WO2008087141A2 (BASF PLANT SCIENCE GMBH), 24 July 2008 (24.07.2008), see the whole document | 1-4,7-24(all partial) |
| A | WO2005054439A2 (NORTH CAROLINA STATE UNIVERSITY), 16 June 2005 (16.06.2005), see the whole document | 1-4,7-24(all partial) |
| A | TAI, Fu-Ju et al. Cellulose biosynthesis in plant and the enzymes involved in it. Chinese Journal of Cell Biology, 20 October 2004 (20.10.2004), Vol. 26, No. 5, pages 490-494, ISSN 0253-9977, see the whole document | 1-4,7-24(all partial) |
| A | BURTON, Rachel A. et al. Over-expression of specific *HvCslF* cellulose synthesis-like genes in transgenic barley increase the levels of cell wall (1,3;1,4)-β-D-glucans and alters their fine structure. Plant Biotechnology Journal, 28 February 2011 (28.02.2011), Vol. 9, No. 2, pages 117-135, ISSN 1467-7644, see the whole document | 1-4,7-24(all partial) |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052999

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-4 and 7-24 (all partial) which relate to SEQ ID NO: 1 or 6.

**Remark on protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052999

**Box No. III Observations where unity of invention is lacking**

This International Searching Authority found multiple inventions in this international application, as follows:

Invention 1 (claims 1-4 and 7-24 (all partial)): relates to methods for producing transgenic or mutant plants having an increased resistance to pathogens, correlative expression constructs, transgenic or mutant plants and uses thereof, wherein the expression constructs and transgenic/mutant plants relate to a cellulose synthase-like protein which is encoded by nucleic acid sequence of SEQ ID No: 1, or a specific target sequence of SEQ ID No: 6;

Invention 2 (claims 1-4 and 7-24 (all partial)): relates to methods for producing transgenic or mutant plants having an increased resistance to pathogens, correlative expression constructs, transgenic or mutant plants and uses thereof, wherein the expression constructs and transgenic/mutant plants relate to a cellulose synthase-like protein which is encoded by nucleic acid sequence of SEQ ID No: 2, or a specific target sequence of SEQ ID No: 6;

Invention 3 (claims 1-6 and 8-24 (all partial)): relates to methods for producing transgenic or mutant plants having an increased resistance to pathogens, correlative expression constructs, transgenic or mutant plants and uses thereof, wherein the expression constructs and transgenic/mutant plants relate to a cellulose synthase-like protein which is encoded by nucleic acid sequence of SEQ ID No: 3, a specific target sequence of SEQ ID No: 7, or an antisense:rga1-intron:sense hairpin RNAi repeat cassette of SEQ ID No: 8;

Invention 4 (claims 1-4 and 7-24 (all partial)): relates to methods for producing transgenic or mutant plants having an increased resistance to pathogens, correlative expression constructs, transgenic or mutant plants and uses thereof, wherein the expression constructs and transgenic/mutant plants relate to a cellulose synthase-like protein which is encoded by nucleic acid sequence of SEQ ID No: 4, or a specific target sequence of SEQ ID No: 9;

Invention 5 (claims 1-6 and 8-24 (all partial)): relates to methods for producing transgenic or mutant plants having an increased resistance to pathogens, correlative expression constructs, transgenic or mutant plants and uses thereof, wherein the expression constructs and transgenic/mutant plants relate to a cellulose synthase-like protein which is encoded by nucleic acid sequence of SEQ ID No: 5, a specific target sequence of SEQ ID No: 10, or an antisense:rga1-intron:sense hairpin RNAi repeat cassette of SEQ ID No: 11.

D1: CN1871352A (ARBORGEN INC), 29 November 2006 (29.11.2006), see claims 12-25, SEQ ID NOs: 4, 5, 8, 9, 11, 12, 14, 16-18, 25-29.

D1 discloses DNA constructs encoding RNA transcripts which induce RNAi targeting for cellulose synthase-like protein, and methods for producing transgenic plants having improved phenotypes such as increased resistance to fungi by transforming the DNA construct into plants (see claims 12-25, SEQ ID NOs: 4, 5, 8, 9, 11, 12, 14, 16-18, 25-29). Therefore, the subject matter of claim 1 is not novel, thus, there are no common or corresponding special technical features within the meaning of Rule 13.2 among the above 5 inventions. The application, hence does not meet the requirements of unity of invention as defined in Rule 13.1.

**A. CLASSIFICATION OF SUBJECT MATTER**

C12N 15/113 (2010.01)i

C12N 15/29 (2006.01)i

C12N 15/82 (2006.01)i

C12N 5/04 (2006.01)i

A01H 5/00 (2006.01)i

# INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB2012/052999

| Patent Documents referred in the Report | Publication Date | Patent Family | Publication Date |
|---|---|---|---|
| CN1871352 A | 29.11.2006 | JP2006526990 A | 30.11.2006 |
| | | US2010186118 A1 | 22.07.2010 |
| | | JP2011036257 A | 24.02.2011 |
| | | EP1639081 A2 | 29.03.2006 |
| | | AU2011201695 A1 | 02.06.2011 |
| | | BR200411028 A | 25.07.2006 |
| | | US2007261137 A1 | 08.11.2007 |
| | | AU2011201695 B2 | 22.12.2011 |
| | | US7671188 B2 | 02.03.2010 |
| | | AU2004252481 B2 | 27.01.2011 |
| | | WO2005001051 A2 | 06.01.2005 |
| | | AU2004252481 A1 | 06.01.2005 |
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