



(86) Date de dépôt PCT/PCT Filing Date: 2009/03/12
(87) Date publication PCT/PCT Publication Date: 2009/09/17
(45) Date de délivrance/Issue Date: 2021/07/13
(85) Entrée phase nationale/National Entry: 2010/09/13
(86) N° demande PCT/PCT Application No.: EP 2009/001805
(87) N° publication PCT/PCT Publication No.: 2009/112270
(30) Priorité/Priority: 2008/03/13 (DE10 2008 014 041.4)

(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01)
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(54) Titre : PROCEDE DE CREATION D'UNE RESISTANCE A LARGE SPECTRE CONTRE DES CHAMPIGNONS DANS
DES PLANTES TRANSGENIQUES
(54) Title: METHOD FOR CREATING BROAD-SPECTRUM RESISTANCE TO FUNGI IN TRANSGENIC PLANTS

(57) **Abrégé/Abstract:**

The present invention relates to the creation of broad-spectrum resistance in transgenic plants by inserting inhibitory nucleic acid sequences inhibiting the expression of fungal genes. The nucleic acid sequences of genes which are crucial to development, growth and proliferation of fungi, i.e. sequences of genes with an essential function in fungi, are often conserved and/or have a high sequence identity. Regions having a particularly high sequence identity between various fungi are used in order to produce inhibitory gene constructs, e.g. on basis of antisense, siRNA, shRNA, ribozyme technology and other technologies imparting the inhibition of the expression and activation of genes. Since said methods base on the sequence-specific hybridization of the inhibitory RNA molecules with respective target sequences in certain fungal genes, the expression of all corresponding genes from various fungi is thus inhibited as well. Due to the high sequence identity of such conserved gene sections, transgenic plants are thus produced, which have broad-spectrum resistance to various fungi.

Abstract

The present invention relates to the creation of broad-spectrum resistance in transgenic plants by inserting inhibitory nucleic acid sequences inhibiting the expression of fungal genes.

The nucleic acid sequences of genes which are crucial to development, growth and profilation of fungi, i.e. sequences of genes with an essential function in fungi, are often conserved and/or have a high sequence identity. Regions having a particularly high sequence identity between various fungi are used in order to produce inhibitory gene constructs, e.g. on basis of antisense, siRNA, shRNA, ribozyme technology and other technologies imparting the inhibition of the expression and activation of genes. Since said methods base on the sequence-specific hybridization of the inhibitory RNA molecules with respective target sequences in certain fungal genes, the expression of all corresponding genes from various fungi is thus inhibited as well. Due to the high sequence identity of such conserved gene sections, transgenic plants are thus produced, which have broad-spectrum resistance to various fungi.

- 1 -

Method for creating broad-spectrum resistance to fungi in transgenic plants

The invention relates to a method for creating broad-spectrum resistance to fungi in transgenic plants, characterized in that a recombinant nucleic acid molecule, comprising at least one nucleic acid sequence, is introduced into the plant, wherein the nucleic acid
5 sequence is identical to and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1 - 16 and/or a part thereof, or has a sequence identity of at least 85% to one or more of the nucleic acids, selected from SEQ ID NOs: 1 - 16, and is transcribed in the plant, so that in case of an infection of the plant with a fungus the nucleic acid sequence or parts thereof interact with one or more of the corresponding
10 and/or complementary nucleic acids of the fungus, such that the expression of the fungal nucleic acid sequence is substantially inhibited.

The invention also relates to the nucleic acid molecule according to the invention, and to plants containing said nucleic acid molecule according to the invention.
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The present invention relates to creating broad-spectrum resistance in transgenic plants by introducing inhibitory nucleic acid sequences inhibiting the expression of fungal genes.

The nucleic acid sequences of genes which are crucial to development, growth and propagation of fungi, i.e. sequences of genes with an essential function in fungi, are often
20 conserved and/or have a high sequence identity. Regions having a particularly high sequence identity between various fungi are used in order to produce inhibitory gene constructs, e.g. on basis of antisense, siRNA, shRNA, ribozyme technology and other technologies imparting the inhibition of the expression and activation of genes. Since
25 said methods are based on on the sequence-specific hybridization of the inhibitory RNA molecules with corresponding target sequences in certain fungal genes, the expression of all corresponding genes from various fungi is thus inhibited as well. On the basis of the high sequence identity of such conserved gene sections, transgenic plants are produced, which have broad-spectrum resistance to various fungi.

- 2 -

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

Since the last century, chemical fungicides have been utilised for controlling fungal diseases. Using these substances has reduced the extent of plant diseases, however, it cannot be excluded to this day that these compounds have adverse effects on humans,
10 animals and the environment. In order to reduce the usage of conventional pesticides to a minimum in the long run, it is important to examine the natural pathogen defence of various plants towards different pathogens and to use the same specifically for the production of pathogen-resistant plants by genetechological manipulation, e.g. by means of introducing external resistance genes or by means of the manipulation of the
15 endogenous gene expression of the plants.

Resistance is the ability of a plant to inhibit any infestation or population of a pest or at least to limit the same. 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.

20 One approach for producing resistant plants is the expression of a plant transgene in said plants, resulting in the formation of said specific defence substances. In this way, 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
25 plants having increased fungal resistance.

Conversely, it could be shown that it is some of the plant genes that enable the 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 in transgenic

- 3 -

plants, which code for example for a polyphenoloxidase (WO 02/061101), NADPH-oxidase (WO 2004/009820) and the Mlo-gene (WO 00/01722).

Another alternative for causing resistance to pathogenic fungi is to introduce gene constructs in plants which inhibit the expression and/or activity of fungal genes that are essential for the proliferation and/or development of fungi (US Patent No. 2007/0061918).

To date, resistance strategies against all fungal pathogens are not available. This is particularly serious, since virtually every agricultural crop may be attacked by a plurality of pathogenic fungi. Thus, there is an urgent need for strategies to confer resistance to a plant against a plurality of potential pathogenic fungi at the same time.

It is thus an object of the present invention to provide a method for producing transgenic plants having broad-spectrum resistance to various fungal pathogens.

The features of the independent claims are to solve said and further objects as resulting from the specification.

Preferred embodiments of the present invention are defined by the features of the subclaims.

One aspect of the present invention relates to a method for creating broad-spectrum resistance to fungi in transgenic plants, characterized in that a recombinant nucleic acid molecule comprising at least one nucleic acid sequence is introduced in the plant, wherein the nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1 - 5 and/or a part thereof, or has a sequence identity of at least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 1 - 5, and/or

- 4 -

- b) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 6 - 11 and/or a part thereof, or has a sequence identity of at least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 6 - 11, or
- 5 c) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 12 - 16 and/or a part thereof, or has a sequence identity of at least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 12 - 16,
- and is transcribed in the plant, so that in case of an infection of the plant with a
- 10 fungus, the nucleic acid sequence or parts thereof interacts with one or more of the corresponding and/or complementary nucleic acids of the fungus such that the expression of the fungal nucleic acid sequence is substantially inhibited.

In the sense of the present invention, "sequence identity" denotes the degree of conformity with regard to the 5' - 3' sequence within a sequence of nucleotide units

15 within a nucleic acid molecule in comparison to another nucleic acid molecule. The sequence identity is determined using a series of programs, which base 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. Here, in addition, the program

20 Sequencher (Gene Codes Corp., Ann Arbor, MI, USA) using the "dirtydata"-algorithm for sequence comparisons was employed.

In a preferred embodiment, the at least one nucleic acid sequence introduced in the plant is at least 50 %, preferably at least 60 %, also preferably at least 70 %, particularly preferably at least 80 %, in particular preferably 90, 92 or 94 %, and most preferably to

25 at least 96, 98 or 99 % or 100 % identical to a nucleic acid sequence from fungi.

In another preferred embodiment, the at least one nucleic acid sequence introduced in the plant is at least 50 %, preferably at least 60 %, also preferably at least 70 %, particularly

- 5 -

preferably at least 80 %, in particular preferably 90, 92 or 94 %, and most preferably at least 96, 98 or 99 % or 100 % identical to a nucleic acid sequence essential for the fungus.

5 A "nucleic acid sequence essential for the fungus" in the sense of the present invention, is a nucleic acid sequence which is crucial to the development, growth and propagation of fungi, so that inhibiting the expression of said nucleic acid leads to the death of the fungus and/or stops the propagation and/or development of the fungus.

10 In a preferred embodiment of the method according to the invention, conserved sections within essential fungal nucleic acid sequences are used as target sequences for the reduction of the expression level of the corresponding fungal nucleic acid sequence, according to the invention.

15 A "corresponding" nucleic acid sequence, in the sense of the present invention, is synonymous with a homologous nucleic acid sequence, i.e. a nucleic acid sequence from a fungal species having identical or almost identical biological function, and usually also a high or very high sequence identity. Corresponding fungal nucleic acid sequences are, according to the invention, at least 50 %, preferably at least 60 %, also preferably at least 70 %, particularly preferably at least 80 %, in particular preferably 85, 90, 92 or 94 %, and most preferably at least 96, 98 or 99 % or 100 % identical to a nucleic acid sequence from another fungus.

20 The term "conserved sections" or target sequences relates to the high identity of nucleic acid sequences, usually the DNA sequence of a gene, or the RNA sequence transcribed therefrom and/or mRNA sequence, upon comparison of organisms which are evolutionary closely related. Within the scope of the present invention, regions are considered suitable conserved gene sections, in which due to a sequence comparison
25 with the genome of other fungal species there is the possibility that the reduction of the expression level of the underlying fungal gene will lead to a resistance to said fungus but also possibly to further fungi.

- 6 -

The inventors were able to determine conserved sections in essential fungal genes by means of sequence comparisons. These were used as target sequences in order to derive corresponding RNAi constructs which create broad-spectrum resistance to fungi in transgenic plants. The essential genes are

- 5 a) the mitochondrial ADP/ATP translocator (AAC)
- b) the elongation factor 1 (EF1)
- c) gamma-actin (GA)
- d) the mitochondrial heat shock protein 70 (Hsp)
- e) the Rho-GTPase (Rho).

- 10 Using the RNAi constructs derived from the corresponding gene sequences of *Fusarium culmorum* the inventors were able to create resistance in barley to both, *Fusarium culmorum* and *Blumeria graminis*.

- 15 Thereby, it was ensured that the corresponding gene sections or a sequence homologous thereto are not endogenous to the plant to be used, as otherwise endogenous processes in the plant may be inhibited as well, which is not desired.

In a preferred embodiment of the method of the present invention, the nucleic acid sequence of the present invention comprises conserved sections within essential fungal genes as target sequences for the reduction of the expression level of the corresponding fungal nucleic acid sequence, according to the invention.

- 20 In another preferred embodiment, the at least one nucleic acid sequence of the present invention has, in comparison with corresponding nucleic acid sequences or target sequences in nucleic acid sequences from fungi and especially preferably from pathogenic fungi, over a range of at least 100 bp, preferably 150 bp, preferably 200 bp, especially preferably 250 bp and also especially preferably 300 bp a sequence identity of
- 25 at least 85 %; at least 90 % or at least 95 %.

In another preferred embodiment, the at least one nucleic acid sequence of the present invention has, in comparison with corresponding nucleic acid sequences from plants,

- 7 -

over a "sliding window" of 100 bp a sequence identity of maximal 80 %, preferably 75 % and most preferably maximal 70 %.

A "sliding window", in the sense of the present invention, is a nucleotide sequence with a length of 100 bp, which is randomly relocatable within a given nucleotide sequence.

5 In an especially preferred embodiment of the method of the present invention, the corresponding and/or complementary nucleic acid of the fungus, the expression of which is basically inhibited by the nucleic acid sequence of the present invention, is

- a) the mitochondrial ADP/ATP translocator (AAC)
- b) the elongation factor 1 (EF1)
- 10 c) gamma-actin (GA)
- d) the mitochondrial heat shock protein 70 (Hsp)
- e) the Rho-GTPase (Rho).

The term "resistance" stands for minimizing or reducing symptoms of disease of a plant due to an infestation by pests and/or pathogens, and preferably by a fungus and especially preferably by several fungi. Said symptoms may be diverse, however, they preferably comprise symptoms that directly or indirectly impair the quality of the plant, the quantity of the yield, the suitability for use as feed or food, or which impede sowing, cultivating, harvesting or processing the harvest. Furthermore, "resistance" also means that pests and/or a pathogen and preferably a fungus and especially preferably several fungi 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 plants or plant cells of the present invention have an increased resistance to pests and/or pathogens or fungi only for a limited period of time, compared to the corresponding wild type.

The term "broad-spectrum resistance" according to the invention denotes that the transgenic plants or plant cells of the present invention are infected or attacked less
 15 strongly and/or less frequently by more than one fungal species in comparison with non-transformed wild type plants or plant cells, which were – apart from that – treated in the same manner (e.g. climate and cultivation conditions, fungal species, etc.). Preferably,

- 8 -

the attack by at least one fungus, by at least two different fungi, especially preferably by at least three different fungi, particularly preferably by at least four different fungi, and most preferably by at least five different fungi is reduced, which results in reducing the formation of disease symptoms. Such fungal resistance may be experimentally proven
5 *inter alia* by a reduction of haustoria formation and/or hyphen growth.

"Substantially inhibited" in the scope of the present invention means that the expression level of the nucleic acid sequence of the fungus in the transgenic plants is reduced to a level, at which the plants show an increased resistance to fungal infection and/or show signs of recovery after initial symptoms of a fungal infection. "Transgenic plants having
10 broad-spectrum resistance" are plants according to the invention which show few symptoms of a fungal infection, however, said symptoms are relatively weak so that the usability and applicability of the infested plant is not questioned. Plants of the present invention show a phenotype which is substantially unchanged compared to wild type plants, i.e. the use as agricultural crop, food plant or feed plant, etc., is not questioned.

15 If, in the scope of the present invention, nucleic acid sequences are mentioned, which code for fungal genes or parts thereof, then both the complete coding DNA sequence of the respective fungal gene as well as the complete mRNA sequence or the respective sections thereof are meant.

The expression level of the nucleic acid of the fungus, the expression of which is
20 substantially inhibited by the nucleic acid transcribed in the transgenic plant, may be determined in the infested wild type 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 the nucleic acid of the fungus. Preferably, the expression level
25 of the nucleic acid of the fungus 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 %.

- 9 -

In another preferred embodiment of the method of the present invention, the at least one nucleic acid sequence comprises at least 19 nucleotides, at least 20 nucleotides, at least 21 nucleotides, at least 22 nucleotides, at least 23 nucleotides, at least 24 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

- 5 In another preferred embodiment of the method of the present invention, the at least one nucleic acid sequence comprises at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides, at least 400 nucleotides or at least 500 nucleotides.

- 10 In a preferred embodiment, the at least one nucleic acid sequence of the present invention comprises at least 19 nucleotides, at least 20 nucleotides, at least 21 nucleotides, at least 22 nucleotides, at least 23 nucleotides, at least 24 nucleotides, at least 25 nucleotides or at least 50 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in
15 particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence, wherein the nucleic acid sequence is preferably derived from fungi.

- In another preferred embodiment, the at least one nucleic acid sequence of the present invention comprises at least 50 nucleotides, at least 100 nucleotides, at least 150
20 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides, at least 400 nucleotides or at least 500 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence, wherein the
25 nucleic acid sequence is preferably derived from fungi.

In the sense of the present invention, the term "identical" relates to the degree of sequence identity of a nucleic acid sequence compared to another nucleic acid sequence.

- 10 -

Identical nucleic acid sequences, in the sense of the present invention have a sequence identity of at least 40 %, at least 50 %, at least 60 %, preferably at least 70 %, especially preferably at least 80 %, also especially preferably at least 90 %, in particular preferably at least 95 % and most preferably at least 98 or 100 % compared to another nucleic acid sequence.

The term "complementary" means the ability of a nucleic acid sequence to hybridize with another nucleic acid sequence due to hydrogen bridges between complementary bases. The skilled person knows that two nucleic acid molecules do not need to have a 100 % complementarity in order to hybridize with each other. Preferably, a nucleic acid sequence, which is to hybridizes with another nucleic acid sequence, is least 40 %, at least 50 %, at least 60 %, at least 70 %, especially preferably at least 80 %, also especially preferably at least 90 %, in particular preferably to at least 95 % and most preferably at least 98 or 100 % complementary to said nucleic acid sequence.

According to the invention, the "recombinant nucleic acid molecule" stands for all vectors, plasmids, cosmids, viruses and other vectors common in genetic engineering, for the transfer/introduction of nucleic acid molecules in plants or plant cells.

A high number of cloning vectors is available for preparing the introduction of foreign genes in higher plants or the cells thereof, which contain a replication signal for *E. coli* and a marker gene for selecting transformed bacteria cells. Examples of such vectors are pBR322, pUC-series, M13mp-series, pACYC184 etc. The desired sequence may be inserted into the vector at a suitable restriction site. The resulting plasmid is used for the transformation of *E. coli* cells. Transformed *E. coli* cells are cultivated in a suitable medium and subsequently harvested and lysed. The plasmid is regained. Analysis methods for characterizing the regained plasmid DNA generally comprise restriction analyses, gel electrophoreses, and other biochemical-molecular biological methods. After each manipulation, the plasmid DNA can be cleaved, and regained DNA fragments can be linked to other DNA sequences. Each plasmid DNA sequence may be cloned into the same or other plasmids. Standard methods for cloning may be taken from Sambrook et

- 11 -

al., 2001 (Molecular cloning: A laboratory manual, 3rd edition, Cold Spring Harbor Laboratory Press).

In the method of the invention for producing transgenic plants having broad-spectrum resistance to fungi, the characteristic of the at least one nucleic acid sequence to impart
5 the suppression of the expression of a nucleic acid sequence of a fungus may also make the plant resistant to all other fungi which are comprising the respective highly conserved region in said gene. As discussed below, said methods for suppressing the expression of a nucleic acid sequence are based on specific complementary base pairing of inhibitory nucleic acid molecules with sequences of a fungal cellular nucleic acid molecule. Due to
10 the high sequence identity of conserved regions in one or more essential genes of various fungi, a nucleic acid sequence derived from fungus A may be used, for example, in order to inhibit the infection of a plant by fungus A and fungus B, and/or by fungi A, B, C and D etc. (see below).

In a preferred embodiment, the at least one nucleic acid sequence comprises several
15 nucleic acid sequences which are homologous and/or complementary to several different nucleic acid sequences of a fungus and which are transcribed in the plant, so that upon infection of the plant with said fungus, the nucleic acid sequences transcribed in the plant interact with the respective homogenous and/or complementary nucleic acid sequences of the fungus or with parts thereof, so that the expression of the nucleic acid sequences of
20 the fungus is substantially inhibited. In this way, the expression of several fungal genes may be inhibited by the plant at the same time, and increased resistance to individual or preferably various fungal pathogens may be imparted to the transgenic plant.

In a preferred embodiment of the method of the invention, the recombinant nucleic acid molecule comprises two nucleic acid sequences.

25 In another preferred embodiment of the method of the invention, the recombinant nucleic acid molecule comprises three nucleic acid sequences.

- 12 -

The inhibition of the expression of a nucleic acid of an organism is referred to as "gene silencing" by those skilled in the art. Said method is also referred to as "host-induced gene silencing" (HIGS), since the "gene silencing" of the fungal gene is caused by a nucleic acid sequence which is formed by the host of the fungus, i.e. the plant.

- 5 Those skilled in the art know various techniques which may cause "gene silencing". The expression of the nucleic acid sequence of a fungus may be substantially inhibited in transgenic plants for example by "silencing". For silencing, a nucleic acid sequence which is identical to a nucleic acid sequence of a fungus and/or which is complementary thereto is transferred to the plant. To ensure that the plants are transgenic for the
- 10 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
- 15 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
- 20 those sequences, which correspond to the coding strand of a nucleic acid sequence of a fungus, or which comprise parts thereof. Such sequences do not have to be 100 % identical to the fungal genes of interest. It should be sufficient if said sequences are at least 50 % identical, preferably at least 60 %, especially preferably at least 70 %, further especially preferably at least 80 %, in particular preferably at least 90 % and most
- 25 preferably at least 95 % 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 acids of fungi or parts thereof may originate from deletion, addition, substitution and/or insertion. The skilled person surely knows that with decreasing identity, it becomes more likely that multiple nucleic acids are

- 13 -

suppressed in a silencing manner. Sequences having such a low degree of identity or homology that the expression of endogenous genes 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.

5 Respectively, if antisense sequences are mentioned, those sequences of the invention are referred to which correspond to the codogenous DNA strand of the fungal genes. Said sequences are preferably complementary to at least 50 %, preferably complementary to at least 60 %, especially preferably complementary to at least 70 %, further especially preferably complementary to at least 80 %, in particular preferably complementary to at
10 least 90 %, and most preferably complementary to at least 95 %, 98 % and/or 99 %. As mentioned above, it is sufficient if the antisense sequences are able to hybridize specifically with the mRNA of the respective fungal gene of interest, but not with the endogenous mRNA of the transgenic plant. Hybridization of an antisense sequence with a mRNA sequence of the fungus typically takes place *in vivo* under cellular conditions,
15 or *in vitro*.

In a preferred embodiment of the above-mentioned method, the recombinant nucleic acid molecule comprises a promoter functional in plants and operatively linked thereto the at least one nucleic acid sequence.

Said promoters of the present invention may be constitutive, but also inducible or tissue
20 and/or development-specific promoters.

In order to carry out the present invention, promoters which are suitable for the expression of genes in plants may be generally obtained from various sources, such as from plants or plant viruses, for example. The selection of the promoter and other
25 regulatory sequences determines the spatial and temporal expression pattern in transgenic plants.

- 14 -

The at least one nucleic acid sequence of the present invention may be present in antisense or sense orientation (identical or complementary to the nucleic acid sequence of the fungus).

In a preferred embodiment of the present invention, the at least one nucleic acid
5 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 of the fungus. By hybridizing the antisense sequence with the nucleic acid sequence of the fungus *in vivo*, the expression of the nucleic acid sequence of the fungus may be suppressed in plant cells, whereby the plant becomes resistant to said
10 fungus.

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 of the fungus. Those skilled in the art know that, upon expression of such
15 sense sequences in plants, antisense RNAs (asRNAs) may be formed which may cause silencing of both, the transgene, i.e. the at least one nucleic acid sequence according to the invention, and the endogenous, corresponding gene (co-suppression). By co-suppressing the nucleic acid sequence of the fungus *in vivo*, the expression of the nucleic acid sequence of the fungus may be suppressed in plant cells, whereby the plant becomes
20 resistant to said fungus.

The nucleic acid sequence of the present invention may consist of or be derived from a naturally occurring nucleic acid sequence or a synthetically produced, by sequence comparison derived or recombinantly produced nucleic acid sequence.

In a preferred embodiment, the at least one nucleic acid sequence of the present
25 invention comprises at least 50 bp, at least 100 bp, at least 150 bp, at least 200 bp, at least 250 bp, or at least 300 bp.

- 15 -

In another embodiment of the method of the present invention, vectors are used for introducing the nucleic acids in the plant cells which comprise a promotor in 5' - 3'-orientation functional in plants, operatively linked thereto a DNA sequence coding for a ribozyme which specifically recognizes the nucleic acid sequence of a fungus, and a
5 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 of the fungus or parts thereof, and
10 thus directs 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 recombinant nucleic acid molecule comprises a promoter which is functional in plants being operatively linked thereto the at least one nucleic acid sequence, another nucleic acid
15 sequence coding for ribonuclease P, and 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 fungal gene, 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
20 complementary to the mRNA of a fungal gene 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 nucleotide 5' from the paired region. Such cleaved mRNA cannot be translated into a functional protein.

25 The skilled person further 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

- 16 -

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, result from double-stranded RNA substrates. Such double-stranded RNA molecules (dicer substrates) must have at least a length of 25 bp, those skilled in the art know, however, 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)
- 10 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 of a fungus. Thereby, the corresponding polypeptide(s) cannot be formed. As discussed in detail above, according to the invention the expression of essential nucleic acid sequences of the fungus is inhibited. Said nucleic acid sequences are highly conserved in various fungi. Accordingly, those skilled in the art know that the sequence-specific inhibition of the expression of such a nucleic acid sequence in a transgenic plant of the present invention extends to all fungi which have a homologous section in the corresponding nucleic acid sequence.

25 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.

- 17 -

In a preferred embodiment of the method of the invention, the recombinant nucleic acid molecule comprises a RNAi construct.

In a preferred embodiment of the above-mentioned method, the recombinant nucleic acid molecule comprises a promotor which is functional in plants, operatively linked thereto
5 is the at least one nucleic acid sequence, wherein said sequence has reverse-complementary regions, and a termination sequence. The skilled person knows respective 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, again double-
10 stranded RNA is being formed, which is also a substrate for the dicer enzyme complex, for example. Accordingly, siRNA molecules are also formed, which lead to the degradation of a corresponding nucleic acid of a fungus. The above-mentioned reverse-complementary nucleic acid sequences are also referred to as *inverted repeats*.

In another preferred embodiment, the recombinant nucleic acid molecule comprises a
15 promoter which is functional in plants, operatively linked thereto is the at least one nucleic acid sequence, a "short hairpin" structure-generating nucleic acid and the nucleic acid sequence which is reverse-complementary to the at least one nucleic acid sequence, and a termination sequence.

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

In another preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct, wherein the at least one nucleic acid sequence comprises a sequence

- 18 -

selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and a sequence reverse-complementary thereto.

In another preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct, wherein the at least one nucleic acid sequence comprises two sequences
5 selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and one or two sequences reverse-complementary thereto.

In another preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct, wherein the at least one nucleic acid sequence comprises three sequences selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof,
10 and one or more sequences reverse-complementary thereto.

In an especially preferred embodiment of the method of the present invention using the RNAi methodology, the recombinant nucleic acid molecule comprises a promoter which is functional in plants, the at least one nucleic acid sequence is operatively linked thereto,
15 an intron comprising splice donor and splice acceptor sequences, the nucleic acid sequence reverse-complementary to the at least one nucleic acid sequence of the present invention, and a termination sequence.

So-called termination sequences are sequences which ensure that the transcription or the translation is terminated properly. If the transmitted nucleic acids are to be translated,
20 said nucleic acids are typically stop codons and corresponding regulatory sequences; if the transmitted nucleic acids are only to be transcribed, said nucleic acids are normally poly-A sequences.

If such vectors 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
25 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

- 19 -

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 same applies of course to the above-mentioned recombinant nucleic acid molecules of the present invention in which several siRNAs are contained. Therefore, e.g. for three various siRNAs, different configurations of the polarity (sense/antisense) are possible, such as:

	siRNA#1	siRNA#2	siRNA#3	(reverse #1	reverse #2	reverse #3)
	5'-3'	5'-3'	5'-3'	(3'-5'	3'-5'	3'-5')
10	5'-3'	5'-3'	3'-5'	(3'-5'	3'-5'	5'-3')
	5'-3'	3'-5'	5'-3'	(3'-5'	5'-3'	3'-5')
	5'-3'	3'-5'	3'-5'	(3'-5'	5'-3'	5'-3')
	3'-5'	5'-3'	5'-3'	(5'-3'	3'-5'	3'-5')
	3'-5'	5'-3'	3'-5'	(5'-3'	3'-5'	5'-3')
15	3'-5'	3'-5'	5'-3'	(5'-3'	5'-3'	3'-5')
	3'-5'	3'-5'	3'-5'	(5'-3'	5'-3'	5'-3')

Of course, the present invention also contains such recombinant nucleic acid molecules in which the 5' - 3' sequence is varied, e. g.

siRNA#2 siRNA#1 siRNA#3

20 etc. (see below)

In an embodiment of the method of the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto.

25 In an embodiment of the method of the invention, the recombinant nucleic acid molecule comprises a RNAi construct, comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one

- 20 -

nucleic acid sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and the sequence reverse-complementary thereto.

5 In an especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct, comprising an intron 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 coding for the mitochondrial ADP/ATP translocator (AAC) from a fungus or a part thereof, and the sequence reverse-complementary thereto.

10 In an especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron 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, coding for the mitochondrial heat shock protein 70 from a fungus or a part thereof, and the sequence reverse-complementary
15 thereto.

In an embodiment of the method according to the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises two nucleic acid sequences, and the sequences
20 reverse-complementary thereto.

In an embodiment of the method according to the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises two nucleic acid sequences selected from the
25 group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and the sequences reverse-complementary thereto.

- 21 -

- In a preferred embodiment of the method according to the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises two nucleic acid sequences in random 5' - 3' sequence selected from the group consisting of nucleic acid sequences, coding for gamma-actin (GA) from a fungus, the mitochondrial ADP/ATP translocator (AAC) from a fungus, the mitochondrial heat shock protein 70 (Hsp) from a fungus, the elongation factor 1 (EF1) from a fungus and the Rho GTPase (Rho) from a fungus or a respective part of each, and the sequences reverse-complementary thereto.
- 5
- 10 In an embodiment of the method according to the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences, and the sequences reverse-complementary thereto.
- 15 In an embodiment of the method according to the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and the sequences reverse-
- 20 complementary thereto.

- In a preferred embodiment of the method of the invention, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences in random 5' - 3' sequence selected from the group consisting of nucleic acid sequences coding for gamma-actin (GA) from a fungus, the mitochondrial ADP/ATP translocator (AAC) from a fungus, the mitochondrial heat shock protein 70 (Hsp) from a fungus, the elongation
- 25

- 22 -

factor 1 (EF1) from a fungus and the Rho GTPase (Rho) from a fungus or a respective part of each, and the sequences reverse-complementary thereto.

In an especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence
5 and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences coding for the gamma-actin (GA) from a fungus or a part thereof, the mitochondrial ADP/ATP translocator (AAC) from a fungus or a part thereof, and the mitochondrial heat shock protein 70 (Hsp) from a fungus or a part thereof, and the sequences reverse-complementary thereto.

10 In another especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences coding for the gamma-actin (GA) from a fungus or a part thereof, the mitochondrial ADP/ATP translocator
15 (AAC) from a fungus or a part thereof, and the elongation factor 1 (EF1) from a fungus or a part thereof, and the sequences reverse-complementary thereto.

In another especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one
20 nucleic acid sequence comprises three nucleic acid sequences coding for the gamma-actin (GA) from a fungus or a part thereof, the Rho GTPase (Rho) from a fungus or a part thereof, and the elongation factor 1 (EF1) from a fungus or a part thereof, and the sequences reverse-complementary thereto.

In another especially preferred embodiment, the recombinant nucleic acid molecule
25 comprises a RNAi construct comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences coding for the gamma-actin (GA) from a fungus or a part thereof, the Rho GTPase (Rho) from a fungus or a

- 23 -

part thereof, and the mitochondrial heat shock protein 70 (Hsp) from a fungus or a part thereof, and the sequences reverse-complementary thereto.

In another especially preferred embodiment, the recombinant nucleic acid molecule comprises a RNAi construct comprising an intron between the at least one nucleic acid
5 sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises three nucleic acid sequences coding for the mitochondrial ADP/ATP translocator (AAC) from a fungus or a part thereof, the Rho GTPase (Rho) from a fungus or a part thereof, and the elongation factor 1 (EF1) from a fungus or a part thereof, and the sequences reverse-complementary thereto.

10 Of course, embodiments, wherein the inhibition and/or prevention of the expression of various fungal genes is achieved by the simultaneous usage of several RNAi constructs, are also part of the present invention.

In an embodiment, the method according to the invention comprises two recombinant nucleic acid molecules comprising RNAi constructs comprising an intron between the at
15 least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises one, two or three nucleic acid sequences selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and the sequences reverse-complementary thereto.

In an embodiment, the method according to the invention comprises three recombinant
20 nucleic acid molecules comprising RNAi constructs comprising an intron between the at least one nucleic acid sequence and the sequence reverse-complementary thereto, wherein the at least one nucleic acid sequence comprises one, two or three nucleic acid sequences selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof, and the sequences reverse-complementary thereto.

25 In another preferred embodiment, the at least one nucleic acid sequence comprises a nucleic acid sequence of at least 19 nucleotides, at least 20 nucleotides, at least 21 nucleotides, at least 22 nucleotides, at least 23 nucleotides, at least 24 nucleotides and at

- 24 -

least 25 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or
5 99 % compared to another nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof.

In a preferred embodiment, the at least one nucleic acid sequence comprises two nucleic acid sequences, each of at least 19 nucleotides, at least 20 nucleotides, at least 21 nucleotides, at least 22 nucleotides, at least 23 nucleotides, at least 24 nucleotides and at
10 least 25 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence selected from the group consisting of
15 SEQ ID NOs: 1 - 16 or parts thereof.

In another preferred embodiment, the at least one nucleic acid sequence comprises three nucleic acid sequences, each of at least 19 nucleotides, at least 20 nucleotides, at least 21 nucleotides, at least 22 nucleotides, at least 23 nucleotides, at least 24 nucleotides and at least 25 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially
20 preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1-16 or parts thereof.

25 In a preferred embodiment, the at least one nucleic acid sequence comprises a nucleic acid sequence of at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides and at least 350 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably

- 25 -

at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof.

- 5 In a preferred embodiment, the at least one nucleic acid sequence comprises two nucleic acid sequences of at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides and at least 350 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof.

- In a preferred embodiment, the at least one nucleic acid sequence comprises three nucleic acid sequences of at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, at least 300 nucleotides and at least 350 nucleotides having a sequence identity of at least 40 %, preferably at least 50 %, further preferably at least 60 %, also preferably at least 70 % and/or 75 %, especially preferably at least 80 %, 82 %, 84 %, 86 % and/or 88 %, in particular preferably at least 90 %, 92 % and/or 94 %, and most preferably at least 95 %, 96 %, 97 %, 98 % and/or 99 % compared to another nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1 - 16 or parts thereof.

- 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) *Nat. Biotechnol.* 20: 446-448).

Another part of the present invention is the method according to the invention, wherein synthetic double-stranded siRNAs are used for inhibiting the expression of a nucleic acid of a fungus, which typically have a length of 19 - 21 nucleotides. Such synthetic siRNAs

- 26 -

may be introduced in the respective plant cell or plant by biolistic transformation techniques. Such synthetic siRNA molecules may activate the PTGS system in plants, and trigger a RNAi effect (Hamilton and Baulcombe (1999) *Science* 286: 950-2).

5 The selection of the target sequence for siRNA inhibition as well as the siRNA sequence motif may be determined 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 inhibition may typically be 70 nucleotides downstream from the start codon in
10 5' - 3' direction and 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
15 sequence.

Further, those skilled in the art know the rules and regulations, established by Reynolds *et al.* ((2004) *Nat 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
- 20 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
- 25 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

- 27 -

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

In another preferred embodiment of the method of the invention, the at least one nucleic acid sequence is selected from the group consisting of

- (i) nucleic acids comprising SEQ ID NOs: 1 - 16 or fragments thereof, and/or
- (ii) nucleic acids which hybridize under stringent conditions with a complementary strand of a nucleic acid of (i).

The term "hybridizing under stringent conditions" denotes in the context of the present invention 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 und Russell (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY). The term "specific hybridization" refers to the circumstance that a molecule, under stringent conditions, 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

- 28 -

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 destabilises 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 non-restrictive 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 fluctuates, e.g. under standard hybridization conditions depending on the type of the nucleic acid, between 42 °C and 58 °C in 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 und 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
5	50 mM NaPO ₄ , pH 6.8
	0.1 % sodium pyrophosphate
	5x Denhardt's solution
	100 µg/mL salmon sperm DNA

Hybridization solution:

10 1×10^6 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:

15	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

20 *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 -

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
 5 modified or have to be modified, depending on the application.

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
 10 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.).

In a preferred embodiment, the corresponding sense or antisense nucleic acid sequences are inserted into a vector by homologous recombination using the GATEWAY[®] system.

15 In another preferred embodiment of the above-mentioned method, the promotor is a constitutive promotor, an inducible promotor or a tissue-specific promotor.

Besides constitutive promotors, such as the ubiquitin promotor (Binet *et al.* (1991) *Plant Science* 79:87-94) and the actin promotor (McElroy *et al.* (1990) *Plant Cell* 2:163-171), the promotors of phosphoenolpyruvate-carboxylase from corn (Hudspeth *et al.* (1989)
 20 *Plant Mol. Biol.* 12:579) or of fructose-1,6-bisphosphatase from potato (WO 98/18940), which impart leaf-specific expression, are considered as tissue-specific promotors.

Wound-, light- or pathogen-induced promotors and other development depending promotors 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*
 25 *Cell* 1:805-813; Puente *et al.* (1996) *EMBO J.* 15:3732-3734; Gough *et al.* (1995) *Mol.*

- 31 -

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.

Suitable promoters also include promoters which ensure expression in tissues only, e.g. epidermis-specific promoters, such as the GSTA1 promotor (Altpeter *et al.* (2005) *Plant Mol Biol.* 57:271-83), or promoters of photosynthetically active tissues, such as the ST-LS1 promotor (Stockhaus *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:7943-7947; Stockhaus *et al.* (1989) *EMBO J.* 8:2445-2451). Promoters which are active during the plant transformation, plant regeneration or certain stages of said processes, e.g. cell division specific promoters, such as the histone H3 promotor (Kaprois *et al.* (1993) *In Vitro Cell Dev. Biol. Plant* 29:27-32), or 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 implementation of the present invention are fungal inducible promoters, and especially those, which are induced by pathogenic fungi and not by useful fungi (e.g. mycorrhiza in the soil). Such promoters, such as the GER4 promotor (WO 2006/128882), enable a highly specific creation of broad-spectrum resistance in transgenic plants whereby undesired side effects are significantly reduced and eliminated.

Further fungal inducible promoters comprise promoters, such as the GAFF-2 promotor (Sa *et al.* (2003) *Plant Cell Rep.* 22:79-84), which, e.g., is induced by the fungus *Trichoderma viride*, or the PAL promotor which is induced by inoculation with *Pyricularia oryzae* (Wang *et al.* (2004) *Plant Cell Rep.* 22:513-518).

Further preferred promoters are those which are in particular active in fruits. Examples of these are the promotor of a polygalacturonase gene, e. g. from tomato, which

- 32 -

mediates expression during the ripening process of tomato fruits (Nicholass *et al.* (1995) *Plant Mol. Biol.* 28:423-435), the promotor 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 promotor from tomato (van Haaren *et al.* (1991) *Plant Mol. Biol.* 17:615-630).

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

The skilled person knows that the use of inducible promotors 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 only show transiently increased fungal resistance. Such transiently increased resistance may be desired, if, for example, there is a 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 ubiquitin promotor is used as constitutive promotor.

In another preferred embodiment of the method according to the invention, the epidermis-specific promotor GSTA1 is used as tissue-specific promotor.

In an especially preferred embodiment of the method according to the invention, the GER4 promotor is used as pathogen-inducible promotor.

The vectors which are used for the silencing of fungal nucleic acids further comprise regulatory elements besides the nucleic acid sequence to be transferred. Which specific

- 33 -

regulatory elements must be included in said vectors depends on the respective procedure which is to be used for said vectors. Those skilled in the art, who are familiar with the above-mentioned various methods for producing transgenic plants in which the expression of a protein is inhibited know which regulatory elements and also other
5 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 "operatively linked" related to nucleic acid sequences or DNA sections in vectors refers to the fact that the nucleic acid sequences are linked to the vectors such
10 that the sequence is under transcriptional and/or translational control of a vector suitable for expression in plants.

The vectors of the present invention may for example also comprise enhancer elements as regulatory elements, further, said vectors may contain resistance genes, replication signals and further DNA regions which allow for a propagation of the vectors in bacteria,
15 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.

20 Those skilled in the art also know that various vectors may be employed for the RNAi method. Such vectors may be designed such that the sense and antisense sequences are transcribed from a U6 promotor each, hybridize in the cell and induce the PTGS system (Tuschl (2002) *Nat. Biotechnol.* 20: 446-448; Miyagishi *et al.* (2002) *Nat. Biotechnol.* 20: 497-500; Lee *et al.* (2002) *Nat. Biotechnol.* 20: 500-505). A plurality of RNAi
25 compatible vectors is commercially available.

In an especially preferred embodiment, the pIPKb007 vector is used.

- 34 -

In another special embodiment, the pIPKb010 vector is used.

In another special embodiment, the p6UGER4deltaSwaIntronRNAi vector is used.

It is obvious to those skilled in the art that the many different kinds of plants may be attacked by fungi.

- 5 A preferred embodiment of the method is a method of creating broad-spectrum resistance to fungi in monocotyledonous plants.

Examples of monocotyledonous plants are plants which belong to the genus avena (oat), triticum (wheat), secale (rye), hordeum (barley), oryza (rice), panicum, pennisetum, setaria, sorghum (millet), zea (corn) and the like.

- 10 In a particularly preferred embodiment of the method, the plant is a cereal plant, in particular wheat or barley.

In another especially preferred embodiment of the method, the plant is barley.

Another preferred embodiment of the method, the method is for creating broad-spectrum resistance to fungi in dicotyledonous plants.

- 15 Dicotyledonous plants or agricultural crops comprise, but are not limited to, cotton, legumes, e.g. pulses, and in particular alfalfa, soy beans, rape, canola, tomato, sugar beet, potato, sunflower, ornamental plants as well as trees. Further agricultural crops may comprise fruit (in particular apples, pears, cherries, grapes, citrus, pineapple and bananas), oil palms, tea, cacao and coffee bushes, tobacco, sisal as well as medicinal
- 20 plants, such as rauwolfia and digitalis. Especially preferred are the cereals wheat, rye, oat, barley, rice, corn and millet as well as the dicotyledonous plants sugar beet, rape, soy, tomato, potato and tobacco. Further agricultural crops may be taken from US Patent No. 6,137,030.

- 35 -

Preferred plants are tagetes, sunflower, arabidopsis, tobacco, red pepper, soy, tomato, aubergine, pepper, carrot, potato, corn, lettuces and types of cabbage, cereals, alfalfa, oat, barley, rye, wheat, triticale, millet, rice, lucerns, flax, cotton, hemp, brassicaceae, such as rape or canola, sugar beet, sugar cane, nut and wine species or woody plants, such as
 5 aspen or yew.

An especially preferred embodiment of the method is a method for creating broad-spectrum resistance to fungi by means of RNAi method for inhibiting expression of a nucleic acid sequence of a fungus, wherein the corresponding transgenic plant is resistant to at least said fungus.

10 An especially preferred embodiment of the method is a method for creating broad-spectrum resistance to fungi by means of RNAi method for inhibiting expression of a nucleic acid sequence of a fungus, wherein the corresponding transgenic plant is resistant to at least another fungus.

In an especially preferred embodiment of the method according to the invention, broad-
 15 spectrum resistance to pathogenic fungi is created.

Especially preferred, the suppression of the expression of a nucleic acid sequence of a fungus creates broad-spectrum resistance to

- Plasmodiophoromycota, such as *Plasmodiophora brassicae* (clubroot of crucifers),
Spongospora subterranea (powdery scab of potato tubers), *Polymyxa graminis*
 20 (root disease of cereals and grasses);
- Oomycota, such as *Bremia lactucae* (downy mildew on lettuce), *Peronospora*
 (downy mildew) on 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 on hop),
 25 *Plasmopara* (downy mildew on grapes) (*P. viticola*) and sunflower (*P. halstedii*),
Sclerophthora macrospora (downy mildew in cereals and grasses), *Pythium* (seed
 rot, seedling damping-off, and root rot and all types of plants, e.g. leak at beta beet

- 36 -

- by *P. debaryanum*), *Phytophthora infestans* (late blight in potatoes, blight in tomatoes etc.), *Albugo spec.* (white rust on cruciferous plants);
- Ascomycota, such as *Microdochium nivale* (snow mould on rye and wheat),
Fusarium graminearum, *Fusarium culmorum* (spike rot, especially on wheat),
5 *Fusarium oxysporum* (fusarium withering on tomato), *Blumeria graminis*
(powdery mildew on barley (*f.sp. hordei*) and wheat (*f.sp. tritici*)), *Erysiphe pisi*
(mildew in peas), *Nectria galligena* (fruit tree cancer), *Unicnula necator* (powdery
mildew in grapevines), *Pseudopeziza tracheiphila* (red fire disease of grapevines),
Claviceps purpurea (ergot on, e.g. rye and grasses), *Gaeumannomyces graminis*
10 (blackleg on wheat, rye, and other grasses), *Magnaporthe grisea* (rice blast
disease), *Pyrenophora graminea* (leaf stripes on barley), *Pyrenophora teres* (net
blotch on barley), *Pyrenophora tritici-repentis* (leaf spots (*tritici*) on wheat),
Venturia inaequalis (apple scab), *Sclerotinia sclerotium* (stem rot, rape cancer),
Pseudopeziza medicaginis (leaf spots on lucerne, white and red clover);
 - 15 - Basidiomycetes, such as *Typhula incarnata* (typhula rot on barley, rye, wheat),
Ustilago maydis (smut on corn), *Ustilago nuda* (loose smut on barley), *Ustilago*
tritici (loose smut on wheat, spelt), *Ustilago avenae* (loose smut on oat),
Rhizoctonia solani (rhizoctonia disease on potatoes), *Sphacelotheca spp.* (head
smut of sorghum), *Melampsora lini* (rust of flax), *Puccinia graminis* (black rust on
20 wheat, barley, rye, oat), *Puccinia recondita* (brown rust on wheat), *Puccinia*
dispersa (brown rust on rye), *Puccinia hordei* (brown rust on barley), *Puccinia*
coronata (crown rust on oat), *Puccinia striiformis* (yellow rust on wheat, barley,
rye and numerous grasses), *Uromyces appendiculatus* (rust), *Sclerotium rolfsii*
(root and stem rots of many plants);
 - 25 - Deuteromycetes (Fungi imperfecti), such as *Septoria nodorum* (rust blotch) on
wheat (*Septoria tritici*), *Pseudocercospora herpotrichoides* (root rot on wheat,
barley, rye), *Rhynchosporium secalis* (leaf spot disease on rye and barley),
Alternaria solani (early blight of potato, tomato), *Phoma betae* (leaf on beta-root),
Cercospora beticola (Cercospora leaf spot disease on beta-root), *Alternaria*
30 *brassicae* (dark leaf spots of crucifers on rape, cabbage, and other cruciferous
plants), *Verticillium dahliae* (verticillium wilt and root rot), *Colletotrichum*
lindemuthianum (bean spot disease), *Phoma lingam* – damping off (take-all on

- 37 -

cabbage; root and stem rot on rape), *Botrytis cinerea* (grey mould on grapevine, strawberry, tomato, hop etc.).

In an especially preferred embodiment of the method according to the invention, broad-spectrum resistance is created in a transgenic monocotyledonous plant to *Fusarium*
 5 *graminearum*, *Fusarium culmorum*, *Septoria tritici*, *Puccinia recondita*, *Puccinia striiformis*, *Puccinia triticina*, *Puccinia hordei*, *Blumeria graminis* ff.ssp., *Rhynchosporium secalis*, *Bipolaris sorokiniana*, *Magnaporthe oryzae* and *Pyrenophora teres*.

In another especially preferred embodiment of the method according to the invention,
 10 broad-spectrum resistance is created in a transgenic dicotyledonous plant to *Phytophthora infestans*, *Uncinula necator*, *Plasmopora viticola*, *Uromyces spec.*, *Phakopsora pachyrhizi* and *Erysiphe sp.*.

In a preferred embodiment for creating broad-spectrum resistance to fungi by means of RNAi method directed against nucleic acid sequences from *Fusarium culmorum*, the
 15 transgenic plant is resistant at least to *Fusarium culmorum*.

In an especially preferred embodiment for creating broad-spectrum resistance to fungi by means of RNAi method directed against nucleic acid sequences from *Fusarium culmorum*, the transgenic plant is resistant at least to *Fusarium culmorum* and *Blumeria graminis*.

20 In the most preferred embodiment, the plant is barley (*Hordeum vulgare*).

Another aspect of the present invention refers to a transgenic plant cell having broad-spectrum resistance being produced according to the above-mentioned method.

In the sense of the present invention, a transgenic plant or plant cell is, as mentioned-above, a plant containing nucleic acid sequences which do not naturally occur in the
 25 plant, but which are homologous and/or complementary to a nucleic acid of a fungus.

- 38 -

Said nucleic acid sequences are not able to interact with endogenous plant sequences, and to influence the expression thereof.

In a preferred embodiment, the transgenic plant cell contains a recombinant nucleic acid molecule comprising a promotor which is operatively linked to at least one nucleic acid
5 sequence.

In a preferred embodiment, the transgenic plant cell contains one of the recombinant nucleic acid molecules, as defined above.

Another aspect of the present invention relates to a transgenic plant which contains one of the plant cells defined above.

10 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, the fusion of protoplasts, the direct gene transfer of isolated DNA in protoplasts, the
15 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, entire plants are to be
20 regenerated from cells which were transformed in such manner, the presence of a selectable marker gene becomes necessary. Those skilled in the art know all common 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,
25 streptomycin, sulfonyl urea, gentamycin or phosphinotricin and the like.

- 39 -

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
5 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 intermediary vector or into a binary vector. The intermediary vectors may be integrated into the Ti or Ri plasmid of the agrobacteria by means of homologous recombination due to sequences which are
10 homologous to sequences in the T-DNA. Same contains the vir region, required for the transfer of the T-DNA. Intermediary vectors are not able to replicate in agrobacteria. By means of a helper plasmid, the intermediary 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
15 which are enframed by the right and left T-DNA border region. Same 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
20 in such manner, is used for the transformation of plant cells.

For the transfer of the DNA into the plant cell, plant explants may be cultivated expediently 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) entire plants may be regenerated in a suitable medium
25 containing antibiotics or biocides for the selection of transformed cells. 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 in respect to the presence of the introduced DNA.

- 40 -

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).

- 5 Whereas transformation of dicotyledonous plants or the cells thereof by Ti plasmid vector systems with the help of *Agrobacterium tumefaciens* has well been established for a period of time, monocotyledonous plants or the cells thereof may now also be transformed using vectors which are based on agrobacteria (see e.g. Chan *et al.* (1993) *Plant Mol. Biol.* 22: 491-506).
- 10 Alternative systems for the transformation of monocotyledonous plants or the cells thereof are transformation by biolistic approach (Wan und 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
- 15 insertion of DNA by means of glass fibres.

- 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
- 20 properties.

Two or more generations should be cultivated in order to ensure that the phenotypical feature is stably maintained and inherited. Seeds should also be harvested in order to ensure that the corresponding phenotype or other characteristics remain conserved.

- According to common procedures, transgenic lines may also be determined, which are
- 25 homozygous for the new nucleic acid molecules, and the phenotypical behaviour thereof

- 41 -

may be examined with respect to a present or absent pathogen responsiveness, and said behaviour may be compared to the behaviour of hemizygous lines.

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 vectors represented above may be transmitted to plant cells in different ways. It depends on the chosen application, whether the vectors must be present in linear or circular form. Those skilled in the art know, if and when to use corresponding linearized vectors.

10 According to the invention, the term "transgenic plant" includes both the plant in its entity as well as all parts of the plant, in which the nucleic acid sequences homologous and/or complementary to fungal genes are transcribed. Such parts of a plant may be plant cells, plant seeds, leaves, petals and pollen. A "transgenic plant" is, according to the invention, also the propagating material of transgenic plants of the present invention, e.g.
15 seeds, fruits, cuttings, tubers, pieces of root, etc., wherein said propagating material contains above-mentioned transgenic plant cells, and if necessary, transgenic parts of said plants, such as protoplasts, plant cells and calli.

Various procedures and possibilities may be used in the production of transgenic plants, as already discussed above. In general, plants or plant cells may be modified by means of
20 common genetically-engineered transformation procedures such that the new nucleic acid molecules are integrated into the plant genome, i.e. that stable transformants are produced and the transmitted nucleic acid molecules are replicated with the plant genome. Depending on the vector system used, according to the invention, also transgenic plants may be produced in which the nucleic acids to be transferred are
25 contained in the plant cell or the plant as autonomously replicating system. The vectors used for transforming the plants, must therefore contain DNA sequences which enable the replication of plasmids used for transformation within the cell.

- 42 -

Another object of the present invention is transgenic plants or plant cells which have been produced according to one of the methods of the present invention, and which have higher resistance to fungi compared to the wild type.

Another object of the present invention is transgenic plants or plant cells which have
5 been produced according to one of the methods of the present invention, and which have broad-spectrum resistance to fungi compared to the wild type.

According to the invention, a "wild type" is the corresponding parental organism which has not been genetically modified.

Transforming plants with the recombinant nucleic acid molecule of the present invention
10 may be performed by any known transformation procedure; suitable are, for example, the biolistic method, the agrobacterium mediated transformation, the protoplast transformation, the electroporation of partially permeabilized cells, and the introduction of DNA by means of glass fibres. Which respective procedure may best be employed for the transformation depends on the used test plant.

15 Preferably, the test plants are transformed by means of the biolistic transformation, since this approach results particularly fast and effectively in a transformation with a plurality of various recombinant nucleic acid molecules.

A transgenic plant or plant cell in the sense of the present invention is, as mentioned-
above, a plant containing nucleic acid sequences which do not naturally occur in the
20 plant but which are homologous and/or complementary to parts of the fungal genome. Said nucleic acid sequences are not able to interact with endogenous plant sequences and to influence the expression thereof.

Another object of the present invention relates to the usage of the transgenic plants of the present invention and the cells, cell cultures, parts and transgenic propagating materials
25 derived thereof for the production of food and feed, pharmaceuticals or fine chemicals.

- 43 -

The infection of test plants with fungal organisms in order to examine potential resistance phenomena is a method well-known to those skilled in the art. The used test plants must be responsive to said used fungus, i.e. they must be able to serve as host plant for said fungus, and the fungal attack must be detectable by simple means.

- 5 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 that fungus the plant is to be infected with, or with the infectious parts thereof, under conditions in which the fungus may enter a wild type plant.

10 The fungal infestation of the plant may then be evaluated by means of a suitable evaluation procedure. The visual evaluation, in which the formed fungal structures are detected in the plant and quantified, is particularly suitable. In order to identify the successfully transformed cells, a reporter gene, such as the β -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 β -galactosidase
15 (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, preferably along with the RNAi construct. 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.

20 Another aspect of the present invention relates to the above-defined recombinant nucleic acid molecules comprising at least one nucleic acid sequence, operatively linked to a promotor functional in plant cells, wherein the nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1 - 5 and/or a part thereof, or has a sequence identity of at
25 least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 1 - 5, and/or
- b) is identical and/or complementary to one or more of the nucleic acids, selected from SEQ ID NOs: 6 - 11 and/or a part thereof, or has a sequence identity of at

- 44 -

least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 6 - 11,
or

- c) is identical and/or complementary to one or more of the nucleic acids, selected
from SEQ ID NOs: 12 - 16 and/or a part thereof, or has a sequence identity of at
least 85 % to one or more of the nucleic acids selected from SEQ ID NOs: 12 -
16.

The nucleic acid molecules used in the method may be isolated using molecular
biological standard techniques and the sequence information provided herein. With the
help of comparison algorithms, which may be found for example on the NCBI homepage
at <http://www.ncbi.nlm.nih.gov>, a homologous sequence or homologous, conserved
sequence regions on DNA or amino acid level may also be identified. Substantial parts of
said sequence or the entire homologous sequence may be used as hybridization probe
using standard hybridization techniques (such as, for example, described in Sambrook *et al.*,
vide supra) for the isolation of further nucleic acid sequences, which are useful in the
method, from other organisms by screening cDNA and/or genomic libraries.
Furthermore, a nucleic acid molecule may be isolated by polymerase chain reaction,
wherein oligonucleotide primers are used on the basis of the sequences, or parts thereof,
contained in the libraries (e.g. a nucleic acid molecule, comprising the entire sequence or
a part thereof, may be isolated by polymerase chain reaction, using oligonucleotide
primers, which have been created on the basis of said same sequence). For example,
mRNA may be isolated from cells (e.g. by the guanidinium thiocyanate extraction
method from Chirgwin *et al.* (1979) Biochemistry 18: 5294 – 5299), and cDNA may be
produced using reverse transcriptase (e.g. Moloney MLV reverse transcriptase, available
from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase, available from
Seikagaku America, Inc, St. Petersburg, FL). A nucleic acid may be amplified using
cDNA or, alternatively, using genomic DNA as matrix and suitable oligonucleotide
primers by means of standard PCR amplification techniques. The thus amplified nucleic
acid may be cloned into a suitable vector, and may be characterized by means of DNA
sequence analysis. Oligonucleotides, which are suitable for amplifying the desired
sequence, may be produced by standard synthesis methods, e.g. with an automatic DNA
synthesis device.

The exemplary identification of fungal genes which are suitable for the method of the present invention and the use thereof for creating broad-spectrum resistance in transgenic plants, will be described in the following. The following examples shall not limit the scope of the present invention.

5

Examples

A. Method for producing triple RNAi constructs

Producing the RNAi constructs of the present invention is based on the modified GATEWAY technology according to Douchkov et al. (2005) *Mol. Plant. Microbe. Interact.* 18:755-61.

10

Conserved proteins, or the genes encoding said conserved proteins, of phytopathogenic fungi serve as a basis for the RNAi constructs of the present invention. Examples here are gamma-actin, the mitochondrial ADP/ATP carrier protein, the RhoGTPase, the elongation factor 1, and the mitochondrial heat shock protein 70 chaperone.

15 Sequence sections within the genes, which are conserved in various fungi, encoding the above-mentioned proteins were determined by sequence comparisons.

For this purpose, corresponding DNA sequence data from various fungi were compared by the Sequencher software (Gene Codes Corp., Aim Arbor, MI, USA) using the "dirtydata" algorithm, and a consensus sequence was derived.

20 The following sequences from the COGEME data base (<http://cogeme.ex.ac.uk>) and from the NCBI database (<http://www.ncbi.nlm.nih.gov/sites/entrez>) were used as basis for the sequence comparisons of the different genes:

- 46 -

Gamma-actin (GA) BfCon[0048], Contig[0009], CpCon[0433], FsCon[0011], FsCon[0012], GzCon[0219], MagCon[0535], MagCon[10153a], MagCon[11943a], mg[1255], MGT_4266, Pi10229566, PiCon[0102], PsCon[0011], UmCon[0226], VD0108G03, YDL029W Chr4, YFL039C Chr6, YHR129C Chr8, YJR065C Chr10.

- 5 Elongation factor 1 (EF1): BfCon[0035], BfCon81924], BfCon[2093], Cf8667247, CtCon[0128], DQ522321, GzCon[0015], Mag3392227, MagCon[2061], MagCon[2896], MagCon[3191], MagCon[4553], MagCon[4838], MagCon[5002], MagCon[5960], MagCon[6041], MagCon[690a], MagCon[10107a], MagCon [12375a], MagCon[12727a], MagCon[13104a], MagCon[13904a], mg[0073], mg[0358],
 10 SSPG1015, Um34332478, UmCon[0014], UmCon[1272], UmCon[2780], VD0110B10, VD0201A10

Mitochondrial ADP/ATP translocator (AAC): BfCon[0014], BfCon[1221], Cf8667327, CfCon[0008], Contig[0005], FsCon[0026], FsCon[0406], GzCon[0025], mg[0104], SSPG985, VD0100D40, VD0203D11.

- 15 Mitochondrial heat shock protein 70 (Hsp70): BfCon[0098], Cf8667122, Cf8667223, FsCon[1028], FsCon[1857], Gz47835892, GzCon[1242], GzCon[2886], GzCon[3587], MagCon[2045], MagCon[10827a], mg[1360], mga0294f, UmCon[1735], UmCon[3134], VD0204G10.

- Rho GTPase (Rho): AF395859, BfCon[0909], Bg13902524, Bg27453832,
 20 BgCon[2092], CfCon[0041], Contig[0007], CpCon[0819], CtCon[0239], FsCon[0840], Gz47836309, GzCon[0911], GzCon[7209], Lm13259684, Mag14180535, Mag30400635, Mag30403197, MagCon[0027a], MagCon[1398], MagCon[4208a], MagCon[6895], MagCon[11651a], mg[1439], mga0839f, SSPG240R, Um34331745, Um37403496, Um37407871, UmCon[0162], UmCon[0987], UmCon[1018],
 25 UmCon[2074], W0AA016ZE09C1.

- 47 -

As a result of the sequence comparisons, a consensus sequence was established for each gene (for nucleotide symbols, see table on page 87):

Consensus sequence of AAC

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GC:AGACCGTTCTTGGGCATGCCCCCTTCGTCGCTGACTTCCTCATGGG
5  TGGTGTCTCCGCCGCTGTCTCCAAGACCGCTGCGCCCCCATYGAGCGTG
   TCAAGCTCCTCATCCAGAACCAGGATGAGATGCTGAAGACCGGTCGTCTY
   GACCGCAAGTACGACGGCATTGGTGASTGCTTCAAGCGYACCACCGCSGA
   TGAGGGGTGTCATGTCCCTCTGGCGWGGHAACACTGCCAACGTCATCCGTT
   ACTTCCCTACCCAGGCCCTGAACTTCGCTTTCGGTGACAAGTTCAAGTCG
10  ATGTTCTGGCTACAAGAAGGACCGTGATGGCTACGCCATGTGGATGGCYGGTA
   ACYTKGCCTCCGGTGGTGCTGCTGGTGCCACTTCCCTCCTCTTCGTCT
   ACTCCCTCGACTACGCCCGTACYCGTCTTGCCAACGACGCCAAGAACGCC
   AAGACCGGTGGTGACCGTCAGTTCAACGGTCTSGTCGATGTCTACAAGAA
   GACCCTCGCCTCTGACGGTATTGCCGGTCTCTACCGTGGTTTCGGT:CCT
15  TCCGTTGCTGGTATCGTCGTYTACCGTGGTCTCTACTTCGGWATGTACGA
   YTCCATCAAGCCAGTCCTCCTCAYYGGWMMYCTYGAGGGYAACTTCCTTGC
   WTCCTTCTTGCTCGGATGGACCGTCACCACTGGTGCCGGTATCGCCTCTTACC
   CATTGGACACCATCCGTCGTCGTATGATGATGACTTCTGGTGAGGCCGTCAA
   GTACAAGTCTTCCTTGGATGCTGSYCGYCARATCGTYGCCAAGGAGGGWGTY
20  AAGTCTCTCTTCAAGGGTGCTGGTGCCAACATTCTCCGTGGTGTGTCAGGTGC
   TGGTGTCTGTCCATCTACGATCARATGCARGTCYTGATGTTCCGAAAGGCA
   TTCAAGTAAAYGWATCGTA:GA:ATGGAGTGAAGG::G::ATGAG:T:TGAKTG:T
   AGA:GGTGGT:TGATGATAATGCGAAATGAG:C:GCCT::TGRGGTCG:GTC:C:G:T
   GGTT:GA:AA::CT::AAAAGGTTG:AG:ATSGTAG:TACAG::ATCSATTGC:ATTG:T
25  G:T:TRSAGATAC:TT:GMC GTG:TAGTCTGMCAC::AR:CAWGATCT:AT:ACRA:T
   YTGAGT:SGCTTMTG:CA:C:AGAGT:::CTT:TAT:CTA:ATCTTKCWTT:GCCTTCC
   GTTCTATGCCCKV:T:GTA:ATT::T::A:TACACCAATTCAAVT::TCC:CTTTA:CTGT
   CTRTTCNCTTA:A:CTGAGDDTTMAATTTKRMKCMSTMKTCATCCTKKTKAK
   TGCTGACYCYTRKTCTMCACTCTRTCTWTTCSYTTTCWTCCATTTGCYMYW
30  CYCMCTCCTATCMKTAGCATACTWCGTAYWATCYKTSTTGCCTAACGTMYK

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- 48 -

WACTACCYCYMTTMYTRCTTWKCGATTACTMTYYCTWTMTYYCTYCGKT
 WCMYTCWCSTWYMTGTTKCATMTCTCTGACTCTCTCTMTYCTCCCTCT
 MTAYACYYKYTTTRYCTKTYGCCMYTMRYCCTMTCTTCTMATTTTCCTACT
 TCTATTCTCTTGCTATTTCTCTCCTATCTGTTCCATCCCTTCATTCATCATTCT
 5 CTCTCTCTGCTTCAACGATTATCATCACTATTCTATCTC

Consensus sequence of EF1

ATGGGTAAAGGAGAAGACTCACATCAACGTCGTCGTCATCGGCCACGTCGAC
 TCCGGCAAGTCGACCACCACCGGTCACTTGATCTACAAGTGCGGTGGTATCG
 ACAAGCGTACCATCGAGAAGTTCGAGAAGGAAGCCGCCGAGCTCGGYAAGG
 10 GTTCCTTCAAGTACGCCTGGGTTCTTGACAAGCTCAAGGCCGAGCGTGAGCG
 TGGTATCACCATCGACATCGCCCTCTGGAAGTTCGAGACYCCCAAGTACTAT
 GTTACCGTCATTGACGCCCCTGGTCACCGTGACTTCATCAAGAACATGATCA
 CTGGTACCTCCCAGGCCGACTGCGCCATTCTCATCATTGCCGCTGGTACTGGT
 GAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTCC
 15 TCGCCTACACCCTCGGTGTCAAGCAGCTCATCGTYGCCATCAACAAGATGGA
 CACCACCAAGTGGTCCGAGGACCGTTWCCAGGAGATCATCAAGGAGACCTC
 CAACTTCATCAAGAAGGTCGGCTACAACCCCAAGACYGTGCGCTTCGTCCCC
 ATCTCCGGTTTCAACGGTGACAACATGMTGACGCGCTCCACCAACTGCCCCT
 GGTACAAGGGTTGGGAGAAGGAGACCAAGKCWGGCAAGGYCACCGGCAAG
 20 ACCCTCCTCGAGGCCATCGACGCCATCGAGCCCCCAAGCGTCCCACCGACA
 AGCCCCTCCGTCTTCCCCTCCAGGATGTCTACAAGATCGGYGGTATTGGMAC
 TGTTCCCGTCGGCCGTRTCGAGACYGGTATCATCAAGCCCGGYATGGTCGTC
 ACCTTCGCTCCCGCCAACGTCACCACTGAAGTCAAGTCCGTCGAGATGCACC
 ACGAGCAGCTCACTGAGGGTSTTCCCGGTGACAACGTCGGTTTCAACGTCAA
 25 GAACGTTTCCGTCAAGGACATTGCGCCGTGGTAACGTCGCTGGTGACTCCAAG
 AACGACCCCCCAAGGGTGCCGCTTCTTCAACGCYCAGGTCATYGTCTTYA
 ACCACCCTGGTCAGGTCGGTGCTGGTTACGCCCCCGTTCTCGATTGCCACACT
 GCCACATTGCCTGCAAGTTCTCCGAGCTCCTCGAGAAGATCGACCGCCGTA
 CCGGTAAGTCCATTGAGGACTCCCCCAAGTTCATCAAGTCTGGTGACGCTGC
 30 CATCGTCAAGATGGTTCCTCCAAGCCCATGTGTGTTGAGGCCTTCACYGAC

- 49 -

TACCCTCCTCTGGGMCGTTTCGCCGTCCGTGACATGCGTCAGACCGTCGCTG
 TCGGTGTCATCAAGTCCGTCGACAAGTCCACGACACTGCCGGTAAGGTCACC
 AAGKCCGCCGYCAAGGCTGGTGCCAAGAAATAAACTTATTTATG:AATGA::C
 GA:CCT:TCA:G:TBGGATSTTGCGASTTTG::WT:TCAYGCTCA:TGAGGCTGATC:
 5 T:ACTGGCTT:GT:ACGCC:GAATCGATCAT:TTG:CA:
 TGAG:C:AC:TC:GKGTAATGACT:TGSA:G:A:KT:C:TTSAGA:G:ATWTT:CAAATG
 CCT:GCTGATS:A:C:GTTGT:G:GCA:CATCA:C:ATA:GAACACT:TMGTCKA::G:A
 A:AT:G:ACTTC:ARAG:AAAAAAAAAAAAAAAAACAW:GT:CATG:ACCAAACGCCG
 AGATCACTTCTGAG:GACTCTA:TGTAG:ACTAAAGC:ACAT:GT:CAAGAATTTT
 10 TTTGAGTTACGAAATCCCAAACCGTCATTTTCATCATGTGCCCCAATAAAAAAC
 CGAGTACTCTTTTGTGATAAAGCCAAAATTGACCGGAAAACCTGCGTTTGC
 AACGAGGAAAGTGCCCAAGATGCTACGACATGCTTCCTGGCTTGTCGTGGCT
 CGACATTAGATGTTTGTGCTGKTTGGTTGATCGCAGCVTYATATTTCTTTKGGCS
 GGCCCCTTGGCCACGCGGGRACCTTGGGTGACCCTTGSCCCTGGGGTACCCT
 15 GGGGACTTGGTCGACGGCTCTTGATGACACCGAACAGCGACGGTCTGACGCA
 TGTCACGGACGGCGAAACGACCAAGGGGAGGGTACTCGGAGAAAGTCTCGA
 CACACATGGGCTTGGACTCGTSCGCBMMGRATMMCTAGCAGACCGGRGG

- 50 -

Consensus sequence of GA

CCACGCGTCCGCCCTTTTCTGCCCAACAACCTTTCCT:CTCYBTCAACGATCTW
 CT::TTTCC:CA:TCAACT:T:GTCGCCTG:CAACA::A:TCTCT:GC:TCTA:CATCT::T
 CAG:A:TCTAT::C::ATTCCCTCC:GCGACTT:TCG:C:GA:TTC:TTCT:WTC:TCCTGA
 5 C:CT:TG:ATT:CA:TC::CG::C:C::AG:C:TCTC:TTM:ATCTCC:CTATTCCACT:A:CA
 CA:CCT:TA:ATCMATCA:CGATGGAGGAAGAAGTCGCWGCTCTCGTCA TYGAC
 AATGGTTCGGGTATGTGCAAGGCCGGTTTCGCCGGTGACGATGCTCCCCGAG
 CTGTCTTCCCTTCCATTGTGCGGTCGCCCCCGTCACCATGGTATCATGATTGGT
 ATGGGCCAGAAGGACTCGTACGTTGGTGATGAGGCCAGTCCAAGCGTGTT
 10 ATCCTCACTCTGCGGTACCCCATCGAGCACGGTGTYGTCACCAACTGGGACG
 A:CATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTGCGTGTCGCCC
 CCGAGGAGCAC:CCCGTCCTGCTGACTGAGG:CTCCCATCAACCCCAAGTCCA
 ACCGTGAGAAGATGACGCAGATCGTC:TTCG:AGACCTTCAACGCCCCCGCCT
 TCTACGTCTCCATCCAGGCCGTKCTGTCCCTGTACGCCTCCGGTCTGTA CCACT
 15 GG:TATCGTGCTCGACTCYGGTGACGGTGTTACTCACGTTGTGCCCATCTACG
 AGGGTTTCKCCCTKCCCCACGCCATTGCCCCGTGTCGACATGGCTGGTC:GTG:A
 :CTTG:A:CCG::AC:T::ACCTG:ATGAAGA:TCTTGGCTGAGCGCGGTTACA:CCTT
 :CTCC:ACCAC::T:GCCGAGCGWGAAATC:GTC:CGTGA:CATCAAGGAGAAGCT
 CT:::GCTACGTCG
 20 CCCTT:::GA:CTTCGAGCAGGAGATCCAGACTGCTGCCCAGTCCTCCAGC:YTG
 GAGAAGTCCTACGAGCTTCCCGACGGACAGGTTATCACCATCG:GCAACGAG
 CGC:T:TCCG:TG:CT:CCTGAGGCTCTGTTCCAGCC::TTCTGT:CC:TGGGTCTTGA
 GA:GCGG:TGGTATCC:ACGT:CACCACTTTCAACTCCATCATGAAGTGTGATGT
 CGA:TGTCCGAAAGGATCT:GTACGGCAACATTGTCATGTCTGGTGG:TACCAC
 25 C::ATG:TACCC:TGGTATCTCCGAC:CGTATGCA:GAAG:GAGATCACTGCT:CTT
 GCCCK:TCCTCSATGAAGGTCAAGATCATTGCTCCTCCCGAGCGCAAGTACT
 CCGTCTGGATCGGTGGTTCCATTCTCGCTT:CG:C::TGTCGACCTT:CCAGCAGA
 TGTG::GATCTCGAAGCAGGAGTACGACGAGAGCGGACCTTCSATCGTSCACC
 GCAAGTGCTTCTAAGC:G:CCTGACCG:A:CGA:TT::GTGC:TCCAACCT:CG::C:TT:
 30 ACGAA:CTG:GAK:CAGAA:ATGTC:GCGA:CAG:ACGC:TA:AGC:KTCGDGAGG:T
 TG:GG:T:GCTT:CGAAG:CTCG:TCCGAAACTCGTRTCTG:CG:AAC:A:TTTA::TTA

- 51 -

CATG:G::TCGA:T:GAGTSAACGTTGCGCATG:TACG:AGAGATG:GATGGCCTG
 WT:T:T:CTTGCAGGTCGAGTA
 C::AATG:CGT::CTAGGGC:TASAAG:BCGA:GGTTGACATAC:GAATA:CG:TGTCT
 ACTW::TCC::G:C::TTAGARCTG:ACGACA:T:AW:TC:GAGGGGTTGA:TCAT:GCA
 5 RCGYGAG::T::CHG:TC:CC:TTGCT:CTAGAGAATCRAMTTAMAWGGTMGWAA
 M:TGA:AAAGAGBTCRGTTGGMCATCCTGSCRMRCARTRAWMSATMYGMYAA
 CGMMAMWMARWARAMRWRDMAMMWWMAAAAAAAAAAAAAAAAAAAAAA
 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGGGCGGCCGCTCTAN

Consensus sequence of Hsp

10 GCACGAGGAGAAGGTTGAGGGTGAGCGCAACGTCCTCATCTTCGATCTTGGY
 GSWSGWRSCTTCGATGTCTCTCTCCTTACCATTGAGGAGGGTATCTTCGAGGT
 CAAGTCTACTGCCGGTGACACTCACTTGGGTGGTGAAGATTTCGACAACCGC
 CTCGTTAACCACTTCGTTAACGAGTTCAAGCGAAAGCATAAGAAGGATCTAA
 GCACCAACGTCCGTGCTCTTCGACGTCTCCGAACCGCTTGTGAGCGCGCCAA
 15 GCGAACTCTCTCTTCTTCCGCTCAGACCTCCATTGAGATCGACTCKCTSTWCG
 AGGGTATTGACTTCTACACCTCCATCACCCGTGCCCGTTTCGAGGAGCTCTGC
 CAGGATCTCTTCCGATCCACCATCCAGCCCGTCGACCGTGTCTTACCGACGC
 CAAGATCGACAAGTCCCTBGTCCACGAGATCGTCCTCGTYGGYGGWTCYAC
 CCGTATCCCCCGTGTCCAGAAGCTCATCACCGACTACTTCAACGGAAGGAG
 20 CCCAACAAGTCCATCAACCCYGATGAGGCTGTTGCCTACGGTGCTGCCGTCC
 AGGCTGCCATTCTCTCTGGTGACACCACCTCCAAGTCCACCAACGAGATCCT
 GCTYCTCGATGTGCCCCSCTSTCTCTCGGTATCGAGACCGCTGGTGGTATGA
 TGACCAAGCTCATCCCCGCAACACCACCATCCCCACCAAGAAGTCYGAGGT
 CTTCTCCACCTTCTCCGACAACCAGCCTGGTGTGCTCATCCAGGTCTACGAGG
 25 GTGAGCGCCAGCGCACCAAGGACAACAACCTSTGGGCAAGTTCGAGCTTAC
 CGGCA:TCCCMCCTGCTCCCCGTGGTGTTCGCCAGATTGAGGTCACCTTCGAC
 CTSGATGCCAACGGTATCATGAACGTCTCCGCCGTCGAGAAGGGCACCGGCA
 AGACCAACAAGATTGTCATCACCAACGACAAGGGCCGYCTGTCCAAGGAGG
 AGATYGAGCGCATGCTTGCGYAGGGCCGAGAAGTACAAGGAGGAGGATGAGG
 30 CYGAGGSCRMCCGTGTCTCTGCCAAGAACGGCCTYG:AGTCKTACGCCTACT

- 52 -

CCCTSCGCAA:CACCCTSTCCG:ACYCCAAGGTCGASG:AGAAGCT:TGAKGCTK
 CCGACAAGGAGAMSCTCAMYGCTGAGATYRACAAGRITYGTCCAGCTGGCTY
 GAYGASARCCAGCAGGCTACYMRKGAGGAGTAYGAGGAGCACCAGAAGGA
 GCTYGAGGSYRWKGCYAACCCCATCATGATGAARTTCTACGGAGCTGSTGGT
 5 GAGGGYGCTCCCGGTGGCATGCCCCGYRYRCCCGGTS GTGCCCTGGTGGCT
 TCCCTGGTG CYGGTGGCCCYGCYCCYGGYGCCGGYGGYGACGATGGYCCCA
 CYGTCGAGGAGGTCGACTAAAYWMTYGMTACYCYWRTAMCATCAKTCYC
 KAMTTSTWKWKYGRKCAYAKMTSSRTKKKTWTWATGCGGWKKYTAGTMGT
 TTKMAYGSKMTGGRTSWGATTASATTGTTCTMTTTTCTYCWRTTTTMTTCGTA
 10 AYGACWTYACGRWTRKRAAAAGTCWMMGGYGVITYYATRYACGTGGKGRW
 TGTATWTYAATTAGRATCWTWCAWTSRDAAMAAMARWWMAAAAWAAAA
 AAAACTCGAAACTAGTTCTCTCTCTCTCTCCTCGTGC

Consensus sequence of Rho

AATCCATMACCATGGCTGAAATCCGCCGAAAGCTCGTCATTGTCGGHGAYG
 15 GTGCTTGTGGTAAGACYTGTTTGTTGATTGTCTTCTCCAAGGGCACHTTCCCM
 GAGGTCTACGTCCCMACCGTCTTCGAGAACTATGTGCGCGATGTGCGAGGTCG
 ATGGCAAGCACGTGCGAGCTCGCGCTATGGGATACBGCTGGTCAGGAGGATTA
 CGACCGTCTTCGACCTCTCTCATACCCCGACTCSCACGTTATCCTGATCTGCT
 TCGCCGTTGACTCTCCCGATTCCCTCGAYAACGTYCAGGAGAAGTGGATCTC
 20 CGAGGTCCTSCAYTTCTGCCAGGGTCTCCCTATCATCCTTGTCGGCTGCAAGA
 AGGATTTGCGAYACGACCAGAAGACCATTGAGGAGCTCCACAAGACCASCC
 AGAASCCCGTCACCCCWGARCAGGGTGAAGAGGTCCGCAAGAAGATTGGTG
 CTTACAAGTATCTTGAGTGCTCAGCCAAGACCAATGAAGGTGTCCGYGAGGT
 GTTCGAGCACGCCACTCGCGCYGCTCTCCTGACGCGGAAGAAGCAGAAGAA
 25 GCGCGCAAG

Employing the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>), the sequence with the highest sequence homology to the respective consensus sequence (SEQ IDs NOs: 1 - 5) was determined from the above-mentioned COGEME or NCBI sequences:

- 53 -

- GA:** GzCon[0219] (95 % identity to GA consensus sequence)
EF1: GzCon[0015] (91 % identity to EF1 consensus sequence)
AAC: FsCon[0026] (93 % identity to AAC consensus sequence)
Hsp: GzCon[2886] (92 % identity to Hsp consensus sequence)
5 **Rho:** FsCon[0840] (93 % identity to Rho consensus sequence)

Suitable regions for the identification of siRNA target sequences within said sequences were – in turn – determined employing the RNAiscan-algorithm (<http://bioinfo2.noble.org>). Thereby, the target sequences were selected such that no or only minimal "cross-silencing" to corresponding sequences from barley and/or wheat was to be expected.

Primer were derived from the respective target sequences, and fragments having about 300 - 350 bp (SEQ ID NOs: 6 - 11) were isolated from *Fusarium* cDNA and/or DNA samples, and cloned in RNAi vectors (see below). In the case of GA, two different GA fragments ("GA":300 bp; "GA-1":350 bp) may be presented.

15 The sections within the consensus sequences, to which the above-mentioned contigs show the described high homology, i.e. the conserved regions within the consensus sequences, are listed as SEQ ID NOs: 12 - 16 further below.

A1. Preparation of the "entry vector" (pIPKTA38)

Bacteria, which contained the plasmid, were grown in LB + kanamycin (50 µg/mL).

20 Plasmid DNA was prepared with the *Jetstar* midi DNA kit.

A control digestion with the restriction enzyme *Apa* I was carried out in the following. The DNA concentration was measured and adjusted to 150 ng/µL subsequently.

A2. Preparation of the "destination vectors"

The vectors pIPKb007, pIPKb010 and GER4 (p6UGLP4deltaSwaIntronRNAi) were
25 used as "destination vectors". Bacteria, which contained the respective plasmid, were grown in LB + spectinomycin (100 µg/mL). Plasmid DNA was prepared with the *Jetstar*

- 54 -

mid DNA kit. The plasmid preparations were digested for control with *EcoR* V (bands: pIPKb007 – 12039, 3953 bp; pIPKb010 – 12039, 3821 and 1148 bp; GER4 – 12039, 3869 and 1721 bp).

The DNA concentration was measured and adjusted to 150 ng/ μ L.

5 A3. PCR amplification of the target sequences of conserved fungal genes

Fragments, which were amplified by PCR from *Fusarium culmorum* DNA/cDNA, of about 300 bp length from the coding sequence regions from the selected conserved fungal genes were first ligated in pIPKTA38 as *entry vector* (see Figs. 1 and 2). For this purpose, various enzymatic cleavage sites (*Swa* I, *EcoR* V, *Sma* I) within the multiple
10 cloning site were used. Said enzymatic cleavage sites were selected according to the desired sequence combination of the RNAi target constructs (see table 5). The ligation of the first sequence took place at the *Swa* I site of pIPKTA38. In insert two further sequences, the pIPKTA38 construct was used, which contained the first successfully inserted sequence. Said resulting construct was obtained by two further ligations to form
15 a construct comprising three different sequences (see Fig. 2).

“Master mixes” for 20 μ L PCR reactions with two specific primers

Specific primers were designed in order to amplify ~300 bp fragments from *Fusarium culmorum* DNA or cDNA. The melting temperature (T_m) of the primer was ~65 °C.

A “master mix” (see table 1) was prepared, and 3.75 μ L/well were added to wells of a
20 96-well PCR plate. 8.5 μ L „target“-specific “forward” and “reverse” primer each were added to each well.

Table 1: Composition of master mix:

Component	1x	10x
<i>Thermal ace buffer (10x)</i>	2 μ L	20 μ L
<i>dNTPs (50x, 10 mM each)</i>	0.5 μ L	5 μ L
<i>Thermal Ace DNA polymerase (2 U/μL)</i>	0.25 μ L	2.5 μ L

- 55 -

Template (<i>Fusarium culmorum</i> DNA)	1.0 μ L	10 μ L
	3.75 μ L Master mix per well	
Target-specific primer - forward (1 μ M)	8.5 μ L	
Target-specific primer - reverse (1 μ M)	8.5 μ L	

PCR cycle conditions

95 °C	3 min	} 30 Cycles
95 °C	30 sec	
65 °C	30 sec	
74 °C	60 sec	
74 °C	10 min	
4 °C	Pause	

Purification of the PCR product

30 μ L H₂O were added to each PCR reaction in order to adjust to a total volume of 50 μ L.

Purification was carried out using the *Qiagen MinElute UF 96-well* kit, wherein 20 μ L

- 5 H₂O were used for elution subsequently (see *Qiagen MinElute* protocol). For control, 2 μ L each of the purified PCR product were separated by agarose gel electrophoresis.

A4. Cloning of the PCR products

A ligation mix was prepared (see table 2), 6 μ L each of this ligation mix was added to each well and 4 μ L of the purified PCR product were added.

- 10 The samples were incubated for 1 h at 25 °C and the reaction was stopped by heating up to 65 °C for 10 min subsequently. 5 μ L *Swa* I / *EcoR* V / *Sma* I-mix (see table 3) each was added to each well, following by an incubation at 25 °C for 1 h. Next, the ligation samples were transformed into competent bacteria and suitable clones were isolated after miniprep and control digestion.

- 56 -

Table 2: Ligation mix for 10 μ L reactions (6 μ L mix + 4 μ L PCR product per reaction)

<i>Components</i>	<i>1x</i>
<i>H₂O</i>	<i>1 μL</i>
1) <i>pIPKTA38 (150 ng/μL)</i> 2) <i>pIPKTA38_target1 (150 ng/μL)</i> 3) <i>pIPKTA38_target1_target2 (150 ng/μL)</i>	<i>1 μL</i>
<i>Ligation buffer (10x)</i>	<i>1 μL</i>
<i>50 % PEG 4000</i>	<i>1 μL</i>
<i>NaCl (0.5 M)</i>	<i>1 μL</i>
1) <i>Swa I (10 U/μL)</i> 2) <i>EcoR V (10 U/μL)</i> 3) <i>Sma I (10 U/μL)</i>	<i>0.5 μL</i>
<i>T4 DNA ligase (5 U/μL)</i>	<i>0.5 μL</i>
<i>PCR product (purified)</i> 1) <i>target 1</i> 2) <i>target 2</i> 3) <i>target 3</i>	<i>4 μL</i>

Table 3: *Swa I* / *EcoR V* / *Sma I* mix (5 μ L per reaction)

<i>Components</i>	<i>1x</i>
1) <i>Swa I buffer (10x)</i> 2) <i>EcoR V buffer (10x)</i> 3) <i>Sma I buffer (10x)</i>	<i>0.5 μL</i>
<i>NaCl (0.5 M)</i>	<i>1 μL</i>
<i>H₂O</i>	<i>3 μL</i>
1) <i>Swa I (10 U/μL)</i> 2) <i>EcoR V (10 U/μL)</i> 3) <i>Sma I (10 U/μL)</i>	<i>0.5 μL</i>

A5. LR reaction

According to their purpose, the constructs with one or three sequence fragments (targets)
 5 were transferred from the intermediate vector pIPKTA38 (entry vector) to a RNAi vector

- 57 -

using the LR clonase system. pIPKb007 (formerly p6UUbiRNAi), pIPKb010 (formerly p6U-pGSTA1-RNAi) and p6UGLP4deltaSwaIntronRNAi (p6UGer4deltaSwaIntronRNAi) were used as RNAi vectors (destination vector).

5 A graphic representation of the components of all three vectors of the *triple target* RNAi constructs can be found in the annex, where as example the sequence combination of gamma-actin, RhoGTPase and the mitochondrial heat shock protein chaperone 70 (GA-Rho-Hsp) is shown.

Master mixes for 6 μ L LR reactions

10 Table 4: LR master mix (5 μ L master mix + 1 μ L IPKTA38_target1_target2_target3 DNA per reaction).

Component	1x	for 96 samples
1) pIPK007 (150 ng/ μ L)	1 μ L	100 μ L
2) pIPK010 (150 ng/ μ L)		
3) GER4 (p6UGLP4deltaSwaIntronRNAi) (150 ng/ μ L)		
H ₂ O	3 μ L	200 μ L
LR clonase mix II	1 μ L	80 μ L
	5 μ L Master mix per well	
pIPKTA38_target1_target2_target3	1 μ L per reaction	

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

- 58 -

Table 5: Overview of the RNAi constructs in combination of three different vector systems, five different conserved fungal proteins, and three different enzyme cleavage sites for the ligation of the respective RNAi targets.

No.	Vector system	Insertion of the RNAi sequence (target) into enzymatic cleavage site		
		<i>Swa</i> I	<i>EcoR</i> V	<i>Sma</i> I
		target 1	target 2	target 3
1	pIPKb007	GA (gamma-actin)		
2	pIPKb007	AAC (mitochondrial ADP/ATP carrier)		
3	pIPKb007	Hsp (mitochondrial heat shock protein chaperone)		
4	pIPKb007	Rho (Rho-GTPase)		
5	pIPKb007	EF1 (elongation factor 1)		
6	pIPKb007	GA	AAC	Rho
7	pIPKb007	GA	AAC	Hsp
8	pIPKb007	GA	AAC	EF1
9	pIPKb007	GA	Rho	EF1
10	pIPKb007	GA	Rho	Hsp
11	pIPKb007	AAC	Rho	EF1
12	GER4 (p6UGLP4deltaSwaIntronRNAi)	GA	AAC	Rho
13	GER4 (p6UGLP4deltaSwaIntronRNAi)	GA	AAC	Hsp
14	GER4 (p6UGLP4deltaSwaIntronRNAi)	GA	AAC	EF1
15	GER4 (p6UGLP4deltaSwaIntronRNAi)	GA	Rho	EF1
16	GER4 (p6UGLP4deltaSwaIntronRNAi)	GA	Rho	Hsp
17	GER4 (p6UGLP4deltaSwaIntronRNAi)	AAC	Rho	EF1
18	pIPKb010	GA	AAC	Rho
19	pIPKb010	GA	AAC	Hsp
20	pIPKb010	GA	AAC	EF1
21	pIPKb010	GA	Rho	EF1
22	pIPKb010	GA	Rho	Hsp
23	pIPKb010	AAC	Rho	EF1

Table 6: Overview of further RNAi constructs in combination of three different vector systems, five different conserved fungal proteins, and three different enzyme cleavage sites for the ligation (4. Cloning of the PCR products) of the respective RNAi targets.

No.	Vector system	Insertion of the RNAi sequence (target) into enzymatic cleavage site		
		Swa I	EcoR V	Sma I
		target 1	target 2	target 3
24	pIPKb007	GA-1 (gamma-actin)		
like 2	pIPKb007	AAC (mitochondrial ADP/ATP carrier)		
like 3	pIPKb007	Hsp (mitochondrial heat shock protein chaperone)		
like 4	pIPKb007	Rho (Rho-GTPase)		
like 5	pIPKb007	EF1 (elongation factor 1)		
25	pIPKb007	AAC	GA-1	Rho
26	pIPKb007	AAC	GA-1	Hsp
27	pIPKb007	AAC	GA-1	EF1
28	pIPKb007	Rho	GA-1	EF1
29	pIPKb007	Rho	GA-1	Hsp
30	pIPKb007	Rho	AAC	EF1
31	GER4 (p6UGLP4deltaSwaIntronRNAi)	AAC	GA-1	Rho
32	GER4 (p6UGLP4deltaSwaIntronRNAi)	AAC	GA-1	Hsp
33	GER4 (p6UGLP4deltaSwaIntronRNAi)	AAC	GA-1	EF1
34	GER4 (p6UGLP4deltaSwaIntronRNAi)	Rho	GA-1	EF1
35	GER4 (p6UGLP4deltaSwaIntronRNAi)	Rho	GA-1	Hsp
36	GER4 (p6UGLP4deltaSwaIntronRNAi)	Rho	AAC	EF1
37	pIPKb010	AAC	GA-1	Rho
38	pIPKb010	AAC	GA-1	Hsp
39	pIPKb010	AAC	GA-1	EF1
40	pIPKb010	Rho	GA-1	EF1
41	pIPKb010	Rho	GA-1	Hsp
42	pIPKb010	Rho	AAC	EF1

A6. Materials

- 5 Jetstar Plasmid Midi prep kit
Genomed
Cat. No. 210250 (250 midipreps)

NucleoSpin Robot-96 plasmid kit

- 60 -

- Macherey-Nagel
Cat. No. 740708.4 (4x96 minipreps)
- Qiagen MinElute UF 96-well
Qiagen
5 Cat. No. 2853 (4x96)
- Thermal ace DNA polymerase kit
Invitrogen
Cat. No. E1000 (1000 U)
- Library Efficient DB3.1 competent cells
10 Invitrogen
Cat. No. 11782-018
- Gateway® LR Clonase™ II enzyme mix
Invitrogen
Cat. No. 11791-100 (100 reactions)
- 15 T4 DNA ligase (5 U/μL)
Fermentas
Cat No. EL0331 (1000 U)
- Swa* I (10U/μL)
New England BioLabs
20 Cat. No. R0604S (2000 U)
- EcoR* V (10U/μL)
Fermentas
Cat. No. #ER0301 (2000 U)
- Sma* I (10U/μL)
25 Fermentas
Cat. No. #ER0661 (1200 U)

Vectors

- pIPKTA38 – Gateway entry vector, kanamycin resistance.
- 30 pIPKb007 – binary RNAi vector, spectinomycin resistance. ccdB negative selection marker (needs DB3.1 cells), chloramphenicol resistance (not tested), ubiquitin promotor.
- pIPKb010 – binary RNAi vector, spectinomycin resistance. ccdB negative selection marker (needs DB3.1 cells), chloramphenicol resistance (not tested), GstA1 promotor.

- 61 -

GER4 (p6UGLP4deltaSwaIntronRNAi) – binary RNAi vector, spectinomycin resistance. ccdB negative selection marker (needs DB3.1 cells), chloramphenicol resistance (not tested), GER4 promotor.

5 **B. TransGen test and TIGS protocol**

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
 10 Petri dish (adaxial side up). Thereby, magnetic stirrers were put onto the leaves such that they repel each other.

Coating of gold particles with DNA or RNA:

7 µL = 7 µg DNA (plasmid) were used per bombardment. A Bio-Rad Hepta-Adaptor (7 macro carrier slides) was used.

7.0 µl	pUbiGUS (reporter gene)
0.7 µl	pUbi-Mlo-nos (resistance neutralization)
7.0 µl	pIPK007_Mlo, pIPK010_Mlo, Ger4_Mlo (resistant control)
Sum: 14.7 µl	

- 15 Per bombardment, N µL 1 M Ca(NO₃)₂ pH 10 were added drop wise to 87.5 µL (gold particles, 25 mg/mL in 50 % of glycerol; storage at 4 °C) coating suspension during vortexing (N = volume of DNA in µL). The particle suspension was left for at least 10 min at room temperature and was occasionally tipped. The suspension was centrifugated (15 sec, 14000 rpm) and the supernatant was removed with a pipette and discarded. The
 20 pellet was washed with 500 µL ethanol (70 %), and the ethanol was removed with a pipette. The pellet was again washed with 500 µL ethanol (absolute) and resuspended in 30 µL ethanol (absolute) subsequently.

- 62 -

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
5 in an ultrasonic bath for 3 sec, and the coating suspension was mixed with a pipette subsequently. 3 µL of the coating suspension were applied to each macro carrier by pipette and the suspension was left to dry for 2 min to 5 min.

Biolistic transformation:

Leaves and macro carrier holder with the treated macro carriers as well as grids (Hepta
10 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.

Incubation and inoculation of the leaves with mildew:

Bombarded leaves were first incubated for 4 h in slightly opened Petri dishes.
15 24 h after the bombardment, 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 thoroughly. For inoculation, open Petri dishes were put in dishes with nylon nets (100 µm aperture mesh width) stretched thereover. The leaves were inoculated with mildew (about 150 - 200 conidia/mm²). For inoculation, conidia as
20 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 were inoculated seven days before. The dishes were placed in the incubation chamber, subsequently.

GUS staining (for staining the transformed cells):

48 h after inoculation, the leaves were collected, the leaf tips were cut off and the
25 resulting leaves were transferred to Greiner tubes containing 10 mL of X-glucose solution. The tubes were placed in a suction bottle and vacuum was applied thereto 2 - 3 times. The infiltration is complete, once 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.

- 63 -

TCA staining:

The leaves were placed in destaining solution (7.5 % TCA, 50 % methanol) for 10 min.

Leaves were washed with aqua destillata, subsequently. Then, the leaves were carefully removed from the tube and were placed onto an object slide with their adaxial side facing

5 upwards.

200 μ L aqua destillata were added to each object slide and the cover glass was carefully applied.

Solutions:**GUS staining solution (X-glucose solution):**

- 100 mM trisodium phosphate, final pH 7.0
- 10 mM sodium EDTA
- 5 1.4 mM potassium hexacyanoferrate(II)
- 1.4 mM potassium hexacyanoferrate(III)
- 0.1 % triton[®] X-100
- 20 % methanol
- 1 mg / mL X-glucose

10 Ca(NO₃)₂

- 23.61 g Ca(NO₃)₂ per 100 mL millipore water = 1M
- by addition of 0.1 M KOH set ph 10

Benzimidazole stock solution

- 40 mg/mL of benzimidazole in ethanol (absolute)

15 Phytoagar

- 0.5 - 1 % (w/v) agarose in millipore water with 50 µL of benzimidazole stock solution
- (see above)

Preparation of gold suspension:

- 27.5 mg of Bio-Rad gold particles (1 µm diameter, prod. No. LLP892) were
- 20 vortexed in 1 mL sterile H₂O and treated with ultrasonic for 20 sec. After
- centrifugation (30 sec. 14.000 rpm), the supernatant was removed with a pipette,
- the particles were again vortexed in 1 mL of sterile H₂O, treated with ultrasonic
- for 20 sec. and centrifugated (as above). The supernatant was removed and 1 mL of
- EtOH absolute was added to the pellet and the sample was treated with ultrasonic for
- 25 20 sec. After another centrifugation (as above), the supernatant was removed with a
- pipette and the pellet was dried with open lid at 40 °C for about

- 65 -

20 min in a thermomixer. After addition of 1 mL 50 % sterile glycerol, the preparation was vortexed and treated with ultrasonic until the pellet was suspended. The suspension was stored at -20 °C.

C. Experiment for primary data acquisition

5

For checking the resistance effect of the RNAi constructs, in particular the *single* and *triple* constructs in combination with the pIPKb007 vector were used in the transient experiments.

10

The barley plants used (*Hordeum vulgare*, cultivar ,*Ingrid BC mlo5*`) were cultivated in soil without fertilisation in a phyto-cultivator (from Sanyo, at constantly 20 °C, 60 - 70 % rel. humidity, 16 h light). 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), 0.7 µg pUbi-Mlo-nos (resistance neutralization) and 7 µg of a control vector (pIPKb007_MLO, pIPKb010_MLO or Ger4_MLO (resistant control) according to the RNAi constructs to be tested) or with a RNAi construct. In closed Petri dishes, the leaves were stored at 20 °C at a north-facing window until inoculation.

15

One day after bombardment, the leaves were transferred to 1 % phytoagar with 2 % benzimidazole. A nylon net (aperture mesh width of 200 µm) was stretched over the leaves, and the same were inoculated with a conidia density of about 200 conidia/mm². The conidia (from the pathogen *Blumeria graminis hordei*) originated from barley plants (cultivar ,*Golden Promise*`), which were 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.

25

About 48 h after inoculation, GUS staining was performed. Said staining was stopped after 20 h by incubation in 7.5 % trichloroacetic acid, 50 % v/v methanol, and the leaves were bleached.

- 66 -

Every experiment contained 3 - 4 parallel bombardments to 7 leaf sections each of the HIGS negative control (respective empty vector). Further, each experiment contained 2 parallel bombardments of a TIGS positive control (pIPKb007_MLO, pIPKb010_MLO, Ger4_MLO, causes resistance by inhibiting the *Mlo* gene of barley). Data per experiment
5 are based on the comparison of the effect of the test constructs with the average value of the 4 negative controls of the respective experiment.

In all test series of the experiments in Ingrid BC mlo5, pUbi-MLO-nos was bombarded as well, which causes a neutralization of the resistance of the Ingrid BCmlo5 cells. This allows for the analysis of haustoria development as well as hyphen development, since
10 hyphens are only able to grow from transformed epithermal cells and thus do not mix with hyphens from adjacent infection events on non-transformed cells.

- 67 -

D. Examples of use**D1 result of the HIGS screening**

For transient experiments, barley leaves of the sort Ingrid BC mlo5 (*Hordeum vulgare*) were used. Spores of the phytopathogen *Blumeria graminis* f. sp. *hordei* (barley powdery mildew) were used as inoculum. In said system, all *single* and *triple target* RNAi constructs were tested in combination with the vector pIPKb007. In these experiments, it could be shown that a significant infestation-reduced effect is detectable in six out of the tested constructs (see table 8). In order to control the functionality of the system and to measure the resistance effect, a construct having a known resistant Mlo sequence (ubi_mlo) was carried along, which showed in all experiments a significant reduction of the haustoria index with a very high significance ($p \leq 0.0001$). The results demonstrate that the RNAi constructs which are directed against highly conserved target genes of a fungus (in this case: *Fusarium culmorum*) are effective against another fungus (in this case: *Blumeria graminis*).

Table 6: Average values, standard deviations and p-values of significant constructs from transient experiments using Ingrid mlo5 (*Hordeum vulgare*) and the pathogen *Blumeria graminis* f. sp. *hordei*

Construct (No. cf. tab.5) (repeats)	pIPKb007 (empty vector)	pIPKb007 _mlo	2 AAC	3 Hsp	8 AAC- GA-EF1	9 Rho- GA- EF1	10 Rho- GA- Hsp	11 Rho- AAC-EF1
n1 (number of evaluated leaves)	84	64	23	27	29	28	30	22
n2 (number of evaluated GUS cells)	5967	4141	1419	1598	1413	1533	1533	1185
Average value in % (of the haustoria index* in relation to the empty ubi vector (=100 %))	100	41.88	56.03	78.02	82.62	74.93	72.65	69.14
Standard deviation	.	36.97	33.50	41.67	44.70	38.66	48.93	51.52
p-value from t-test, two tailed	.	<0.0001	<0.0001	0.011	0.0455	0.002	0.0047	0.0105

* the haustoria index corresponds to the ratio between the number of haustoria formed and the number of the evaluated GUS cells

- 68 -

D2. Production of transgenic barley plants

By way of the above-mentioned vectors or RNAi constructs, transgenic barley plants have been produced by the *Agrobacterium* methodology. Transgenic plants were selected by hygromycin resistance. The following constructs were used:

<u>Vector (promotor)</u>	<u>RNAi insert(s)</u>
pIPKb007(ubiquitin)	AAC GA - AAC - Rho GA - AAC - Hsp GA - AAC - EF1 GA - Rho - EF1 GA - Rho - Hsp AAC - Rho - EF1 Empty vector
pIPKb010 (GSTA1)	GA - Rho - Hsp AAC - Rho - EF1 Empty vector
p6UGLP4deltaSwalIntronRNAi (GER4)	GA - Rho - Hsp AAC - Rho - EF1 Empty vector

- 5 In initial inoculation experiments, plants transgenic for pIPKb007_GA-AAC-Hsp and pIPKb007_GA-AAC-Rho showed resistance to *Fusarium culmorum* and *Blumeria graminis*, compared to wild type plants.

D3. Effect of RNAi construct against barley mildew and fusarium head blight in transgenic plants

Sample	Fusarium assay (% infected blossoms)		Mildew assay (% infected leaf area, rel. to GP)	
	MW $\pm$ SDM	p	MW $\pm$ SDM	p
GP (wt)	5.1 $\pm$ 0.5		100	
K26 transgenic	1.57 $\pm$ 0.5	0.0256	74.8 $\pm$ 9.3	0.0092
K26 azygous	3.45 $\pm$ 2.56	NS	122.8 $\pm$ 24.4	Precipitation

- 10 Four independent transgenic barley lines, which were transformed with construct No. 26 (K26), showed increased resistance to both, fusarium head blight and barley mildew. Azygous segregants of the examined transgenic lines (T1 generation) showed no significant resistance phenotype.

- 69 -

D4. Sequences of the vectors and *triple target* RNAi constructs

In the following, the sequences of the *triple* RNAi-*targets* are listed, which are inserted into the various vectors using the above-described GATEWAY procedure. The GATEWAY cleavage sites in the binary RNAi vectors are marked in colour code in all 6
5 different triple combinations (see table 6).

The sequences as well as the components of the three different vectors (pIKPb007, pIPKb010, GER4 (p6UGLP4deltaSwaIntronRNAi)) are annexed as genebank library file.

As an example of the *triple target* RNAi constructs, the combination of gamma-actin,
10 RhoGTPase and mitochondrial heat shock protein 70 chaperone is presented
schematically in all three vector systems.

- 70 -

Colour code within the insert sequences:

GATEWAY site

***EcoR* V site**

Swa I site

5 *Sma* I site

Targets of the conserved proteins:

GA

AAC

Rho

10 **EF1**

Hsp

>AAC insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACC
 TGCGCGGCCGCGAATTCGATATCATTCTTGTAGACGTCGACGAGACCGTT
 15 GAACTGACGGTCACCACCGGACTTGGCGTTCTTGGCATCGTTGGCAAGAC
 GAGTACGGGCGTAGTCCAGAGAGTAGACGAAGAGCAGAGAAGTGGCAC
 CAGCAGCACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCGTAGCC
 ATCCTTGTCTTCTTGTAGCCGAACATCTTCTTGAAGTTGTCACGGAAAGC
 GAAGTTCAGGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGGTGTTT
 20 CCTCGCCAGAGGGACATGACACCCTCATCGAAATGCCCGGGCGAATTCTC
 TAGACCCAGCTTT

The above-mentioned sequence of the AAC siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 6.

25 **>EF1 insert**

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACC
 TGCGCGGCCGCGAATTCGATATCATTGTCAGGCAATGTGGGCAGTGTGGC
 AATCGAGGACGGGAGCGTAACCAGCACCGACCTGACCGGGGTGGTTGAG
 GACGATGACCTGGGCGGTGAAAGAAGCGGCACCCATGGGGGGGGTCGT
 30 TCTTGGAGTCACCAGCGACGTTACCACGTCGGATGTCCTTGACGGAAACG
 TTCTTACGTTGAAACCAACGTTGTCACCGGGCTGTCCCTCAGTGAGCTG
 CTCGTGGTGCATCTCAACGGACTTGACTTCAGTGGTGACGTTGGAAGGAG
 CGAAGGTAACGACCATACCGGGCTTGATAAATGCCCGGGCGAATTCTCTA
 GACCCAGCTTT

35 The above-mentioned sequence of the EF1 siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 7.

- 71 -

>GA insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACC
 TGC GCGGCCGCGAATTCGATATCATTTGGAAGAAGGAGCAAGAGCAGTGA
 TCTCCTTCTGCATACGGTCGGAGAGACCGGGGTACATGGTGGTACCACC
 5 AGACATGACAATGTTTCCGTAGAGATCCTTTCGGACATCGACATCACAC
 TTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTCTCAAGAC
 CAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCTCGT
 TACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTCCAA
 GCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATGCCCCGGG
 10 CGAATTCTCTAGACCCAGCTTT

The above-mentioned sequence of the GA siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 8

>GA-1 insert

TTAAGGAACCAATTCAGTCGACCTCGAGCACC
 TGC GCGGCCGCTAATTCGATATCATTTGGAAGAAGGAGCAAGAGCAGTGA
 TCTCCTTCTGCATACGGTCGGAGAGACCGGGGTACATGGTGGTACCACC
 AGACTAAAGACATGTTAGAACAGTTGGAGTGCTTTCGATTTGCAGAACT
 TACCATGACAATGTTTCCGTAGAGATCCTTTCGGACATCGACATCACACT
 20 TCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTCTCAAGACC
 AAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCTCGTT
 ACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTCCAAG
 CTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATGCCCCGGG
 CGAATTCTCTAG

25 The above-mentioned sequence of the GA-1 siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 9.

>Hsp insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACC
 30 TGC GCGGCCGCGAATTCGATATCATTTACTTGCCCATGAGGTTGTTGTCCTT
 GGTGCGCTGGCGCTCACCCCTCGTAGACCTGGATAAGGACACCAGGCTGG
 TTGTCGGAGAAGGTAGAGAAGACCTCGGACTTCTTGGTGGGAATGGTGGT
 GTTGCGGGGGGATGAGCTTGGTCATCATACCACCAGCGGTCTCGATACCGA
 GAGAGAGGGGGGCGACGTCGAGGAGCAGAATCTCGTTGGTGGCCTTGCT
 35 AGAGGTGTCACCAGAGAGAATAGCAGCCTGGACAGCGGCACCGTAGGCA
 ACAGCCTCATCAGGATTGATGGACTTGTTGGGCTCCTTTCCGTTGAAGTA
 GTCGGTGATGAGCTTCTGGACACGGGGGATACGGAAATGCCCCGGGCGAAT
 TCTCTAGACCCAGCTTT

40 The above-mentioned sequence of the Hsp siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 10.

- 72 -

>Rho insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 TGCGCGGCCGCGAATTCGATATCATTTTGCTTGTGGTAAAACCTGTTTGTG
 ATCGTTTTCTCCAAGGGCACTTTCCCCGAGGTCTACGTCCCCACCGTCTTC
 5 GAGAACTATGTCGCCGATGTCGAGGTTGACGGCAAGCACGTGAGCTCG
 CCCTATGGGATACTGCTGGTCAGGAGGATTACGACCGTCTTCGACCTCTC
 TCTTACCCCGACTCCCACGTTATCTTGATCTGCTTCGCTGTTGACTCTCCC
 GACTCTCTCGACAACGTCCAGGAGAAGTGGATCTCTGAGGTTCTGCACTT
 CTGCCAGGGKCTCCCTATCATCCTTGTCGGCTAAATGCCCGGGCGAATTCT
 10 CTAGACCCAGCTTT

The above-mentioned sequence of the Rho siRNA insert is in antisense orientation (3' - 5'), the complementary "sense" sequence (5' - 3') corresponds to SEQ ID NO: 11.

>GA_AAC_Rho insert

15 GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 GCGCGGCCGCGAATTCGATCTTGTAGACGTCGACGAGACCGTTGAACTGA
 CGGTCACCACCGGACTTGGCGTTCTTGGCATCGTTGGCAAGACGAGTACG
 GGCGTAGTCCAGAGAGTAGACGAAGAGCAGAGAAGTGGCACCAGCAGC
 ACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCGTAGCCATCCTTGT
 20 CCTTCTTGTAGCCGAACATCTTCTTGAAGTTGTCACGGAAAGCGAAGTTCA
 GGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGGTGTTTCCTCGCCA
 GAGGGACATGACACCCTCATCGATCATTTGGAAGAAGGAGCAAGAGCAG
 TGATCTCCTTCTGCATACGGTCGGAGAGACCGGGGTACATGGTGGTACC
 ACCAGACATGACAATGTTTCCGTAGAGATCCTTTCGGACATCGACATCA
 25 CACTTCATGATGGAGTTGAAAGTGGTGACGTGGATACCACCGCTCTCAA
 GACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCT
 CGTTACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTC
 CAAGCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATGCC
 TCGTTG/GGTAAAACCTGTTTGTGATCGTTTCTGCAAGGGCACTTTCCC
 30 CGAGGTCTACGTCCCCACCGTCTTCGAGAACTATGTGCGCGATGTGAGC
 TTGACGGCAAGCACGTGAGCTCGCCCTATGGGATACTGCTGGTCAGGA
 GGATTACGACCGTCTTCGACCTCTCTCTTACCCCGACTCCACGTTATCTT
 GATCTGCTTCGCTGTTGACTCTCCCGACTCTCTCGACAACGTCCAGGAGA
 AGTGGATCTCTGAGGTTCTGCACTTCTGCCAGGGKCTCCCTATCATCCTTG
 35 TCGGGTGGGCGAATTCTCTAGACCCAGCTTT

>GA_AAC_mHspCh insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 GCGCGGCCGCGAATTCGATCTTGTAGACGTCGACGAGACCGTTGAACTGA
 CGGTCACCACCGGACTTGGCGTTCTTGGCATCGTTGGCAAGACGAGTACG
 40 GGCGTAGTCCAGAGAGTAGACGAAGAGCAGAGAAGTGGCACCAGCAGC
 ACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCGTAGCCATCCTTGT
 CCTTCTTGTAGCCGAACATCTTCTTGAAGTTGTCACGGAAAGCGAAGTTCA

- 73 -

GGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGGTGTTTCCTCGCCA
 GAGGGACATGACACCCTCATCGATCATTTGGAAGAAGGAGCAAGAGCAG
 TGATCTCCTTCTGCATACGGTCGGAGAGACCGGGGTACATGGTGGTACC
 ACCAGACATGACAATGTTTCCGTAGAGATCCTTTCGGACATCGACATCA
 5 CACTTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTCTCAA
 GACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCT
 CGTTACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTC
 CAAGCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATGCCC
 CTCACCCTCGTAGACCTGGATAAAGGACACCAGGCTGGTTGTGCGGAGAAG
 10 GTAGAGAAGACCTCGGACTTCTTGGTGGGAATGGTGGTGTTCGCGGGGA
 TGAGCTTGGTCATCATACCACCAGCGGTCTCGATACCGAGAGAGAGGGG
 GCGGACGTGAGGAGCAGAATCTCGTTGGTGGCCTTGCTAGAGGTGTCA
 CCAGAGAGAATAGCAGCCTGGACAGCGGCACCGTAGGCAACAGCCTCAT
 CAGGATTGATGGACTTGTTGGGCTCCTTTCCGTTGAAGTAGTCGGTGATG
 15 AGCTTCTGGGGGCGAATTCTCTAGACCCAGCTTT

>GA_AAC_EF1 insert

TACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 GCGCGGCCGCGAATTCGATCTTGTAGACGTGACGAGACCGTTGAACTGA
 CGGTCAACACCGGACTTGGCGTTCTTGGCATCGTTGGCAAGACGAGTACG
 20 GGCGTAGTCCAGAGAGTAGACGAAGAGCAGAGAAGTGGCACCAGCAGC
 ACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCGTAGCCATCCTTGT
 CCTTCTTGTAGCCGAACATCTTCTTGAAGTTGTACGGAAAGCGAAGTTCA
 GGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGGTGTTTCCTCGCCA
 GAGGGACATGACACCCTCATCGATCATTTGGAAGAAGGAGCAAGAGCAG
 25 TGATCTCCTTCTGCATACGGTCGGAGAGACCGGGGTACATGGTGGTACC
 ACCAGACATGACAATGTTTCCGTAGAGATCCTTTCGGACATCGACATCA
 CACTTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTCTCAA
 GACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCT
 CGTTACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTC
 30 CAAGCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATGCCC
 GCAGGCAATGTGGGCAGTGTGGCAATCGAGGACGGGAGCGTAACCAGC
 ACCGACCTGACCGGGGTGGTTGAGGACGATGACCTGGGCGGTGAAAGAA
 GCGGCACCCATGGGGGGGGTCTGTTCTTGGAGTCACCAGCGACGTTACCA
 CGTCGGATGTCCTTGACGGAAACGTTCTTCACGTTGAAACCAACGTTGTC
 35 ACCGGGCTGTCCCTCAGTGAGCTGCTCGTGGTGCATCTCAACGGACTTGA
 CTTCAGTGGTGACGTTGGAAGGAGCGAAGGTAACGACCATAACGGGGCTT
 GATGGGCGAATTCTCTAGACCCAGCTTT

>GA_Rho_EF1 insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACC
 40 TGCGCGGCCGCGAATTCGATTGGGCTGTTCCCTACTATCCCTCKGGGACCGT
 CTTCAAGTCTTGGAGTCTCTAGGTGAAGAGGACCTGCAACAGCTCTCTCA
 GCCCTCTCAGTTGTCGCTTCGTCTAGTTCTATTGCACCCCTCAGCCCCATTC
 TCTCTCCAGCTTCTGCCAGCATTAGGAGGACTGGTCGTCATAGGGTATCC
 CGCTCGAGCTGCACGAACGGCAGTTGGAGCTGTAGCCGCTGTATCAAGA
 45 GCTTCTGCCACCCCTGCATCTGGAGCCCCCTTTCACGGGAACCTCTTTTGCT

- 74 -

AGTTGTTTGTCCAAAATGGTGTTCGTATCATTTGGAAGAAGGAGCAAGAG
 CAGTGATCTCCTTCTGCATACGGTTCGGAGAGACCGGGGTACATGGTGGT
 ACCACCAGACATGACAATGTTTCCGTAGAGATCCTTTTCGGACATCGACA
 TCACACTTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTCT
 5 CAAGACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATC
 GCTCGTTACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTT
 CTCCAAGCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCGAAAATG
 CCCGCAGGCAATGTGGGCAGTGTGGCAATCGAGGACGGGAGCGTAACCA
 GCACCGACCTGACCGGGGTGGTTGAGGACGATGACCTGGGCGGTGAAAG
 10 AAGCGGCACCCATGGGGGGGGTTCGTTCTTGAGTACACGACGACGTTAC
 CACGTCGGATGTCCTTGACGGAAACGTTCTTCACGTTGAAACCAACGTTG
 TCACCGGGCTGTCCCTCAGTGAGCTGCTCGTGGTGCATCTCAACGGACTT
 GACTTCAGTGGTGACGTTGGAAGGAGCGAAGGTAACGACCATAACGGGC
 TTGATGGGCGAATTCTCTAGACCCAGCTTT

15 >GA_Rho_mHspCh insert
 GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 GCGCGGCCGCGAATTCGATAGCCGACAAGGATGATAGGGAGMCCCTGGC
 AGAAGTGCAGAACCTCAGAGATCCACTTCTCCTGGACGTTGTTCGAGAGAG
 TCGGGAGAGTCAACAGCGAAGCAGATCAAGATAACGTGGGAGTCGGGGT
 20 AAGAGAGAGGTCGAAGACGGTTCGTAATCCTCCTGACCAGCAGTATCCCA
 TAGGGCGAGCTCGACGTGCTTGCCGTC AACCTCGACATCGGCGACATAG
 TTCTCGAAGACGGTGGGGACGTAGACCTCGGGGAAAGTGCCCTTGGAGA
 AAACGATCAACAAACAGGTTTACCAGAAGCAATCATTTGGAAGAAGGAG
 CAAGAGCAGTGATCTCCTTCTGCATACGGTTCGGAGAGACCGGGGTACAT
 25 GGTGGTACCACCAGACATGACAATGTTTCCGTAGAGATCCTTTTCGGACA
 TCGACATCACACTTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCAC
 CGCTCTCAAGACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCAC
 GGAATCGCTCGTTACCAATGGTGATAACCTGACCGTCAGGAAGCTCGTA
 AGACTTCTCCAAGCTGGAGCTCTGGGCAGCAGTCTGGATCTCCTGCTCG
 30 AAAATGCCCTCACCTCGTAGACCTGGATAAAGGACACCAGGCTGGTTGT
 CGGAGAAGGTAGAGAAGACCTCGGACTTCTTGGTGGGAATGGTGGTGT
 GCGGGGGATGAGCTTGGTCATCATACCACCAGCGGTCTCGATACCGAGA
 GAGAGGGGGGCGACGTCGAGGAGCAGAATCTCGTTGGTGGCCTTGCTAG
 AGGTGTCACCAGAGAGAATAGCAGCCTGGACAGCGGCACCGTAGGCAAC
 35 AGCCTCATCAGGATTGATGGACTTGTGGGCTCCTTTCCGTTGAAGTAGTC
 GGTGATGAGCTTCTGGGGCGAATTCTCTAGACCCAGCTTT

>AAC_Rho_EF1 insert
 GTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACCTCGAGCACCT
 GCGCGGCCGCGAATTCGATTGCTTGTGGTAAACCTGTTTGTGATCGTTTT
 40 CTCCAAGGGCACTTTCCCGAGGTCTACGTCCCCACCGTCTTCGAGA
 ATGTGCGCGATGTCGAGGTTGACGGCAAGCACGTTCGAGCTCGCCCTATG
 GGATACTGCTGGTCAGGAGGATTACGACCGTCTTCGACCTCTCTTACC
 CCGACTCCACGTTATCTTGATCTGCTTCGCTGTTGACTCTCCCGACTCTC

- 75 -

TCGACAACGTCCAGGAGAAGTGGATCTCTGAGGTTCTGCACTTCTGCCAG
 GGGKCTCCCTATCATCCTTGTCTGGCTATCATTTCTTGTAGACGTGACGAGA
 CCGTTGAACTGACGGTCACCACCGGACTTGGCGTTCTTGGCATCGTTGGC
 AAGACGAGTACGGGCGTAGTCCAGAGAGTAGACGAAGAGCAGAGAAGT
 5 GGCACCAGCAGCACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCG
 TAGCCATCCTTGTCTTCTTGTAGCCGAACATCTTCTTGAACCTTGTCACGG
 AAAGCGAAGTTCAGGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGG
 TGTTCCTCGCCAGAGGGACATGACACCCTCATCGAAATGCCCATCAAGC
 CCGGTATGGTCGTTACCTTCGCTCCTTCCAACGTCACCACTGAAGTCAAG
 10 TCCGTTGAGATGCACCACGAGCAGCTCACTGAGGGACAGCCCGGTGACA
 ACGTTGGTTTCAACGTGAAGAACGTTTCCGTCAAGGACATCCGACGTGGT
 AACGTCGCTGGTGACTCCAAGAACGACCCCCCATGGGTGCCGCTTCTT
 TCACCGCCCAGGTCATCGTCCTCAACCACCCCGGTCAGGTCGGTGCTGGT
 TACGCTCCCGTCCTCGATTGCCACACTGCCACATTGCCTGCGGGCGAAT
 15 TCTCTAGACCCAGCTTT

>AAC_GA-1_Rho insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTTCAGTCGACCTCGAGCACCTGCGCGGCC
 GCGAATTCGATCTTGTAGACGTGACGAGACCGTTGAACTGACGGTCACCACCGGACTT
 GCGGTTCTTGGCATCGTTGGCAAGACGAGTACGGGCGTAGTCCAGAGAGTAGACGAAGA
 20 GCAGAGAAGTGGCACCAGCAGCACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCG
 TAGCCATCCTTGTCTTCTTGTAGCCGAACATCTTCTTGAACCTTGTCACGGAAAGCGAA
 GTTCAGGGCCTGGGTGGGGAAGTATCGGATGACGTTGGCGGTGTTTCTCGCCAGAGGG
 ACATGACACCCTCATCGATCATTTTGGGAAGAAGGAGCAAGAGCAGTGATCTCCTTCTGCA
 TACGGTTCGGAGAGACCGGGGTACATGGTGGTACCACCAGACTAAAGACATGTTAGAACA
 25 GTTGGAGTGCTTTCGATTTGCAGAACTTACCATGACAATGTTTCCGTAGAGATCCTTTC
 GGACATCGACATCACACTTCATGATGGAGTTGAAAGTGGTGACGTGGATAACCACCGCTC
 TCAAGACCAAGGACAGAAGGCTGGAAGAGAGCCTCAGGAGCACGGAATCGCTCGTTACC
 AATGGTGATAACCTGACCGTCAGGAAGCTCGTAAGACTTCTCCAAGCTGGAGCTCTGGG
 CAGCAGTCTGGATCTCCTGCTCGAAATGCCCTGCTGTGGTAAACCTGTTTGTJGAT
 30 GGTCTCTCCAAAGGACACTTTCCTCCGAGGCTCTAGCTCCCAACCGTCTTCGAGAACTATG
 TCCCGATGTTGAGGTTGACGGCAAGCAGCTCGACTCCGCCCTATGGGATACTGCTGGT
 CAGCAGGATTACGAACGCTCTCGACCTCTCTCTTACCCCGACTCCCACGTTATCTTGAT
 CTCTTCGCTGTTGACTCTCCCGACTCTCTCGACAACGTCCAGGAGAAGTGGATCTCTG
 AGGTTCTGCACTTCTGCCAGGGKCTCCCTATCATCCTTGTCTGGCTGGGCGAATTCTCTA
 35 GACCCAGCTTT

>AAC_GA-1_mHspCh insert

GTACAAAAAAGCAGGCTTTAAAGGAACCAATTTCAGTCGACCTCGAGCACCTGCGCGGCC
 GCTAATTCGATTGTAGACGTGACGAGACCGTTGAACTGACGGTCACCACCGGACTTGG
 CGTTCTTGGCATCGTTGGCAAGACGAGTACGGGCGTAGTCCAGAGAGTAGACGAATAGA
 40 AGAGAAGTGGCACCAGCAGCACCACCGGAGGCAAGGTTACCAGCCATCCACATGGCGTA
 GCCATCCTTGTCTTCTTGTAAACCGAACATCTTCTTGAACCTTGTCACGGAAAGCGAAGT
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 ATGACACCCTCATCGATCATTTTGGGAAGAAGGAGCAAGAGCAGTGATCTCCTTCTGCATA
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- 76 -

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10 >AAC_GA-1_EF1 insert

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- 77 -

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40 GGTGCTGGTTACGCTCCCGTCTCGATTGCCACACTGCCACATTGCCTGGGGCGAATT
CTCTAGACCCAGCTTT

Description of the figures

- Fig. 1: Flow diagram for the high-throughput production of RNAi constructs. I, PCR amplification of cDNA fragments of fungal genes 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 of all re-ligated vector molecules; III, Recombination of the cloned cDNA fragments in the RNAi vector pIPKTA30 by means of LR clonase.
- Fig. 2: Schematic representation of the synthesis pathway of simple (single) as well as combined (triple) RNAi constructs by means of the modified GATEWAY technology as to Douchkov *et al.* (2005) using the example of a sequence from gamma-actin (*single RNAi construct*), and a sequence combination of three components (gamma-actin, ADP/ATP carrier as well as RhoGTPase; *triple RNAi construct*). pIPKb007 (ubi), p6UGER4deltaSwaIntronRNAi (Ger4) and pIPKb010 (GSTA1) were used as binary RNAi *destination vectors*.
- Fig. 3: Schematic representation of an alternative synthesis pathway of simple (single) and combined (triple) RNAi constructs by means of the modified GATEWAY technology as to Douchkov *et al.* (2005) using the example of a sequence from gamma-actin (*single RNAi construct*), and a sequence combination of three components (gamma-actin, ADP/ATP carrier as well as RhoGTPase; *triple RNAi construct*). pIPKb007 (ubi), p6UGER4deltaSwaIntronRNAi (Ger4) and pIPKb010 (GSTA1) were used as binary RNAi *destination vectors*.
- Fig. 4: Flow diagram of the HIGS system. The RNAi constructs are directed against fungal transcripts.

- 79 -

Fig. 5: Transient transformation experiments with barley leaves of the sort Ingrid BC mlo5 (*Hordeum vulgare*). Spores of the phytopathogen *Blumeria graminis* f. sp. *hordei* (barley powdery mildew) served as inoculum. In said system, *single* and *triple target* RNAi constructs were tested in combination with the vector pIPKb007.

5

Fig. 6 to 11: Schematic representation of various triple siRNA vectors.

- 80 -

In the nucleotide sequences, the below-mentioned symbols were used for nucleic acids:

Symbol	Meaning	Derivation of the designation
a	a	<u>a</u> denine
g	g	<u>g</u> uanine
c	c	<u>c</u> ytosine
t	t	<u>t</u> hymine
u	u	<u>u</u> racil
r	g or a	purine
y	t/u or c	pyrimidine
m	a or c	<u>a</u> mino
k	g or t/u	<u>k</u> eto
s	g or c	<u>s</u> trong bonds 3 H bridges
w	a or t/u	<u>w</u> weak bonds 2 H bridges
b	b or c or t/u	not a
d	a or g or t/u	not c
h	a or c or t/u	not g
v	a or g or c	not t, not u
n	a or g or c or t/u, unknown or other	<u>a</u> ny

- 81 -

SEQ ID NO: 1

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>FsCon[0026]

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- 82 -

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- 83 -

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- 84 -

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SEQ ID NO: 5

FsCon[0840] Rho

5 >FsCon[0840]
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- 86 -

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GA-1

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- 87 -

SEQ ID NO: 12

AAC consensus shortened

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15 SEQ ID NO: 13

EF1 consensus shortened

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 GAGTTCGAGGCTGGTATCTCCAAGGATGGCCAGACTCGTGAGCACGCTCTCC
 25 TCGCCTACACCCTCGGTGTCAAGCAGCTCATCGTYGCCATCAACAAGATGGA
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 GGTACAAGGGTTGGGAGAAGGAGACCAAGKCWGGCAAGGYCACCGGCAAG
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 AACGACCCCCCAAGGGTGCCGCTTCCTTCAACGCYCAGGTCAATYGTCTTYA
 ACCACCCTGGTCAGGTCGGTGCTGGTTACGCCCCCGTTCTCGATTGCCACACT
 GCCCACATTGCCTGCAAGTTCTCCGAGCTCCTCGAGAAGATCGACCGCCGTA
 CCGGTAAGTCCATTGAGGACTCCCCCAAGTTCATCAAGTCTGGTGACGCTGC
 40 CATCGTCAAGATGGTTCCCTCCAAGCCCATGTGTGTTGAGGCCTTCACYGAC

- 88 -

TACCCTCCTCTGGGMC GTTTTCGCCGTCCGTGACATGCGTCAGACCGTCGCTG
TCGGTGT CATCAAGTCCGTCGACAAGTCCACGACACTGCCGGTAAGGTCACC
AAG

SEQ ID NO: 14

5 GA consensus shortened

ATGGAGGAAGAAGTCGCWGCTCTCGTCATYGACAATGGTTCGGGTATGTGC
AAGGCCGGTTTCGCCGGTGACGATGCTCCCCGAGCTGTCTTCCCTTCCATTGT
CGGTCGCCCCCGTCACCATGGTATCATGATTGGTATGGGCCAGAAGGACTCG
TACGTTGGTGATGAGGCCAGTCCAAGCGTGGTATCCTCACTCTGCGGTACC
10 CCATCGAGCACGGTGTGTCACCAACTGGGACGACATGGAGAAGATCTGGC
ACCACACCTTCTACAACGAGCTGCGTGTCGCCCCCGAGGAGCACCCCGTCCT
GCTGACTGAGGCTCCCATCAACCCCAAGTCCAACCGTGAGAAGATGACGCA
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TKCTGTCCCTGTACGCCTCCGGTTCGTACCACTGGTATCGTGCTCGACTCYGGT
15 GACGGTGTTACTCACGTTGTGCCCATCTACGAGGGTTTCKCCCTKCCCCACGC
CATTGCCCGTGTGACATGGCTGGTTCGTGACTTGACCGACTACCTGATGAAG
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TCCGTGACATCAAGGAGAAGCTCTGCTACGTGCGCCCTTGACTTCGAGCAGGA
GATCCAGACTGCTGCCCAGTCCCTCCAGCYTGGAGAAGTCCCTACGAGCTTCCC
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25 CTCTCCCGAGCGCAAGTACTCCGTCTGGATCGGTGGT:TCCATTCTC

SEQ ID NO: 15

Hsp consensus shortened

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30 GATGAGGCTGTTGCCTACGGTGCTGCCGTCCAGGCTGCCATTCTCTCTGGTGA
CACCACCTCCAAGTCCACCAACGAGATCCTGCTYCTCGATGTCGCCCCSCTST
CTCTCGGTATCGAGACCGCTGGTGGTATGATGACCAAGCTCATCCCCGCAA
CACCACCATCCCCACCAAGAAGTCYAGGTTCTTCTCCACCTTCTCCGACAAC
CAGCCTGGTGTGCTCATCCAGGTCTACGAGGGTGAGCGCCAGCGCACCAAGG
35 ACAACAACCTSCTGGGCAAGTTCGAGCTTACCGGCATCCCMCCTGCTCCCCG
TGGTGTTCGCCAGATTGAGGTACCTTCGACCTSGATGCCAACGGTATCATGA
ACGTCTCCGCCGTGAGAAGGGCACCGGCAAGACCAACAAGATTGTCATCA

- 89 -

CCAACGACAAGGGCCGYCTGTCCAAGGAGGAGATYGAGCGCATGCTTGCTG
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SEQ ID NO: 16

Rho consensus shortened

5 AATCCATMACCATGGCTGAAATCCGCCGAAAGCTCGTCATTGTCGGHGAYG
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GAGGTCTACGTCCCMACCGTCTTCGAGAACTATGTCGCCGATGTCGAGGTCG
ATGGCAAGCACGTCGAGCTCGCGCTATGGGATACBGCTGGTCAGGAGGATTA
CGACCGTCTTCGACCTCTCTCATACCCCGACTCSCACGTTATCCTGATCTGCT
10 TCGCCGTTGACTCTCCCGATTCCCTCGAYAACGTYCAGGAGAAGTGGATCTC
CGAGGTCCTSCAYTTCTGCCAGGGTCTCCCTATCATCCTTGTCGGCTGCAAGA
AGGATTTGCGAYACGACCAGAAGACCATTGAGGAGCTCCACAAGACCA

Claims:

1. Method for creating broad-spectrum resistance to fungi in transgenic plants, comprising introducing a recombinant nucleic acid molecule into a plant, wherein the nucleic acid molecule comprises one or more consensus sequences wherein each of the one or more the consensus sequences is:

a) identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or

b) identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or

c) identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 12-16, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 12-16,

wherein said recombinant nucleic acid molecule is transcribed in the plant, so that in case of an infection of the plant with a fungus, the nucleic acid sequence interacts with one or more of the corresponding and/or complementary nucleic acids of the fungus such that the expression of the fungal nucleic acid sequence is reduced by at least 80 %.

2. Method according to claim 1, wherein the recombinant nucleic acid molecule comprises:

(a) a promoter functional in plants,

(b) operatively linked thereto the at least one nucleic acid sequence.

3. Method according to claim 1, wherein the recombinant nucleic acid molecule comprises:

- (a) a promoter functional in plants,
- (b) operatively linked thereto the at least one nucleic acid sequence,
- (c) an intron comprising splice donor and splice acceptor sequences,
- (d) a nucleic acid sequence being reverse-complementary to the nucleic acid sequence mentioned in b),
- (e) a termination sequence.

4. Method according to claim 1, wherein the recombinant nucleic acid molecule comprises:

- (a) a promoter functional in plants,
- (b) operatively linked thereto the at least one nucleic acid sequence, wherein said sequence has reverse-complementary regions, so that after transcription and self-hybridization within the nucleic acid sequence with the reverse-complementary regions a double-stranded RNA is formed; and
- (c) a termination sequence.

5. Method according to claim 1, wherein the recombinant nucleic acid molecule comprises:

- (a) a promoter functional in plants,
- (b) operatively linked thereto the at least one nucleic acid sequence,
- (c) another nucleic acid sequence encoding a ribonuclease P, and
- (d) a termination sequence.

6. Method according to any one of claims 1 to 5, wherein the promoter is a constitutive promoter, an inducible promoter or a tissue-specific promoter.

7. Method according to any one of claims 1 to 6, wherein the at least one nucleic acid sequence is selected from the group consisting of
 - (i) nucleic acids, comprising SEQ ID NOs: 1-16, and
 - (ii) nucleic acids which hybridize under stringent conditions with a complementary strand of a nucleic acid of (i), wherein the stringent hybridization conditions comprise hybridization in 6x 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° C to 65° C.
8. Method according to any one of claims 1 to 7, wherein the transgenic plants are dicotyledonous or monocotyledonous plants.
9. Method according to claim 8, wherein the plants are cereal plants.
10. Method according to claim 9, wherein the cereal plants are wheat or barley.
11. Method according to any one of claims 1 to 10, wherein the broad-spectrum resistance is against pathogenic fungi.
12. A transgenic plant cell having broad-spectrum resistance being produced according to the method of any one of claims 1 to 11, wherein the recombinant nucleic acid molecule comprises two nucleic acid sequences, wherein one nucleic acid sequence is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, and wherein a further nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or
- b) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 12-16, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 12-16.

13. Transgenic plant cell according to claim 12 containing a recombinant nucleic acid molecule comprising a promoter operatively linked to at least one nucleic acid sequence, wherein the recombinant nucleic acid molecule comprises two nucleic acid sequences, wherein one nucleic acid sequence is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, and wherein a further nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or
- b) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 12-16, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 12-16.

14. Transgenic plant cell containing the recombinant nucleic acid molecule as defined in any one of claims 1 to 7, wherein the recombinant nucleic acid molecule comprises two nucleic acid sequences, wherein one nucleic acid sequence is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, and wherein a further nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or
- b) is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 12-16, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 12-16.

15. Recombinant nucleic acid molecule comprising one or more consensus sequences, the nucleic acid molecule comprising at least two nucleic acid sequences operatively linked to a promoter functional in plant cells, wherein one nucleic acid sequence is identical and/or complementary to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 1-5, and wherein a further nucleic acid sequence

- a) is identical and/or complementary to one or more of the nucleic acids, selected from SEQ ID NOs: 6-11, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 6-11, or
- b) is identical and/or complementary to one or more of the nucleic acids, selected from SEQ ID NOs: 12-16, or has a sequence identity of at least 85 % over a range of at least 300 bp to one or more of the nucleic acids selected from SEQ ID NOs: 12-16;

wherein the sequences inhibit the expression of fungal DNA, but do not suppress the expression of endogenous genes when expressed in a transgenic plant.

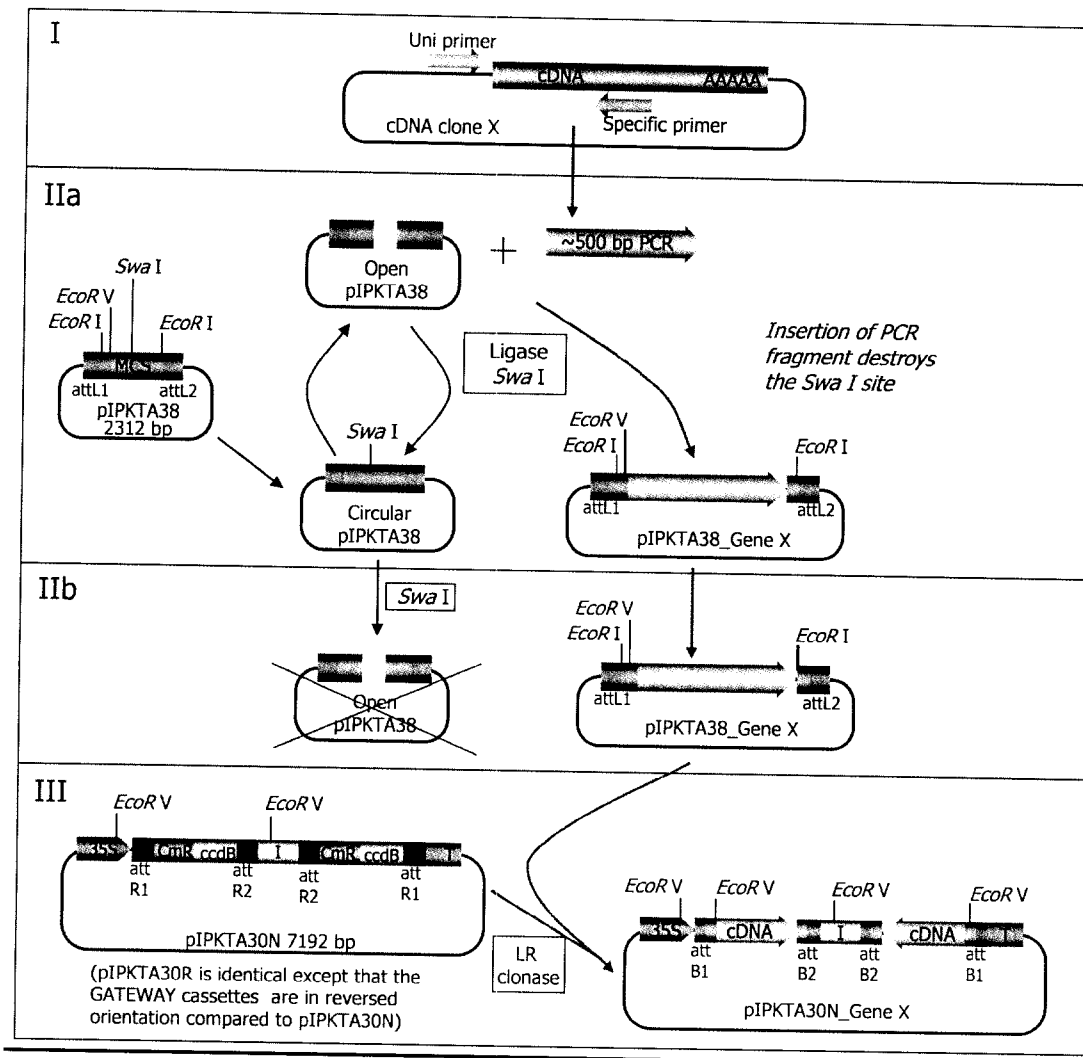


Fig. 1: Flow diagram for the high-throughput production of RNAi constructs. I, PCR amplification of cDNA fragments of fungal genes of interest; IIa, Ligation of the PCR fragments in the intermediate vector pIPKTA38 in the presence of the restriction endonuclease, *Swa* I, which inhibits the re-ligation 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.

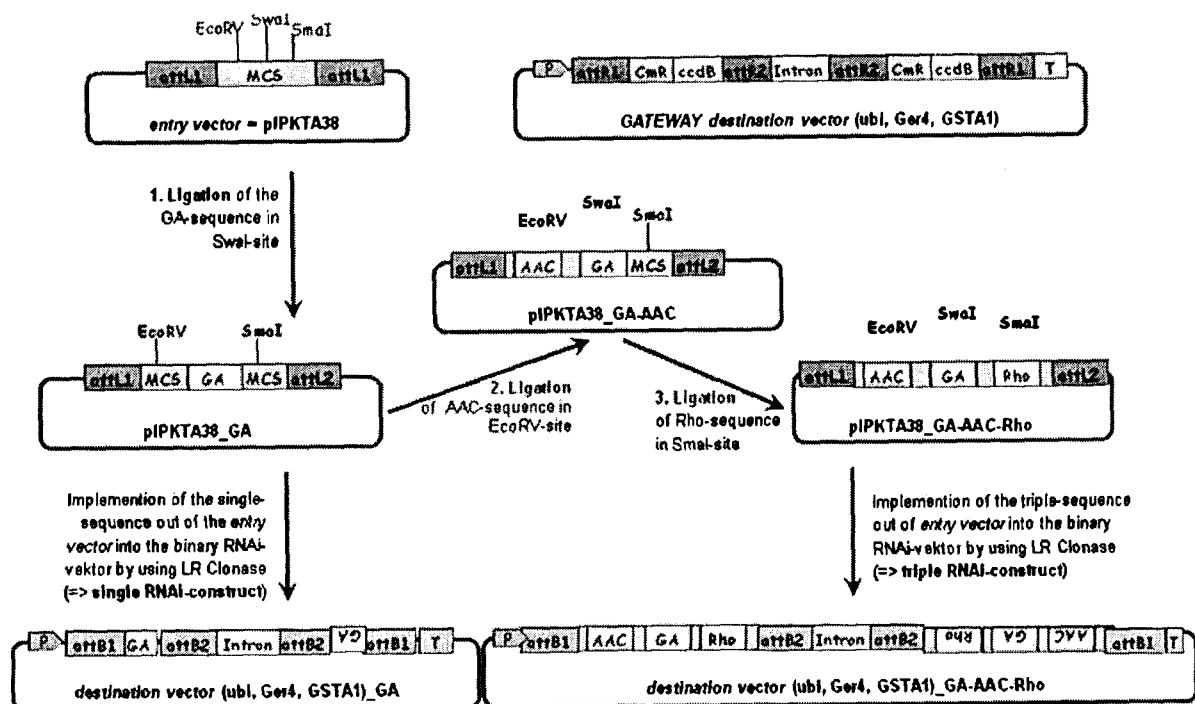


Fig. 2: Schematic representation of the synthesis pathway of simple (single) as well as combined (triple) RNAi constructs by means of the modified GATEWAY technology as to Douchkov *et al.* (2005) using the example of a sequence from gamma-actin (*single RNAi construct*), and a sequence combination of three components (gamma-actin, ADP/ATP carrier as well as RhoGTPase; *triple RNAi construct*). pIPKb007 (ubi), p6UGER4deltaSwaIntronRNAi (Ger4) and pIPKb010 (GSTA1) were used as binary RNAi *destination vectors*.

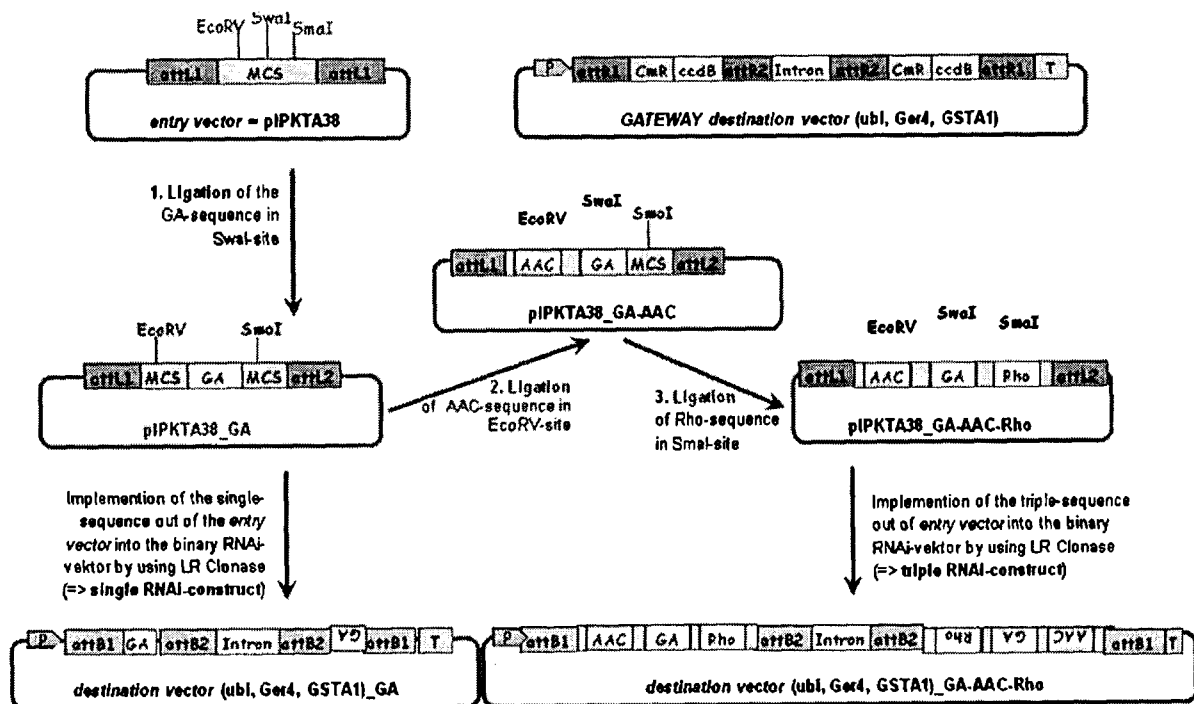


Fig. 3: Schematic representation of an alternative synthesis pathway of simple (single) as well as combined (triple) RNAi constructs by means of the modified GATEWAY technology as to Douchkov *et al.* (2005) using the example of a sequence from gamma-actin (*single RNAi construct*), and a sequence combination of three components (ADP/ATP carrier, gamma-actin as well as RhoGTPase; *triple RNAi construct*). pIPKb007 (ubi), p6UGER4deltaSwaIntronRNAi (Ger4) and pIPKb010 (GSTA1) were used as binary RNAi *destination vectors*.

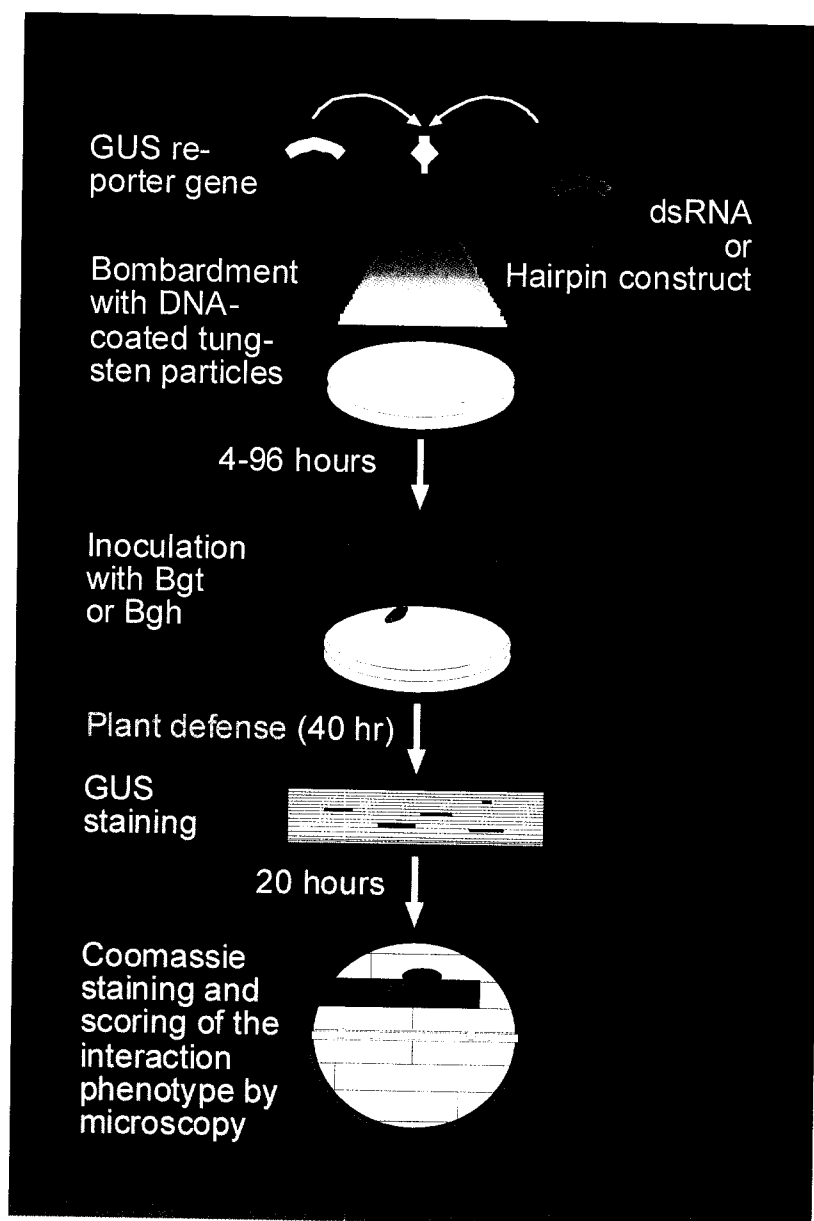


Fig.4: Flow diagram of the HIGS system. The RNAi constructs are directed against fungal transcripts.

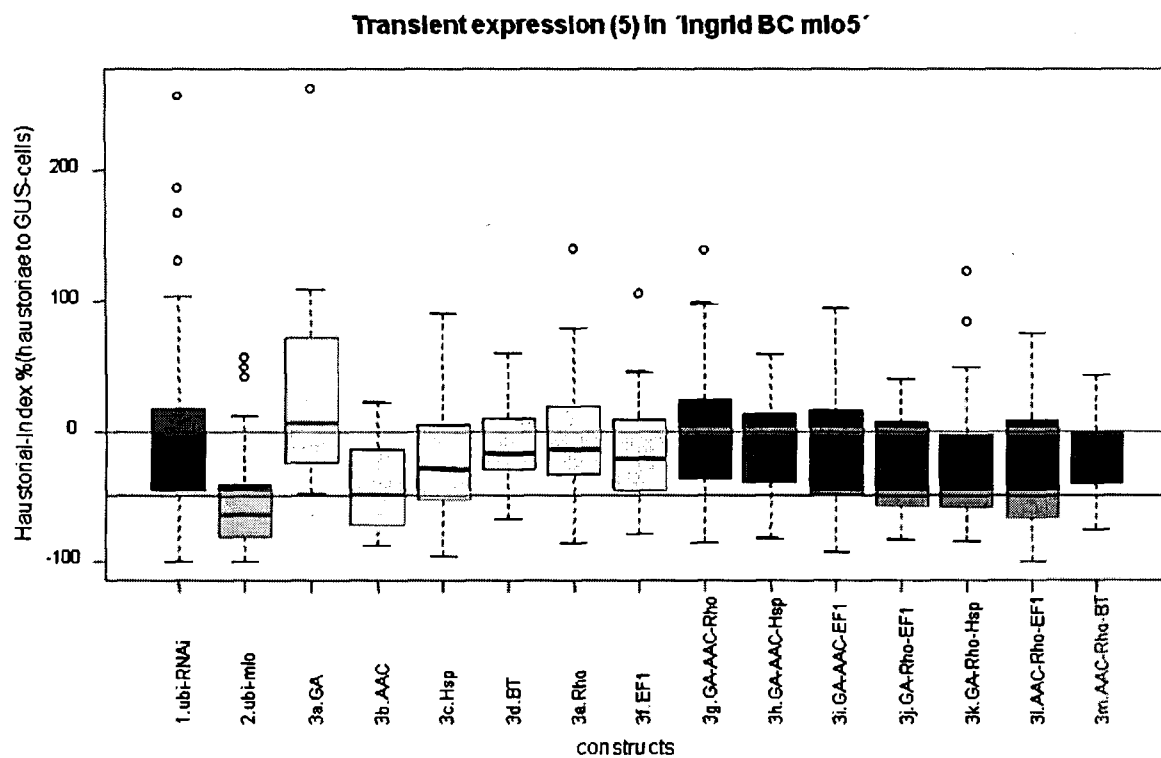
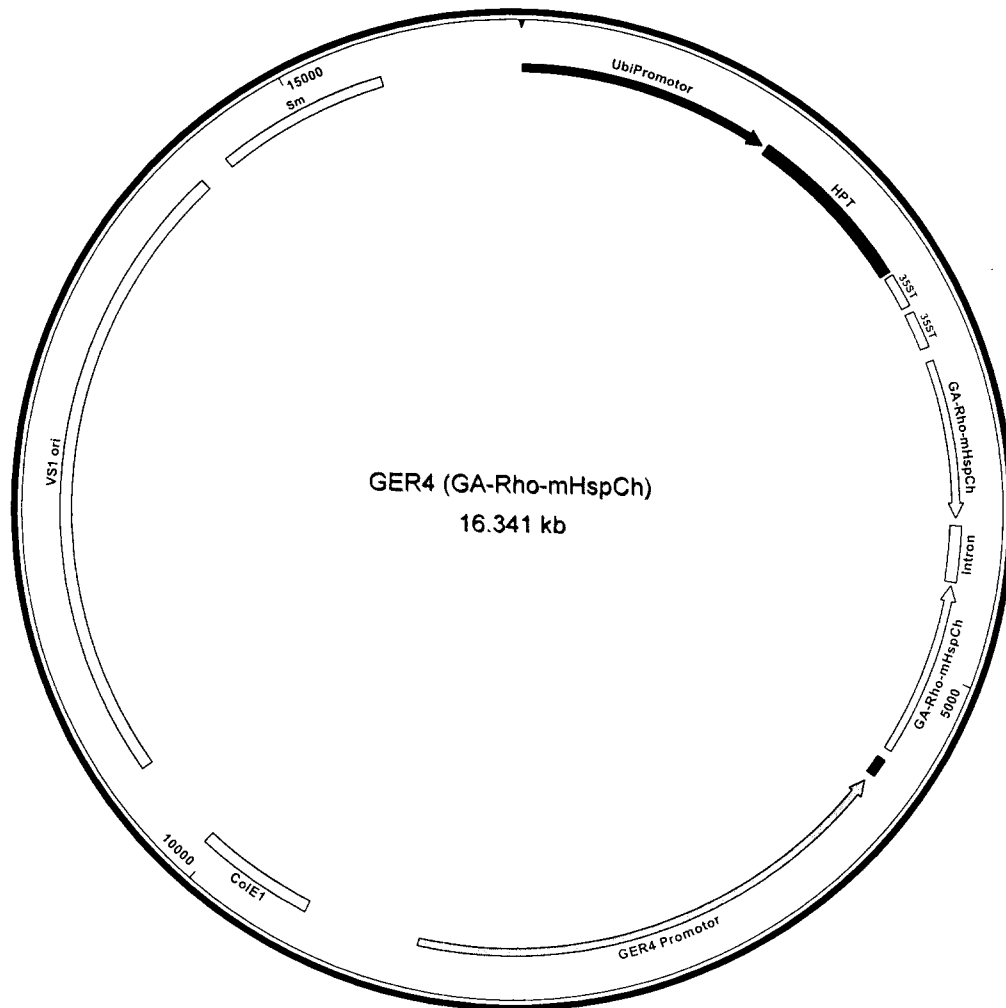


Fig. 5: Transient transformation experiments with barley leaves of the sort Ingrid BC mlo5 (*Hordeum vulgare*). Spores of the phytopathogenic *Blumeria graminis* f. sp. *hordei* (barley powdery mildew) were used as inoculum. In said system, *single* and *triple target* RNAi constructs were tested in combination with the vector pIPKb007.

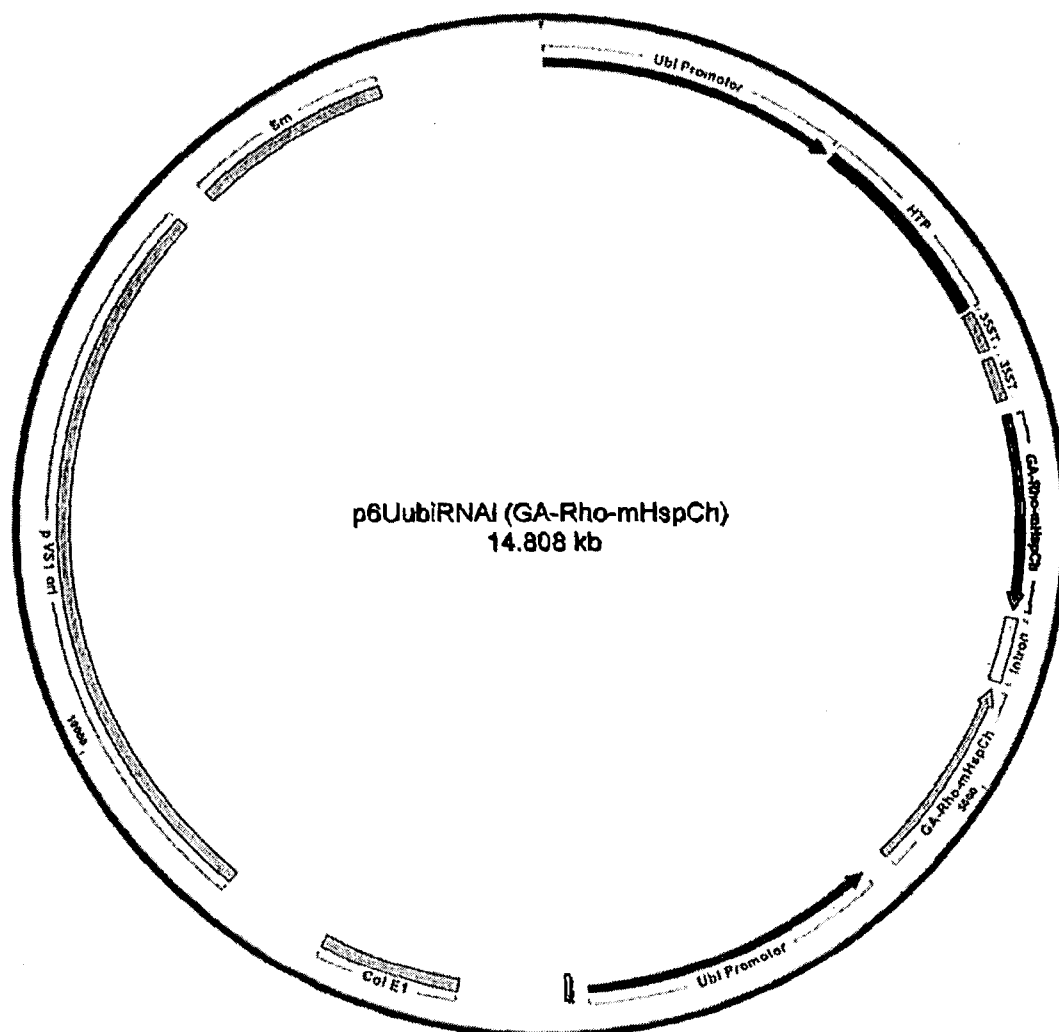
Fig. 6: Schematic structure of a construct having the GER4 promotor as well as an insert of a triple sequence combination (e.g. gamma-actin, RhoGTPase and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 6:

- LB (16041-16063 bp)
- ori Col E1 (9374-10071 bp)
- ori p VS1 (10620-14325 bp)
- Sm (14509-15519 bp)
- Ubi promotor (10-1517 bp)
- HPT (1544-2569 bp)
- 35ST (2592-2795 bp)
- 35ST (2835-3058 bp)
- Inverted repeat 1: gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (3140-4083 bp)
- Intron (5579-5692 bp)
- Inverted repeat 2: gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (4478-5516 bp)
- GER4 promotor (5742-8694 bp)
-

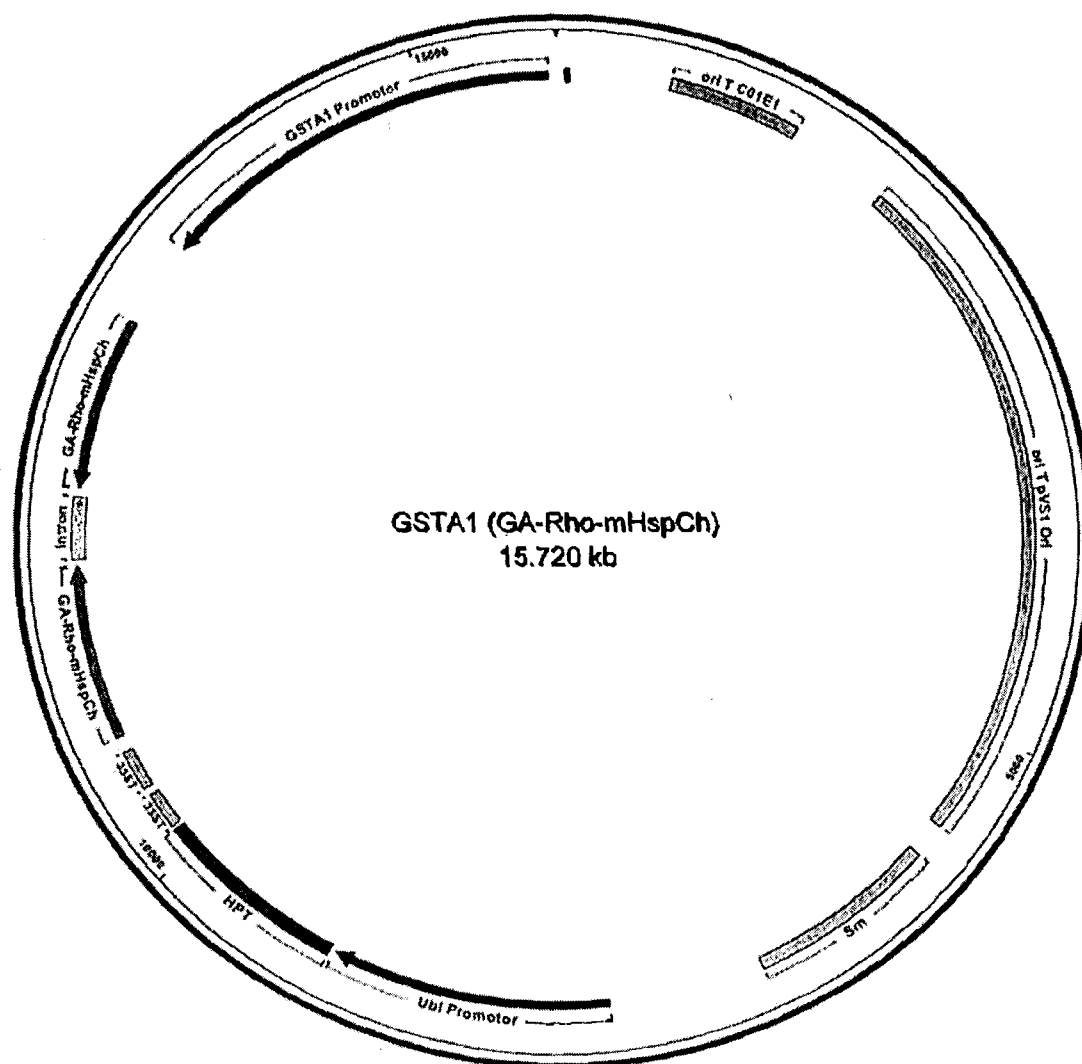
Fig. 7: Schematic structure of a construct having the p6UubiRNAi promotor as well as an insert of a triple sequence combination (e.g. gamma-actin, RhoGTPase and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 7:

- RB (7283-7306 bp)
- ori T Col E1 (7841-8538 bp)
- ori p VS1 (9087-14792 bp)
- Sm (12976-13986 bp)
- Ubi promotor (10-1517 bp)
- HPT (1544-2569)
- 35ST (2592-2795 bp)
- 35ST (2835-3058 bp)
- Inverted repeat 1: gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (3141-4135 bp)
- Intron (4178-4508 bp)
- Inverted repeat 2 : gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (4554-5548 bp)
- Ubi promotor (5688-7194 bp)
-

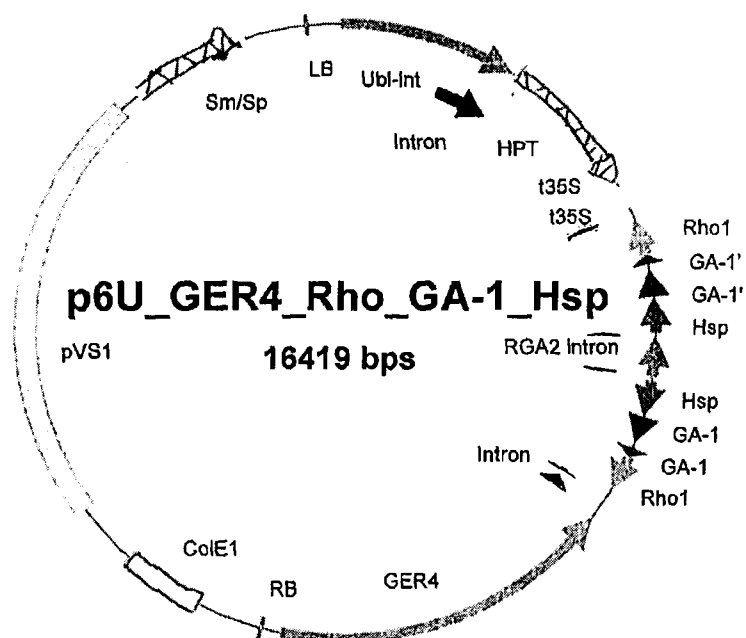
Fig. 8: Schematic structure of a construct having the GSTA1 promotor and an insert of a triple sequence combination (e.g. gamma-actin, RhoGTPase and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 8:

- RB (48-71 bp)
- ori T Col E1 (606-1303 bp)
- ori T pVS1 (1852-5557 bp)
- Sm (5741-6751 bp)
- Ubi promotor (7583-9090 bp)
- HPT (9117-10142 bp)
- 35ST (1048-10631 bp)
- 35ST (10165-10368 bp)
- Inverted repeat 1: gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (10714-11656 bp)
- Intron (11699-12029 bp)
- Inverted repeat 2: gamma-actin – RhoGTPase – mitochondrial heat shock protein 70 chaperone (12075-13017 bp)
- GSTA1 promotor (13483-15680 bp)
-

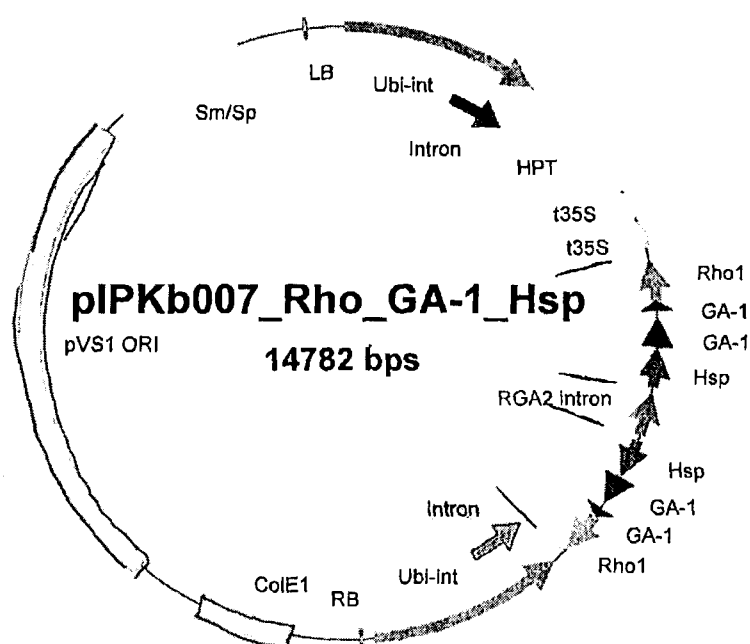
Fig. 9: Schematic structure of the construct No. 35 (p6U_GER4_Rho_GA-1_Hsp) having the GER4 promotor and an insert of a triple sequence combination (e.g. RhoGTPase, gamma-actin and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 9:

- LB (16118-16141 bp)
- ori ColE1 (9452-10149 bp)
- ori pVS1 (10698-14403 bp)
- Sm/Sp (14587-15597 bp)
- Ubi promotor (10-1517 bp)
- HPT (1544-2569 bp)
- 35ST (2594-2795 bp)
- 35ST (2840-3030 bp)
- Inverted repeat 1: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (3141-4122 bp)
- Intron (4165-4500 bp)
- Inverted repeat 2: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (4448-5529 bp)
- Intron (5657-5770 bp)
- GER4 promotor (5820-8729 bp)
- RB (8894-8917 bp)
-

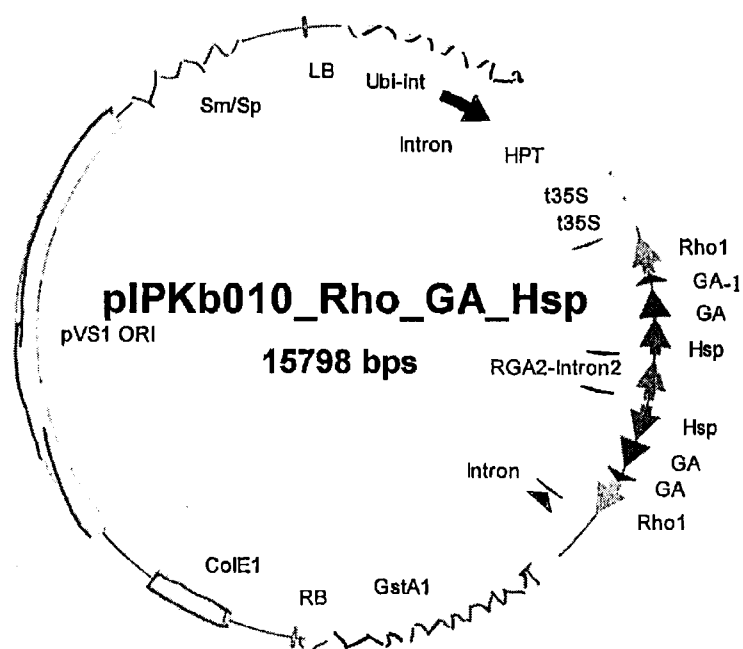
Fig. 10: Schematic structure of the construct No. 29 (pIPKb007_Rho_GA-1_Hsp) having the ubiquitin1 promotor and an insert of a triple sequence combination (e. g. RhoGTPase, gamma-actin and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 10:

- LB (14481-14504 bp)
- ori T ColE1 (7815-8512 bp)
- ori pVS1 (9061-12766 bp)
- Sm/Sp (12950-13960 bp)
- Ubi promotor (10-1517 bp)
- HPT (1544-2569 bp)
- 35ST (2594-2795 bp)
- 35ST (2840-3030 bp)
- Inverted repeat 1: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (3141-4122 bp)
- Intron (4165-4500 bp)
- Inverted repeat 2: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (4548-5529 bp)
- Ubi promotor (5662-7169 bp)
- RB (7257-7280 bp)
-

Fig. 11: Schematic structure of the construct No. 41 (pIPKb010_Rho_GA-1_Hsp) having the GstA1 promotor and an insert of a triple sequence combination (e. g. RhoGTPase, gamma-actin and mitochondrial heat shock protein 70 chaperone):



Components of the construct in Fig. 11:

- LB (15497-15520 bp)
- ori T ColE1 (8831-9528 bp)
- ori T pVS1 (10077-13782 bp)
- Sm/Sp (13966-14976 bp)
- Ubi promotor (10-1517 bp)
- HPT (1544-2569 bp)
- 35ST (2594-2795 bp)
- 35ST (2834-3058 bp)
- Inverted repeat 1: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (3141-4122 bp)
- Intron (4165-4500 bp)
- Inverted repeat 2: RhoGTPase – gamma-actin – mitochondrial heat shock protein 70 chaperone (4548-5529 bp)
- GSTA1 promotor (5988-8185 bp)
- RB (8273-8296 bp)