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(54) **PROCEDE INDUISANT UNE RESISTANCE AUX PATHOGENES  
DANS LES PLANTES**  
(54) **METHOD FOR THE INDUCTION OF PATHOGEN RESISTANCE  
IN PLANTS**

(57) L'invention concerne un procédé permettant d'induire une résistance aux pathogènes dans les plantes, caractérisé par la transformation d'une plante via une séquence polynucléotidique renfermant un promoteur à induction pathogénique qui régule l'expression d'une protéine de transduction de signal dans les plantes ou qui régule un homologue de ladite protéine. Cette expression constitutive induit une réaction d'hypersensibilité dans les plantes. Selon une variante, l'invention concerne un procédé permettant d'induire une résistance aux pathogènes dans les plantes, caractérisé par la transformation d'une plante via une séquence polynucléotidique renfermant un promoteur à induction pathogénique qui régule l'expression d'un composé capable d'atténuer l'effet inhibiteur d'une protéine sur le trajet de transduction de signal, engendrant ainsi une réaction d'hypersensibilité dans les plantes.

(57) The invention comprises a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a plant signal transduction protein or a homologue thereof which when constitutively expressed gives rise to a hypersensitive response in plants. Alternatively, the invention comprises a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a compound which is able to alleviate the inhibitory effect of a protein on the signal transduction pathway leading to a hypersensitive response in plants.

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<b>(21) International Application Number:</b> PCT/EP99/01672  <b>(22) International Filing Date:</b> 8 March 1999 (08.03.99)  <b>(30) Priority Data:</b> 98104076.9                   6 March 1998 (06.03.98)             EP  <b>(71) Applicant (for all designated States except US):</b> MOGEN INTERNATIONAL N.V. [NL/NL]; Einsteinweg 97, NL-2333 CB Leiden (NL).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> STUIVER, Maarten, Hendrik [NL/NL]; Groenhoevelaan 71, NL-2343 BR Oegstgeest (NL). CUSTERS, Jérôme, Hubertina, Henricus, Victor [NL/NL]; Fazantstraat 32, NL-2406 VK Alphen a/d Rijn (NL). SIMONS, Lambertus, Henrus [NL/NL]; Anne de Vrieslaan 20, NL-1187 WL Amstelveen (NL).  <b>(74) Agent:</b> VAN WEZENBEEK, L., A., C., M.; Mogen International, P.O. Box 628, NL-2300 AP Leiden (NL).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title: METHOD FOR THE INDUCTION OF PATHOGEN RESISTANCE IN PLANTS</b>		
<b>(57) Abstract</b>  <p>The invention comprises a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a plant signal transduction protein or a homologue thereof which when constitutively expressed gives rise to a hypersensitive response in plants. Alternatively, the invention comprises a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a compound which is able to alleviate the inhibitory effect of a protein on the signal transduction pathway leading to a hypersensitive response in plants.</p>		

## Method for the induction of pathogen resistance in plants

The present invention relates, inter alia, to recombinant polynucleotides, proteins and methods of imparting to and/or enhancing an organisms ability to resist pathogens.

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### Background

Plants resistant to pathogens often are found to evoke their resistance through a mechanism which eventually yields a hypersensitive response (HR) resulting in rapid cell death of the infected plant cells. This rapid cell death or necrosis inhibits the pathogen from further growth and thus stops the infection. This mechanism is known within the art (Klement, Z., In: *Phytopathogenic Prokaryotes*, Vol. 2, eds.: Mount, M.S. and Lacy, G.H., New York, Academic Press, 1982, pp. 149-177). The HR is often confused with other lesion-like phenomena, but a typical HR gives local cell death and is associated with secondary responses such as callus deposition, generation of active oxygen species, induction of phytoalexins, changes in ion fluxes across membranes and induction of acquired resistance (AR) (Hammond-Kosack, K.E., *et al.*, *Plant Physiol.* 110, 1381-1394, 1996).

Pathogen resistance can be elicited by response to elicitor compounds, which are frequently found to be of proteinaceous nature (Arlat, M., *et al.*, *EMBO J.*, 13, 543-553, 1994; Baker, C.J. *et al.*, *Plant Physiol.* 102, 1341-1344, 1993; Staskawicz, B.J. *et al.*, *Proc. Natl. Acad. Sci. USA* 81, 6024-6028, 1984; Vivian, A. *et al.*, *Physiol. Mol. Plant Pathol.* 35, 335-344, 1989; Keen, N.T., *Ann. Rev. Gen.* 24, 447-463, 1990; Ronald, P.C. *et al.*, *J. Bacteriol.* 174, 1604-1611, 1992; Whitham, S. *et al.*, *Cell* 78, 1-20, 1994; Kobe, B. and Deisenhofer, J., *Trends Biochem. Sci.* 19, 415, 1994; and Honée G. *et al.*, *Plant Mol. Biol.* 29, 909-920, 1995). These elicitor proteins (encoded by avirulence genes) are thought to bind to a resistance protein available in the plant, therewith starting a cascade of events resulting in the HR. The elicitor proteins are characterized by the fact that they are race-specific and only are able to elicit the response with a corresponding (also specific) resistance protein. The concept of avirulence-gene based resistance is also known under the name of the gene-for-gene response. Avirulence genes have been cloned from bacterial pathogens (such as

*Pseudomonas* and *Xanthomonas*) and from fungal pathogens (such as *Cladosporium fulvum*, *Rhynchosporium secalis* and *Phytophthora parasitica*). Also plant genes coding for some of the corresponding resistance genes have been cloned (such as the tomato gene Cf9 corresponding to the avirulence gene *avr9* from *Cladosporium fulvum*,  
5 and the tomato Pto-gene corresponding to the avirulence gene *avrPto* from *Pseudomonas*).

It has recently become clear that the plant resistance proteins when activated by interaction with the pathogen-derived elicitor proteins are capable of inducing a signal transduction pathway. It has been established that some interactions at least partly use a  
10 common pathway (Century, K.S., *et al.*, *Science* 278, 1963-1965, 1997). In this publication the NDR1 locus has been shown to be required for resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and to the fungal pathogen *Peronospora parasitica*. Similarly Parker, J.E., *et al.* (*The Plant Cell* 8, 2033-2046, 1996) have shown that the product encoded by the *eds1*-locus in *Arabidopsis thaliana*  
15 also has a key function in the signal transduction pathway after infection with *Peronospora parasitica*, but not after infection with *Pseudomonas syringae* pv *glycinae*.

Recently a report has been published stating that also plant derived proteins can elicit cell-death like phenomena (Karrer, E.E. *et al.*, *Plant Mol. Biol.* 36, 681-690, 1998). In  
20 this publication 11 clones have been described which were able to produce lesions in tobacco plants.

Methods to use resistance genes to confer pathogen resistance to plants are often hampered by the fact that the resistance is only limited to a few specific  
25 pathotypes.

Thus there is still need for a system which can convert a general pathogen resistance to plants which is silent when no pathogens are infecting.

### Summary of the invention

The invention now concerns a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a plant signal transduction protein or a homologue thereof which when expressed gives rise to a hypersensitive response in plants.

Specifically the signal transducing protein is *ndr1* or a homologue thereof, *eds1* or a homologue thereof, or *Xa21* or a homologue thereof.

It is also possible that the signal transduction protein is selected from the group consisting of a G-protein, a protein kinase, an AMP-cyclase and a protein phosphatase. Another embodiment of this part of the invention is where the signal transduction protein is a mutant from a signal transduction protein, which, when expressed, yields a hypersensitive response. These mutants preferably are encoded on the *acd* locus or the *lsd* locus. Specifically mutants would be *ndr1*-PKC, *ndr1*-cDPK and truncated *Xa21*.

An other embodiment of the invention is a method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a compound which is able to alleviate the inhibitory effect of a protein on the signal transduction pathway leading to a hypersensitive response in plants. An example of such a method is a method wherein the compound is an mRNA which is coding for the inhibitory protein in an anti-sense orientation, a method wherein the compound is interacting sterically with the inhibitory protein or a method wherein the compound is an antibody, a ribozyme or an RNA molecule which is able to suppress translation of the mRNA coding for the inhibitory protein.

Also part of the invention are polynucleotides comprising a pathogen inducible promoter sequence operably linked to a protein encoding sequence which encodes a plant signal transduction protein which is active in the signal transduction pathway of a plants hypersensitive response. Preferably these transduction proteins are selected from

the group comprising of *ndr1*, *eds1* and *Xa21*. More generally, the signal transduction proteins are selected from the group consisting of a G-protein, a protein kinase, an AMP-cyclase and a protein phosphatase. Another example of these signal transduction proteins are mutants from a signal transduction protein, which when expressed gives rise to a hypersensitive response in plants, especially those where the mutant protein is encoded on the *acd* locus or the *lsd* locus. Specifically preferred mutants are selected from the group of *ndr1*-PKC, *ndr1*-cDPK and truncated *Xa21*.

Yet another embodiment of the invention are polynucleotides comprising a pathogen inducible promoter sequence operably linked to a sequence which is able to alleviate the inhibitory effect of a protein which is active in the signal transduction pathway of a plants hypersensitive response.

The pathogen-inducible promoters for these polynucleotides can be selected from the group comprising of the promoters of *prp1*, *Fis1*, *Bet v 1*, *Vst1*, *gstA1*, and sesquiterpene cyclase, but any pathogen-inducible promoter which is switched on after pathogen infection can be used.

Also part of the invention is a method using a polynucleotide as described above to transform a plant, making said plant at least partially resistant to pathogens.

### Legends to the figures

Figure 1. Typical transient expression assay in Onion for wt and truncated XA21 function. Panel A: bombardment with 35S-uidA alone; panel B: cobombardment of 35S-uidA and pMOG1468; panel C: cobombardment of 35S-uidA and pMOG1470; panel D: cobombardment of 35S-uidA and pMOG1475.

### Detailed description

Although the invention is illustrated in detail for some transgenic plants, it should be understood that any plant species that is subject to some form of pathogen attack, especially from fungi or bacteria, may be provided with one or more plant expressible gene constructs, which when expressed are capable of inducing a HR. The invention can even be practiced in plant species that are presently not amenable for transformation, as the amenability of such species is just a matter of time and because transformation as such is of no relevance for the principles underlying the invention. Hence, plants for the purpose of this description shall include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants, be they for feed, food or industrial processing purposes; included are plants used for any agricultural or horticultural purpose including forestry and flower culture, as well as home gardening or indoor gardening, or other decorative purposes.

The invention is illustrated using signal transduction proteins which are known to play a role in the cascade of events after pathogen infection and which can induce a HR upon such an infection. In order to provide a quick and simple test if the constructs which are described here or any new constructs which are obvious to the person skilled in the art after reading this application indeed can yield a hypersensitive response the person skilled in the art can perform a rapid transient expression test known under the name of ATTA (*Agrobacterium tumefaciens* Transient expression Assay). In this assay (of which a detailed description can be found in Van den Ackerveken, G., *et al.*, Cell 87, 1307-1316, 1996) the nucleotide sequence coding for the signal transduction protein which is to be tested is placed under control of the CaMV 35S promoter and

introduced into an *Agrobacterium* strain which is also used in protocols for stable transformation. After incubation of the bacteria with acetosyringon or any other phenolic compound which is known to enhance *Agrobacterium* T-DNA transfer, 1 ml of the *Agrobacterium* culture is infiltrated into an *in situ* plant leaf (from e.g. a tobacco or tomato plant) by injection after which the plants are placed in a greenhouse. After 2-5 days the leaves can be scored for occurrence of HR symptoms.

### Overexpression of proteins.

One of the possibilities that is available to use plant proteins with a role in hypersensitive lesion formation is through direct overexpression. There are a number of ways in which a person skilled in the art can achieve over expression of a particular protein. These methods are well known and frequently used within the art.

The protein that is overexpressed can be the receptor of a ligand that normally triggers the hypersensitive response or a positive acting component in the signal transduction pathway leading to a hypersensitive response. Overexpression of a positive regulator of the pathway, which can be e.g. a G-protein, AMP-cyclase, kinase or phosphatase can upset the balance present between components of the signalling pathway leading to a hypersensitive response. This in fact leads to inadvertent signalling in the absence of the ligand normally responsible for triggering of the pathway.

### Downregulation, inhibition or inactivation of proteins.

Another way to reach a similar effect is through downregulation, inhibition or inactivation of negative acting components within the signal transduction pathway leading to a hypersensitive response. There are a number of ways in which a person skilled in the art can achieve under expression or down regulation of a particular protein. These methods are well known and frequently used within the art.



The negative acting proteins may have a variety of functions. Well known examples are phosphatases and kinases. These represent common regulators of enzyme- and signal transduction component activity.

Pathogen-induced removal of such a protein can effectively be mediated through induced expression of antisense RNA (as outlined in Kumria et al., 1998, Current Science 74, 35-41), (short stretches of) sense RNA (sense suppression, van Blokland et al., 1994 Plant Journal 6, 861-877) or through the expression of ribozymes, sequence specific RNA-based ribonucleases (outlined in e.g. Wegener et al., 1994, Mol. Gen. Genet. 245, 465-470; Perriman et al., 1995, Proc. Natl. Acad. Sci. USA 92, 6175-6179).

More directly, expression of proteins that interfere with the normal inhibitory function of these proteins, thereby alleviating their inhibitory effect, is a possibility that is known to those skilled in the art. A specific example of such compounds are antibodies that can be expressed in plants, which may e.g. interfere sterically with the inhibitory function.

#### **Dominant interfering proteins.**

It is known to those skilled in the art, that mutant proteins, such as point mutants and deletion mutants derived from proteins with a role in signal transduction, can have altered properties relating to the way they are regulated. As an example, the activity of mutant proteins can be expressed in an active form continuously, whereas the activity of its non-mutated counterpart is tightly regulated (Chang & Meyerowitz, 1995, Proc. Natl. Acad. Sci. USA 92, 4129-4133; Miloso et al., 1995, J. Biol. Chem. 270, 19557-19562). When such a mutant protein can be identified/constructed from one having a positively acting role in the signal transduction pathway leading to the hypersensitive reaction, it then can be used as a tool to obtain broad-spectrum resistance. By coupling the open reading frame encoding such active mutant protein to a pathogen-inducible promoter in a functional manner, activation of the signal transduction pathway leading to the HR is directly mediated through promoter activation by pathogen infection. Likewise, dominant interfering negative acting proteins are described, (Boylan et al., 1994, Plant Cell 6, 449-460; Okamoto et al.,

1997 Plant Physiol. 115, 79-85; McNellis et al., 1996, Plant Cell 8, 1491-1503; Emmeler et al., 1995, Planta 197, 103-110).

Examples of mutants described to give an HR are the spontaneous HR, accelerated cell death (acd) and lesion simulating mutants (lsd) (Neuffer & Calvert, 1975, J. Hered. 66, 265-270; Dietrich et al., 1994 Cell 77, 565-577).

In both cases described above, the mutant protein used may be derived from the same plant, or from a heterologous source.

### 2nd messenger generating systems

Yet another way to induce the signal transduction pathway leading to the hypersensitive response is through second messengers. Signal transduction leading to the hypersensitive response is known to be mediated by second messenger molecules. As an example, influx of  $Ca^{2+}$  -ions appears to play an important role (Cho, Abstract ISPMB congress Singapore, 1997). It is possible to generate such a stimulus by introducing a heterologous protein that allows unregulated  $Ca^{2+}$  influx into the cytoplasm, setting off the downstream sequence of events that eventually lead to the hypersensitive reaction.

### Method of generating a mutant protein that activates the hypersensitive response

When a protein is identified that plays a role in the hypersensitive response, mutants can be made that are thought to be refractory to inhibitory regulation. Commonly used procedures to identify sequences of interest are known to those skilled in the art. Database searches such as Prosite, containing known modification sites are frequently used.

In the NDR1 protein, which plays a role in defense response of plants, two putative CDPK ( $Ca^{2+}$ -dependent protein kinase) and three PKC (protein kinase C) phosphorylation sites can be identified, that could provide a good starting point searching for suitable mutants (Subramanian et al., 1997, Plant Cell 9, 653-664). Mutation of threonine and serine residues to aspartic acid and glutamic acid residues frequently leads to activation, as was shown in many proteins of which the activity is

modulated by phosphorylation, e.g. in a MAPK-activated protein (Engel et al., 1995, J. Biol. Chem. 270, 27213-27221), and in a MAP-kinase-kinase protein (Huang et al., 1995 Mol. Biol. Cell 6, 237-245). Also C- and N-terminal as well as internal deletion mutants of these proteins can be tested for suitable mutants.

5 A more undirected way of identifying interesting mutants of which constitutive activity is induced is through propagation of the protein-encoding DNA in so-called E. coli 'mutator' strains.

Another protein active in the hypersensitive response is Xa21 (Wen-Yuan Song et al. 1995, science 270: 1804-1806) which is thought to be a resistance gene in rice. It has a  
10 leucine rich region and a cytoplasmatic serine-threonine kinase domain.

A rapid way of testing all made mutants for their suitability to elicit an HR is through the ATTA assay, as described above. Many mutants can be screened with low effort to identify those that will elicit an HR upon expression.

15 Proteins of the invention, also denominated as signal transduction proteins, include all proteins known or to be detected which play a role in the signal transduction pathway(s) of plants which lead to a hypersensitive response. These include proteins that are components of this pathway up from the first proteins that transfer the signal which is generated by the elicitor protein interacting with the receptor moiety down to  
20 the proteins which actually cause the HR, regulatory proteins in this pathway, proteins which can affect the inhibitory effects of pathway inhibitors and 2nd messenger generating enzymes. It should be understood that anti-sense suppression or co-suppression of inhibitory components should also be caught under this definition.

The word protein means a sequence of amino acids connected through peptide  
25 bonds. Polypeptides or peptides are also considered to be proteins. Homologues of the signal transduction proteins which are part of the invention are proteins that are encoded by DNA sequences found to hybridize under stringent conditions with nucleotide sequences coding for said signal production proteins. Such homologues can be found in heterologous species but they can also be made by protein  
30 engineering *in vivo*, e.g. by changing the open reading frame capable of encoding the signal transduction proteins. Mutations are made by replacing, adding and/or deleting one or more amino acids, while still retaining their HR inducing activity. As long as

the changes in the amino acid sequences do not altogether abolish the HR inducing activity such homologues are embraced in the present invention. In addition to this, conservative amino acid replacements may be made within these mutated proteins which do not affect activity. In particular conservative amino acid replacements may  
5 be made between the following groups, *Viz.*

- (a) Alanine, Serine, Glycine and Threonine
- (b) Glutamic acid and Aspartic acid
- (c) Arginine and Lysine
- 10 (d) Isoleucine, Leucine, Valine and Methionine
- (e) Phenylalanine, Tyrosine and Tryptophan

Further, it should be understood that mutations should be derivable from the proteins or the DNA sequences encoding these proteins depicted in the sequence  
15 listing while retaining biological activity, i.e. all, or a great part of the intermediates between the mutated protein and the protein depicted in the sequence listing should have HR-response inducing activity. A great part would mean 30% or more of the intermediates, preferably 40% or more, more preferably 50% or more, more preferably 60% or more, more preferably 70% or more, more preferably 80% or more,  
20 more preferably 90% or more, more preferably 95% or more, more preferably 99% or more.

The present invention provides a chimeric DNA sequence which comprises a pathogen inducible promoter which regulates the expression of a plant protein or a  
25 homologue thereof which is capable of eliciting a hypersensitive response. The expression chimeric DNA sequence shall mean to comprise any DNA sequence which comprises DNA sequences not naturally found in nature. The said open reading frame may be incorporated in the plant genome wherein it is not naturally found, or in a replicon or vector where it is not naturally found, such as a bacterial plasmid or a viral  
30 vector. Chimeric DNA shall not be limited to DNA molecules which are replicable in a host, but shall also mean to comprise DNA capable of being ligated into a replicon, for

instance by virtue of specific adaptor sequences, physically linked to the nucleotide sequence according to the invention.

The open reading frame coding for the signal transduction protein may be derived from a genomic library. In this latter it may contain one or more introns separating the exons making up the open reading frame that encodes the protein. The open reading frame may also be encoded by one uninterrupted exon, or by a cDNA to the mRNA encoding the signal transduction protein. Open reading frames according to the invention also comprise those in which one or more introns have been artificially removed or added. Each of these variants is embraced by the present invention.

Pathogen inducible promoters are known in the art and are responsive to a large number of pathogens and to aspecific elicitors produced by these pathogens. Examples of such pathogen inducible promoters are: the *prp1* promoter (Martini, N., *et al.*, Mol. Gen. Genet. 236, 179-186, 1993), the *Fis1* promoter (WO 96/34949), the *Bet v 1* promoter (Swoboda, I., *et al.*, Plant, Cell and Env. 18, 865-874, 1995), the *Vst1* promoter (Fischer, R., Dissertation, Univ. of Hohenheim, 1994; Schubert, R., *et al.* Plant Mol. Biol. 34, 417-426, 1997), the sesquiterpene cyclase promoter (Yin, S., *et al.*, Plant Physiol. 115, 437-451, 1997) and the *gstA1* promoter (Mauch, F. and Dudler, R., Plant Physiol. 102, 1193-1201, 1993). Several other promoters are known in the art and can be used for the nucleotide sequences of this invention.

In eukaryotic cells, an expression cassette usually further comprises a transcriptional termination region located downstream of the open reading frame, allowing transcription to terminate and polyadenylation of the primary transcript to occur. In addition, the codon usage may be adapted to accepted codon usage of the host of choice. The principles governing the expression of a chimeric DNA construct in a chosen host cell are commonly understood by those of ordinary skill in the art and the construction of expressible chimeric DNA constructs is now routine for any sort of host cell, be it prokaryotic or eukaryotic.

In order for the open reading frame to be maintained in a host cell it will usually be provided in the form of a replicon comprising said open reading frame according to the invention linked to DNA which is recognised and replicated by the chosen host cell. Accordingly, the selection of the replicon is determined largely by the host cell of

choice. Such principles as govern the selection of suitable replicons for a particular chosen host are well within the realm of the ordinary skilled person in the art.

A special type of replicon is one capable of transferring itself, or a part thereof, to another host cell, such as a plant cell, thereby co-transferring the open reading frame according to the invention to said plant cell. Replicons with such capability are herein referred to as vectors. An example of such vector is a Ti-plasmid vector which, when present in a suitable host, such as *Agrobacterium tumefaciens*, is capable of transferring part of itself, the so-called T-region, to a plant cell. Different types of Ti-plasmid vectors (*vide*: EP 0 116 718 B1) are now routinely being used to transfer chimeric DNA sequences into plant cells, or protoplasts, from which new plants may be generated which stably incorporate said chimeric DNA in their genomes. A particularly preferred form of Ti-plasmid vectors are the so-called binary vectors as claimed in (EP 0 120 516 B1 and US 4,940,838). Other suitable vectors, which may be used to introduce DNA according to the invention into a plant host, may be selected from the viral vectors, *e.g.* non-integrative plant viral vectors, such as derivable from the double stranded plant viruses (*e.g.* CaMV) and single stranded viruses, gemini viruses and the like. The use of such vectors may be advantageous, particularly when it is difficult to stably transform the plant host. Such may be the case with woody species, especially trees and vines.

The expression "host cells incorporating a chimeric DNA sequence according to the invention in their genome" shall mean to comprise cells, as well as multicellular organisms comprising such cells, or essentially consisting of such cells, which stably incorporate said chimeric DNA into their genome thereby maintaining the chimeric DNA, and preferably transmitting a copy of such chimeric DNA to progeny cells, be it through mitosis or meiosis. According to a preferred embodiment of the invention plants are provided, which essentially consist of cells which incorporate one or more copies of said chimeric DNA into their genome, and which are capable of transmitting a copy or copies to their progeny, preferably in a Mendelian fashion. By virtue of the transcription and translation of the chimeric DNA according to the invention in some or all of the plant's cells, those cells that produce the antifungal protein will show enhanced resistance to fungal infections.

Transformation of plant species is now routine for an impressive number of plant species, including both the *Dicotyledoneae* as well as the *Monocotyledoneae*. In principle any transformation method may be used to introduce chimeric DNA according to the invention into a suitable ancestor cell, as long as the cells are capable of being regenerated into whole plants. Methods may suitably be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, 1982, *Nature* 296, 72-74; Negrutiu I. *et al.*, June 1987, *Plant Mol. Biol.* 8, 363-373), electroporation of protoplasts (Shillito R.D. *et al.*, 1985 *Bio/Technol.* 3, 1099-1102), microinjection into plant material (Crossway A. *et al.*, 1986, *Mol. Gen. Genet.* 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. *et al.*, 1987, *Nature* 327, 70), infection with (non-integrative) viruses and the like. A preferred method according to the invention comprises *Agrobacterium*-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP A 120 516 and U.S. Patent 4,940,838.

Generally, after transformation plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant expressible genes co-transferred with the nucleic acid sequence according to the invention, whereafter the transformed material is regenerated into a whole plant.

Although considered somewhat more recalcitrant towards genetic transformation, monocotyledonous plants are amenable to transformation and fertile transgenic plants can be regenerated from transformed cells or embryos, or other plant material. Presently, preferred methods for transformation of monocots are microprojectile bombardment of embryos, explants or suspension cells, and direct DNA uptake or electroporation (Shimamoto, *et al.*, 1989, *Nature* 338, 274-276).

Transgenic maize plants have been obtained by introducing the *Streptomyces hygroscopicus bar*-gene, which encodes phosphinothricin acetyltransferase (an enzyme which inactivates the herbicide phosphinothricin), into embryogenic cells of a maize suspension culture by microprojectile bombardment (Gordon-Kamm, 1990, *Plant Cell*, 2, 603-618). The introduction of genetic material into aleurone protoplasts of other monocot crops such as wheat and barley has been reported (Lee, 1989, *Plant Mol. Biol.* 13, 21-30). Wheat plants have been regenerated from embryogenic suspension culture by selecting only the aged compact and nodular embryogenic callus tissues for the

establishment of the embryogenic suspension cultures (Vasil, 1990 Bio/Technol. 8, 429-434). The combination with transformation systems for these crops enables the application of the present invention to monocots.

Monocotyledonous plants, including commercially important crops such as rice  
5 and corn are also amenable to DNA transfer by *Agrobacterium* strains (*vide* WO 94/00977; EP 0 159 418 B1; Gould J, Michael D, Hasegawa O, Ulian EC, Peterson G, Smith RH, (1991) Plant. Physiol. 95, 426-434).

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the chimeric  
10 DNA according to the invention, copy number and/or genomic organization. After the initial analysis, which is optional, transformed plants showing the desired copy number and expression level of the newly introduced chimeric DNA according to the invention may be tested for resistance levels against a pathogen.

Other evaluations may include the testing of pathogen resistance under field  
15 conditions, checking fertility, yield, and other characteristics. Such testing is now routinely performed by persons having ordinary skill in the art.

Following such evaluations, the transformed plants may be grown directly, but usually they may be used as parental lines in the breeding of new varieties or in the creation of hybrids and the like.

20 These plants, including plant varieties, with improved resistance against pathogens may be grown in the field, in the greenhouse, or at home or elsewhere. Plants or edible parts thereof may be used for animal feed or human consumption, or may be processed for food, feed or other purposes in any form of agriculture or industry. Agriculture shall mean to include horticulture, arboriculture, flower culture, and the like. Industries  
25 which may benefit from plant material according to the invention include but are not limited to the pharmaceutical industry, the paper and pulp manufacturing industry, sugar manufacturing industry, feed and food industry, enzyme manufacturers and the like.

The advantages of the plants, or parts thereof, according to the invention are the  
30 decreased need for pesticide treatment, thus lowering costs of material, labour, and environmental pollution, or prolonging shelf-life of products (*e.g.* fruit, seed, and the like) of such plants. Plants for the purpose of this invention shall mean multicellular



organisms capable of photosynthesis, and subject to some form of pathogen induced disease. They shall at least include angiosperms as well as gymnosperms, monocotyledonous as well as dicotyledonous plants.

5 The invention will now be described by way of the following non-limiting examples. It will be apparent to the skilled artisan that the following techniques can be varied and modified without detracting from the gist of the present invention.

### EXPERIMENTAL PART

10 Standard methods for the isolation, manipulation and amplification of DNA, as well as suitable vectors for replication of recombinant DNA, suitable bacterium strains, selection markers, media and the like are described for instance in Maniatis *et al.*, molecular cloning: A Laboratory Manual 2nd. edition (1989) Cold Spring Harbor Laboratory Press; DNA Cloning: Volumes I and II (D.N. Glover ed. 1985); and in:  
15 From Genes To Clones (E.-L. Winnacker ed. 1987).

### EXAMPLE 1

#### Isolation and cloning of the ndr1 gene

20

The sequence of the ndr1 gene (non-race specific disease resistance) from *Arabidopsis thaliana* was published (Genbank accession number AF021346 Century K.S., Science, Vol.278, 12 December 1997 pp.1964-1965). Based on this sequence (SEQ ID NO:13 ) the primers FR-Ndr-297 (SEQ ID NO:1) 5'-CAA GAA TTC GGT CAT GAA TAA TCA AAA  
25 TGA AGA CAC-3', introducing a EcoRI site and, downstream, a RcaI site at the start codon and FR-Ndr-298 (SEQ ID NO:2) 5'-ATT AGG ATC CTT AAC GAA TAG CAA AGA ATA  
CGA G-3', introducing a BamHI site behind the stopcodon were synthesised to amplify Ndr1  
with Pfu turbo DNA polymerase (Stratagene) from genomic DNA of *Arabidopsis thaliana*  
colombia (PCR program 1' 95°C, 1'43°C, 2' 72°C, 4 cycles; 1' 95°C, 1' 55°C, 2' 72°C, 25  
30 cycli). The PCR product was digested with EcoR1 and BamH1 yielding a 660 bp fragment that was cloned in pBKS (Stratagene) that was digested with the same restriction enzymes. The sequence of the PCR product was confirmed by DNA sequencing. The ndr1 ORF was

subsequently ligated between the 35S promoter joined with the *GluII* 5' untranslated region (Linthorst et al., 1990, Proc. Natl. Acad. Sci. USA 87: 8756-8760) (SEQ ID NO:17) and the 3' untranslated region of the potato proteinase inhibitor II gene (Thornburg et al., 1987, Proc. Natl. Acad. Sci. USA 84, 744-748) which contains sequences needed for polyadenylation (An et al., 1989, Plant cell 1, 115-122). The complete expression unit was then transferred as a *SacI*-*EcoRI* fragment to the binary vector pMOG800 (described *inter alia* in WO 98/13478) resulting in pMOG1456. This construct was electroporated in EHA105 for ATTA experiments.

10

## EXAMPLE 2

### Constructing of modified *ndr1*

15 To make a constitutive active *Ndr1* protein potential PKC (protein kinase C) or CDPK (Ca<sup>2+</sup>-dependent protein kinase) sites were modified by replacing the codon Serine or Threonine for an Aspartate codon.

Potential PKC sites were changed in the *ndr1* gene at amino acid Ser-35, Thr-94 and Thr-164 (SEQ ID NO:14) using the following primers :

20

FR-Ndr-297 (SEQ ID NO:1) : 5'-

CAAGAATTCGGTCATGAATAATCAAAATGAAGACAC-3'

FR-Ndr-298 (SEQ ID NO:2) : 5'-ATTAGGATCCTTAACGAATAGCAAAGAATACGAG-3'

25 (for amplification of the *ndr1* ORF)

FR-Ndr-303 (SEQ ID NO:7) : 5'-CTTATGGCTTGATCTCCGTGCGGAC-3'

FR-Ndr-304 (SEQ ID NO:8) : 5'-CGCACGGAGATCAAGCCATAAGAAAAGA-3'

(ser-35=>asp-35)

30

FR-Ndr-305 (SEQ ID NO:9) : 5'-CCACCATCAACGATACCAAGATCAATTCC-3'

FR-Ndr-306 (SEQ ID NO:10): 5'-GATCTTGGTATCGTTGATGGTGGAAAAATTAAGG-3'

(thr-94=>asp-94)

5 FR-Ndr-307 (SEQ ID NO:11) : 5'-GGAAAGATAAGAGGTATGGGGTTG-3'

FR-Ndr-308 (SEQ ID NO:12) : 5'-CCATACCTCTTATCTTTCCAAAAAAC-3'

(thr-164=>asp-164)

Using these primers and pMOG1456 as a template ndr1 was mutated on all three PKC sites  
 10 in one construct using Pfu turbo DNA polymerase (PCR 1' 95°C, 1'43°C, 1.5' 72°C, 4 cycli,  
 1' 95°C, 1'51°C, 1.5' 72°C, 25 cycli). In a first PCR step the following primer combinations  
 were used to generate the mutations: 1) FR-Ndr-297 (SEQ ID NO:1) + FR-Ndr-304 (SEQ ID  
 NO:8); 2) FR-Ndr-303 (SEQ ID NO:7) + FR-Ndr-306 (SEQ ID NO:10); 3) FR-Ndr-305 (SEQ  
 ID NO:9) + FR-Ndr-308 (SEQ ID NO:12); 4) FR-Ndr-307 (SEQ ID NO:11) + FR-Ndr-298  
 15 (SEQ ID NO:2). In the next PCR steps the fragments were joined with the SOE technique  
 (Splicing by Overlap Extension; Methods in Molecular Biology, Vol67: PCR Cloning  
 Protocols; From Molecular Cloning to Genetic Engineering) and after digestion with BamHI  
 and EcoRI the resulting PCR fragment was cloned in pBKS. After sequencing this clone,  
 ndr1-PKC (SEQ ID NO:15) contained one point mutation at position 297 in which an A was  
 20 substituted for a C. This mistake was introduced by primer FR-Ndr-305 but did not result in  
 an amino acid change. Therefore it was decided to continue with this construct. The ndr1-  
 PKC ORF was subsequently ligated between the 35S promoter/GluII-leader (SEQ ID NO:17)  
 and the 3' untranslated region of the potato proteinase inhibitor II gene (Thornburg et al.,  
 1987, Proc. Natl. Acad. Sci. USA 84, 744-748) which contains sequences needed for  
 25 polyadenylation (An et al., 1989, Plant cell 1, 115-122).

The complete expression unit was then transferred as a SacI-EcoRI fragment to binary vector  
 pMOG800 resulting in pMOG1457. This construct was electroporated in EHA105 for ATTA  
 experiments.

30 Potential CDPK sites were changed at ser-20 and ser-207 (SEQ ID NO.14) using the  
 following primers:

FR-Ndr-297 (SEQ ID NO.1): 5'-

CAAGAATTCGGTCATGAATAATCAAATGAAGACAC-3'

FR-Ndr-298 (SEQ ID NO.2): 5'-ATTAGGATCCTTAACGAATAGCAAAGAATACGAG-  
3'

5 (for amplification of the ndr1 ORF)

FR-Ndr-299 (SEQ ID NO.3): 5'-TGCTTAGATTTTCATCTTCACAGC-3'

FR-Ndr-300 (SEQ ID NO.4): 5'-TGTGAAGATGAAATCTAAGCAGCAAG-3'

(ser-20=>asp-20)

10

FR-Ndr-301 (SEQ ID NO.5): 5'-CCGATTGATGTTTTGATGAATTTAC-3'

FR-Ndr-302 (SEQ ID NO.6): 5'-TTCATCAAACATCAATCGGAAAAGAG-3'

(ser-207=>asp-207)

15

With these primers and pMOG1456 as a template ndr1 was mutated on the two CDPK sites using PCR as described for ndr1-PKC. The resulting fragment was digested with with BamHI and EcoRI and cloned in pBKS. The correct sequence of the PCR product was confirmed with DNA sequencing (SEQ ID NO.16). The Ndr1-CDPK ORF was subsequently  
20 ligated between the 35S promoter/35s-GluII leader (SEQ ID NO:17) and 3' untranslated region of the potato proteinase inhibitor II gene (Thornburg et al., 1987, Proc. Natl. Acad. Sci. USA 84, 744-748) which contains sequences needed for polyadenylation (An et al., 1989, Plant cell 1, 115-122). The complete expression unit was then transferred as a SacI-EcoRI fragment to the binary vector pMOG800.

25 This construct was electroporated in EHA105 for ATTA experiments.

### EXAMPLE 3

#### ATTA experiments

30 ATTA (Agrobacterium tumefaciens Transient expression assay) was carried out essentially as described by Van den Ackerveken G., et al., Cell 87, 1307-1316, 1996, with the exception that plants were incubated in the phytochamber after infiltration without a transparent bag.

Experiments were done with pMOG1456 (ndr1), pMOG1457(ndr1-PKC), pMOG1458(ndr1-cDPK) and pMOG1047 (35S-GUS) as a negative control. These constructs were all available in EHA105.

- 5 The results of the ATTA experiment using the ndr1 clone and the two mutants, ndr1-PKC and ndr1-CDPK, are presented in Table 1.

Table 1: ATTA in leaves of several crops to score for HR development caused by expression of ndr1 and mutants thereof.

10

	pMOG1456 ndr1-WT	pMOG1457 ndr1-PKC	pMOG1458 ndr1-cDPk	pMOG1047 GUS
SR1	+	+	+	-
Tomato	-	+	+	-
Brassica napus	-	+	+	-

#### EXAMPLE 4

15

#### **Construction of ndr1 and derivatives under control of a pathogen inducible promoter**

The openreading frame of the ndr1 gene and the modified ndr1 genes were ligated between the prp1 promoter (Martini et al., MOL. Gen. Genet. (1993) 236:179-185) and the 3' untranslated region of the potato proteinase inhibitor II gene (Thornburg et al., 1987, Proc. Natl. Acad. Sci. USA 84, 744-748) which contains sequences needed for polyadenylation (An et al., 1989, Plant cell 1, 115-122) in the high copy vector pBS (Stratagene). The complete expression unit was then transferred as a BamHI-EcoRI fragment to the binary vector pMOG800 resulting in pMOG1459(ndr1), pMOG1460(ndr1-PKC) and pMOG1461(ndr1-cDPK). The Agrobacterium strain MOG 101 was produced according to the method described in WO96/21030.

20

25

The constructs pMOG1459, pMOG1460 and pMOG1461 were then electroporated into MOG101 in preparation for transformation of tobacco.

#### **EXAMPLE 5**

5

#### **Transformation of tobacco with the constructs pMOG1459, pMOG1460 and pMOG1461.**

Tobacco was transformed by co-cultivation of plant tissue with *Agrobacterium tumefaciens* strain MOG101 containing the vector of interest as described above. Transformation was carried out using co-cultivation of tobacco leaf disks as described by Horsch et al. (1985) Science 227, 1229. Transgenic plants were regenerated from shoots that grow on selection medium containing kanamycin, rooted and then transferred to soil.

15

#### **EXAMPLE 6**

#### **Infection assay of ndr1, ndr1-PKC and ndr1-CDPK tobacco transgenics with *Oidium lycopersicon***

Tobacco plants with prp1-ndr1 (pMOG1459), prp1-ndr1-PKC (pMOG1460), prp1-ndr1-CDPK (pMOG1461) and 2 control constructs, prp1-½-GUS (pMOG1058) and Ferredoxin-rolD-GUS (pMOG1059) were tested for resistance to *Oidium lycopersicon*.

*In vitro* grown plants were transplanted to potting soil and moved to a growthroom for 14 days. After these two weeks, the plants were placed in a testroom with 16h light/8h dark, 20°C and 80% RH and were inoculated with the pathogen when they had been in the testroom for 3 days (17 days after potting).

Inoculation was done by spraying at least 10 plants per construct with a spore suspension of *O. Lycopersicon* containing  $3 \times 10^4$  spores/ml (total volume used: 1 litre).

Disease severity (% leaf area covered by powdery mildew) was measured as soon as the first infection symptoms were visible. The infection started 9 days after inoculation and was

equally distributed over all the plants but too low to give a clear disease severity. The first disease severity data were collected 14 days after inoculation.

From the results it can be learned that one plant transformed with pMOG1461 showed to be resistant to fungal infection.

### Example 7

#### **Isolation of the Xa21 gene from *O.sativa* ssp. Indica line, IRBB21**

The predicted amino acid sequence (SEQ ID NO: 18) and the deduced protein domains of Xa21 have been published (Wen-Yuan Song et al., 1995, Science 270: 1804-1806). The GenBank accession number for Xa21 genomic and cDNA sequences is U37133.

For the isolation of the Xa21 gene mRNA was isolated from leafmaterial of *O.sativa* ssp. Indica line, IRBB21 using the QuickPrep® Micro mRNA Purification Kit of Pharmacia. First strand cDNA was synthesised using SuperScript™ Rnase H- Reverse Transcriptase from GibcoBRL following the instructions of the manufacture. Primers FR-Xa21-362 (SEQ ID NO: 19) 5'CCG GTA CCT CAT GAT ATC ACT CCC ATT ATT GC 3' and FR-Xa21-363 (SEQ ID NO: 20) 5' GGA GAT CTT CAG AAT TCA AGG CTC CCA CC 3' were used to PCR the complete CDS of Xa21. Primer FR-Xa21-362 introduces a Rca I site on the ATG of Xa21 and a Kpn I site at the 5' end of the PCR-product. Primer FR-Xa21-363 introduces a Bgl II site directly behind the stopcodon of Xa21. Using PfuTurbo™ DNA Polymerase (Stratagene) and the primers FR-Xa21-362 and FR-Xa21-363 the Xa21 gene was amplified from first strand cDNA. Amplification was started by melting for 2' at 95°C, followed by 30 cycli for 1' at 95°C, 1' at 55°C and 6' at 72°C and ended with an extension period at 72°C for 10'. The PCR yielded a 3.1 kb fragment that was digested with Bgl II and Kpn I and ligated in pUC28 degested with the same enzymes. The vector pUC28 (SEQ ID NO: 27) was constructed from pUC18 (J.Messing, 1983, New M13 vectors for cloning, Methods Enzymology, vol. 101:20) by ligation of adapter

5'           CATCGATCCATGGAGATCTG           3'  
3'           TCGAGTAGCTAGGTACCTCTAGACTTAA       5'

in the EcoR I and Sst I site of pUC18. A Cla I, Nco I and Bgl II site are thus added to the multiple cloning site of pUC18.

The correct sequence of the PCR-product was confirmed by sequence analysis.

- 5 The complete CDS of Xa21 was cut out from the pUC28 vector by digestion with Rca I and Bgl II. This fragment was cloned between the 35S promoter/gluII leader (SEQ ID NO:17) and the 3' untranslated region of the potato proteinase inhibitor II gene. The resulting plasmid was named pMOG1468.

10

### Example 8

#### **Cloning of truncated versions of Xa21**

Two truncated versions of the Xa21 gene were both cloned between the 35S promoter/gluII leader (SEQ ID NO:17) and the 3' untranslated region of the potato proteinase inhibitor II  
15 gene.

For cloning of truncation 1 two primers were developed: FR-Xa21-369 (SEQ ID NO: 21) 5' CCT TCA AGA ACT TTC ATG AAA GGC CAC CC 3' and FR-Xa21-370 (SEQ ID NO: 22) 5' CTC CCG GAT CCT CAC ACT GGA AAC AAT CC 3'. Truncation 1 uses M690 of  
20 Xa21 as a start codon and V1017 of Xa21 (see SEQ ID NO:18 for amino acid references) as last codon. Primer FR-Xa21-369 introduces an Rca I site at the ATG of M690 and primer FR-Xa21-370 introduces a stopcodon and a BamH I site directly behind V1017. Using PfuTurbo™ DNA Polymerase (Stratagene) and the primers FR-Xa21-369 and FR-Xa21-370 truncation 1 was amplified from pMOG1468. Amplification was started by melting for 1' at  
25 95°C, followed by 25 cycli for 1' at 95°C, 1' at 60°C and 1'30'' at 72°C and ended with an extension period at 72°C for 10'. PCR yielded the expected 1015 bp fragment. This fragment was cloned between the 35S promoter/gluII leader (SEQ ID NO:17) and the 3' untranslated region of the potato proteinase inhibitor II gene. The resulting plasmid was named pMOG1470.

30

For cloning of truncation 2 three primers were developed: FR-PR1a-371 (SEQ ID NO: 23) 5' CAA TTA TCA TGA GAT TTG TTC TCT TTT C 3', FR-Xa21-372 (SEQ ID NO: 24) 5'



GGC AGA TGT AGA TCG GCA CGG CAA GAG TG 3' and FR-Xa21-373 (SEQ ID NO: 25) 5' CAC TCT TGC CGT GCC GAT CTA CAT CTG CC 3'. In this truncation part of the Xa21 CDS (D635- V1017) is fused to the C-terminus of the PR-1a signal peptide (SEQ ID NO:26)(Cornelissen et al., 1987, Nucleic Acid Research 15: 6799-6811). The fusion is

5 established by overlap extension as described.

Primer FR-PR1a-371 introduces a Rca I site on the ATG of M1 of the PR-1a signal sequence. As a consequence the second amino acid changes from G to R. Primer FR-Xa21-372 has homology with the last 5 amino acids of the signal peptide PR-1a and with the first 4 2/3 amino acids of Xa21. Using PfuTurbo™ DNA Polymerase (Stratagene) and the primers FR-

10 PR1a-371 and FR-Xa21-372 the tobacco PR-1a signal peptide was amplified from tobacco genomic DNA.

Amplification was started by melting for 1' at 95°C, followed by 35 cycli for 1' at 95°C, 1' at 50°C and 20'' at 72°C and ended with an extension period at 72°C for 10'. The expected 114 bp PCR-product was cut from a 2% agarose gel and DNA was extracted with QIAGEN

15 Gelextraction Kit.

Primer FR-Xa21-370 introduces a stopcodon and a BamH I site directly behind V1017 of Xa21 and primer FR-Xa21-373 is homologous to the sequence encoding the last 5 amino acids of the signal peptide PR-1a and to the first 4 2/3 amino acids of Xa21. Using PfuTurbo™ DNA Polymerase (Stratagene) and the primers FR-PR1a-370 and FR-Xa21-373

20 the Xa21 part was amplified from pMOG1468. Amplification was started by melting for 1' at 95°C, followed by 25 cycli for 1' at 95°C, 1' at 40°C and 1'30'' at 72°C and ended with an extension period at 72°C for 10'. The expected 1.2 kb PCR-product was purified with QIAGEN PCR Purification Kit.

The fusion between the signal peptide PR-1a and the Xa21 was accomplished by overlap

25 extension with primers FR-PR1a-371 and FR-Xa21-370. Amplification, with PfuTurbo™ DNA Polymerase, was started by melting for 1' at 95°C, 3 cycli for 1' at 95°C, 1' at 50°C and 1'30'' at 72°C followed by 22 cycli for 1' at 95°C, 1' at 55°C and 1'30'' at 72°C and ended with an extension period at 72°C for 10'. The expected 1265 bp PCR-product was purified with QIAGEN PCR Purification Kit and digested with BamH I and Rca I. This

30 fragment was then cloned between the 35S promoter/gluII leader (SEQ ID NO:17) and the 3' untranslated region of the potato proteinase inhibitor II gene. The resulting plasmid was named pMOG1475.

**Example 9****Transient expression assay for Xa21, and derivatives, function**

- 5 A biolistic system was chosen for gene delivery in monocot tissue (Onion) to study whether Xa21 and truncated versions of Xa21 (Example 8) can elicit a HR in monocot tissue. The assay was carried out essentially as described by Mindrinos et al. (1994, Cell 78: 1089-1099). Figure 1 shows a typical result of such a biolistic experiment. Compared to the GUS control construct (35S-uidA), co-bombardment of the 35S-uidA construct with either pMOG1468,
- 10 pMOG1470 or pMOG1475 resulted in a reduced density of blue spots after GUS staining. Construct pMOG1475 shows the strongest reduction in blue spots, which was in all experiments (at least 3 repeats) less than 10% of the 35S-uidA control.

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## SEQUENCE LISTING

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 aggccaagaa gtgggggtcaa gtaaagccgc taaacaacca gacggtttta cgagcggttt 420  
 20 tgcctaattg atcggctggt ttcagggttg atctcaagac tcaagttaga ttcaagattg 480  
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25 <210> 16  
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 <212> DNA  
 <213> Artificial Sequence

30 <220>  
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 aacccaaatg ctcaatccaa aactttttca ttctgacct cggaaaagac ccaaattcac 180  
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 40 tctactacga cgatgtccac cttaattttt ccaccatcaa cagaccaag atcaattcat 300  
 ctgctcttgt cttagttggt aactacacag tgcctaagtt ctatcaagga cacaagaaga 360  
 aggccaagaa gtgggggtcaa gtaaagccgc taaacaacca gacggtttta cgagcggttt 420  
 tgcctaattg atcggctggt ttcagggttg atctcaagac tcaagttaga ttcaagattg 480  
 ttttttgtaa aactaagagg tatgggggtg aagttggagc tgatgttgaa gtcaacgggtg 540  
 atggagttaa agctcagaag aaaggaatta agatgaagaa atctgattct tcttttccat 600  
 45 taagaagctc ttttccgatt gatgttttga tgaatttact cgtattcttt gctattcgtt 660  
 aaggatcc 668

50 <210> 17  
 <211> 907  
 <212> DNA  
 <213> Artificial Sequence

55 <220>  
 <223> Description of Artificial Sequence:Combination of  
 35S Promoter with the Glucanase II leader

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 agctatctgt cactttattg tgaagatagt ggaaaaggaa ggtggctcct acaaagcca 180  
 tcattgcgat aaaggaaagg ccacgttga agatgcctct gccgacagtg gtcccaaaga 240  
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 gaactcggcg taaagactgg cgaacagttc atacagagtc tcttactgact caatgacaag 420  
 65 aagaaaatct tegtcaacat ggtggagcac gacacacttg tctactcaa aatatcaaa 480  
 gatacagtct cagaagacca aagggtcaat gagacttttc acaaagggt aatatccgga 540  
 aacctctcgt gattccattg cccagctatc tgtcacttta ttgtgaagat agtggaaaag 600  
 gaagggtggt cctacaaatg ccatcattgc gataaaggaa aggccatcgt tgaagatgcc 660  
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 70 gacgttcaa ccacgtcttc aaagcaagtg gattgatgtg atatctccac tgacgtaagg 780  
 gatgagcac aatcccacta tctctcga gacccttct ctatataagg aagttcattt 840  
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5

gccatgg

907

<210> 18  
 <211> 1026  
 5 <212> PRT  
 <213> Oryza sativa

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 1 5 10 15  
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 20 25 30  
 15 Glu Leu Ala Leu Leu Ser Phe Lys Ser Ser Leu Leu Tyr Gln Gly Gly  
 35 40 45  
 Gln Ser Leu Ala Ser Trp Asn Thr Ser Gly His Gly Gln His Cys Thr  
 20 50 55 60  
 Trp Val Gly Val Val Cys Gly Arg Arg Arg Arg Arg His Pro His Arg  
 65 70 75 80  
 25 Val Val Lys Leu Leu Leu Arg Ser Ser Asn Leu Ser Gly Ile Ile Ser  
 85 90 95  
 Pro Ser Leu Gly Asn Leu Ser Phe Leu Arg Glu Leu Asp Leu Gly Asp  
 100 105 110  
 30 Asn Tyr Leu Ser Gly Glu Ile Pro Pro Glu Leu Ser Arg Leu Ser Arg  
 115 120 125  
 Leu Gln Leu Leu Glu Leu Ser Asp Asn Ser Ile Gln Gly Ser Ile Pro  
 130 135 140  
 35 Ala Ala Ile Gly Ala Cys Thr Lys Leu Thr Ser Leu Asp Leu Ser His  
 145 150 155 160  
 40 Asn Gln Leu Arg Gly Met Ile Pro Arg Glu Ile Gly Ala Ser Leu Lys  
 165 170 175  
 His Leu Ser Asn Leu Tyr Leu Tyr Lys Asn Gly Leu Ser Gly Glu Ile  
 180 185 190  
 45 Pro Ser Ala Leu Gly Asn Leu Thr Ser Leu Gln Glu Phe Asp Leu Ser  
 195 200 205  
 Phe Asn Arg Leu Ser Gly Ala Ile Pro Ser Ser Leu Gly Gln Leu Ser  
 210 215 220  
 50 Ser Leu Leu Thr Met Asn Leu Gly Gln Asn Asn Leu Ser Gly Met Ile  
 225 230 235 240  
 Pro Asn Ser Ile Trp Asn Leu Ser Ser Leu Arg Ala Phe Ser Val Arg  
 245 250 255  
 Glu Asn Lys Leu Gly Gly Met Ile Pro Thr Asn Ala Phe Lys Thr Leu  
 260 265 270  
 60 His Leu Leu Glu Val Ile Asp Met Gly Thr Asn Arg Phe His Gly Lys  
 275 280 285  
 Ile Pro Ala Ser Val Ala Asn Ala Ser His Leu Thr Val Ile Gln Ile  
 290 295 300  
 65 Tyr Gly Asn Leu Phe Ser Gly Ile Ile Thr Ser Gly Phe Gly Arg Leu  
 305 310 315 320  
 Arg Asn Leu Thr Glu Leu Tyr Leu Trp Arg Asn Leu Phe Gln Thr Arg  
 325 330 335  
 70 Glu Gln Asp Asp Trp Gly Phe Ile Ser Asp Leu Thr Asn Cys Ser Lys

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5	Leu	Gln	Thr	Leu	Asn	Leu	Gly	Glu	Asn	Asn	Leu	Gly	Gly	Val	Leu	Pro
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	Asn	Ser	Phe	Ser	Asn	Leu	Ser	Thr	Ser	Leu	Ser	Phe	Leu	Ala	Leu	Glu
		370					375					380				
10	Leu	Asn	Lys	Ile	Thr	Gly	Ser	Ile	Pro	Lys	Asp	Ile	Gly	Asn	Leu	Ile
	385					390					395					400
	Gly	Leu	Gln	His	Leu	Tyr	Leu	Cys	Asn	Asn	Asn	Phe	Arg	Gly	Ser	Leu
					405					410					415	
15	Pro	Ser	Ser	Leu	Gly	Arg	Leu	Lys	Asn	Leu	Gly	Ile	Leu	Leu	Ala	Tyr
				420					425					430		
	Glu	Asn	Asn	Leu	Ser	Gly	Ser	Ile	Pro	Leu	Ala	Ile	Gly	Asn	Leu	Thr
20			435					440					445			
	Glu	Leu	Asn	Ile	Leu	Leu	Leu	Gly	Thr	Asn	Lys	Phe	Ser	Gly	Trp	Ile
		450					455					460				
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	465					470					475					480
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					485					490					495	
30	Thr	Leu	Ser	Ile	Met	Ile	Asn	Val	Ser	Lys	Asn	Asn	Leu	Glu	Gly	Ser
				500					505					510		
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35			515					520					525			
	Glu	Ser	Asn	Arg	Leu	Ser	Gly	Lys	Ile	Pro	Asn	Thr	Leu	Gly	Asp	Cys
		530					535					540				
40	Gln	Leu	Leu	Arg	Tyr	Leu	Tyr	Leu	Gln	Asn	Asn	Leu	Leu	Ser	Gly	Ser
	545					550					555					560
	Ile	Pro	Ser	Ala	Leu	Gly	Gln	Leu	Lys	Gly	Leu	Glu	Thr	Leu	Asp	Leu
					565					570					575	
45	Ser	Ser	Asn	Asn	Leu	Ser	Gly	Gln	Ile	Pro	Thr	Ser	Leu	Ala	Asp	Ile
				580					585					590		
	Thr	Met	Leu	His	Ser	Leu	Asn	Leu	Ser	Phe	Asn	Ser	Phe	Val	Gly	Glu
50			595					600					605			
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	625					630					635					640
	Cys	Cys	Pro	Leu	Leu	Glu	Asn	Arg	Lys	His	Phe	Pro	Val	Leu	Pro	Ile
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				660					665					670		
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65			675					680					685			
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		690					695					700				
70	Thr	Asp	Gly	Phe	Ala	Pro	Thr	Asn	Leu	Leu	Gly	Ser	Gly	Ser	Phe	Gly
	705					710					715					720
	Ser	Val	Tyr	Lys	Gly	Lys	Leu	Asn	Ile	Gln	Asp	His	Val	Ala	Val	Lys



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	725	730	735
5	Val Leu Lys Leu Glu Asn Pro Lys Ala Leu Lys Ser Phe Thr Ala Glu 740 745 750		
	Cys Glu Ala Leu Arg Asn Met Arg His Arg Asn Leu Val Lys Ile Val 755 760 765		
10	Thr Ile Cys Ser Ser Ile Asp Asn Arg Gly Asn Asp Phe Lys Ala Ile 770 775 780		
	Val Tyr Asp Phe Met Pro Asn Gly Ser Leu Glu Asp Trp Ile His Pro 785 790 795 800		
15	Glu Thr Asn Asp Gln Ala Asp Gln Arg His Leu Asn Leu His Arg Arg 805 810 815		
	Val Thr Ile Leu Leu Asp Val Ala Cys Ala Leu Asp Tyr Leu His Arg 820 825 830		
20	His Gly Pro Glu Pro Val Val His Cys Asp Ile Lys Ser Ser Asn Val 835 840 845		
	Leu Leu Asp Ser Asp Met Val Ala His Val Gly Asp Phe Gly Leu Ala 850 855 860		
	Arg Ile Leu Val Asp Gly Thr Ser Leu Ile Gln Gln Ser Thr Ser Ser 865 870 875 880		
30	Met Gly Phe Ile Gly Thr Ile Gly Tyr Ala Ala Pro Glu Tyr Gly Val 885 890 895		
	Gly Leu Ile Ala Ser Thr His Gly Asp Ile Tyr Ser Tyr Gly Ile Leu 900 905 910		
35	Val Leu Glu Ile Val Thr Gly Lys Arg Pro Thr Asp Ser Thr Phe Arg 915 920 925		
	Pro Asp Leu Gly Leu Arg Gln Tyr Val Glu Leu Gly Leu His Gly Arg 930 935 940		
	Val Thr Asp Val Val Asp Thr Lys Leu Ile Leu Asp Ser Glu Asn Trp 945 950 955 960		
45	Leu Asn Ser Thr Asn Asn Ser Pro Cys Arg Arg Ile Thr Glu Cys Ile 965 970 975		
	Val Trp Leu Leu Arg Leu Gly Leu Ser Cys Ser Gln Glu Leu Pro Ser 980 985 990		
50	Ser Arg Thr Pro Thr Gly Asp Ile Ile Asp Glu Leu Asn Ala Ile Lys 995 1000 1005		
	Gln Asn Leu Ser Gly Leu Phe Pro Val Cys Glu Gly Gly Ser Leu Glu 1010 1015 1020		
	Phe Glx 1025		
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65	<220> <223> Description of Artificial Sequence:Primer		
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5 <220>  
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10 <210> 21  
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15 <220>  
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<210> 22  
 <211> 29  
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30 <400> 22  
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<210> 23  
 <211> 28

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<210> 24  
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45 <213> Artificial Sequence

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 <223> Description of Artificial Sequence:Primer

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55 <210> 25  
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 <223> Description of Artificial Sequence:Primer

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65 <210> 26  
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 <212> PRT  
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70 <220>  
 <223> Description of Artificial Sequence:PR-1a signal

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peptide from *Nicotiana tabacum*

&lt;400&gt; 26

5 Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu Leu Val Ser  
 1 5 10 15

Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg Ala  
 20 25 30

10

&lt;210&gt; 27

&lt;211&gt; 2704

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence: pUC 28 high copy cloning vector

15

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Polynucleotide

&lt;400&gt; 27

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&lt;210&gt; 28

&lt;211&gt; 3078

&lt;212&gt; DNA

70 <213> *Oryza sativa*

&lt;400&gt; 28

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&lt;210&gt; 29

55 &lt;211&gt; 90

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

60 &lt;223&gt; Description of Artificial Sequence:PR 1a signal peptide from Nicotiana tabacum

&lt;400&gt; 29

65 atgggatttg ttctcttttc acaattgcct tcatttcttc ttgtctctac acttctctta 60  
ttcctagtaa tatcccactc ttgccgtgcc 90

## AMENDED CLAIMS

1. Method for the induction of pathogen resistance in plants, comprising:
  - (a) providing a polynucleotide sequence encoding a constitutively active mutated plant signal transduction protein or a constitutively active homologue thereof;
  - (b) producing a chimeric DNA sequence by coupling said polynucleotide sequence to a pathogen-inducible promoter in a functional manner;
  - (c) transforming a plant with said chimeric DNA sequence,wherein expression of said constitutively active mutated plant signal transduction protein or constitutively active homologue thereof gives rise to a hypersensitive response.
2. Method according to claim 1, characterized in that the signal transduction protein is encoded on the *acd* locus or the *lsd* locus.
3. Method according to claim 1, characterized in that the signal transducing protein is *ndr1* or a homologue thereof.
4. Method according to claim 1, characterized in that the signal transducing protein is *eds1* or a homologue thereof.
5. Method according to claim 1, characterized in that the signal transducing protein is *Xa21* or a homologue thereof.
6. Method according to claim 1, characterized in that the signal transduction protein is selected from the group consisting of a G-protein, a protein kinase, an AMP-cyclase and a protein phosphatase.
7. Method for the induction of pathogen resistance in plants characterized by transforming a plant with a polynucleotide sequence comprising a pathogen inducible promoter which regulates the expression of a compound which is able

to alleviate the inhibitory effect of a protein on the signal transduction pathway leading to a hypersensitive response in plants.

8. Method according to claim 7, characterized in that the compound is an mRNA which is coding for the inhibitory protein in an anti-sense orientation.
9. Method according to claim 7, characterized in that the compound is interacting sterically with the inhibitory protein.
10. Method according to claim 9, characterized in that the compound is an antibody.
11. Method according to claim 7, characterized in that the compound is a ribozyme.
12. Method according to claim 7, characterized in that the compound is an RNA molecule which is able to suppress translation of the mRNA coding for the inhibitory protein.
13. Method according to any of claims 1-12 wherein the transformed plant is at least partially resistant to pathogens.
14. A polynucleotide comprising a pathogen-inducible promoter sequence operably linked to a protein encoding sequence which encodes a constitutively active mutated plant signal transduction protein which is active in the plant-signal transduction pathway of a plants hypersensitive response.
15. A polynucleotide according to claim 14, characterised in that the mutated plant signal transduction protein is selected from the group comprising of mutants of *ndr1*, *eds1* and *Xa21*.
16. A polynucleotide according to claim 15, characterised in that the mutant from the signal transduction protein is selected from the group of *ndr1*-PKC, *ndr1*-cDPK and truncated *Xa21*.

17. A polynucleotide according to claim 14, 15 or 16, characterised in that the pathogen inducible promoter is selected from the group comprising of the promoters of *prp1*, *Fis1*, *Ber v 1*, *Vst1*, *gstA1*, and sesquiterpene cyclase.
18. A polynucleotide according to claim 14, characterised in that the signal transduction protein is selected from the group consisting of a G-protein, a protein kinase, an AMP-cyclase and a protein phosphatase.
19. A polynucleotide according to claim 14, characterised in that the mutant protein is encoded on the *acd* locus or the *lsd* locus.
20. A polynucleotide comprising a pathogen inducible promoter sequence operably linked to a sequence which is able to alleviate the inhibitory effect of a protein which is active in the signal transduction pathway of a plants hypersensitive response.
21. A polynucleotide comprising a pathogen inducible promoter sequence operably linked to a protein encoding sequence which is an antibody binding to an inhibitory protein which is active in the signal transduction pathway of a plants hypersensitive response.

#2322382

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA  
PRÉPARATION DES DOSSIERS)