(54) Titre : PROCEDE DE GENERATION D'UNE RESISTANCE A DES AGENTS PATHOGENES DANS DES PLANTES

(54) Title: METHOD FOR OBTAINING THE PATHOGENIC RESISTANCE IN PLANTS

(57) Abrégé/Abstract:
The invention relates to a method for obtaining or increasing the pathogenic resistance in plants by reducing the expression, activity or the functioning of a NADPH oxidase.
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(57) Zusammenfassung: Die Erfindung betrifft Verfahren zur Erzeugung oder Erhöhung einer Pathogenresistenz in Pflanzen durch Verminderung der Expression, Aktivität oder Funktion einer NADPH Oxidase.
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METHOD FOR OBTAINING THE PATHOGENIC RESISTANCE IN PLANTS

The invention relates to methods for generating or increasing a pathogen resistance in plants by reducing the expression, activity or function of an NADPH oxidase.

The aim of plant biotechnology work is the generation of plants with advantageous novel properties, for example for increasing agricultural productivity. The plants' natural defense mechanisms against pathogens are frequently insufficient. Fungal diseases alone result in annual yield losses of many billions of US$. The introduction of foreign genes from plants, animals or microbial sources can increase the defenses. Examples are the protection against feeding damage by insects by expressing Bacillus thuringiensis endotoxins (Vaeeck et al. (1987) Nature 328:33-37) or the protection against fungal infection by expressing a bean chitinase (Brogle et al. (1991) Science 254:1194-1197). However, most of the approaches described only offer resistance to a single pathogen or a narrow spectrum of pathogens.

Only a few approaches exist which impart a resistance to a broader spectrum of pathogens to plants. Systemic acquired resistance (SAR) - a defense mechanism in a variety of plant/pathogen interactions - can be conferred by the application of endogenous messenger substances such as jasmonic acid (JA) or salicylic acid (SA) (Ward, et al. (1991) Plant Cell 3:1085-1094; Uknese, et al. (1992) Plant Cell 4(6):645-656). Similar effects can also be achieved by synthetic compounds such as 2,6-dichloroisonicotinic acid (INA) or S-methyl benzo(1,2,3)thiadiazole-7-thiocarboxylate (BTH; Bion®) (Friedrich et al. (1996) Plant J 10(1):61-70; Lawton et al. (1996) Plant J. 10:71-82). The expression of pathogenesis-related (PR) proteins, which are upregulated in the case of SAR, may also cause pathogen resistance in some cases.

In barley, the Mlo locus has been described as a negative regulator of the defense against pathogens. The loss of the Mlo gene causes an increased and, above all, race-unspecific resistance against a large number of mildew species (Büsches R et al. (1997) Cell 88:695-705; Jorgensen JH (1977) Euphytica 26:55-62; Lyngkjaer MF et al. (1995) Plant Pathol 44:786-790). Mlo-deficient barley varieties obtained by conventional breeding are already being used in agriculture. Despite intensive cultivation, the resistance has proved to be durable, presumably due to the fact that it is recessive. Mlo-like resistances in other plants,
in particular in cereal species, are not described. The Mlo gene and various homologs from other cereal species have been identified and cloned (Buschges R et al. (1997) Cell 88:695-705; WO 98/04586; Schulze-Lefert P, Vogel J (2000) Trends Plant Sci. 5:343-348). Various methods using these genes for obtaining a pathogen resistance are described (WO 98/04586; WO 00/01722; WO 99/47552). The disadvantage is that the Mlo-mediated defense mechanism comprises a spontaneous die-off of leaf cells (Wolter M et al. (1993) Mol Gen Genet 239:122-128). Another disadvantage is that the Mlo-deficient genotypes show hypersensitivity to hemibiotrophic pathogens such as Magnaporthe grisea (M. grisea) and Cochliobolus sativus (Bipolaris sorokiniana) (Jarosch B et al. (1999) Mol Plant Microbe Interact 12:508-514; Kumar J et al. (2001) Phytopathology 91:127-133).

The liberation of reactive oxygen species (ROS; for example superoxide (O_2^-), hydroxyl radicals and H_2O_2) is ascribed an important protection function in the reaction on plant pathogens (Wojtaszek P (1997) Biochem J 322:681-692). A variety of ways of how a cell can produce ROS are known. In the macrophages of mammals, it is in particular the enzyme NADPH oxidase, which is able to transfer electrons to molecular oxygen, which must be mentioned. Homologous enzymes have also been identified in plants (Lamb & Dixon (1997) Annu Rev Plant Physiol Plant Mol Biol 48:251).

It has been shown that mutations in the catalytic subunit of NADPH oxidase in Arabidopsis thaliana show a reduced accumulation of reactive oxygen intermediates (ROI). With regard to the hypersensitive reaction (HR), the results were heterogeneous: while infection with the avirulent and bacterium Pseudomonas syringae showed a reduced HR in a double mutant, the virulent oomycete Peronospora parasitica showed an increased HR. Growth - both of virulent and of avirulent P. syringae strains - was not changed in comparison with wild-type plants, however (Torres MA et al. (2002) Proc Natl Acad Sci USA 99:517-522). Likewise, the inhibition of NADPH oxidase by means of the inhibitor diphenyleneiodonium chloride (DPI) - at physiologically relevant concentrations - had no effect on the development of pathogenic fungi (Huckelhoven R & Kogel KH (1998) Mol Plant Microbe Interact 11:292-300). A cDNA fragment of a phagocytic barley NADPH oxidase (pNAox, homolog of the large subunit gp91phox of a phagocytic NADPH oxidase) is described under the GenBank Acc.-No.: AJ251717).

The present invention aims at providing novel compounds for the defense against pathogens in plants, which compounds bring about an efficient defense against as broad as possible a pathogen spectrum in as many different plant species as possible, prefer-
ably the crop plants used in agriculture. We have found that this object is achieved by the present method.

A first aspect of the invention comprises a method for generating or increasing the resistance to at least one pathogen in plants, which comprises the following operating steps:

a) reduction of the protein quantity, activity or function of an NADPH oxidase in a plant or a tissue, organ, part or cell thereof, and

b) selection of the plants in which — in contrast or in comparison with the starting plant — the resistance to at least one pathogen exists or is increased.

Surprisingly, the reduction of the expression of a barley NADPH oxidase (pNAox) in the epidermal cell by a sequence-specific RNA interference approach using double-stranded pNAox-dsRNA ("gene silencing") shows a significantly reduced disease level following Bgh infection (measured with reference to the formulation of Haustoria). This finding is particularly surprising because the release of reactive oxygen species ("oxidative burst"), which is associated with NADPH oxidase, is generally ascribed a protective function.

Similar to Mlo, the reduction of the NADPH oxidase expression mediates a broad resistance to various isolates of Blumeria graminis f.sp. hordei. In transient gene silencing experiments, the penetration efficiency (development of Haustoria) of Bgh is reduced significantly by more than 35% — an effect which, in its intensity, corresponds to the effect obtained by means of Mlo-dsRNA (Schweizer P et al. (2000) Plant J 24:895-903). In the wild-type barley variety Pallas, approximately 40% of the fungal penetrations result in the development of haustoria, while the penetration rate in the case of reduced NADPH oxidase expression by introduction of a double-stranded RNA of NADPH oxidase (pNAox-dsRNA) only amounts to approximately 25%. The fact that even in pathogen-sensitive wild-type varieties such as Pallas only a penetration rate of approximately 40 to 50% can be observed can be attributed to the basal resistance, which is always present. Owing to these findings, the enzyme NADPH oxidase can be considered a key element for the successful penetration of a pathogen such as Bgh into the plant cell. In addition, the method is superior to all those methods where a pathogen-resistant phenotype is generated by overexpression of a resistance-mediating protein. Switching off a gene can be done without expression of a (foreign) protein. In the ideal case, only the endogenous gene is de-
activated. This has not inconsiderable advantages regarding approval and acceptance by the consumer, who is frequently unsure about plants with foreign proteins. Very especially advantageous in this context is the use of inducible promoters for reducing the NADPH oxidase quantity, activity or function, which, for example in the case of pathogen-inducible promoters, makes possible an expression only when required (i.e. attack by pathogens).

In principle, the method according to the invention can be applied to all plant species, preferably to those in which an NADPH oxidase or a functional equivalent thereof is expressed naturally.

For the purposes of the invention, "plant" means all genera and species of higher and lower plants of the Plant Kingdom. Included in this expression are the mature plants, seed, shoots and seedlings, and parts, propagation material, plant organs, tissues, protoplasts, callus and other cultures, for example cell cultures, derived from them, and all other types of groups of plant cells which give functional or structural units. Mature plants refers to plants at any developmental stage beyond that of the seedling. Seedling means a young, immature plant in an early developmental stage. "Plant" comprises all annual and perennial monocotyledonous and dicotyledonous plants and includes by way of example, but not by limitation, those of the genera Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solarium, Petunia, Digitalis, Majoreana, Cicoria, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaelia, Glycine, Pisum, Phaseolus, Lolium, Oryza, Zea, Avena, Hordeum, Secale, Triticum, Sorghum, Picea and Populus.

The term "plant" preferably comprises monocotyledonous crop plants, such as, for example, cereal species such as wheat, barley, millet, rye, triticale, maize, rice, sorghum or oats, and sugar cane.

The term furthermore comprises dicotyledonous crop plants such as, for example

- Brassicaceae such as oilseed rape, canola, cress, Arabidopsis, cabbages or canola, Leguminosae such as soybean, alfalfa, pea, beans or peanut
5 - Solanaceae such as potato, tobacco, tomato, egg plant or paprika, Asteraceae such as sunflower, Tagetes, lettuce or Calendula,

5 - Cucurbitaceae such as melon, pumpkin/squash or zucchini,

and linseed, cotton, hemp, clover, spinach, flax, red pepper, carrot, beet, radish, sugar beet, sweet potato, cucumber, chicory, cauliflower, broccoli, asparagus, onion, garlic, celeriac, strawberry, raspberry, blackberry, pineapple, avocado, and the various tree, bush, nut and vine species. Tree species preferably comprises plum, cherry, peach, nectarine, apricot, banana, paw paw, mango, apple, pear, quince.

Furthermore comprised are ornamental plants, useful or ornamental trees, flowers, cut flowers, shrubs or lawn, by way of example but not by way of limitation, the families of the Rosaceae such as rose, Ericaceae such as rhododendrons and azaleas, Euphorbiaceae such as poinsettias and croton, Caryophyllaceae such as carnations, Solanaceae such as petunias, Gesneriaceae such as African violets, Balsaminaceae such as touch-me-not, Orchidaceae such as orchids, Iridaceae such as gladioli, iris, freesia and crocus, Compositae such as calendula, Geraniaceae such as geraniums, Liliaceae such as dracaena, Moraceae such as ficus, Araceae such as philodendron, and many others.

Preferred for the purposes of the invention are those plants which are employed as food or feedstuff, very especially preferably monocotyledonous genera and species, such as the above-described cereal species.

The method is very especially preferably applied to monocotyledonous plants, most preferably to plants with agricultural importance such as wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed or sugar cane.

"Pathogen resistance" denotes the reduction or weakening of disease symptoms of a plant following infection by a pathogen. The symptoms can be manifold, but preferably comprise those which directly or indirectly have an adverse effect on the quality of the plant, the quantity of the yield, the suitability for use as feedstuff or foodstuff, or else which make sowing, planting, harvesting or processing of the crop difficult.

45 "Conferring", "existing", "generating" or "increasing" a pathogen resistance means that the defense mechanisms of a specific plant species or variety is increasingly resistant to one or more
pathogens due to the use of the method according to the invention in comparison with the wild type of the plant ("original plant"), to which the method according to the invention has not been applied, under otherwise identical conditions (such as, for example, climatic conditions, growing conditions, pathogen species and the like). The increased resistance manifests itself preferably in a reduced manifestation of the disease symptoms, disease symptoms comprising - in addition to the abovementioned adverse effects - for example also the penetration efficiency of a pathogen into the plant or plant cells or the proliferation efficiency in or on the same. In this context, the disease symptoms are preferably reduced by at least 10% or at least 20%, especially preferably by at least 40% or 60%, very especially preferably by at least 70% or 80% and most preferably by at least 90% or 95%.

"Selection" with regard to plants in which - as opposed or as compared to the original plant - resistance to at least one pathogen exists or is increased means all those methods which are suitable for recognizing an existing or increased resistance to pathogens. These may be symptoms of pathogen infection (for example the development of haustoria in the case of fungal infection), but may also comprise the above-described symptoms which relate to the quality of the plant, the quantity of the yield, the suitability for use as feedstuff or foodstuff and the like.

"Pathogen" within the scope of the invention means by way of example but not by limitation viruses or viroids, bacteria, fungi, animal pests such as, for example, insects or nematodes. Especially preferred are fungi, such as mildew. However, it can be assumed that the expression of an NADPH oxidase, its activity or its function also brings about resistance to other pathogens. The following pathogens may be mentioned by way of example but not by limitation:

1. Fungal pathogens and fungus-like pathogens:

Fungal pathogens and fungus-like pathogens (such as, for example, Chromista) are preferably from the group comprising Plasmodiophoramyctia, Oomycota, Ascomycota, Chytridiomycetes, Zygomycetes, Basidiomycota and Deuteromycetes (Fungi imperfecti). The pathogens mentioned in Tables 1 and 2 and the diseases with which they are associated may be mentioned by way of example but not by limitation.
Table 1: Fungal plant diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf rust</td>
<td>Puccinia recondita</td>
</tr>
<tr>
<td>Yellow rust</td>
<td>P. striiformis</td>
</tr>
<tr>
<td>Powdery mildew</td>
<td>Erysiphe graminis / Blumeria graminis</td>
</tr>
<tr>
<td>Glume blotch</td>
<td>Septoria nodorum</td>
</tr>
<tr>
<td>Leaf blotch</td>
<td>Septoria tritici</td>
</tr>
<tr>
<td>Ear fusarioses</td>
<td>Fusarium spp.</td>
</tr>
<tr>
<td>Eyespot</td>
<td>Pseudocercospora herpotrichoides</td>
</tr>
<tr>
<td>Smut</td>
<td>Ustilago spp.</td>
</tr>
<tr>
<td>Bunt</td>
<td>Tilletia caries</td>
</tr>
<tr>
<td>Take-all</td>
<td>Gaemannomyces graminis</td>
</tr>
<tr>
<td>Anthrocnose leaf blight</td>
<td>Colletotrichum graminicola (teleomorph: Glomerella graminicola Politis); Glomerella tucumanensis (anamorph: Glomerella falcatum Went)</td>
</tr>
<tr>
<td>Anthracnose stalk rot</td>
<td></td>
</tr>
<tr>
<td>Aspergillus ear and kernel rot</td>
<td>Aspergillus flavus</td>
</tr>
<tr>
<td>Banded leaf and sheath spot</td>
<td>Rhizoctonia solani Kuhn = Rhizoctonia microscerotia J. Matz (teleomorph: Thanatephorus cucumeris)</td>
</tr>
<tr>
<td>Black bundle disease</td>
<td>Acremonium strictum W. Gams = Cephalosporium acremonium Auct. non Corda</td>
</tr>
<tr>
<td>Black kernel rot</td>
<td>Lasiodiplodia theobromae = Botryodiplodia theobromae</td>
</tr>
<tr>
<td>Borde blanco</td>
<td>Marasmiellus sp.</td>
</tr>
<tr>
<td>Brown spot (black spot, stalk rot)</td>
<td>Physoderma maydis</td>
</tr>
<tr>
<td>Cephalosporium kernel rot</td>
<td>Acremonium strictum = Cephalosporium acremonium</td>
</tr>
<tr>
<td>Charcoal rot</td>
<td>Macrophomina phaseolina</td>
</tr>
<tr>
<td>Corticium ear rot</td>
<td>Thanatephorus cucumeris = Corticium sasakii</td>
</tr>
<tr>
<td>Curvularia leaf spot</td>
<td>Curvularia clavata, C. eragrostidis = C. maculans (teleomorph: Cochliobolus eragrostidis), Curvularia inaequalis, C. intermedia (teleomorph: Cochliobolus intermedius), Curvularia lunata (teleomorph: Cochliobolus lunatus), Curvularia pallescens (teleomorph: Cochliobolus pallescens), Curvularia senegalensis, C. tuberculata (teleomorph: Cochliobolus tuberculatus)</td>
</tr>
<tr>
<td>Didymella leaf spot</td>
<td>Didymella exitalis</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Diplodia ear rot and stalk rot</td>
<td>Diplodia frumenti (teleomorph: Botryosphaeria festucae)</td>
</tr>
<tr>
<td>Diplodia ear rot, stalk rot, seed rot and seedling blight</td>
<td>Diplodia maydis = Stenocarpella maydis</td>
</tr>
<tr>
<td>Diplodia leaf spot or streak</td>
<td>Stenocarpella macrospora = Diplodia leaf macrospora</td>
</tr>
</tbody>
</table>

Table 2: Downy mildew

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown stripe downy mildew</td>
<td>Sclerophthora rayssiae var. zeae</td>
</tr>
<tr>
<td>Crazy top downy mildew</td>
<td>Sclerophthora macrospora = Sclerospora macrospora</td>
</tr>
<tr>
<td>Green ear downy mildew (graminicola downy mildew)</td>
<td>Sclerospora graminicola</td>
</tr>
<tr>
<td>Java downy mildew</td>
<td>Peronosclerospora maydis = Sclerospora maydis</td>
</tr>
<tr>
<td>Philippine downy mildew</td>
<td>Peronosclerospora philippinensis = Sclerospora philippinensis</td>
</tr>
<tr>
<td>Sorghum downy mildew</td>
<td>Peronosclerospora sorghi = Sclerospora sorghi</td>
</tr>
<tr>
<td>Spontaneum downy mildew</td>
<td>Peronosclerospora spontanea = Sclerospora spontanea</td>
</tr>
<tr>
<td>Sugarcane downy mildew</td>
<td>Peronosclerospora sacchari = Sclerospora sacchari</td>
</tr>
<tr>
<td>Dry ear rot (cob, kernel and stalk rot)</td>
<td>Nigrospora oryzae (teleomorph: Khuskia oryzae)</td>
</tr>
<tr>
<td>Ear rots, minor</td>
<td>Alternaria alternata = A. tenuis, Aspergillus glaucus, A. niger, Aspergillus spp., Botrytis cinerea (teleomorph: Botryotinia fuckeliana), Cunninghamella sp., Curvularia pallescens, Doratomyces stemonitis = Cephalotrichum stemonitis, Fusarium culmorum, Gonatabotrys simplex, Pithomyces maydicus, Rhizopus microsporus Tiegh., R. stolonifer = R. nigricans, Scopulariopsis brumptii</td>
</tr>
<tr>
<td>Ergot (horse’s tooth)</td>
<td>Claviceps gigantea (anamorph: Sphaecelia sp.)</td>
</tr>
<tr>
<td>Eyespot</td>
<td>Aureobasidium zeae = Kabatiella zeae</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Fusarium ear and stalk rot</td>
<td>Fusarium subglutinans = P. moniliforme var.subglutinans</td>
</tr>
<tr>
<td>Fusarium kernel, root and stalk rot, seed rot and seedling blight</td>
<td>Fusarium moniliforme (teleomorph: Gibberella fujikuroi)</td>
</tr>
<tr>
<td>Fusarium stalk rot, seedling root rot</td>
<td>Fusarium avenaceum (teleomorph: Gibberella avenacea)</td>
</tr>
<tr>
<td>Gibberella ear and stalk rot</td>
<td>Gibberella zeae (anamorph: Fusarium graminearum)</td>
</tr>
<tr>
<td>Gray ear rot</td>
<td>Botryosphaeria zeae = Physalospora zeae (anamorph: Macrophoma zeae)</td>
</tr>
<tr>
<td>Gray leaf spot (Cercospora leaf spot)</td>
<td>Cercospora sorghi = C. sorghi var. maydis, C. zeae-maydis</td>
</tr>
<tr>
<td>Helminthosporium root rot</td>
<td>Exserohilum pedicellatum = Helminthosporium pedicellatum (teleomorph: Setosphaeria pedicellata)</td>
</tr>
<tr>
<td>Hormodendrum ear rot (Cladosporium rot)</td>
<td>Cladosporium cladosporioides = Hormodendrum cladosporioides, C. herbarum (teleomorph: Mycosphaerella tassiana)</td>
</tr>
<tr>
<td>Hyalothyridium leaf spot</td>
<td>Hyalothyridium maydis</td>
</tr>
<tr>
<td>Late wilt</td>
<td>Cephalosporium maydis</td>
</tr>
<tr>
<td>Northern corn leaf blight (white blast, crown stalk rot, stripe)</td>
<td>Setosphaeria turcica (anamorph: Exserohilum turcicum = Helminthosporium turcicum)</td>
</tr>
<tr>
<td>Northern corn leaf spot Helminthosporium ear rot (race 1)</td>
<td>Cochliobolus carbonum (anamorph: Bipolaris zeicola = Helminthosporium carbonum)</td>
</tr>
<tr>
<td>Penicillium ear rot (blue eye, blue mold)</td>
<td>Penicillium spp., P. chrysogenum, P. expansum, P. oxalicum</td>
</tr>
<tr>
<td>Phaeocytostroma stalk rot and root rot</td>
<td>Phaeocytostroma ambiguum, = Phaeocytostromella zeae</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Phaeosphaeria leaf spot</td>
<td>Phaeosphaeria maydis = Sphaerulina maydis</td>
</tr>
<tr>
<td>Physalospora ear rot (Botryosphaeria ear rot)</td>
<td>Botryosphaeria festucae = Physalospora zeicola (anamorph: Diplodia frumenti)</td>
</tr>
<tr>
<td>Purple leaf sheath</td>
<td>Hemiparasitic bacteria and fungi</td>
</tr>
<tr>
<td>Pyrenochaeta stalk rot and root rot</td>
<td>Phoma terrestris = Pyrenochaeta terrestris</td>
</tr>
<tr>
<td>Pythium root rot</td>
<td>Pythium spp., P. arrhenomanes, P. graminicola</td>
</tr>
<tr>
<td>Pythium stalk rot</td>
<td>Pythium aphanidermatum = P. butleri L.</td>
</tr>
<tr>
<td>Red kernel disease (ear mold, leaf and seed rot)</td>
<td>Epicoccum nigrum</td>
</tr>
<tr>
<td>Rhizoctonia ear rot (sclerotial rot)</td>
<td>Rhizoctonia zeae (teleomorph: Waitea circinata)</td>
</tr>
<tr>
<td>Rhizoctonia root rot and stalk rot</td>
<td>Rhizoctonia solani, Rhizoctonia zeae</td>
</tr>
<tr>
<td>Rostratum leaf spot (Helminthosporium leaf disease, ear and stalk rot)</td>
<td>Setosphaeria rostrata, (anamorph: Exserohilum rostratum = Helminthosporium rostratum)</td>
</tr>
<tr>
<td>Rust, common corn</td>
<td>Puccinia sorghi</td>
</tr>
<tr>
<td>Rust, southern corn</td>
<td>Puccinia polysora</td>
</tr>
<tr>
<td>Rust, tropical corn</td>
<td>Physopella pallescens, P. zeae = Angiopsora zeae</td>
</tr>
<tr>
<td>Sclerotium ear rot (southern blight)</td>
<td>Sclerotium rolfsii Sacc. (teleomorph: Athelia rolfsii)</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Selenophoma leaf spot</td>
<td>Selenophoma sp.</td>
</tr>
<tr>
<td>Sheath rot</td>
<td>Gaemannomyces graminis</td>
</tr>
<tr>
<td>Shuck rot</td>
<td>Myrothecium gramineum</td>
</tr>
<tr>
<td>Silage mold</td>
<td>Monascus purpureus, M ruber</td>
</tr>
<tr>
<td>Smut, common</td>
<td>Ustilago zeae = U. maydis</td>
</tr>
<tr>
<td>Smut, false</td>
<td>Ustilaginoidea virens</td>
</tr>
<tr>
<td>Smut, head</td>
<td>Sphacelotheca reiliana = Sporisorium holcisorghi</td>
</tr>
<tr>
<td>Southern corn leaf blight and stalk rot</td>
<td>Cochliobolus heterosporus (anamorph: Bipolaris maydis = Helminthosporium maydis)</td>
</tr>
<tr>
<td>Southern leaf spot</td>
<td>Stenocarpella macrospora = Diplodia macrospora</td>
</tr>
<tr>
<td>Stalk rots, minor</td>
<td>Cercospora sorghi, Fusarium episphaeria, F. merismoides, F. oxysporum Schlechtend, F. poae, F. roseum, F. solani (teleomorph: Nectria haematoecocca), F. tricinctum, Mariannae elegans, Mucor sp., Rhopogonhae zeae, Spicaria sp.</td>
</tr>
<tr>
<td>Storage rots</td>
<td>Aspergillus spp., Penicillium spp. and other fungi</td>
</tr>
<tr>
<td>Tar spot</td>
<td>Phyllachora maydis</td>
</tr>
<tr>
<td>Trichoderma ear rot and root rot</td>
<td>Trichoderma viride = T. lignorum teleomorph: Hypocrea sp.</td>
</tr>
<tr>
<td>White ear rot, root and stalk rot</td>
<td>Stenocarpella maydis = Diplodia macrospora</td>
</tr>
<tr>
<td>Yellow leaf blight</td>
<td>Ascochyta ischaemi, Phyllosticta maydis (teleomorph: Mycosphaerella zeae-maydis)</td>
</tr>
<tr>
<td>Zonate leaf spot</td>
<td>Gloeocercospora sorghi</td>
</tr>
</tbody>
</table>

The following are especially preferred

- Plasmodiophoromycota such as Plasmodiophora brassicae (clubroot of crucifers), Spongospora subterranea (powdery scab of potato tubers), Polymyxa graminis (root disease of cereals and grasses),

- Oomycota such as Bremia lactucae (downy mildew of lettuce), Peronospora (downy mildew) in snapdragon (P. antirrhini), onion (P. destructor), spinach (P. effusa), soybean (P. manchurica), tobacco ("blue mold"; P. tabacina), alfalfa and clover (P. trifolium), Pseudoperonospora humuli (downy mildew of hops), Plasmopara (downy mildew in grapevines) (P.
viticola) and sunflower (P. halstedii), Sclerophtohra macrospora (downy mildew in cereals and grasses), Pythium (seed rot, seedling damping-off, and root rot of all types of plants, for example damping-off of Beta beet caused by P. debaryanum), Phytophthora infestans (blight in potato, brown rot in tomato and the like), Albugo spec. (white rust on cruciferous plants).

- Ascomycota such as Microdochium nivale (snow mold of rye and wheat), Fusarium graminearum, Fusarium culmorum (partial ear sterility mainly in wheat), Fusarium oxysporum (Fusarium wilt of tomato), Blumeria graminis (powdery mildew of barley (f.sp. hordei) and wheat (f.sp. tritici)), Erysiphe pisi (powdery mildew of pea), Nectria galligena (Nectria canker of fruit trees), Uncinula necator (powdery mildew of grapevine), Pseudopeziza tracheiphila (red fire disease of grapevine), Claviceps purpurea (ergot on, for example, rye and grasses), Gaeumannomyces graminis (take-all on wheat, rye and other grasses), Magnaporthe grisea (rice blast disease), Pyrenophora graminea (leaf stripe of barley), Pyrenophora teres (net blotch of barley), Pyrenophora tritici-repentis (leaf blight of wheat), Venturia inaequalis (apple scab), Sclerotinia sclerotium (stalk break, stem rot). Pseudopeziza medicaginis (leaf spot of alfalfa, white and red clover).

- Basidiomycetes such as Typhula incarnata (typhula blight on barley, rye, wheat), Ustilago maydis (blister smut on maize), Ustilago nuda (loose smut on barley), Ustilago tritici (loose smut on wheat, spelt), Ustilago avenae (loose smut on oats), Rhizoctonia solani (rhizoctonia root rot of potato), Sphacelotheca spp. (head smut of sorghum), Melampsora lini (rust of flax), Puccinia graminis (stem rust of wheat, barley, rye, oats), Puccinia recondita (leaf rust on wheat), Puccinia dispersa (brown rust on rye), Puccinia hordei (leaf rust of barley), Puccinia coronata (crown rust of oats), Puccinia striiformis (yellow rust of wheat, barley, rye and a large number of grasses), Uromyces appendiculatus (brown rust of bean), Sclerotium rolfsii (root and stem rots of many plants).

- Deuteromycetes (Fungi imperfecti) such as Septoria nodorum (glume blotch) of wheat (Septoria tritici), Pseudocercospora herpotrichoides (eyespot of wheat, barley, rye), Rhyhchosporium secalis (leaf spot on rye and barley), Alternaria solani (early blight of potato, tomato), Phoma betae (blackleg on Beta beet), Cercospora beticola (leaf spot on Beta beet), Alternaria brassicae (black spot on
oilseed rape, cabbage and other crucifers), Verticillium dahliae (verticillium wilt), Colletotrichum lindemuthianum (bean anthracnose), Phoma lingam (blackleg of cabbage and oilseed rape), Botrytis cinerea (gray mold of grapevine, strawberry, tomato, hops and the like).

Most preferred are Phytophthora infestans (potato blight, brown rot in tomato and the like), Microdochium nivale (previously Fusarium nivale; snow mold of rye and wheat), Fusarium graminearum, Fusarium culmorum (partial ear sterility of wheat), Fusarium oxysporum (Fusarium wilt of tomato), Blumeria graminis (powdery mildew of barley (f. sp. hordei) and wheat (f. sp. tritici)), Magnaporthe grisea (rice blast disease), Sclerotinia sclerotium (stalk break, stem rot), Septoria nodorum and Septoria tritici (glume blotch of wheat), Alternaria brassicae (black spot of oilseed rape, cabbage and other crucifers), Phoma lingam (blackleg of cabbage and oilseed rape).

2. Bacterial pathogens:

The pathogens and diseases associated with them, all of which are mentioned in table 3, may be mentioned by way of example, but not by limitation.

### Table 3: Bacterial diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial leaf blight and stalk rot</td>
<td>Pseudomonas avenae subsp. avenae</td>
</tr>
<tr>
<td>Bacterial leaf spot</td>
<td>Xanthomonas campestris pv. holcicola</td>
</tr>
<tr>
<td>Bacterial stalk rot</td>
<td>Enterobacter dissolvens = Erwinia dissolvens</td>
</tr>
<tr>
<td>Bacterial stalk and top rot</td>
<td>Erwinia carotovora subsp. carotovora, Erwinia chrysanthemi pv. zeeae</td>
</tr>
<tr>
<td>Bacterial stripe</td>
<td>Pseudomonas andropogonis</td>
</tr>
<tr>
<td>Chocolate spot</td>
<td>Pseudomonas syringae pv. coronafaciens</td>
</tr>
<tr>
<td>Goss’s bacterial wilt and blight (leaf freckles and wilt)</td>
<td>Clavibacter michiganensis subsp. nebraskensis = Corynebacterium michiganense pv.nebraskense</td>
</tr>
<tr>
<td>Holcus spot</td>
<td>Pseudomonas syringae pv. syringae</td>
</tr>
<tr>
<td>Purple leaf sheath</td>
<td>Hemiparasitic bacteria</td>
</tr>
<tr>
<td>Seed rot-seedling blight</td>
<td>Bacillus subtilis</td>
</tr>
</tbody>
</table>
Very especially preferred are the following pathogenic bacteria: Corynebacterium sepedonicum (potato bacterial ring rot), Erwinia carotovora (potato bacterial soft rot), Erwinia amylovora (fire blight on pear, apple, quince), Streptomyces scabies (potato scab), Pseudomonas syringae pv. tabaci (tobacco black fire), Pseudomonas syringae pv. phaseolicola (bean grease spot), Pseudomonas syringae pv. tomato (tobacco bacterial speck), Xanthomonas campestris pv. malvacearum (cotton bacterial blight) and Xanthomonas campestris pv. oryzae (bacterial leaf blight on rice and other grasses).

3. Viral pathogens:

"Viral pathogens" includes all plant viruses such as, for example, tobacco or cucumber mosaic virus, ringspot virus, necroses virus, maize dwarf mosaic virus and the like.

Pathogens and the diseases associated with them may be mentioned in table 4 by way of example, but not by limitation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>American wheat striate (wheat striate mosaic)</td>
<td>American wheat striate mosaic virus (AWSMV)</td>
</tr>
<tr>
<td>Barley stripe mosaic</td>
<td>Barley stripe mosaic virus (BSMV)</td>
</tr>
<tr>
<td>Barley yellow dwarf</td>
<td>Barley yellow dwarf virus (BYDV)</td>
</tr>
<tr>
<td>Brome mosaic</td>
<td>Brome mosaic virus (BMV)</td>
</tr>
<tr>
<td>Cereal chlorotic mottle</td>
<td>Cereal chlorotic mottle virus (CCMV)</td>
</tr>
<tr>
<td>Corn chlorotic vein banding (Brazilian maize mosaic)</td>
<td>Corn chlorotic vein banding virus (CCVBV)</td>
</tr>
<tr>
<td>Corn lethal necrosis</td>
<td>Virus complex of Maize chlorotic mottle virus (MCMV) and Maize dwarf mosaic virus (MDMV) A or B or Wheat streak mosaic virus (WSMV)</td>
</tr>
<tr>
<td>Cucumber mosaic</td>
<td>Cucumber mosaic virus (CMV)</td>
</tr>
<tr>
<td>Cynodon chlorotic streak</td>
<td>Cynodon chlorotic streak virus (CCSV)</td>
</tr>
<tr>
<td>Johnsongrass mosaic</td>
<td>Johnsongrass mosaic virus (JGMV)</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Maize bushy stunt</td>
<td>Mycoplasma-like organism (MLO) associated</td>
</tr>
<tr>
<td>Maize chlorotic dwarf</td>
<td>Maize chlorotic dwarf virus (MCDV)</td>
</tr>
<tr>
<td>Maize chlorotic mottle</td>
<td>Maize chlorotic mottle virus (MCMV)</td>
</tr>
<tr>
<td>Maize dwarf mosaic</td>
<td>Maize dwarf mosaic virus (MDMV) strains A, D, E and F</td>
</tr>
<tr>
<td>Maize leaf fleck</td>
<td>Maize leaf fleck virus (MLFV)</td>
</tr>
<tr>
<td>Maize line</td>
<td>Maize line virus (MLV)</td>
</tr>
<tr>
<td>Maize mosaic (corn leaf stripe, enanismo rayado)</td>
<td>Maize mosaic virus (MMV)</td>
</tr>
<tr>
<td>Maize mottle and chlorotic stunt</td>
<td>Maize mottle and chlorotic stunt virus</td>
</tr>
<tr>
<td>Maize pellucid ringspot</td>
<td>Maize pellucid ringspot virus (MPRV)</td>
</tr>
<tr>
<td>Maize raya gruesa</td>
<td>Maize raya gruesa virus (MRGV)</td>
</tr>
<tr>
<td>maize rayado fino (fine striping disease)</td>
<td>Maize rayado fino virus (MRFV)</td>
</tr>
<tr>
<td>Maize red leaf and red stripe</td>
<td>Mollicute</td>
</tr>
<tr>
<td>Maize red stripe</td>
<td>Maize red stripe virus (MRSV)</td>
</tr>
<tr>
<td>Maize ring mottle</td>
<td>Maize ring mottle virus (MRMV)</td>
</tr>
<tr>
<td>Maize rio IV</td>
<td>Maize rio cuarto virus (MRCV)</td>
</tr>
<tr>
<td>Maize rough dwarf (nanismo ruvido)</td>
<td>Maize rough dwarf virus (MRDV) (Cereal tillering disease virus)</td>
</tr>
<tr>
<td>Maize sterile stunt</td>
<td>Maize sterile stunt virus (strains of barley yellow striate virus)</td>
</tr>
<tr>
<td>Maize streak</td>
<td>Maize streak virus (MSV)</td>
</tr>
<tr>
<td>Maize stripe (maize chlorotic stripe, maize hoja blanca)</td>
<td>Maize stripe virus</td>
</tr>
<tr>
<td>Maize stunting</td>
<td>Maize stunting virus</td>
</tr>
<tr>
<td>Maize tassel abortion</td>
<td>Maize tassel abortion virus (MTAV)</td>
</tr>
<tr>
<td>Maize vein enation</td>
<td>Maize vein enation virus (MVEV)</td>
</tr>
<tr>
<td>Maize wallaby ear</td>
<td>Maize wallaby ear virus (MWEV)</td>
</tr>
<tr>
<td>Maize white leaf</td>
<td>Maize white leaf virus</td>
</tr>
<tr>
<td>Maize white line mosaic</td>
<td>Maize white line mosaic virus (MWLMV)</td>
</tr>
<tr>
<td>Millet red leaf</td>
<td>Millet red leaf virus (MRLV)</td>
</tr>
<tr>
<td>Northern cereal mosaic</td>
<td>Northern cereal mosaic virus (NCMV)</td>
</tr>
<tr>
<td>Oat pseudorosette (zakuklivanie)</td>
<td>Oat pseudorosette virus</td>
</tr>
<tr>
<td>Oat sterile dwarf</td>
<td>Oat sterile dwarf virus (OSDV)</td>
</tr>
<tr>
<td>Rice black-streaked dwarf</td>
<td>Rice black-streaked dwarf virus (RBSDV)</td>
</tr>
<tr>
<td>Rice stripe</td>
<td>Rice stripe virus (RSV)</td>
</tr>
<tr>
<td>Disease</td>
<td>Pathogen</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Sorghum mosaic</td>
<td>Sorghum mosaic virus (SrMV) (also: sugarcane mosaic virus (SCMV) strains H, I and M)</td>
</tr>
<tr>
<td>Sugarcane Fiji disease</td>
<td>Sugarcane Fiji disease virus (FDV)</td>
</tr>
<tr>
<td>Sugarcane mosaic</td>
<td>Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B)</td>
</tr>
<tr>
<td>Wheat spot mosaic</td>
<td>Wheat spot mosaic virus (WSMV)</td>
</tr>
</tbody>
</table>

4. Animal pests

4.1 Insect pathogens:

Insects such as, for example, beetles, caterpillars, lice or mites may be mentioned by way of example, but not by limitation. Preferred are insects of the genera Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thyssanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, and the like. Especially preferred are Coleoptera and Lepidoptera insects such as, for example, the European corn borer (ECB), Diabrotica barberi (“northern corn rootworm”), Diabrotica undecimpunctata (“southern corn rootworm”), Diabrotica virgifera (“Western corn rootworm”), Agrotis ipsilon (“black cutworm”), Crymodes devastator (“glassy cutworm”), Feltia ducens (“dingy cutworm”), Agrotis gladiaria (“claybacked cutworm”), Melanotus spp., Aeolus mellillus (“wireworm”), Aeolus mancus (“wheat wireworm”), Horistonotus uhleri (“sand wireworm”), Sphenophorus maidis (“maize billbug”), Sphenophorus zeae (“timothy billbug”), Sphenophorus parvulus (“bluegrass billbug”), Sphenophorus callus (“southern corn billbug”), Phyllophaga spp. (“white grubs”), Anuraphis maidiradicus (“corn root aphid”), Delia platura (“seedcorn maggot”), Colaspis brunnea (“grape colaspis”), Stenolophus lecontei (“seedcorn beetle”) and Clivinia impressifrons (“lender seedcorn beetle”).

Others which may be mentioned are: the cereal leaf beetle (Oulema melanopus), the frit fly (Oscinella frit), wireworms (Agrotis lineatus) and aphids (such as, for example, the oat grain aphid Rhopalosiphum padi, the blackberry aphid Sitobion avenae).

4.2 Nematodes:
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Pathogens and the diseases associated with them may be mentioned by way of example, but not by way of limitation, in table 6.

Table 6: Parasitic nematodes

<table>
<thead>
<tr>
<th>Disease</th>
<th>Pathogenic Nematode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awl</td>
<td>Dolichodorus spp., D. heterocephalus</td>
</tr>
<tr>
<td>Bulb and stem nematode disease; Europe</td>
<td>Ditylenchus dipsaci</td>
</tr>
<tr>
<td>Burrowing</td>
<td>Radopholus similis</td>
</tr>
<tr>
<td>Cyst nematode disease</td>
<td>Heterodera avenae, H. zeae, Punctodera chalcoensis</td>
</tr>
<tr>
<td>Dagger</td>
<td>Xiphinema spp., X. americanum, X. mediterraneum</td>
</tr>
<tr>
<td>False root-knot</td>
<td>Nacobbus dorsalis</td>
</tr>
<tr>
<td>Lance, Columbia</td>
<td>Hoplolaimus columbus</td>
</tr>
<tr>
<td>Lance</td>
<td>Hoplolaimus spp., H. galeatus</td>
</tr>
<tr>
<td>Lesion</td>
<td>Pratylenchus spp., P. brachyurus, P. crenatus, P. hexincisus, P. neglectus, P. penetrans, P. scribneri, P. thornei, P. zeae</td>
</tr>
<tr>
<td>Needle</td>
<td>Longidorus spp., L. breviannulatus</td>
</tr>
<tr>
<td>Ring</td>
<td>Criconemella spp., C. ornata</td>
</tr>
<tr>
<td>Root-knot disease</td>
<td>Meloidogyne spp., M. chitwoodi, M. incognita, M. javanica</td>
</tr>
<tr>
<td>Spiral</td>
<td>Helicotylenchus spp.</td>
</tr>
<tr>
<td>Sting</td>
<td>Belonolaimus spp., B. longicaudatus</td>
</tr>
<tr>
<td>Stubby-root</td>
<td>Paratrichodorus spp., P. christiei, P. minor, Quinisolcius acutus, Trichodorus spp.</td>
</tr>
<tr>
<td>Stunt</td>
<td>Tylenchorhynchus dubius</td>
</tr>
</tbody>
</table>

Very especially preferred are Globodera rostochiensis and G. pallida (cyst eelworm on potato, tomato and other Solanaceae), Heterodera schachtii (beet eelworm on sugar and fodder beet, oilseed rape, cabbage and the like), Heterodera avenae (cereal cyst nematode on oat and other cereal species), Ditylenchus dipsaci (stem or bulb eelworm, stem eelworm of rye, oats, maize, clover, tobacco, beet), Anguina tritici (ear-cockle nematode, cockle disease of wheat (spelt, rye), Meloidogyne hapla (root-knot nematode of carrot, cucumber, lettuce, tomato, potato, sugar beet, alfalfa).
Examples of fungal or viral pathogens which are preferred for the individual varieties are the following:

1. Barley:

- fungal, bacterial and viral pathogens: Puccinia graminis f.sp. hordei (barley stem rust), Blumeria (Erysiphe) graminis f.sp. hordei (Barley Powdery Mildew), barley yellow dwarf virus (BYDV),

Pathogenic insects / nematodes: Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Schizaphis graminum (greenbug); Blissus leucopterus leucopterus (chinch bug); Acrosternum hilare (green stink bug); Euschistus servus (brown stink bug); Deliaplatura (seedcorn maggot); Mayetiola destructor (Hessian fly); Petrobia latens (brown wheat mite).

2. Soybean:

- Fungal, bacterial or viral pathogens: Phytophthora megasperma f.sp. glycinea, Macrophomina phaseolina, Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium oxysporum, Diaporthe phaseolorum var. sojae (Phomopsis sojae), Diaporthe phaseolorum var. caulivora, Sclerotium rolfsii, Cercospora kikuchii, Cercospora sojina, Peronospora manchurica, Colletotrichum dematium (Colletotrichum truncatum), Corynespora cassiicola, Septoria glycines, Phyllosticta soicola, Alternaria alternata, Pseudomonas syringae p.v. glycinea, Xanthomonas campestris p.v. phaseoli, Microsphaera diffusa, Fusarium semitectum, Phialophora gregata, Soybean mosaic virus, Glomerella glycines, Tobacco Ring spot virus, Tobacco Streak virus, Phakopsorapachyrhizi, Pythium aphanidermatum, Pythium ultimum, Pythium debaryanum, Tomato spotted wilt virus, Heterodera glycines, Fusarium solani.

Pathogenic insects / nematodes: Pseudoplusia includens (soybean looper); Anticarsia gemmatalis (velvetbean caterpillar); Plathypena scabra (green cloverworm); Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Spodoptera exigua (beet armyworm); Heliothis virescens (cotton bollworm); Helicoverpa zea (cotton bollworm); Epilachna varivestis (Mexican bean beetle); Myzus persicae (green peach aphid); Empoasca fabae (potato leaf hopper); Acrosternum hilare (green stink bug); Melanoplus femurbrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Hylemya platura (seedcorn maggot); Sericothrips variabilis (soybean thrips); Thrips tabaci (onion thrips); Te-
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Tranynchus turkestani (strawberry spider mite); Tetranychus urticae (two-spotted spider mite).

3. Canola:

Fungal, bacterial or viral pathogens: Albigo candida, Alternaria brassicae, Leptosphaeria maculans, Rhizoctonia solani, Sclerotinia sclerotiorum, Mycosphaerella brassicicola, Pythium ultimum, Peronospora parasitica, Fusarium roseum, Alternaria alternata.

4. Alfalfa:

Fungal, bacterial or viral pathogens: Clavibacter michiganense subsp. insidiosum, Pythium ultimum, Pythium irregulare, Pythium splendens, Pythium debaryanum, Pythium aphanidermatum, Phytophthora megasperma, Peronospora trifoliorum, Phoma medicaginis var. medicaginis, Cercospora medicaginis, Pseudopeziza medicaginis, Leptotricha medicaginis, Fusarium, Xanthomonas campestris p.v. alfalfae, Aphanomyces euteiches, Stemphylium herbarum, Stemphylium alfalfae.

5. Wheat:

Pathogenic insects / nematodes: Pseudoletia unipunctata (armyworm); Spodoptera frugiperda (fall armyworm); Elasmopalpus lignosellus (lesser cornstalk borer); Agrotis orthogonia (western cutworm); Elasmopalpus Zigosellus (lesser cornstalk borer); Oulema melanopus (cereal leaf beetle); Hypera punctata (clover leaf weevil); Diabrotica undecimpunctata howardi (southern corn rootworm); Russian wheat aphid; Schizaphis graminum (greenbug); Macrosiphum avenae (English grain aphid); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Melanoplus sanguinipes (migratory grasshopper); Mayetiola destructor (Hessian fly); Sitodiplosis mosellana (wheat midge); Meromyza americana (wheat stem maggot); Hylemya coarctata (wheat bulb fly); Frankliniella fusca (tobacco thrips); Cephus cinctus (wheat stem sawfly); Aceria tulipae (wheat curl mite).

Sunflower:


Pathogenic insects / nematodes: Suleima helianthana (sunflower bud moth); Homoeosoma electellum (sunflower moth); zygo-gramma exclamationis (sunflower beetle); Bothyrus gibbosus (carrot beetle); Neolasioptera murtfeldtiana (sunflower seed midge).

Maize:

Fungal, bacterial or viral pathogens: Fusarium moniliforme var. subglutinans, Erwinia stewartii, Fusarium moniliforme, Gibberella zeae (Fusarium graminearum), Stenocarpella maydi (Diplodia maydis), Pythium irregularare, Pythium debaryanum, Pythium graminicola, Pythium splendens, Pythium ultimum, Pythium aphanidermatum, Aspergillus flavus, Bipolaris maydis 0, T (Cochliobolus heterostrophus), Helminthosporium carbonum I, II & III (Cochliobolus carbonum), Exserohilum turcicum I, II & III, Helminthosporium pedicellatum, Physoherma maydis, Phyllosticta maydis, Kabatiella maydis, Cercospora sorghi, Ustilago maydis, Puccinia sorghi, Puccinia polysora, Macro-

Pathogenic insects / nematodes: Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Helicoverpa zea (corn earworm); Spodoptera frugiperda (fall armyworm); Diatraea grandiosella (southwestern corn borer); Elasmopalpus lignosellus (lesser cornstalk borer); Diatraea saccharalis (surgarcane borer); Diabrotica virgifera (western corn rootworm); Diabrotica longicornis barberi (northern corn rootworm); Diabrotica undecimpunctata howardi (southern corn rootworm); Melanotus spp. (wireworms); Cyclocephala borealis (northern masked chafer; white grub); Cyclocephala immaculata (southern masked chafer; white grub); Popillia japonica (Japanese beetle); Chaetocnema plicaria (corn flea beetle); Sphenophorus maidis (maize billbug); Rhopalosiphum maidis (corn leaf aphid); Anuraphis maidaridicis (corn root aphid);
Blissus leucopterus leucopterus (chinsh bug); Melanoplus femurrum (redlegged grasshopper); Melanoplus sanguinipes (migratory grasshopper); Hylemya platura (seedcorn maggot); Agromyza parvicornis (corn blot leafminer); Anaphothrips obscurus (grass thrips); Solenopsis milesta (thief ant); Tetranychus urticae (twospotted spider mite).

8. Sorghum:

Fungal, bacterial or viral pathogens: Exserohilum turcicum, Colletotrichum graminicola (Glomerella graminicola), Cercospora sorghi, Gloecercospora sorghi, Ascochyta sorghina, Pseudomonas syringae p.v. syringae, Xanthomonas campestris p.v. holcicola, Pseudomonas andropogonis, Puccinia purpurea, Macrophomina phaseolina, Perconia circinata, Fusarium moniliforme, Alternaria alternata, Bipolaris sorghicola, Helminthosporium sorghicola, Curvularia lunata, Phoma insidiosa, Pseudomonas avenae (Pseudomonas alboprecipitans), Ramulispora

Pathogenic insects / nematodes: Chilo partellus (sorghum borer); Spodoptera frugiperda (fall armyworm); Helicoverpa zea (corn earworm); Elasmopalpus lignosellus (lesser cornstalk borer); Feltia subterranea (granulate cutworm); Phyllaphaga crinita (white grub); Eleodes, Conoderus and Aeolus spp. (wireworm); Oulema melanopus (cereal leaf beetle); Chaetocnema punicaria (corn flea beetle); Sphenophorus maidis (maize billbug); Rhopalosiphum maidis (corn leaf aphid); Siphaflava (yellow sugarcane aphid); Blissus leucopterus leucopterus (chinch bug); Contarinia sorghicola (sorghum midge); Tetranychus cinnabarinus (carmine spider mite); Tetranychus urticae (two-spotted spider mite).

Pathogenic insects / nematodes: Heliothis virescens (cotton budworm); Helicoverpa zea (cotton bollworm); Spodoptera exigua (beet armyworm); Pectinophora gossypiella (pink bollworm); Anthonomus grandis grandis (boll weevil); Aphis gossypii (cotton aphid); Pseudatomoscelis seriatus (cotton fleahopper); Trialeurodes abutilonea (bandedwinged whitefly); Lygus lineolaris ( tarnished plant bug); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Thrips tabaci (onion thrips); Frankliniella fusca (tobacco thrips); Tetranychus cinnabarinus (carmine spider mite); Tetranychus urticae (twospotted spider mite).

Pathogenic insects / nematodes: Diatraea saccharalis (sugarcane borer); Spodoptera frugiperda (fall armyworm); Helicoverpa zea (corn earworm); Colaspis brunnea (grape colaspis); Lissorhoptrus oryzophilus (rice water weevil); Sitophilus oryzae (rice weevil); Nephotettix nigropictus (rice leafhopper); Blissus leucopterus leucopterus (chinch bug); Acrosternum hilare (green stink bug).
11. Oilseed rape:

Pathogenic insects / nematodes: Brevicoryne brassicae (cabbage aphid); Phylotreta cruciferae (Flea beetle); Mamestra conjugrata (Bertha armyworm); Plutella xylostella (Diamond-back moth); Delia ssp. (Root maggots).

For the purposes of the invention, "NADPH oxidase" means all those enzymes whose essential characteristic is that they are capable, by means of a single electron transfer, of converting molecular oxygen (O₂) into superoxide (O₂⁻). Preferred are those enzymes which are described by the EC class 1.1.1.4. In this context, the NADPH oxidases can consist of one or more polypeptides which may be identical or different.

Preferably, the NADPH oxidase is a flavocytochrome protein and comprises, as prosthetic groups, a cytochrome b and/or an FAD unit. The NADPH oxidase may consist of an αβββ heterodimer, the β subunit being the functional subunit of the flavocytochrome, which may comprise, as glycoprotein, the electron transport components (a hydrophilic, cytosolic, C-terminal domain, comprising NADPH and FAD, and 4 to 6 N-terminal, putative transmembrane α-helices, comprising two histidine-complexed prosthetic heme groups). The α-subunit may comprise a C-terminal, prolin-rich sequence which is capable of binding potential cytosolic, activating factors of the NADPH oxidase. Activation may take place by binding the cytosolic phox proteins (for example p47-phox, p67-phox, p40-phox) and p21rac, a GTP-binding protein.

The skilled worker is familiar with a large number of NADPH oxidases from plant organisms (Torres MA et al. (1998) Plant J 14: 365-370, inter alia). Sequences which may be mentioned by way of example, but not by limitation, are those with the following GenBank Acc. Nos.: AJ251171 (Hordeum vulgare), AP003560 (Oryza sativa var. japonica), AJ320505 (Nicotiana tabacum), AB050660 (Solanum tuberosum), AF088276 (Lycopersicon esculentum), AB008111 (Arabidopsis thaliana; Atrboh F), AF055357 (Arabidopsis thaliana; RbohD), AJ309006 (Nicotiana tabacum; rboh), AP003271 (Oryza sativa cv. japonica), AP055355 (Arabidopsis thaliana; RbohC), AF055353 (Arabidopsis thaliana; RbohA). Especially preferred are the NADPH oxidases which comprise a sequence as shown in SEQ ID: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

The sequences from other plants which are homologous to the NADPH oxidase sequences disclosed within the present invention can be found readily for example by database search or by screening genetic libraries using the NADPH oxidase sequences as search se-
sequence or probe. Examples which may be mentioned are sequences with the following GenBank Acc. Nos.: CAC51517.1, AJ251717, T03973, BAB68079.1, AP003560, T02024, CAC87256.1, AJ320505, BAB70750.1, AB050660, AF088276_1, NP_564821.1, NM_105079, T00265
AC007764_16, NP_192862.1, NM_117194, AF147783_1, AAM28891.1, AF506374, CAC84140.1, AJ309006, T51804, NP_199602.1, NM_124165, BAB89740.1, AP003271, AAC39477.1, AF055355, NP_199919.1, NM_124485, AAC39475.1, AF055353, NP_196356.1, NM_120821, NP_194239.1, NM_118641, BAB08369.1, AB015475, AAC39478.1,
AF055356, AC069143_9, NP_173357.1, NM_101781, NP_172383.1, NM_100780, AAB70398.1, AC000106, AAC39476.1, AF055354, BAB70751.1, AB050661, BAB63664.1, AP003275, AAD24966.1, AF109150.

The polypeptide sequence of the NADPH oxidase especially preferably comprises at least one sequence motif selected from the group of sequence motifs consisting of

i) AL(K/R)GL(K/R)
ii) DK(N/D)XDG(R/K)(I/L/V)(T/N/E
iii) LSASAN
iv) IMEELDP
v) K(F/L)NMA(I/L)(I/V)LXPCRN
vi) (E/Q)WHPPSIT
vii) S(A/S)PXDD(Q/Y)(L/I)S(I/V)H(V/I/L)R

The peptide sequence very especially preferably comprises at least 2 or 3, very especially preferably at least 4 or 5, most preferably all of the sequence motifs selected from the group of the sequence motifs i), ii), iii), iv), v), vi), vii), viii), ix) x) and xi). (Letters in brackets mean alternative amino acids which are possible at this position, for example (V/I) means that valine or isoleucine are possible at this position).

NADPH oxidase may also mean any other unit of an NADPH oxidase enzyme complex which is essential for activity of the NADPH oxi-

dase.

"Protein quantity" means the amount of a NADPH oxidase polypeptide in an organism, a tissue, a cell or a cell compartment. "Reduction" of the protein quantity means the quantitative reduction of the amount of an NADPH oxidase in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild-type of the
same genus and species to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably by at least 40% or 60%, very especially preferably by at least 70% or 80%, most preferably by at least 90% or 95%.

"Activity" means the ability of an NADPH oxidase of converting molecular oxygen (O₂) into superoxide (O₂⁻). "Reduction" of the activity means the reduction of the total activity of an NADPH oxidase protein in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild type of the same genus and species, to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably to at least 40% or 60%, very especially preferably to at least 70% or 80%, most preferably to at least 90% or 95%.

"Function" preferably means the substrate binding capacity of an NADPH oxidase in an organism, a tissue, a cell or a cell compartment. Suitable substrates are low-molecular-weight compounds such as NADPH or FAD, but also the protein interaction partners of an NADPH oxidase.

"Reduction" of the function means, for example, the quantitative reduction of the binding capacity or binding strength of an NADPH oxidase for at least one substrate in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild-type of the same genus and species to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). "Reduction" is also understood as meaning the change in substrate specificity as expressed, for example, by the kcat/Km value. In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably to at least 40% or 60%, very especially preferably to at least 70% or 80%, most preferably to at least 90% or 95%. Binding partners for NADPH oxidase can be identified for example by the yeast-2-hybrid system in the manner with which the skilled worker is familiar.
Methods for determining the protein quantity, the activity of NADPH oxidases or the substrate binding capacity are known to the skilled worker. For example, it is possible to measure the NADPH-dependent O₂- or H₂O₂ production which can be inhibited by DPI (for example via Nitro Blue Tetrazolium [NBT] or cytochrome c reduction). The protein quantity can be determined for example immunologically, using suitable antibodies. Suitable methods are described (Yu L et al. (1999) Blood 94(7):2497-504; Doke N (1983a) Physiol Plant Pathol 23:345-357; Levine A et al. (1994) Cell 79:583-593; Tenhaken R et al. (1995) Proc Nat Acad Sci USA 92: 4158-4163; Sagi M & Fluhr R. (2001) Plant Physiol 126(3):1281-90; Hückelhoven R & Kogel KH (1998) Mol Plant Microbe Interact 11:292-300; and references cited in the above papers).

"Functional equivalents" of an NADPH oxidase protein preferably means those sequences which are derived from an NADPH oxidase comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 which are homologous with the former and which have the same essential characteristics.

In this context, the efficiency of the pathogen resistance may deviate both upwards and downwards in comparison with a value obtained when reducing one of the NADPH oxidases comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22. Preferred functional equivalents are those where the efficiency of the pathogen resistance - measured for example with the aid of the penetration efficiency of a pathogen (development of haustora) - differs by not more than 50%, preferably 25%, especially preferably 10%, from a comparative value obtained by reducing an NADPH oxidase comprising a polypeptide sequence as described in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22. Especially preferred are those sequences whose reduction has the result that the efficiency of the pathogen resistance quantitatively exceeds a comparative value obtained by reducing one of the NADPH oxidases comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 by more than 50%, preferably 100%, especially preferably 500%, very especially preferably 1000%.

The comparison is preferably carried out under analogous conditions. "Analogous conditions" means that all framework conditions such as, for example, culture or growth conditions, assay conditions (such as buffer, temperature, substrates, pathogen concentration and the like) between the experiments to be compared are kept identical and that the set-ups differ only by the sequence of the NADPH oxidases to be compared, their organism of origin and, if appropriate, the pathogen. When selecting the
pathogen for the comparison, the pathogen to be selected for the
comparison is that which is most similar to the corresponding
other pathogen, taking into consideration the species specifici-
ty.

In particular, "functional equivalents" means natural or artifi-
cial mutations of the NADPH oxidases comprising a polypeptide se-
quence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20
or 22 and homologous polypeptides from other plants which contin-
ue to have essentially identical characteristics. Homologous
polypeptides from the above-described preferred plants are pre-
ferred. The sequences from other plants (for example Arabidopsis
thaliana) which are homologous to the NAPDH oxidase sequences
disclosed within the scope of the present invention can be found
readily for example by database search or screening genetic li-
braries, using the NADPH oxidase sequences as search sequence or
probe. Such sequences are detailed above by way of example to-
gether with their GenBank Acc No.

Mutations comprise substitutions, additions, deletions, inver-
sions or insertions of one or more amino acid residues. Thus, the
present invention also comprises for example those polypeptides
which are obtained by modification of a polypeptide comprising a
polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12,
14, 16, 18, 20 or 22.

Homology between two nucleic acid sequences is understood as
meaning the identity of the two nucleic acid sequences over in
each case the entire sequence length which is calculated by
comparison with the aid of the program algorithm GAP (Wisconsin
Package Version 10.0, University of Wisconsin, Genetics Computer
Group (GCG), Madison, USA), setting the following parameters:

    Gap weight: 50          Length weight: 3
    Average match: 10      Average mismatch: 0

For example a sequence which has at least 80% homology with
sequence SEQ ID NO: 1 at the nucleic acid level is understood as
meaning a sequence which, upon comparison with the sequence SEQ
ID NO: 1 by the above program algorithm with the above parameter
set, has at least 80% homology.

Homology between two polypeptides is understood as meaning the
identity of the two nucleic acid sequences over in each case the
entire sequence length which is calculated by comparison with the
aid of the program algorithm GAP (Wisconsin Package Version 10.0,
28
University of Wisconsin, Genetics Computer Group (GCG), Madison, USA; Altschul et al. (1997) Nucleic Acids Res. 25:3389 et seq.), setting the following parameters:

5 Gap weight: 8	Length weight: 2
Average match: 2,912	Average mismatch: -2,003

For example a sequence which has at least 80% homology with a sequence SEQ ID NO: 2 at the protein level is understood as meaning a sequence which, upon comparison with the sequence SEQ ID NO: 2 by the above program algorithm with the above parameter set, has at least 80% homology.

15 Functional equivalents derived from an NADPH oxidase comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 by substitution, insertion or deletion have at least 50%, preferably at least 70%, by preference at least 90%, especially preferably at least 95%, very especially preferably at least 98% homology with a polypeptide comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 and are distinguished by identical essential characteristics as the former.

25 Functional equivalents derived from an NAPDH oxidase nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 by substitution, insertion or deletion, have at least 50%, preferably at least 70%, by preference at least 90%, especially preferably at least 95%, very especially preferably at least 98% homology with one of the polypeptides according to the invention as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 and encode polypeptides with the same essential characteristics as a polypeptide comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

35 Also, screening cDNA libraries or genomic libraries of other organisms, preferably of the plant species which are mentioned further below as being suitable hosts for the transformation, using the nucleic acid sequences described under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 or parts of these as probe, is a method known to the skilled worker for identifying homologs in other species. In this context, the probes derived from the nucleic acid sequences as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 have a length of at least 20 bp, preferably at least 50 bp, especially preferably at least 100 bp, very especially preferably at least 200 bp, most preferably at least 400 bp. A DNA strand which is complementary to the sequences de-
scribed under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 may also be employed for screening the libraries.

Functional equivalents comprises DNA sequences which hybridize under standard conditions with the NAPDH oxidase nucleic acid sequences described under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21, the nucleic acid sequence complementary thereto or parts of the above and which, as complete sequences, encode proteins which have the same essential characteristics as a polypeptide comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.


For example, the conditions during the wash step can be selected from the range of conditions delimited by low-stringency conditions (approximately 2X SSC at 50°C) and high-stringency conditions (approximately 0.2X SSC at 50°C, preferably at 65°C) (20X SSC: 0.3M sodium citrate, 3M NaCl, pH 7.0). In addition, the temperature during the wash step can be raised from low-stringency conditions at room temperature, approximately 22°C, to higher-stringency conditions at approximately 65°C. Both of the parameters, salt concentration and temperature, can be varied simultaneously, or else one of the two parameters can be kept constant while only the other is varied. Denaturants, for example formamide or SDS, may also be employed during the hybridization. In the presence of 50% formamide, hybridization is preferably effected at 42°C. Some examples of conditions for hybridization and wash step are shown hereinbelow:

(1) Hybridization conditions can be selected, for example, from the following conditions:

a) 4X SSC at 65°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA)

b) 6X SSC at 45°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA),

c) 6X SSC, 0.5% SDS, 50% formamide at 42°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA)
d) 4X SSC, 50% formamide at 42°C (with – optionally – 100 µg/ml denatured fragmented fish sperm DNA)
e) 2X or 4X SSC at 50°C (low-stringency condition),
f) 30 to 40% formamide, 2X or 4X SSC at 42°C (low-stringency condition).

(2) Wash steps can be selected, for example, from the following conditions:

a) 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.
b) 0.1X SSC at 65°C.
c) 0.1X SSC, 0.5% SDS at 68°C.
d) 0.1X SSC, 0.5% SDS, 50% formamide at 42°C.
e) 0.2X SSC, 0.1% SDS at 42°C.
f) 2X SSC at 65°C (low-stringency condition).

The reduction of the expression of an NADPH oxidase protein, the NADPH oxidase activity or the NADPH oxidase function can be performed in many ways.

"Reduction" or "to reduce" in connection with an NADPH oxidase, an NADPH oxidase activity or NADPH oxidase function is to be interpreted in the broad sense and comprises the partial or essentially complete prevention or blocking (due to a variety of cell-biological mechanisms) of the functionality of an NADPH oxidase in a plant or a part, tissue, organ, cells or seed derived therefrom. A reduction for the purposes of the invention also comprises a quantitative reduction of an NADPH oxidase down to an essentially complete absence of the NADPH oxidase (i.e. lack of detectability of NADPH oxidase activity or NADPH oxidase function, or lack of immunological detectability of the NADPH oxidase protein). In this context, one or more essential units of the NADPH oxidase can be reduced. In this context, the expression of a certain NADPH oxidase or the NADPH oxidase activity or NADPH oxidase function in a cell or an organism is reduced by preferably more than 50%, especially preferably more than 80%, very especially preferably more than 90%.

A variety of strategies for reducing the expression of an NADPH oxidase protein, the NADPH oxidase activity or NADPH oxidase function are comprised in accordance with the invention. Strategies which may be mentioned by way of example, but not by limitation, are:
a) Introducing a double-stranded NADPH oxidase RNA nucleic acid sequence (NAox-dsRNA) or (an) expression cassette(s) ensuring its expression;

b) Introducing an NADPH oxidase antisense nucleic acid sequence or an expression cassette ensuring its expression. Comprised are those methods in which the antisense nucleic acid sequence is directed against an NADPH oxidase gene (that is to say, genomic DNA sequences) or an NADPH oxidase gene transcript (that is to say, RNA sequences). Also comprised are α-anomeric nucleic acid sequences.

c) Introducing an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme or an expression cassette ensuring its expression

d) Introducing NADPH oxidase sense nucleic acid sequences for inducing a cosuppression or an expression cassette ensuring their expression

e) Introducing DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins or an expression cassette ensuring their expression

f) Introducing viral nucleic acid sequences and expression constructs bringing about the degradation of NADPH oxidase RNA, or an expression cassette ensuring their expression

g) Introducing constructs for inducing a homologous recombination at endogenous NADPH oxidase genes, for example for the generation of knock-out mutants.

h) Introducing mutations into endogenous NADPH oxidase genes for generating a loss of function (for example generation of stop codons, reading frame shifts and the like)

Here, each and every one of these methods can bring about a reduction of the NADPH oxidase expression, NADPH oxidase activity or NADPH oxidase function in the sense of the invention. A combined use is also feasible. Further methods are known to the skilled worker and can comprise hindering or preventing the processing of the NADPH oxidase protein, the transport of the NADPH oxidase protein or its mRNA, inhibition of the attachment of ribosomes, inhibition of RNA splicing, induction of an NADPH oxidase RNA degrading enzyme and/or inhibition of the translational elongation or termination.
The individual methods which are preferred shall be described briefly hereinbelow:

a) Introducing a double-stranded NADPH oxidase RNA nucleic acid sequence (NAox-dsRNA)

The method of regulating genes by means of double-stranded RNA (double-stranded RNA interference; dsRNAi) has been described many times in animal and plant organisms (for example Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al (1998) Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364). The processes and methods described in the abovementioned references are expressly referred to. Efficient gene suppression can also be shown in the case of transient expression or after transient expression, for example as the result of a biolistic transformation (Schweizer P et al. (2000) Plant J 2000 24:895-903). dsRNAi methods are based on the phenomenon that the simultaneous introduction of complementary strand and counterstrand of a gene transcript brings about a highly-efficient suppression of the expression of the gene in question. The phenotype which results is very similar to a corresponding knock-out mutant (Waterhouse PM et al. (1998) Proc Natl Acad Sci USA 95:13959-64).

The dsRNAi method has proved to be particularly efficient and advantageous when reducing the NADPH oxidase expression. As described in WO 99/32619, inter alia, dsRNAi approaches are markedly superior to traditional antisense approaches.

A further aspect of the invention therefore relates to double-stranded RNA molecules (dsRNA molecules) which, when introduced into a plant (or a cell, tissue, organ or seed derived therefrom), bring about the reduction of an NADPH oxidase.

The double-stranded RNA molecule for reducing the expression of an NADPH oxidase protein comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of an NADPH oxidase nucleic acid sequence, and

b) an antisense RNA strand which is essentially completely complementary to the RNA sense strand of a).
In a furthermore preferred embodiment, the double-stranded RNA molecule for reducing the expression of an NADPH oxidase protein comprises

5 a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of the sense RNA transcript of a nucleic acid sequence encoding an NADPH oxidase protein, and

10 b) an antisense RNA strand which is essentially - preferably completely - complementary to the RNA sense strand of a).

With regard to the double-stranded RNA molecules, NADPH oxidase nucleic acid sequence preferably means a sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21.

"Essentially identical" means that the dsRNA sequence may also have insertions, deletions and individual point mutations in comparison with the NADPH oxidase target sequence or a functional equivalent target sequence while still bringing about an efficient reduction of the expression. Preferably, the homology as defined above between the sense strand of an inhibitory dsRNA and at least part of the sense RNA transcript of a nucleic acid sequence encoding an NADPH oxidase protein or functional equivalent thereof (or between the antisense strand of the complementary strand of a nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof) amounts to at least 75%, preferably at least 80%, very especially preferably at least 90%

25 most preferably 100%.

The length of the part-segment amounts to at least 10 bases, preferably at least 25 bases, especially preferably at least 50 bases, very especially preferably at least 100 bases, most preferably at least 200 bases or at least 300 bases.

Alternatively, an "essentially identical" dsRNA may also be defined as a nucleic acid sequence which is capable of hybridizing with a part of a storage protein gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the antisense RNA strand may also have insertions, deletions and individual point mutations in comparison with the complement of the sense RNA strand. Preferably, the homology between the antisense RNA strand and the complement of the sense RNA strand amounts to at least 80%, pre-
ferably at least 90%, very especially preferably at least 95%, most preferably 100%.

"Part of the sense RNA transcript" of a nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof of means fragments of an RNA or mRNA transcribed from a nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof, preferably an NADPH oxidase gene. Here, the fragments preferably have a sequence length of at least 20 bases, preferably at least 50 bases, especially preferably at least 100 bases, very especially preferably at least 200 bases, most preferably at least 500 bases. Also comprised is the complete transcribed RNA or mRNA.

Also comprised is the use of the dsRNA molecules according to the invention in the methods according to the invention for generating a pathogen resistance in plants.

The dsRNA can consist of one or more strands of polymerized ribonucleotides. Furthermore, modifications both of the sugar-phosphate skeleton and of the nucleosides may be present. For example, the phosphodiester bonds of the natural RNA can be modified in such a way that they comprise at least one nitrogen or sulfur heteroatom. Bases can be modified in such a way that the activity of, for example, adenosine deaminase is limited. These and further modifications are described hereinbelow in the methods for stabilizing antisense RNA.

To achieve the same purpose it is, of course, also possible to introduce, into the cell or the organism, a plurality of individual dsRNA molecules, each of which comprises one of the above-defined ribonucleotide sequence segments.

The dsRNA can be produced enzymatically or, fully or in parts, by chemical synthesis.

The double-stranded dsRNA structure can be formed starting from two complementary, separate RNA strands or - preferably - starting from a single, autocomplementary RNA strand.

In the case of a single, autocomplementary strand, sense and antisense sequence can be linked by a linking sequence (linker) and form for example a hairpin structure. Preferably, the linking sequence may be an intron, which is spliced out after the dsRNA has been synthesized.
The nucleic acid sequence encoding a dsRNA may comprise further elements such as, for example transcription termination signals or polyadenylation signals.

If the two strands of the dsRNA are to be combined in a cell or plant, this may take place in various ways, for example:

a) transformation of the cell or plant with a vector which comprises both expression cassettes,

b) cotransformation of the cell or plant with two vectors, where one comprises the expression cassettes with the sense strand and the other comprises the expression cassettes with the antisense strand.

c) hybridizing two plants, each of which has been transformed with a vector, where one comprises the expression cassettes with the sense strand and the other comprises the expression cassettes with the antisense strand.

The formation of the RNA duplex can be initiated either outside the cell or within the same. As in WO 99/53050, the dsRNA may also comprise a hairpin structure by linking sense and antisense strand by a linker (for example an intron). The autocomplementary dsRNA structures are preferred since they merely require the expression of a construct and comprise the complementary strands always in an equimolar ratio.

The expression cassettes encoding the antisense or sense strand of a dsRNA or the autocomplementary strand of the dsRNA are preferably inserted into a vector and, using the methods described hereinbelow, stably (for example using selection markers) inserted into the genome of a plant in order to ensure durable expression of the dsRNA.

The dsRNA can be introduced using such an amount that at least one copy per cell is made possible. Larger amounts (for example at least 5, 10, 100, 500 or 1000 copies per cell) may, if appropriate, bring about a more efficient reduction.

As already described, 100% sequence identity between dsRNA and an NADPH oxidase gene transcript or the gene transcript of a functionally equivalent gene is not necessarily required in order to bring about an efficient reduction of the NADPH oxidase expression. Accordingly, there is the advantage that the method tolerates sequence deviations, as may be present as the result of genetic mutations, polymorphisms or evolutionary divergences. Using
the dsRNA which has been generated starting from the NADPH oxidase sequence of an organism, it is thus, for example, possible to suppress the NAPDH oxidase expression in another organism. The high degree of sequence homology between the NADPH oxidase sequences from rice, maize and barley allows the conclusion that this protein is highly conserved within plants, so that the expression of a dsRNA derived from one of the NADPH oxidase sequences comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 probably also has an advantageous effect in other plant species.

Owing to the high degree of homology between the individual NADPH oxidase proteins and their functional equivalents, it is also possible to suppress the expression of further homologous NADPH oxidase proteins and/or their functional equivalents of the same organism or else the expression of NAPDH oxidase proteins in other related species, using a single dsRNA which has been generated starting from a specific NADPH oxidase sequence of an organism. For this purpose, the dsRNA preferably comprises sequence regions of NADPH oxidase gene transcripts which correspond to conserved regions. Said conserved regions can be deduced readily from sequence alignments.

The dsRNA can be synthesized either in vivo or in vitro. To this end, a DNA sequence encoding a dsRNA can be introduced into an expression cassette under the control of at least one genetic control element (such as, for example, promoter, enhancer, silencer, splice donor or acceptor, polyadenylation signal). Suitable advantageous constructions are described hereinbelow. A polyadenylation is not necessary, nor do elements for initiating a translation have to be present.

A dsRNA can be synthesized chemically or enzymatically. To this end, cellular RNA polymerases or bacteriophage RNA polymerases (such as, for example, T3, T7 or SP6 RNA polymerase) can be used. Such methods for the in-vitro expression of RNA are described (WO 97/32016; US 5,593,874; US 5,698,425; US 5,712,135, US 5,789,214, US 5,804,693). A dsRNA which has been synthesized in vitro, either chemically or enzymatically, can be isolated fully or in part from the reaction mixture, for example by extraction, precipitation, electrophoresis, chromatography or combination of these methods, before it is introduced into a cell, tissue or organism. The dsRNA can be introduced directly into the cell or else by applied extracellularly (for example into the interstitial space).
However, the plant is preferably transformed stably using an expression construct which brings about the expression of the dsRNA. Suitable methods are described hereinbelow.

5 b) Introduction of an NADPH oxidase antisense nucleic acid sequence

Methods for suppressing a particular protein by preventing the accumulation of its mRNA by antisense technology have been described many times, also in plants (Sheehy et al. (1988) Proc Natl Acad Sci USA 85: 8805-8809; US 4,801,340; Mol JN et al. (1990) FEBS Lett 268(2):427-430). The antisense nucleic acid molecule hybridized with, or binds to, the cellular mRNA and/or genomic DNA encoding the NADPH oxidase target protein to be suppressed, which suppresses the transcription and/or translation of the target protein. The hybridization can be brought about in the traditional manner via the formation of a stable duplex or - in the case of genomic DNA - by binding the antisense nucleic acid molecule with the duplex of the genomic DNA by specific interaction in the large groove of the DNA helix.

An antisense nucleic acid sequence suitable for reducing an NADPH oxidase protein can be derived using the nucleic acid sequence which encodes this protein, for example the nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21, following the Watson-Crick base pair rules. The antisense nucleic acid sequence can be complementary to all of the transcribed mRNA of said protein, may be limited to the coding region or may consist of one oligonucleotide only, which is complementary to part of the coding or noncoding sequence of the mRNA. Thus, the oligonucleotide may, for example, be complementary to the region which comprises the translation start for said protein. Antisense nucleic acid sequences can have a length of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides, but may also be longer and comprise at least 100, 200, 500, 1000, 2000 or 5000 nucleotides. Antisense nucleic acid sequences can be expressed recombinantly or synthetized chemically or enzymatically using methods known to the skilled worker. In the case of chemical synthesis, natural or modified nucleotides may be used. Modified nucleotides can impart an increased biochemical stability to the antisense nucleic acid sequence and may lead to an increased physical stability of the duplex formed of antisense nucleic acid sequence and sense target sequence. Nucleotides which can be used are, for example, phosphorothioate derivatives and acridine-substituted nucleotides such as 5-fluourouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthin, xanthin, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil,
5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, β-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, β-D-mannosylqueosine, 5′-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil and 2,6-diaminopurine.

In a further preferred embodiment, the expression of an NADPH oxidase protein can be inhibited by nucleotide sequences which are complementary to the regulatory region of an NADPH oxidase gene (for example an NADPH oxidase promoter and/or enhancer) and form triple-helical structures with the DNA double helix therein, so that the transcription of the NADPH oxidase gene is reduced.


In a further embodiment, the antisense nucleic acid molecule can be an α-anomeric nucleic acid. Such α-anomeric nucleic acid molecules form specific double-stranded hybrids with complementary RNA in which — in contrast to the conventional β-nucleic acids — the two strands are parallel to one another (Gautier C et al. (1987) Nucleic Acids Res 15:6625-6641). The antisense nucleic acid molecule can furthermore also comprise 2′-O-methylribonucleotides (Inoue et al. (1987) Nucleic Acids Res 15:6131-6148) or chimeric RNA-DNA analogs (Inoue et al. (1987) FEBS Lett 215:327-330).

c) Introduction of an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme

The above-described antisense strategy can advantageously be coupled with a ribozyme method. Catalytic RNA molecules or ribozymes can be adapted to any target RNA and cleave the phosphodiester backbone at specific positions, whereby the target DNA is functionally deactivated (Tanner NK (1999) FEBS Microbiol Rev 23(3):257-275). The ribozyme itself is not modified thereby, but is capable of cleaving further target RNA molecules in an analogous manner, whereby it gains the properties of an enzyme. The incorporation of ribozyme sequences into antisense RNAs imparts to these antisense-RNAs this enzyme-like, RNA-cleaving property
and thus increases their efficiency in the inactivation of the target RNA. The preparation and use of suitable ribozyme antisense RNA molecules is described for example by Haseloff et al. (1988) Nature 334:585-591.


d) Introducing an NADPH oxidase sense nucleic acid sequence for inducing a cosuppression

The expression of an NADPH oxidase nucleic acid sequence in sense orientation can lead to a cosuppression of the corresponding homologous, endogenous gene. The expression of sense RNA with homology to an endogenous gene can reduce or switch off the expression of same, similarly to what has been described for antisense approaches (Jorgensen et al. (1996) Plant Mol Biol 31(5):957-973; Goring et al. (1991) Proc Natl Acad Sci USA 88:1770-1774; Smith et al. (1990) Mol Gen Genet 224:447-481; Napoli et al. (1990) Plant Cell 2:279-289; Van der Krol et al. (1990) Plant Cell 2:291-99). Here, the introduced construct can fully or only partly represent the homologous gene to be reduced. The possibility of translation is not required. The application of this technology to plants is described for example by Napoli et al. (1990) The Plant Cell 2: 279-289 and in US 5,034,323.
Preferably, cosuppression is carried out using a sequence which is essentially identical to at least a part of the nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof, for example the nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21.

e) Introducing DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins


These factors may be selected using any desired portion of an NADPH oxidase gene. Preferably, this segment is located within the promoter region. For gene suppression, however, it may also be located in the region of the coding exons or introns. The corresponding segments are obtainable for the skilled worker by means of database search from the genetic library or - starting from an NADPH oxidase cDNA whose gene is not present in the genetic library, by screening a genomic library for corresponding genomic clones. The methods required for this purpose are known to the skilled worker.

Furthermore, it is possible to introduce, into a cell, factors which inhibit the NADPH oxidase target protein itself. The protein-binding factors may be for example aptamers (Famulok M and
Mayer G (1999) Curr Top Microbiol Immunol 243:123-36) or antibodies or antibody fragments or single-chain antibodies. The way in which these factors are obtained is described and known to the skilled worker. For example, a cytoplasmic scFv antibody has been employed for modulating the activity of the phytochrome A protein in genetically modified tobacco plants (Owen M et al. (1992) Biotechnology (N Y) 10(7):790-794; Franken E et al. (1997) Curr Opin Biotechnol 8(4):411-416; Whitelam (1996) Trend Plant Sci 1:286-272).


f) Introducing viral nucleic acid sequences and expression constructs which bring about the degradation of NADPH oxidase RNA

g) Introducing constructs for the induction of a homologous recombination on endogenous NADPH oxidase genes, for example for the generation of knock-out mutants.

5 To generate a homologously recombinant organism with reduced NADPH oxidase activity, for example a nucleic acid construct is used which comprises at least a part of the endogenous NADPH oxidase gene which is modified by a deletion, addition or substitution of at least one nucleotide in such a way that the functionality is reduced or nullified completely. The modification may also affect the regulatory elements (for example the promoter) of the gene, so that the coding sequence remains unaltered, but expression (transcription and/or translation) does not take place and is reduced.

15 In the case of conventional homologous recombination, the modified region is flanked at its 5' and 3' end by further nucleic acid sequences which must have a sufficient length for making possible the recombination. The length is, as a rule, in the range of from several hundred bases to several kilobases (Thomas KR and Capecchi MR (1987) Cell 51:503; Strepp et al. (1998) Proc Natl Acad Sci USA 95(8):4368-4373). For the homologous recombination, the host organism – for example a plant – is transformed with the recombination construct using the methods described hereinbelow, and clones which have undergone successful recombination are selected using, for example, an antibiotic or herbicide resistance.

Homologous recombination is a relatively rare event in higher eukaryotes, especially in plants. Random integrations into the host genome predominate. A possibility of removing the randomly integrated sequences and thus of enriching cell clones with a correct homologous recombination consists in using a sequence-specific recombination system as described in US 6,110,736, by which unspecifically integrated sequences can be deleted, which facilitates the selection of events which have integrated successfully via homologous recombination. A multiplicity of sequence-specific recombination systems can be used, examples which may be mentioned being the Cre/lox system of the bacteriophage P1, the FLP/FRT system of yeast, the Gin recombinase of the phage Mu, the Pin recombinase from E. coli and the R/RS system of the plasmid pSR1. Preferred are the bacteriophage P1 Cre/lox and the yeast FLP/FRT system. The FLP/FRT and cre/lox recombinase system has already been employed in plant systems (Odell et al. (1990) Mol Gen Genet 223: 369-378)
h) Introducing mutations into endogenous NADPH oxidase genes for generating a loss of function (for example generation of stop codons, reading frame shifts and the like)


The methods of dsRNAi, cosuppression by means of sense RNA and VIGS (virus-induced gene silencing) are also referred to as post-transcriptional gene silencing (PTGS). PTGS methods, including the reduction of the NADPH oxidase function or activity with dominant-negative NADPH oxidase variants are especially advantageous since the requirements to the homology between the endogenous gene to be suppressed and the transgenically expressed sense or dsRNA nucleic acid sequence (or between the endogenous gene and its dominant-negative variant, respectively) are lower than, for example in the case of a traditional antisense approach. Suitable homology criteria are mentioned in the description of the dsRNAi method and applied generally to PTGS methods or dominant-negative approaches. Owing to the high degree of homology between the NADPH oxidase proteins from maize, rice and barley, a high degree of conservation of these protein in plants can be deduced. Thus, using the NADPH oxidase nucleic acid sequences from barley, maize or rice, it is probably also possible efficiently to suppress the expression of homologous NADPH oxidase proteins in other species, without the isolation and structural elucidation of the NADPH oxidase homologs in these species being necessarily required. This substantially reduces the labor involved. Analogously, using dominant-negative variants of an NADPH oxidase protein from rice, maize or barley, it is presumably also possible efficiently to reduce or suppress the function/activity of its homolog in other plant species.

All substances and compounds which directly or indirectly bring about a reduction of the protein quantity, RNA quantity, gene activity or protein activity of an NADPH oxidase protein, shall hereinbelow be grouped together under the term "anti-NADPH oxi-
dase" compounds. The term "anti-NADPH oxidase" compound explicitly includes the nucleic acid sequences, peptides, proteins or other factors employed in the above-described methods.

For the purposes of the invention, "introduction" comprises all those methods which are suitable for introducing an anti-NADPH oxidase compound directly or indirectly into a plant or a cell, compartment, tissue, organ or seed thereof, or generating it therein. Direct and indirect methods are comprised. The introduction can lead to a transient presence of an anti-NADPH-oxidase compound (for example a dsRNA) or else to a stable presence.

In accordance with the different nature of the above-described approaches, the anti-NADPH-oxidase compound can exert its function directly (for example by insertion into an endogenous NADPH oxidase gene). However, the function can also be exerted indirectly after transcription into an RNA (for example in the case of antisense approaches) or after transcription and translation into a protein (for example binding factors). Both directly and indirectly acting anti-NADPH-oxidase compounds are comprised in accordance with the invention.

"Introducing" comprises for example methods such as transfection, transduction or transformation.

Thus, for example, anti-NADPH-oxidase compounds also comprise recombinant expression constructs which bring about an expression (i.e. transcription and, if appropriate, translation) of, for example, an NADPH oxidase dsRNA or an NADPH oxidase antisense RNA — preferably in a plant or a part, tissue, organ or seed thereof.

In said expression constructs, a nucleic acid molecule whose expression (transcription and, if appropriate, translation) generates an anti-NADPH-oxidase compound, is preferably in functional linkage with at least one genetic control element (for example a promoter) which ensures expression in an organism, preferably in plants. If the expression construct is to be introduced directly into the plant and the anti-NADPH-oxidase compound (for example the NADPH oxidase dsRNA) is to be generated therein in plantae, then plant-specific genetic control elements (for example promoters) are preferred. However, the anti-NADPH-oxidase compound can also be generated in other organisms or in vitro and then be introduced into the plant (as described in Example 6 and 7). Here, preferred control elements are all those prokaryotic or eu-

karyotic genetic control elements (for example promoters) which
permit the expression in the organism selected in each case for
the production.

Functional linkage is to be understood as meaning, for example,
the sequential arrangement of a promoter with the nucleic acid
sequence to be expressed (for example an anti-NAox compound) and, if
appropriate, further regulatory elements such as, for example, a
terminator in such a way that each of the regulatory elements
can fulfill its function when the nucleic acid sequence is
expressed recombinantly depending on the arrangement of the
nucleic acids into sense on antisense RNA. To this end, direct
linkage in the chemical sense is not necessarily required.
Genetic control sequences such as, for example, enhancer
sequences, can also exert their function on the target sequence
from positions which are further away, or indeed from other DNA
molecules. Preferred arrangements are those in which the nucleic
acid sequence to be expressed recombinantly is positioned behind
the sequence acting as promoter, so that the two sequences are
linked covalently to each other.

Here, the distance between the promoter sequence and the nucleic
acid sequence to be expressed recombinantly is less than 200 base
pairs, especially preferably less than 100 base pairs, very
especially preferably less than 50 base pairs.

Functional linkage, and an expression cassette, can be generated
by means of customary recombination and cloning techniques as are
described, for example, in Maniatis T, Fritsch EF and Sambrook J
Laboratory, Cold Spring Harbor (NY), in Silhavy TJ, Berman ML and
Harbor Laboratory, Cold Spring Harbor (NY), in Ausubel FM et al.
Assoc. and Wiley Interscience and in Gelvin et al. (1990) In:
Plant Molecular Biology Manual. However, further sequences which,
for example, act as a linker with specific cleavage sites for
restriction enzymes or as a signal peptide, may also be
positioned between the two sequences. The insertion of sequences
may also lead to the expression of fusion proteins. Preferably,
the expression cassette, consisting of a linkage of promoter and
nucleic acid sequence to be expressed, can exist in a
vector-integrated form and be inserted into a plant genome, for
example by transformation.

However, an expression cassette also denotes those constructions
in which a promoter is placed behind an endogenous NADPH oxidase
gene - for example by a homologous recombination - and the
reduction according to the invention, of an NADPH oxidase protein, is brought about by expressing an antisense NADPH oxidase RNA. Analogously, an anti-NADPH-oxidase compound (for example a nucleic acid compound encoding an NADPH oxidase dsRNA or an NADPH oxidase antisense RNA) can be placed behind an endogenous promoter in such a way that the same effect occurs. Both approaches lead to expression cassettes in the sense of the invention.

The term plant-specific promoters is understood as meaning, in principle, any promoter which is capable of governing the expression of genes, in particular foreign genes, in plants or plant parts, plant cells, plant tissues, or plant cultures. Here, expression may be, for example, constitutive, inducible or development-dependent.

The following are preferred:

a) Constitutive promoters

Preferred vectors are those which make possible a constitutive expression in plants (Benfey et al. (1989) EMBO J 8:2195-2202). "Constitutive" promoter is understood as meaning those promoters which ensure expression in a large number of, preferably all, tissues over a substantial period of plant development, preferably at all stages of plant development. In particular a plant promoter or a promoter derived from a plant virus are preferably used. Particularly preferred is the promoter of the CaMV cauliflower mosaic virus 35S transcript (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. (1986) Plant Mol Biol 6:221-228) or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202). Another suitable constitutive promoter is the Rubisco small subunit (SSU) promoter (US 4,962,028), the leguminB promoter (GenBank Acc. No. X03677), the Agrobacterium nopaline synthase promoter, the TR dual promoter, the Agrobacterium OCS (octopine synthase) promoter, the ubiquitin promoter (Holmorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the ATPase subunits or the promoter of a proline-rich protein from wheat (WO 91/13991), and further promoters of genes whose constitutive expression in plants is known to the skilled worker. Especially preferred as constitutive promoter is the promoter of the nitrilase-1 (nit1)

b) Tissue-specific promoters

Preferred are furthermore promoters with specificity for the anthers, ovaries, flower, leaves, stems, roots and seeds.

Seed-specific promoters comprise, for example, the phaseolin promoter (US 5,504,200; Bustos MM et al. (1989) Plant Cell 1(9):839-53), the 2S albumin promoter (Joseffson LG et al. (1987) J Biol Chem 262:12196-12201), the legumin promoter (Shirsat A et al. (1989) Mol Gen Genet 215(2): 326-331), the USP (unknown seed protein) promoter (Bäumlein H et al. (1991) Mol Gen Genet 225(3):459-467, the napin promoter (US 5,608,152; Stalberg K et al. (1996) L Planta 199:515-519), the sucrose binding protein promoter (WO 00/26388), the legumin B4 promoter (LeB4; Bäumlein H et al. (1991) Mol Gen Genet 225: 121-128; Bäumlein H et al. (1992) Plant J 2(2):233-239; Fiedler U et al. (1995) Biotechnology (NY) 13(10):1090f), the Arabidopsis oleosin promoter (WO 98/45461), the Brassica Bce4 promoter (WO 91/13980). Further suitable seed-specific promoters are those of the genes encoding the high-molecular-weight glutenin (HMWG), gliadin, branching enzyme, ADP glucose pyrophosphatase (AGPase) or starch synthase. Furthermore preferred promoters are those which permit seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. The following can be employed advantageously: the promoter of the lpt2 or lpt1 gene (WO 95/15389, WO 95/23230) or the promoters described in WO 99/16890 (promoters of the hordein gene, the glutelin gene, the cryzyn gene, the prolamin gene, the gliadin gene, the glutelin gene, the zein gene, the kasirin gene, or the secalin gene).

Tuber-, storage-root- or root-specific promoters comprise, for example, the class I patatin promoter (B33), the potato cathepsin D inhibitor promoter.

Leaf-specific promoters comprise the potato cytosolic FBPase promoter (WO 97/05900), the Rubisco (ribulose-1,5-bisphosphate carboxylase) SSU (small subunit) promoter or the ST-LSI promoter from potato (Stockhaus et al. (1989) EMBO J 8:2445-2451). Very especially preferred are epidermis-specific promoters such as, for example, the OXLP gene (oxalate-oxidase-like protein) promoter (Wei et al. (1998) Plant Mol Biol 36:101-112).
Flower-specific promoters comprise, for example, the phytoene synthase promoter (WO 92/16635) or the promoter of the P-rr gene (WO 98/22593).

Anther-specific promoters comprise, for example, the 5126 promoter (US 5,689,049, US 5,689,051), the glob-1 promoter and the γ-zein promoter.

c) Chemically inducible promoters

The expression cassettes can also comprise a chemically inducible promoter (review article: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108), by which the expression of the exogenous gene in the plant at a particular point in time can be controlled. Examples which may be mentioned are the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), a salicylic-acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic-acid-inducible promoter (EP 0 335 528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334).

d) Stress- or pathogen-inducible promoters

Further preferred promoters are those which are induced by biotic or abiotic stress such as, for example, the pathogen-inducible promoter of the PRP1 gene (or gst1 promoter), for example from potato (WO 96/28561; Ward et al. (1993) Plant Mol Biol 22:361-366), the tomato high-temperature-inducible hsp70 or hsp80 promoter (US 5,187,267), the potato low-temperature-inducible alpha-amylase promoter (WO 96/12814) or the light-inducible PPDK promoter. Further pathogen-inducible promoters comprise the flax Fis1 promoter (WO 96/34949), the Vst1 promoter (Schubert et al. (1997) Plant Mol Biol 34:417-426) and the tobacco EAS4 sesquiterpene cyclase promoter (US 6,100,451).


A source of further pathogen-inducible promoters is the PR gene family. A series of elements in these promoters has proved to be advantageous. Thus, the region -364 to -288 in the promoter of PR-2d mediates salicylate specificity (Buchel et al. (1996) Plant Mol Biol 30, 493-504). The sequence 5′-TCATCTTCTT-3′ occurs repeatedly in the promoter of the barley β-1,3-glucanase and in more than 30 further stress-induced genes. In tobacco, this region binds a nuclear protein whose abundance is increased by salicylate. The PR-1 promoters from tobacco and Arabidopsis (EP-A 0 332 104, WO 98/03536) are likewise suitable as pathogen-inducible promoters. Preferred, since especially specifically pathogen-induced, are the acidic PR-5 (aPR5) promoters from barley (Schweizer et al. (1997) Plant Physiol 114:79-88) and wheat (Rebmann et al. (1991) Plant Mol Biol 16:329-331). aPR5 proteins accumulate in approximately 4 to 6 hours after pathogen attack and show only very little background expression (WO 99/66057). An approach for achieving an increased pathogen-induced specificity is the generation of synthetic promoters from combinations of known pathogen-responsive elements (Rushton et al. (2002) Plant Cell 14, 749-762; WO 00/01830; WO 99/66057). Further pathogen-inducible promoters from different species are known to the skilled worker (EP-A 1 165 794; EP-A 1 062 356; EP-A 1 041 148; EP-A 1 032 684).

e) Development-dependent promoters

Further suitable promoters are, for example, fruit-maturation-specific promoters such as, for example, the tomato fruit-maturation-specific promoter (WO 94/21794, EP 409 625). Development-dependent promoters comprise partly the tissue-specific promoters since individual tissues develop by nature in a development-dependent fashion.
Especially preferred are constitutive promoters and also leaf- and/or stem-specific, pathogen-inducible and epidermis-specific promoters, with pathogen-inducible and epidermis-specific promoters being most preferred.

Furthermore, further promoters may be linked functionally to the nucleic acid sequence to be expressed, which promoters make possible an expression in further plant tissues or in other organisms, such as, for example, E. coli bacteria. Suitable plant promoters are, in principle, all of the above-described promoters.

The nucleic acid sequences present in the expression cassettes according to the invention can be linked operably to further genetic control sequences in addition to a promoter. The term “genetic control sequences” is to be understood in the broad sense and refers to all those sequences which have an effect on the generation or the function of the expression cassette according to the invention. For example, genetic control sequences modify the transcription and translation in prokaryotic or eukaryotic organisms. Preferably, the expression cassette according to the invention comprise the promoter with specificity for the embryonal epidermis and/or the flower 5′-upstream of the nucleic acid sequence in question to be expressed recombinantly, and 3′-downstream a terminator sequence as additional genetic control sequence and, if appropriate, further customary regulatory elements, in each case linked functionally to the nucleic acid sequence to be expressed recombinantly.

Genetic control sequences also comprise further promoters, promoter elements or minimal promoters, all of which can modify the expression-governing properties. Thus, for example, the tissue-specific expression may additionally depend on certain stress factors, owing to genetic control sequences. Such elements have been described, for example, for water stress, abscisic acid (Lam E and Chua NH (1991) J Biol Chem 266(26): 17131-17135) and heat stress (Schoffl F et al. (1989) Mol Gen Genetics 217(2-3):246-53).

In principle, all natural promoters together with their regulatory sequences such as those mentioned above may be used for the method according to the invention. In addition, synthetic promoters may also be used advantageously.

Genetic control sequences furthermore also comprise the 5′-untranslated regions, introns or noncoding 3′-region of genes, such as, for example, the actin-1 intron, or the Adh1-S introns.
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1, 2 and 6 (general reference: The Maize Handbook, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)). It has been demonstrated that they may play a significant role in the regulation of gene expression. Thus, it has been demonstrated that 5′-untranslated sequences can enhance the transient expression of heterologous genes. Examples of translation enhancers which may be mentioned are the tobacco mosaic virus 5′ leader sequence (Gallie et al. (1987) Nucl Acids Res 15:8693-8711) and the like. Furthermore, they may promote tissue specificity (Rouster J et al. (1998) Plant J 15:435-440).

The expression cassette may advantageously comprise one or more of what are known as enhancer sequences, linked functionally to the promoter, which make possible an increased recombinant expression of the nucleic acid sequence. Additional advantageous sequences, such as further regulatory elements or terminators, may also be inserted at the 3′ end of the nucleic acid sequences to be expressed recombinantly. One or more copies of the nucleic acid sequences to be expressed recombinantly may be present in the gene construct.

Polyadenylation signals which are suitable as control sequences are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from Agrobacterium tumefaciens, in particular to gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACHS (Gielen et al. (1984) EMBO J 3:835 et seq.) or functional equivalents thereof. Examples of terminator sequences which are especially suitable are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

Control sequences are furthermore to be understood as those which make possible homologous recombination or insertion into the genome of a host organism or which permit removal from the genome. Upon homologous recombination, for example the natural promoter of a particular gene can be exchanged to a promoter with specificity for the embryonal epidermis and/or the flower. Methods such as the cre/lox technology permit a tissue-specific, if appropriate inducible, removal of the expression cassette from the genome of the host organism (Sauer B (1998) Methods. 14(4):381-92). Here, certain flanking sequences (lox sequences), which later make possible a removal by means of the cre recombinase, are added to the target gene.

An expression cassette and vectors derived therefrom may comprise further functional elements. The term functional element is to be understood in the broad sense and refers to all those elements
which have an effect on the generation, amplification or function of the expression cassettes, vectors or transgenic organisms according to the invention. The following may be mentioned by way of example, but not by limitation:

a) Selection markers which confer a resistance to metabolism inhibitors (such as 2-deoxyglucose-6-phosphate (WO 98/45456)), antibiotics or biocides, preferably herbicides, such as, for example, kanamycin, G 418, bleomycin, hygromycin or phosphinothricin etc. Especially preferred selection markers are those which confer resistance to herbicides. Examples which may be mentioned are: DNA sequences which encode phosphinothricin acetyl transferases (PAT) and which inactivate glutamin synthase inhibitors (bar and pat genes), 5-enolpyruvylshikimate-3-phosphate synthase genes (EPSP synthase genes), which confer resistance to Glyphosate® (N-(phosphonomethyl)glycine), the gox gene, which encodes Glyphosate®-degrading enzymes (glyphosate oxidoreductase), the deh gene (encoding a dehalogenase which inactivates Dalapon), sulfonylurea- and imidazolinone-inactivating acetolactate synthases, and bxm genes, which encode bromoxynil-degrading nitrilase enzymes, the aasa gene, which confers resistance to the antibiotic apectinomycin, the streptomycin phosphotransferase (spt) gene, which allows resistance to streptomycin, the neomycin phosphotransferase (nptII) gene, which confers resistance to kanamycin or geneticin, the hygromycin phosphotransferase (hpt) gene, which mediates resistance to hygromycin, the acetolactate synthase gene (ALS), which confers resistance to sulfonylurea herbicides (for example mutated ALS variants with, for example, the S4 and/or Hra mutation).


c) Origins of replication, which ensure amplification of the expression cassettes or vectors according to the invention in, for example, E. coli. Examples which may be mentioned are ORI (origin of DNA replication) the pBR322 ori or the P15A ori (Sambrook et al.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

d) Elements which are necessary for Agrobacterium-mediated plant transformation, such as, for example, the right or left border of the T-DNA or the vir region.

The introduction of an expression cassette according to the invention into an organism or cells, tissues, organs, parts or seeds thereof (preferably into plants or plant cells, tissue, organs, parts or seeds) can be effected advantageously using vectors which comprise the expression cassettes. The expression cassette can be introduced into the vector (for example a plasmid vector) via a suitable restriction cleavage site. The plasmid formed is first introduced into E. coli. Correctly transformed E. coli are selected, grown, and the recombinant plasmid is obtained by the methods familiar to the skilled worker. Restriction analysis and sequencing may serve to verify the cloning step.

Examples of vectors may be plasmids, cosmids, phages, viruses or else agrobacteria. In an advantageous embodiment, the expression cassette is introduced by means of plasmid vectors. Preferred vectors are those which make possible a stable integration of the expression cassette into the host genome.

The generation of a transformed organism (or of a transformed cell or tissue) requires that the DNA, RNA or protein in question is introduced into the corresponding host cell.
A multiplicity of methods are available for this procedure, which is termed transformation (or transduction or transfection) (Keown et al. (1990) Methods in Enzymology 185:527-537). For example, the DNA or RNA can be introduced directly by microinjection or by bombardment with DNA-coated microparticles. Also, the cell can be permeabilized chemically, for example using polyethylene glycol, so that the DNA can enter the cell by diffusion. The DNA can also be introduced by protoplast fusion with other DNA-containing units such as minicells, cells, lysosomes or liposomes. Another suitable method of introducing DNA is electroporation, where the cells are permeabilized reversibly by an electrical pulse. Suitable methods have been described (for example by Bilang et al. (1991) Gene 100:247-250; Scheid et al. (1991) Mol Gen Genet 228:104-112; Guerche et al. (1987) Plant Science 52:111-116; Neuhause et al. (1987) Theor Appl Genet 75:30-36; Klein et al. (1987) Nature 327:70-73; Howell et al. (1980) Science 208:1265; Horsch et al. (1985) Science 227:1229-1231; DeBlock et al. (1989) Plant Physiology 91:694-701; Methods for Plant Molecular Biology (Weissbach and Weissbach, eds.) Academic Press Inc. (1988); and Methods in Plant Molecular Biology (Schuler and Zielinski, eds.) Academic Press Inc. (1989)).

In plants, the above-described methods of transforming and regenerating plants from plant tissues or plant cells are exploited for transient or stable transformation. Suitable methods are especially protoplast transformation by polyethylene-glycol-induced DNA uptake, the biolistic method with the gene gun, what is known as the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, and microinjection.

In addition to these "direct" transformation techniques, transformation can also be effected by bacterial infection by means of Agrobacterium tumefaciens or Agrobacterium rhizogenes. The Agrobacterium-mediated transformation is best suited to dicotyledonous plant cells. The methods are described, for example, by Horsch RB et al. (1985) Science 225: 1229f.

When agrobacteria are used, the expression cassette must be integrated into specific plasmids, either into a shuttle or intermediate vector, or into a binary vector. If a Ti or Ri plasmid is to be used for the transformation, at least the right border, but in most cases the right and left border, of the Ti or Ri plasmid T-DNA is linked to the transgenic expression construct to be introduced in the form of a flanking region.
Binary vectors are preferably used. Binary vectors are capable of replication both in E. coli and in Agrobacterium. As a rule, they comprise a selection marker gene for the selection of transformed plants (see above) and a linker or polylinker flanked by the right and left T-DNA border sequence. They can be transformed directly into Agrobacterium (Holsters et al. (1978) Mol Gen Genet 163:181-187). Apart from the T-DNA region, they can additionally comprise elements such as a selection marker gene for the selection of transformed E. coli or agrobacteria (e.g. the nptIII gene). The Agrobacterium which acts as host organism in this case should already contain a plasmid with the vir region. The latter is required for transferring the T-DNA to the plant cell. An Agrobacterium transformed in this way can be used for transforming plant cells. The use of T-DNA for transforming plant cells has been studied and described intensively (EP 120 516; Hoekema, In: The Binary Plant Vector System, Osssetdrukkerij Kanters B.V., Alblaserdam, Chapter V; An et al. (1985) EMBO J 4:277-287). Various binary vectors are known, some of which are commercially available such as, for example, pBII01.2 or pBIN19 (Clontech Laboratories, Inc. USA).


Direct transformation techniques are suitable for any organism and cell type.

The plasmid used need not meet any particular requirements in the case of the injection or electroporation of DNA or RNA into plant cells. Simple plasmids such as those of the pUC series can be used. If complete plants are to be regenerated from the transformed cells, it is advantageous for an additional selectable marker gene to be located on the plasmid.

Stably transformed cells, i.e. those which contain the introduced DNA integrated into the DNA of the host cell, can be selected from untransformed cells when a selectable marker is part of the DNA introduced. Examples of genes which can act as markers are all those which are capable of conferring resistance to a biocide (for example an antibiotic, herbicide or a metabolism inhibitor such as 2-deoxyglucose-6-phosphate WO 98/45456) (see above). Transformed cells which express such marker genes are capable of surviving in the presence of concentrations of a corresponding antibiotic or herbicide which kill an untransformed wild type. Examples are mentioned above and preferably comprise the bar
gene, which confers resistance to the herbicide phosphinothricin (Rathore RS et al. (1993) Plant Mol Biol 21(5):871-884), the nptII gene, which confers resistance to kanamycin, the hpt gene, which confers resistance to hygromycin, or the EPSP gene, which confers resistance to the herbicide glyphosate. The selection marker permits the selection of transformed cells from untransformed cells (McCormick et al. (1986) Plant Cell Reports 5:81-84). The resulting plants can be bred and hybridized in the customary fashion. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary.


As soon as a transformed plant cell has been generated, a complete plant can be obtained using methods known to the skilled worker. For example, callus cultures are used as starting material. The development of shoot and root can be induced from this as yet undifferentiated cell biomass in a known fashion. The shoots obtained can be planted out and bred.


The method according to the invention can advantageously be combined with further methods which bring about a pathogen resistance (for example against insects, fungi, bacteria, nematodes and the like), stress resistance or another improvement of the plant's properties. Examples are mentioned in Dunwell JM, Transgenic approaches to crop improvement, J Exp Bot. 2000;51 Spec No; pages 487-96, inter alia.

With regard to, for example, a nucleic acid sequence, an expression cassette or a vector comprising said nucleic acid sequence or an organism transformed with said nucleic acid sequence, expression cassette or vector, "transgenic" means all those
constructs which have been generated by recombinant methods in which either

a) the NADPH oxidase nucleic acid sequence, or

b) a genetic control sequence which is functionally linked with the NADPH oxidase nucleic acid sequence, for example a promoter, or

c) (a) and (b)

are not located in their natural genetic environment or have been modified by recombinant methods, an example of a modification being a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment refers to the natural chromosomal locus in the source organism, or to the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably at least partially retained. The environment flanks the nucleic acid sequence at at least one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, very especially preferably at least 5000 bp. A naturally occurring expression cassette - for example the naturally occurring combination of the NADPH oxidase promoter and the corresponding NADPH oxidase gene - becomes a transgenic expression cassette when the latter is modified by nonnatural, synthetic ("artificial") methods such as, for example, mutagenization. Suitable methods are described (US 5,565,350; WO 00/15815; see also above).

Another aspect of the invention relates to transgenic organisms transformed with at least one nucleic acid sequence, expression cassette or vector according to the invention, and to cells, cell cultures, tissues, parts - such as, for example in the case of plant organisms, leaves, roots and the like - or propagation material derived from such organisms. Organism is to be understood in the broad sense and means prokaryotic and eukaryotic organisms, preferably bacteria, yeasts, fungi, animal and plant organisms.

The following are preferred:

a) fungi such as Aspergillus, Eremothecium, Trichoderma, Ashbya, Neurospora, Fusarium, Beauveria or further fungi described in Indian Chem Eng. Section B. Vol 37, No 1,2 (1995) on page 15,
table 6. Especially preferred is the filamentous Hemiascomycete Ashbya gossypii or Eremothecium ashbyii,

b) yeasts such as Candida, Saccharomyces, Hansenula or Pichia, with Saccharomyces cerevisiae or Pichia pastoris (ATCC Accession No. 201178) being especially preferred,

c) plants in accordance with the abovementioned definition for "plants",

d) vertebrates and invertebrates. Especially preferred vertebrates are nonhuman mammals such as dogs, cats, sheep, goats, chickens, mice, rats, cattle or horses. Preferred animal cells comprise CHO, COS, HEK293 cells. Preferred invertebrates comprise insect cells such as Drosophila S2 and Spodoptera Sf9 or Sf21 cells,

e) prokaryotic organisms such as Gram-positive or Gram-negative bacteria such as Acetobacter, Gluconobacter, Corynebacterium, Brevibacterium, Bacillus, Clostridium, Cyanobacterium, Escherichia (especially Escherichia coli), Serratia, Staphylococcus, Aerobacter, Alcaligenes, Penicillium or Klebsiella.

Host or starting organisms which are preferred as transgenic organisms are especially plants in accordance with the abovementioned definition. Included within the scope of the invention are all genera and species of higher and lower plants of the Plant Kingdom. Furthermore included are the mature plants, seeds, shoots and seedlings, and parts, propagation materials and culture derived from them, for example cell cultures. Mature plants means plants at any developmental stage beyond the seedling stage. Seedling means a young immature plant in an early developmental stage. Plants which are especially preferred as host organisms are plants to which the method according to the invention for obtaining a pathogen resistance in accordance with the abovementioned criteria can be applied. Very especially preferred are monocotyledonous plants such as wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed, sugar cane, and dicotyledonous crop plants such as oilseed rape, canola, cress, Arabidopsis, cabbages, soybeans, alfalfa, pea, bean plants, peanut, potato, tobacco, tomato, egg plant, capsicum, sunflower, tagetes, lettuce, Calendula, melon, pumpkin/squash or zucchini.
The transgenic organisms can be generated with the above-described methods for the transformation or transfection of organisms.

5 A further aspect of the invention relates to the use of transgenic organisms according to the invention and of the cells, cell cultures, parts - such as for example in the case of transgenic plant organisms roots, leaves and the like - and transgenic propagation material such as seeds or fruits derived from these organisms for the production of foodstuffs, feedstuffs, pharmaceuticals or fine chemicals.

Furthermore preferred is a method for the recombinant production of pharmaceuticals or fine chemicals in host organisms, where a host organism is transformed with one of the above-described expression cassettes and this expression cassette comprises one or more structural genes which encode the desired fine chemical or catalyze the biosynthesis of the desired fine chemical, the transformed host organism is cultured, and the desired fine chemical is isolated from the culture medium. This method can be applied widely for fine chemicals such as enzymes, vitamins, amino acids, sugars, fatty acids, natural and synthetic flavorings, aroma substances and colorants. Especially preferred is the production of tocopherols and tocotrienols and of carotenoids. Culturing the transformed host organisms, and the isolation from the host organisms or from the culture medium, are carried out with methods known to the skilled worker. The production of pharmaceuticals, such as, for example, antibodies or vaccines, is described by Hood EE, Jilka JM (1999) Curr Opin Biotechnol 10(4):382-6; Ma JK, Vine ND (1999) Curr Top Microbiol Immunol 236:275-92.

Sequences

35 1. SEQ ID NO: 1 nucleic acid sequence encoding a barley (Hordeum vulgare) NADPH oxidase.

2. SEQ ID NO: 2 amino acid sequence encoding a barley (Hordeum vulgare) NADPH oxidase.

3. SEQ ID NO: 3 nucleic acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase
4. SEQ ID NO: 4 amino acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase

5. SEQ ID NO: 5 nucleic acid sequence encoding a Nicotiana tabacum NADPH oxidase

6. SEQ ID NO: 6 amino acid sequence encoding a Nicotiana tabacum NADPH oxidase

7. SEQ ID NO: 7 nucleic acid sequence encoding a potato (Solanum tuberosum) NADPH oxidase

8. SEQ ID NO: 8 amino acid sequence encoding a potato (Solanum tuberosum) NADPH oxidase

9. SEQ ID NO: 9 nucleic acid sequence encoding a tomato (Lycopersicon esculentum) NADPH oxidase

10. SEQ ID NO: 10 amino acid sequence encoding a tomato (Lycopersicon esculentum) NADPH oxidase

11. SEQ ID NO: 11 nucleic acid sequence encoding a NADPH oxidase aus Arabidopsis thaliana (RbohF)

12. SEQ ID NO: 12 amino acid sequence encoding a NADPH oxidase aus NADPH oxidase Arabidopsis thaliana (RbohF)

13. SEQ ID NO: 13 nucleic acid sequence encoding an Arabidopsis thaliana (RbohD) NADPH oxidase

14. SEQ ID NO: 14 amino acid sequence encoding an Arabidopsis thaliana (RbohD) NADPH oxidase

15. SEQ ID NO: 15 nucleic acid sequence encoding a Nicotiana tabacum (rboh) NADPH oxidase

16. SEQ ID NO: 16 amino acid sequence encoding a Nicotiana tabacum (rboh) NADPH oxidase
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17. SEQ ID NO: 17 nucleic acid sequence encoding a
rice (Oryza sativa var. japonica)
NADPH oxidase

5 18. SEQ ID NO: 18 amino acid sequence encoding a
rice (Oryza sativa var. japonica)
NADPH oxidase

19. SEQ ID NO: 19 nucleic acid sequence encoding an
Arabidopsis thaliana (RbohC) NADPH oxidase

20. SEQ ID NO: 20 amino acid sequence encoding an
Arabidopsis thaliana (RbohC) NADPH oxidase

15 21. SEQ ID NO: 21 nucleic acid sequence encoding an
Arabidopsis thaliana (RbohA) NADPH oxidase

22. SEQ ID NO: 22 amino acid sequence encoding an
Arabidopsis thaliana (RbohA) NADPH oxidase

20 23. SEQ ID NO: 23 oligonucleotide primer 5’ NAOX
5’-GARCAAGGCTCTTTTGATTG-3’

24. SEQ ID NO: 24 oligonucleotide primer 3’ Naox
5’-GAAATGCTCCTTATGGAATTG-3’

Figures

Fig. 1: RNA interference with pNAox-dsRNA reduces the penetration
efficiency of powdery mildew of barley BghA6 in barley.

The relative penetration efficiency (RPE) was determined in five
individual experiments with inoculation with Bgh from barley cv
Pallas. The RPE is calculated as the difference between the pene-
tration efficiency of pNAox-dsRNA-transformed cells and the pene-
tration efficiency of control-dsRNA-transformed cells (here: aver-
age penetration efficiency 38.74%). The percent RPE (% RPE) is
calculated from the RPE minus 1, multiplied by 100.

\[ \text{RPE} = \frac{\text{PE in pNAox-dsRNA-transformed cells}}{\text{PE in control-dsRNA transformed cells}} \]

\[ \% \text{ RPE} = 100 \times (\text{RPE}-1) \]

The columns (1) to (5) represent the % RPE (i.e. the deviation of
the penetration efficiency from the average of the penetration
efficiency of the control) when evaluating at least 100 interac-
tion sites for in each case one independent experiment. The column (m) represents the average % RPE of the experiments. The error bar indicates the standard error.

"Control dsRNA" represents the parallel experiments with a control dsRNA. "pNAox" dsRNA represents the experiments with the dsRNA of the barley NADPH oxidase.

In cells which have been bombarded with pNAox-dsRNA, the % RPE was markedly (significance p=0.0054) reduced in comparison with cells bombarded with a control dsRNA (TR: human thyroid receptor dsRNA).

Examples

General methods:

The chemical synthesis of oligonucleotides can be effected for example in the known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The cloning steps carried out within the scope of the present invention, such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of E. coli cells, bacterial cultures, phage multiplication and sequence analysis of recombinant DNA, are carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6. Recombinant DNA molecules are sequenced with a laser fluorescence DNA sequencer from MWG Licor following the method of Sanger (Sanger et al. (1977) Proc Natl Acad Sci USA 74:5463-5467).

Example 1: Plants, pathogens and inoculation

The variety Pallas was provided by Lisa Munk, Department of Plant Pathology, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Its production is described (Kölster P et al. (1986) Crop Sci 26: 903-907).

Unless otherwise described, the seed, which had been pregerminated for 12 to 36 hours in the dark on damp filter paper, was placed at a rate of 5 kernels along the edge of a square pot (8 × 8 cm) in Fruehstorfer soil, type P, covered with soil and watered regularly with tap water. All plants were grown in controlled-environment cabinets or chambers at 16 to 18°C, 50 to 60% relative atmospheric humidity and a 16-hour-light/8-hour-dark photoperiod at 3000 or 5000 lux (photon flux density 50 or
60 μmol·m⁻² for 5 to 8 days and used in the experiments during the seedling stage. In experiments in which primary leaves were treated, the latter were fully developed.

Prior to carrying out the transient transfection experiments, the plants were grown in controlled environment cabinets or chambers at 24°C daytime temperature, 20°C nighttime temperature, 50 to 60% relative atmospheric humidity and a 16-hour-light/8-hour-dark photoperiod at 30 000 lux.

Powdery mildew of barley Blumeria graminis (DC) Speer f.sp. hordae Em. Marchal race A6 (Wiberg A (1974) Hereditas 77: 89-148) (BghA6) was used for the inoculation of barley plants. The fungus was provided from the Department of Biometry, JLU Gießen. The inoculum was propagated in controlled-environment chambers under identical conditions as described above for the plants by transferring the conidia of infected material to regularly grown 7-day-old barley plants cv. Golden Promise at a density of 100 conidia/mm².

The inoculation with BghA6 was carried out using 7-day-old seedlings by shaking off the conidia from infected plants in an inoculation tower at approximately 100 conidia/mm² (unless otherwise specified).

Example 2: Cloning of the barley pNAox cDNA sequence

The cDNA fragments required for the isolation of the HvpNAox cDNA, its cloning, sequencing and generation of probes were obtained by RT-PCR using the "One Step RT-PCR Kit" (Life Technologies, Karlsruhe, Germany, or Qiagen, Hilden, Germany). To this end, a total RNA from barley seedlings was used as template. The RNA was isolated from Pallas 3, 5 and 7 days after germination. In addition, RNA was isolated from Pallas and from the backcrossed lines with mlo5, Mlg or Mla12 1, 2 and 5 days after inoculation with BghA6 on day 7 after germination. The RT-PCR was carried out using primers which are derived from conserved regions of the gp91phox homologs from rice and Arabidopsis thaliana (GenBank Acc. No.: X93301 and AB008111):

5' NAOX: 5'-GARCAAGGCTCTTTTGTATTG-3' (SEQ ID NO: 23) and

3' NAOX: 5' GAAATGCTCTTTATGGAATTG 3' (SEQ ID NO: 24)
In each case 1000 ng of total DNA, 0.4 mM dNTPs, in each case 0.6 mM OPN-1 and OPN-2 primer, 10 µl of RNase inhibitor and 1 µl of enzyme mix in 1x RT buffer (one step RT-PCR Kit, Qiagen, Hilden) were employed for the reaction.

The following temperature program is used (PTC-100™ model 96V; MJ Research, Inc., Watertown, Massachusetts):

1 cycle of 30 minutes at 50°C
1 cycle of 150 seconds at 94°C
30 cycles of 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes
1 cycle of 72°C for 7 minutes

The PCR products were separated by means of 2% w/v agarose gel electrophoresis. This gave a 378 bp RT-PCR product (SEQ ID NO: 1) which encodes a part of the open reading frame of the barley NADPH oxidase. The corresponding cDNA was isolated from an agarose gel and cloned in the pGEM-T vector (Promega, Mannheim, Germany) by means of T-overhang ligation. The cDNAs were sequenced starting from the plasmid DNA using the “Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit” (Amersham, Freiburg, Germany). The construct was named pGEM-T-pNAox.

Example 3: In-vitro synthesis of the pNAox dsRNA

The plasmid, which had been employed for the in-vitro RNA transcription, comprises the T7 and SP6 promoters at the respective ends of the inserted nucleic acid sequence, which makes possible the synthesis of sense RNA and antisense RNA. The plasmid can be linearized with suitable restriction enzymes (ApaI for SP6 polymerase and PstI for T7 polymerase) in order to ensure correct transcription of the inserted nucleic acid sequence and to prevent read-through into vectorial sequences. To this end, in each case 10 µg of pGEM-T-pNAox plasmid DNA were cut with ApaI for SP6 polymerase and with and PstI for T7 polymerase. The cut plasmids are extracted in 200 µl of water with the same volume phenol/chloroform/isooamy1 alcohol, transferred into a fresh Eppendorf vessel (RNAase-free) and centrifuged for 5 minutes at 20 000 g.

180 µl of the plasmid solution were treated with 420 µl of ethanol, placed on ice and subsequently precipitated by centrifugation for 30 minutes at 20 000 g and -4°C. The precipitate was taken up in 10 µl of TE buffer. The preparations in question were employed directly in an in-vitro transcription with T7-RNA polymerase and with SP6-RNA polymerase, respectively. RNA polymerases were obtained from Roche Molecular Biology, Mannheim, Germany.
Each transcription mixture contained the following in a volume of 40 μl:

2 μl linearized plasmid DNA (1 μg)
2 μl NTPs (25 mM) (1.25 mM of each NTP)
4 μl 10x reaction buffer (Roche Molecular Biology),
1 μl RNAsin RNAsin (27 units; Roche Molecular Biology),
2 μl RNA polymerase (40 units)
29 μl DEPC water

After 2 hours of incubation at 37°C, in each case some of the reaction mixtures from the transcription of the sense and antisense strands were mixed, denatured for 5 minutes at 95°C and thereafter hybridized with one another (annealed) by cooling over 30 minutes to a final temperature of 37°C. As an alternative, the mixture of sense and antisense strand can also be cooled for 30 minutes at -20°C after the denaturation. The protein precipitate which formed during denaturation and hybridization was removed by briefly centrifuging at 20,800 g, and the supernatant was used directly for coating tungsten particles (see hereinbelow). For the analysis, in each case 1 μl of each RNA strand and of the dsRNA were separated on a non-denaturing agarose gel. Successful hybridization is evident by a band shift towards higher molecular weight in comparison with the individual strands.

4 μl of the dsRNA were precipitated with ethanol (by addition of 6 μl of water, 1 μl of 3M sodium acetate solution and 25 μl of ethanol, and centrifugation for at least 5 minutes at 20,000 g and 4°C) and resuspended in 500 μl of water. The absorption spectrum between 230 and 300 nm was measured or the absorption at 280 and 260 nm was determined to determine the purity and the concentration of the dsRNA. As a rule, 80 to 100 μg of dsRNA with an OD260/OD280 ratio of 1.80 to 1.95 were obtained. If desired, a digestion with DNase I may be carried out, but this has no substantial effect on subsequent results.

The dsRNA of the human thyroid receptor (starting vector pT7betaSal (Norman C et al. (1988) Cell 55(6):989-1003), provided by Dr. Baniahmad, Department of Genetics, Gießen, Germany; the sequence of the insert is described under the GenBank Acc. No.: NM_000461) acted as control dsRNA. The plasmid was digested with PvuII to generate the sense RNA and with HindIII to generate the antisense RNA, and the RNA was then transcribed using T7 or SP6 RNA polymerase. The individual process steps for the generation of the control dsRNA are carried out analogously to those described above for the pNAox-dsRNA.
Example 4: Transient transformation, RNAi and evaluation of the
development of the fungal pathogen

Barley cv Pallas leaf segments were transformed with a pNAox
dsRNA together with a GFP expression vector. Thereafter the leaves
were inoculated with Bgh and the result was analyzed after 48 h
by means of light and fluorescence microscopy. The penetration
into GFP-expressing cells was assessed by detecting haustoria in
live cells and by assessing the fungal development in precisely
those cells. In all five experiments, the bombardment of barley
cv Pallas with pNAox dsRNA resulted in a reduced number of cells
which were successfully penetrated by Bgh in comparison with
cells which had been bombarded with foreign control dsRNA (human
thyroid hormone receptor dsRNA, TR). The resistance-inducing ef-
fect of the pNAox dsRNA resulted in an average reduction of the
Bgh penetration efficiency by 35% (Fig. 4).

A method which had already been described for the biolistic
introduction of dsRNA into epidermal cells of barley leaves was
employed for the transient transformation (Schweizer P et al.
diameter (particle density 25 mg/ml) were coated with dsRNA
(preparation see above) together with plasmid DNA of the vector
pgFP (GFP under the control of the CaMV 35S promoters) as trans-
formation marker. To this end, the following amounts of dsRNA and
reporter plasmid were used for the coating per shot: 1 μg pgFP
and 2 μg dsRNA. Double-stranded RNA was synthesized by annealing
sense and antisense RNA in vitro (see above).

To prepare microcarriers, 55 mg of tungsten particles (M 17, di-
ameter 1.1 μm; Bio-Rad, Munich) were washed twice with 1 ml of
autoclave-distilled water and once with 1 ml of absolute ethanol,
dried and taken up in 1 ml of 50% strength glycerol (approximate-
ly 50 mg/ml stock solution). The solution was diluted with 50%
glycerol to 25 mg/ml, mixed thoroughly prior to use and suspended
in an ultrasonic bath. To coat microcarriers, 1 μg of plasmid,
2 μg of dsRNA (1 μl), 12.5 μl of tungsten particle suspension
(25 mg/ml), 12.5 μl of 1 M Ca(NO3)2 solution (pH 10) per shot were
combined dropwise with constant mixing, left to stand for 10 min-
utes at RT, centrifuged briefly, and 20 μl of the supernatant
were removed. The remainder with the tungsten particles is resus-
spended (ultrasonic bath) and employed in the experiment.

Barley primary leaf segments approximately 4 cm in length were
used. The tissues were placed on 0.5% Phytagar (GibcoBRL™ Life
Technologies™, Karlsruhe) supplemented with 20 μg/ml benzimida-
zole in Petri dishes (diameter 6.5 cm) and, immediately before the particle bombardment, the edges were covered with a stencil with a rectangular opening of dimensions 2.2 cm × 2.3 cm. One after the other, the dishes were placed on the bottom of the vacuum chamber (Schweizer P et al. (1999) Mol Plant Microbe Interact 12:647-54) over which a nylon mesh (mesh size 0.2 mm, Millipore, Eschborn) had been inserted on a perforated sheet to act as diffusor (5 cm above the bottom, 11 cm underneath the macrocarriers, see hereinbelow) in order to diffuse particle clumps and to slow down the particle stream. The macrocarrier attached at the top of the chamber (plastic sterile filter holder, 13 mm, Gelman Sciences, Swinney, UK) was loaded with 5.8 μl of DNA-coated tungsten particles per shot (microcarriers, see hereinbelow). Using a diaphragm vacuum pump (Vacuubrand, Wertheim), the pressure in the chamber was reduced by 0.9 bar, and the tungsten particles were fired at the surface of the plant tissue at a helium-gas pressure of 9 bar. Immediately thereafter, the chamber was aerated. To label transformed cells, the leaves were bombarded with the plasmid (pGFP; vector on pUC18-basis, CaMV 35S promoter/terminator cassette with inserted GFP gene; Schweizer P et al. (1999) Mol Plant Microbe Interact 12:647-54; provided by Dr. P. Schweizer, Department of Plant Genetics IPK, Gatersleben, Germany). Each time a different plasmid was used for the bombardments, the macrocarrier was cleaned thoroughly with water beforehand. After incubation for four hours after the bombardment with slightly open Petri dishes at RT and with daylight, the leaves were incubated with 100 conidia/mm² of powdery mildew of barley (race A6) and incubated under identical conditions for a further 40 to 48 hours.

Leaf segments were bombarded with the coated particles using a particle inflow gun. For each shot, 312 μg of tungsten particles were applied. 4 hours after the bombardment, the leaves were inoculated with Blumeria graminis f.sp. hordei mildew (race A6) and, after a further 40 hours, evaluated for symptoms of infection. The result (for example the penetration efficiency, defined as percentage of attacked cells with a mature haustorium and a secondary elongating hypha were analyzed by means of fluorescence and light microscopy. An inoculation with 100 conidia/mm² results in an infection frequency of approximately 50% of the transformed cells. A minimum of 100 interaction sites was evaluated for each individual experiment. Transformed (GFP-expressing) cells were identified under excitation with blue light. Three different categories of transformed cells were distinguished:
1. Penetrated cells containing a readily recognizable haustorium. A cell with more than one haustorium was considered as one cell.

2. Cells which, while attacked by a fungal appressorium, contain no haustorium. A cell which has been attacked more than once by Bgh, but which contains no haustorium, was considered as one cell.

3. Cells which are not infected by Bgh.

Stomatal cells and guard cells were excluded from the assessment. Surface structures of Bgh were analyzed by means of light microscopy or fluorescence staining of the fungus with 0.1% Calcofluor (w/v in water) for 30 seconds. The fungal development can be evaluated readily by fluorescence microscopy following staining with Calcofluor. In pNAox-dsRNA-transformed cells, the fungus develops a primary and apressorial germ tube, but no haustorium. The development of a haustorium is a condition for the development of a secondary hypha.

The relative penetration efficiency (RPE) is calculated as the difference between the penetration efficiency of transformed cells (transformation with pNAox or control dsRNA) and the penetration efficiency of untransformed cells (here: average penetration efficiency 38.74%). The percent RPE (% RPE) is calculated from the RPE minus 1, multiplied by 100.

\[
\text{RPE} = \frac{[\text{PE in pNAox-dsRNA-transformed cells}]}{[\text{PE in control-dsRNA transformed cells}]} \]

\[
\% \text{ RPE} = 100 \times (\text{RPE}-1) \]

The % RPE value (deviation of the average penetration efficiency of the control) is used to determine the susceptibility of cells transfected with pNAox-dsRNA (fig. 4).

In the case of the control dsRNA, five different experiments reveal no difference between the transfection with the control dsRNA and water with regard to the penetration efficiency of Bgh.

To rule out an effect of the dsRNA and the transformation rate or survival rate of the attacked cells, the number of GFP-expressing cells in control experiments and pNAox-dsRNA experiments was compared. The pNAox-dsRNA had no effect on the total number or the number of the attacked GFP-expressing cells.
Example 5: NADPH oxidase inhibition with diphenyleneiodonium chloride

The results were supported by further experiments with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; table 1). In general, the experiments were carried out as described by Hückelhoven and Kogel, 1998.

Tab. 1: Effect of DPI on the defense against pathogens in Palulas

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<th>Type of interaction</th>
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<th>Control</th>
<th>200 μM DPI</th>
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<td>Penetration</td>
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<td>Nonpenetration</td>
<td>24.25 ± 6.3</td>
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<td>7.5 ± 3.7</td>
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a The DPI treatment was carried out 12 hours after inoculation with the pathogen and the evaluation 36 hours after inoculation.

b Control with 10 mM potassium phosphate buffer, pH 7.8, with DMSO content as in the DPI treatment.

c DPI dissolved in 10 mM potassium phosphate buffer, pH 7.8, starting from a 10 mg/ml DPI stock solution in DMSO.
SEQUENCE LISTING

BASF Plant Science GmbH
Method for obtaining a pathogen resistance in plants
AE20020416
DNA
Hordeum vulgare
CDS
(2) . (337)
coding for NADPH oxidase (fragment)

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Hordeum vulgare
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180 185 190

cct cgt aga ct gaa ttc att agc aat aac aaa acc aat gga tgg aat 624
Leu Arg Arg Leu Lys Phe Ile Ser Asn Asn Lys Thr Asn Gly Trp Asn
195 200 205

gaa gtt gaa aac aat ttc tca aag ctc gct aac gac ggt tat ctt tac 672
Glu Val Glu Asn Asp Ser Lys Leu Ala Lys Asp Gly Tyr Leu Tyr
210 215 220

cgt tcc gat ttc gca caa tgc ata ggt atg aag gat tcc aag gaa ttt 720
Arg Ser Asp Phe Ala Glu Cys Ile Gly Met Lys Asp Ser Lys Glu Phe
225 230 235 240

gca ttg gaa tta ttt gat gct tgg aag gta aag aga aga tta aag gtt 768
Ala Leu Glu Leu Phe Asp Ala Arg Leu Arg Arg Ser Leu Arg Leu Val
245 250 255

gat aaa att agc aag gag gaa ttg tat gat tac tgg tct caa atc acc 816
Asp Lys Ile Ser Lys Glu Glu Leu Tyr Glu Tyr Trp Ser Glu Ile Thr
260 265 270

gat cag aeg ttc gat tct cgg ctt cag atc tcc tct gac atg gtt gac 864
Asp Gln Ser Phe Asp Ser Arg Leu Glu Ile Ser Phe Asp Met Val Asp
275 280 285

aag aat gaa gat ggt cga att gct gaa gag gaa gta aas gaa gtc atc 912
Lys Asn Glu Asp Gly Arg Ile Ala Glu Glu Val Lys Glu Ile Ile
290 295 300

atg cta ggt ctc gca aac gaa tta tca aga tta aaa gaa caa gca 960
Met Leu Ser Ala Ser Ala Lys Leu Ser Arg Leu Lys Glu Glu Ala
305 310 315 320

gag gag tat gca gct tta atc atg gaa tta gat cct gaa aga ctc 1008
Glu Tyr Ala Ala Leu Ile Met Glu Leu Asp Pro Glu Arg Leu
325 330 335

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Gly Tyr Ile Glu Leu Trp Glu Glu Thr Leu Leu Leu Glu Lys Asp
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Thr Tyr Leu Asn Tyr Ser Glu Ala Leu Ser Tyr Thr Ser Glu Ala Leu
355 360 365

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Ser Glu Asn Leu His Gly Leu Arg Lys Ser Pro Ile Lys Arg Met
370 375 380
AGC ACA AAA CTT GTC TAT TCA TTT CAA GAA AAC TGG AAG AGA ATT TGG
Ser Thr Lys Leu Val Tyr Ser Leu Gln Glu Asn Trp Lys Arg Ile Trp
385 390 395 400

GTT CTC ACT TTA TGG ATT TGG ATA ATG ATT GGG CTT TTT CTT TGG AAG
Val Leu Thr Leu Trp Ile Leu Ile Met Ile Gly Leu Leu Trp Lys
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TTC TAT CAG TAC AAA AAC AAG AGT GCA TTC CGT GTC ATG GGT TAT TGC
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Thr Lys Leu Ser His Phe Val Pro Phe Asp Asp Asn Ile Asn Phe His
465 470 475 480

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Lys Thr Val Ala Ala Ile Val Thr Gly Ile Ile Leu His Ala Gly
485 490 495

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Asn His Leu Val Leu Cys Arg Pro Arg Leu Ile His Ala Asp Asp Gln
500 505 510

GAT TAT CAA AGT TTT TTT TCG AAT GAT TTT GGC CAA AGT AAG CCT GGA
Asp Tyr Gln Ser Phe Leu Ser Asn Phe Gly Glu Ser Lys Pro Gly
515 520 525

TAC ATA GAC CTT GTT AAA GGA GTG GAG GTG ACG GGA ATA ATA ATG
Tyr Ile Asp Leu Val Lys Gly Val Glu Gly Val Thr Gly Ile Met
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Val Ile Leu Met Ala Ile Ala Phe Thr Leu Ala Thr Arg Trp Phe Arg
545 550 555 560

CGG AGC CTC ATT AAG TTG CCC AAA CCT TTT GAT AGA CTC ACT GGC TTC
Arg Ser Leu Ile Lys Leu Pro Lys Pro Phe Asp Arg Leu Thr Gly Phe
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Asn Ala Phe Thr Tyr Ser His His Leu Leu Val Ile Val Tyr Ile Leu
580 585 590

CTG ATC ATC CAT GCC AGC TCG TTC TTC CTT GTG CAT AAA TGG TAC TCC
Leu Ile Ile His Gly Thr Leu Leu Leu Val His Lys Trp Tyr Ser
595 600 605

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Lys Thr Thr Trp Met Tyr Leu Val Leu Tyr Leu Tyr Ala Gly
610 615 620

GAA AGA ACT AGA TCT TCC CGG TCA GCC TGG TAC ACT GTC CGG TCT
Glu Arg Thr Leu Arg Phe Phe Arg Ser Gly Leu Tyr Thr Val Arg Leu
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Cys Pro Ala Val Pro Phe Glu Trp His Pro Phe Ser Ile Thr Ser
675  680  685

gct cct ggg gat gac tac ttg agc att cac atc cgg caa ctt ggt gac
Ala Pro Gly Asp Asp Tyr Leu Ser Ile His Ile Arg Glu Leu Gly Asp
690  700

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Trp Thr Gln Glu Leu Lys Arg Val Phe Ser Ala Cys Glu Arg Pro
705  710  715  720

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725  730  735

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Ser Leu Pro Lys Leu Leu Ile Asp Gly Pro Tyr Gly Ala Pro Ala Gln
740  745  750

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Asp Tyr Arg Lys Tyr Asp Val Leu Leu Val Gly Leu Gly Ile Gly
755  760  765

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Ala Thr Pro Phe Ile Ser Ile Leu Lys Asp Leu Leu Val Asn Ile Val
770  775  780

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Lys Met Glu Glu Gln Ala Asp Leu Ala Ser Asp Phe Ser Gly Asn Ser
785  790  795  800

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Asp Met Ser Val Ala Thr Ser Glu Gln Pro Ala Leu Asn Lys Ile Ser
805  810  815

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Leu Lys Arg Arg Lys Ser Thr Leu Arg Thr Thr Asn Ala Tyr Phe Tyr
820  825  830

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835  840  845

aac gaa g tg gct gaa ctt gat caa agg ggg gtc atc gag atg cat aac
Asn Glu Val Ala Glu Leu Asp Gln Arg Gly Val Ile Glu Met His Asn
850  855  860

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Tyr Leu Thr Ser Val Tyr Glu Gly Asp Ala Arg Ser Ala Leu Ile
865  870  875  880

acc atg gtc cag gca ctt aac cat gct aag aat ggg gtt gat att gta
Thr Met Val Gln Ala Leu Asn His Ala Lys Asn Gly Val Asp Ile Val
885  890  895

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Ser Gly Thr Arg Val Arg Thr His Phe Ala Arg Pro Asn Trp Lys Lys
900  905  910

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Val Phe Ser Lys Thr Leu Thr Lys His Ala Asn Ala Arg Ile Gly Val
915  920  925
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Phe Tyr Cys Gly Ala Pro Val Leu Ala Lys Glu Leu Ser Lys Leu Cys
930 935 940

aaa gag tat aat caa aag ggt gca aca aag ttc gag ttt cac aaa gaa 2880

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945 950 955 960

cat ttt tag

His Phe

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Gly Tyr Asn Ser Gly Phe Ala Ser Glu Glu Phe Val Glu Val Thr Leu
35 40 45

Asp Leu Gln Asp Asp Thr Ile Ile Leu Arg Ser Val Glu Pro Ala
50 55 60

Thr Val Ile Asn Ile Asp Ala Pro Asp Leu Pro Ala Gly Val Gly Ile
65 70 75 80

Ser Gly Val Ser Ile Glu Thr Pro Thr Ser Ala Ser Val Ser Glu Ser
85 90 95

Arg Ser Pro Thr Ile Arg Arg Ser Ser Ser Ser Lys Leu Arg Gln Phe
100 105 110

Ser Gln Glu Leu Lys Ala Glu Ala Val Ala Lys Ala Arg Gln Phe Ser
115 120 125

Gln Glu Leu Lys Ala Glu Leu Arg Arg Phe Ser Trp Ser His Gly His
130 135 140

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Gly Thr Gly Asn Gly Val Asp Ser Ala Leu Ala Ala Arg Ala Leu Arg
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Arg Gln Arg Ala Gln Leu Asp Arg Thr Arg Ser Ser Ala His Arg Ala
180 185 190

Leu Arg Arg Leu Lys Phe Ile Ser Asn Asn Lys Thr Asn Gly Trp Asn
195 200 205

Glu Val Glu Asn Phe Ser Lys Leu Ala Lys Asp Gly Tyr Leu Tyr
210 215 220

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225 230 235 240

Ala Leu Glu Leu Phe Asp Ala Leu Ser Arg Arg Arg Arg Leu Lys Val
245 250 255

Asp Lys Ile Ser Lys Glu Glu Leu Tyr Glu Tyr Trp Ser Gln Ile Thr
260 265 270

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275 280 285
Lys Asn Glu Asp Gly Arg Ile Ala Glu Glu Glu Val Lys Glu Ile Ile
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Met Leu Ser Ala Ser Ala Asn Lys Leu Ser Arg Leu Lys Glu Gln Ala
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Glu Glu Tyr Ala Ala Leu Ile Met Glu Glu Leu Asp Pro Glu Arg Leu
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Gly Tyr Ile Glu Leu Trp Gln Leu Glu Thr Leu Leu Leu Gln Lys Asp
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Thr Tyr Leu Asn Tyr Ser Gln Ala Leu Ser Tyr Thr Ser Gln Ala Leu
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Ser Gln Asn Leu His Gly Leu Arg Lys Lys Ser Pro Ile Lys Arg Met
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Ser Thr Lys Leu Val Tyr Ser Leu Gln Glu Asn Trp Lys Arg Ile Trp
         385     390      395      400
Val Leu Thr Leu Trp Ile Leu Ile Met Ile Gly Leu Phe Leu Trp Lys
         405     410      415
Phe Tyr Gln Tyr Lys Asn Lys Ser Ala Phe Arg Val Met Gly Tyr Cys
         420     425      430
Leu Val Thr Ala Lys Gly Ala Ala Glu Thr Leu Lys Phe Asn Met Ala
         435     440      445
Leu Ile Leu Leu Pro Val Cys Arg Asn Thr Ile Thr Trp Leu Arg Ser
         450     455      460
Thr Lys Leu Ser His Phe Val Pro Phe Asp Asp Asn Ile Asn Phe His
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Lys Thr Val Ala Ala Ile Val Thr Gly Ile Ile Leu His Ala Gly
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Asn His Leu Val Cys Asp Phe Pro Arg Leu Ile His Ala Asp Asp Gln
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Asp Tyr Gln Ser Phe Leu Ser Asn Asp Phe Gly Gln Ser Lys Pro Gly
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Val Ile Leu Met Ala Ile Ala Phe Thr Leu Ala Thr Arg Trp Phe Arg
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Asn Ala Phe Trp Tyr Ser His His Leu Leu Val Ile Val Tyr Ile Leu
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Leu Ile Ile His Gly Thr Phe Leu Phe Leu Val His Lys Trp Tyr Ser
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Lys Thr Thr Trp Met Tyr Leu Ala Val Pro Val Leu Leu Tyr Ala Gly
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Glu Arg Thr Leu Arg Phe Phe Arg Ser Gly Leu Tyr Thr Val Arg Leu
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Leu Lys Val Ala Ile Tyr Pro Gly Asn Val Leu Thr Leu Gln Met Ser
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Lys Pro Pro Gln Phe Arg Tyr Lys Ser Gly Gln Tyr Met Phe Val Gln
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1 5

112

160

Glu Arg Arg Thr Ser Asp Thr Val Ser Ser Gly Lys Asp Leu Ser
10 15 20

160

Gly Glu Ser Ser Pro Gly Thr Asp Ser Gly Asn Ile Ser Gly Phe Ala
25 30 35

208

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40 45 50 55

256

att att cta cgg agc gtt gaa ccg gct act gta atc aac att gat gct
Ile Ile Leu Arg Ser Val Glu Pro Ala Thr Val Ile Asn Ile Asp Ala
60 65 70

304

tct gat cct gct acc gga gtc gtt att ggt gga gta tcg att gaa act
Ser Asp Pro Ala Thr Gly Val Gly Ile Gly Gly Val Ser Ile Glu Thr
75 80 85

352

ccg tcg ctg act tcg acg tcg gga act cga tcg ccc acg atg cgt
Pro Ala Ser Leu Thr Ser Thr Ser Gly Thr Arg Ser Pro Thr Met Arg
90 95 100

400

cgg agt aca tcg aat aaa tta cgt cag ttt tca cag gag ttg aaa gct
Arg Ser Thr Ser Asn Lys Leu Gln Pro Ala Thr Val Lys Ala
105 110 115

448

gag gct gtc gcc aaa gcg aag cat ttc tcg cca gag ctt aaa gcg gag
Glu Ala Val Ala Lys Ala Lys His Phe Ser Gln Leu Lys Ala Glu
120 125 130 135

496

tca agg aga ttc tca tcg ggc cag gcg act gta gaa ccc cgt
cgg gtc cag ttc ccc cca aac gcc gtc gtt aca gcc aac ggt gta
Pro Ala Ser Phe Phe Gln Asn Ala Val Val Gly Thr Gly Asn Gly Val
140 145 150 155 160 165

592

gat tcg gct tta gca gct cga gca tta cag ccc cgg cgg cgt cag ctc
Asp Ser Ala Leu Ala Ala Arg Ala Leu Arg Asp Gln Gln Ala Gln Leu
170 175 180

640

gat cgg act cgt tcc agc gct cac aag gct ctt cgt gga ctc aaa ttc
Asp Arg Thr Arg Ser Ser Ala Leu Arg Gly Leu Lys Phe
185 190 195 200 205 210 215

736

atc agc aat aac aac cag gga tgg aat gaa gtt gaa aac aat ttt
Ile Ser Asn Asn Lys Thr Asn Gly Trp Asn Glu Val Glu Asn Phe
200 205 210 215

784

gct aag ctc gct aaa gac ggt tac ctt tat cgc tcc gat ttc gca caa
Ala Lys Leu Ala Lys Asp Gly Tyr Leu Tyr Arg Ser Asp Phe Ala Gln
220 225 230

832

tgc atc ggt agt aag gat tca aag gaa ttt gca tgg gaa tgt ttt gat
Cys Ile Gly Met Lys Asp Ser Lys Glu Phe Ala Leu Glu Leu Phe Asp
235 240 245 250 255 260

880
gaa ttg tat gag tat tgg tct caa atc acc gat cag agt ttc gat tct
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265 270 275

cgg ctt cag atc ttc ttc gac atg gtt gac aag aat gaa gat ggt cga
Arg Leu Gln Ile Phe Phe Asp Met Val Asp Lys Asn Glu Asp Gly Arg
280 285 290 295

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Ile Met Glu Leu Asp Pro Glu Arg Leu Gly Tyr Ile Glu Leu Trp
300 305 310

aac aaa tta cta aga tta aaa gaa caa gca gag gag tat gca gct cta
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315 320 325

atc atg gaa gaa tta gat cct gaa aga ctt ggc tac att gag cta tgg
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330 335 340

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380 385 410

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395 400 405

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Leu Ile Met Ile Gly Leu Phe Leu Trp Lys Phe Tyr Leu Tyr Lys Gln
410 415 420

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425 430 435

gct gct gag act cta aag ttc aac atg gct ttg ata tgg ttg cca gtt
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440 445 450 455

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Val Pro Phe Asp Asn Ile Phe His Lys Thr Val Ala Ala Leu
475 480 485

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490 495 500

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Phe Pro Lys Leu Ile His Ala Asn Thr Asn Tyr Glu Lys Tyr Leu
505 510 515

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555 560 565

Pro Lys Pro Phe Asp Arg Leu Thr Gly Phe Asn Ala Phe Trp Tyr Ser
570 575 580

Hist His Leu Leu Ile Ile Val Tyr Ile Val Leu Ile Ile His Gly Thr
585 590 595

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730 735 740

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760 765 770 775

Arg Val Leu Leu Gly Leu Gly Ile Gly Thr Pro Phe Ile Ser
790 795 800 805

Glu Val Val Thr Gly Ile Ile Met Val Ile Leu Met Ala Ile
1744

Gly Val Val Thr Gly Ile Ile Met Val Ile Leu Met Ala Ile
1792

Phe Leu Tyr Leu Val His Asn Trp Tyr Ser Lys Thr Thr Trp Met Tyr
1936

Tyr Lys Ser Gly Glu Tyr Met Phe Val Gln Cys Ala Ser Val Pro Ser
2128

Phe Glu Trp His Pro Phe Ser Ile Thr Ser Ala Pro Gly Asp Asp Tyr
2176

Arg Val Phe Ser Glu Ala Cys Glu Gln Pro Glu Ala Gly Lys Ser Gly
2224

Arg Val Leu Leu Gly Leu Gly Ile Gly Thr Pro Phe Ile Ser
2272

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890 895 900
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Thr His Phe Ala Arg Pro Asn Trp Arg Lys Val Phe Ser Lys Thr Leu
905 910 915
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Ile Leu Ala Glu Leu Ser His Leu Cys Gly Phe Asn Gln Lys
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gaaaaaaaaaaaaaaaaaaa aaaaaaa 3733

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35 40 45
Asp Leu Gln Asp Asp Thr Ile Ile Leu Arg Ser Val Glu Pro Ala
50 55 60
Thr Val Ile Asn Ile Asp Ala Ser Asp Pro Ala Thr Gly Val Gly Ile
65 70 75 80
Gly Gly Val Ser Ile Glu Thr Pro Ala Ser Leu Thr Ser Thr Ser Gly
85 90 95
Thr Arg Ser Pro Thr Met Arg Arg Ser Thr Ser Asn Lys Leu Arg Gln
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Phe Ser Gln Glu Leu Lys Ala Glu Ala Val Ala Lys Ala Lys His Phe
115 120 125
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         15     20    25
Ser Phe Asp Ser Val Ser Ala Gly Lys Thr Ala Val Gly Ser Ala Ser

act tca ccg gga act gaa tac tcc att aac ggt gat caa gag ttc gtt
        30    35    40
Thr Ser Pro Gly Thr Glu Tyr Ser Ile Asn Gly Asp Gln Glu Phe Val

gaa gtc aca atc gat ctt cca gac gat gac aca atc gtt ctt cgt agc
        45   50    55    60
Glu Val Thr Ile Asp Leu Gln Asp Asp Thr Ile Val Leu Arg Ser

gtc gag cca gca acc gcc att aat gtc atc gga gat atc tcc gag gac
        65   70    75
Val Glu Pro Ala Thr Ala Ile Asn Val Ile Gly Asp Ile Ser Asp

aac acc gga ata atg act ccg gtt tcg att tcg aga tct ccg acg atg
        80   85    90
Asn Thr Gly Ile Met Thr Pro Val Ser Ile Ser Arg Ser Pro Thr Met

aaa cga act tca tct aat ccg ttc cga caa ttc cca cag cgg ttt aaa
        95  100   105
Lys Arg Thr Ser Ser Asn Arg Phe Arg Gln Phe Ser Gln Glu Leu Lys

gcc gaa gct gtg gcg aaa ggg aaa cag tta tct cag gag ttg aaa cga
        110  115  120
Ala Glu Ala Val Ala Lys Ala Lys Gln Leu Ser Gln Glu Leu Lys Arg

ttc tca tgg tct cgt tct ttc ggt aac tta acc act act aqt acc
        125  130   135   140
Phe Ser Trp Ser Arg Ser Phe Ser Gly Asn Leu Thr Thr Thr Ser Thr

gcc gct aat cca aqc ggc gct ggt ggt ggt gtg ttg gtg aac tcc gct
        145  150  155
Ala Ala Asn Gln Ser Gly Gly Ala Gly Gly Gly Leu Val Asn Ser Ala
29

tta gaa gcg cga gcg ttg cga aag caa cgt gct cag tta gat cgg act 530
Leu Glu Ala Arg Ala Leu Arg Lys Gln Arg Ala Gln Leu Asp Arg Thr
160 165 170

cgg tct agt gct caa aga gct ctt cgt ggt ttg aga ttc att agc aat 578
Arg Ser Ser Ala Gln Arg Ala Leu Arg Gly Leu Arg Phe Ile Ser Asn
175 180 185

aag caa aag aac gtt gat ggt tgg aac gat gtt cca tca aat ttc gaa 626
Lys Gln Lys Asn Val Asp Gly Trp Asn Asp Val Gln Ser Asn Phe Glu
190 195 200

aaa ttc gaa aaa aat gtt tac atc tat cgc tcc gat ttc gct caa tgc 674
Lys Phe Glu Lys Asn Gly Tyr Ile Tyr Arg Ser Asp Phe Ala Gln Cys
205 210 215 220

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Ile Gly Met Lys Asp Ser Lys Glu Phe Ala Leu Glu Leu Phe Asp Ala
225 230 235

ttg agt aga aga aga tta aaa gta gag aaa atc aat cac gat gag 770
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240 245 250

ctt tat gag tat tgg tca cca atc aac gac gag agt ttc gat tct cgt 818
Leu Tyr Glu Tyr Trp Ser Gln Ile Asn Asp Glu Ser Phe Asp Ser Arg
255 260 265

ctc cag atc ttc gac ata gtg gac aag aat gaa gat ggg aga att 866
Leu Gln Ile Phe Phe Asp Ile Val Asp Lys Asp Gly Arg Ile
270 275 280

aca gaa gag gaa gta aaa gag ata ata atg ttc agt gca tct gca aat 914
Thr Glu Glu Glu Val Lys Ile Ile Met Leu Ser Ala Ser Ala Asn
285 290 295 300

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Lys Leu Ser Arg Leu Lys Gln Ala Glu Glu Tyr Ala Ala Leu Ile
305 310 315

atg gaa gag tta gat cct gaa aga ctt gcc tac ata gag cta tgg caa 1010
Met Glu Glu Leu Asp Pro Glu Arg Leu Gly Tyr Ile Glu Leu Trp Gln
320 325 330

cta gag act ttg ctt cta cca aaa gag aca tac ctc aat tac agt caa 1058
Leu Glu Thr Leu Leu Gln Lys Asp Thr Tyr Leu Asn Tyr Ser Gln
335 340 345

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agg gga aag agt cga ata cat aga atg agt tgc gat ttc gtc tac att 1154
Arg Gly Lys Ser Arg Ile His Arg Met Ser Ser Asp Phe Val Tyr Ile
365 370 375 380

atg caa gag aat tgg aaa agg ata tgg gtt ttc tta tgg atc atg 1202
Met Gln Glu Asn Trp Lys Arg Ile Trp Val Leu Ser Leu Trp Ile Met
385 390 395

atc atg atc gga tta ttc ttg tgg aaa ttc ttc tca aag cca aag 1250
Ile Met Ile Gly Leu Phe Leu Trp Lys Phe Phe Glu Tyr Lys Gln Lys
400 405 410

gat gca ttt cat gtg atg gga tat tgt tta ctc aca gcc aaa gga gca 1298
Asp Ala Phe His Val Met Gly Tyr Cys Leu Leu Thr Ala Lys Gly Ala
415 420 425
PF 53765

30

gct gaa aca ctt aac ttc aac atg gct cta ata ctt ttc cca gtt tgc
Ala Glu Thr Leu Lys Phe Asn Met Ala Leu Ile Leu Phe Pro Val Cys
430  435  440

aga aac acc att act tgg ctt aga tcc aca aga ctc tct tac ttc gtt
Arg Asn Thr Ile Thr Trp Leu Arg Ser Thr Arg Leu Ser Tyr Phe Val
445  450  455  460

cct ttt gat gat aat atc aac ttc cac aag aca att gct gga gcc att
Pro Phe Asp Asp Ile Asn Phe His Lys Thr Ile Gly Ala Ile
465  470  475

gta gct gtt gtc ctt cat att gga gac cat ctt gct tgt gat ttc
Val Val Ala Val Ile Leu His Ile Gly Asp His Leu Ala Cys Asp Phe
480  485  490

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His Tyr Phe Gln Thr Lys Gln Pro Thr Tyr Phe Asp Leu Val Lys Gly
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Pro Glu Gly Ile Thr Gly Ile Leu Met Val Ile Leu Met Ile Ile Ser
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His Leu Phe Val Ile Val Tyr Ile Leu Leu Ile His Gly Ile Phe
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Glu Trp His Pro Phe Ser Ile Thr Ser Ala Pro Glu Asp Asp Tyr Ile
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Ser Ile His Ile Arg Gln Leu Gly Asp Trp Thr Gln Glu Leu Lys Arg
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Val Gly 710 715
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Leu Leu 725 730
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Asp Asp 740 745
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Phe Phe 820 825

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Val Leu 835 840
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His Leu 850 855 860
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Leu Ile Thr Met Val Glu Ala Leu Asn His Ala Lys Asn Gly Val Asp 865
Leu Gly 870 875
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Leu Gly 885 890
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Lys Leu 900 905
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Gly Gly 915 920
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Lys Glu His Phe

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tctaggttata ttaatacgcgag ggcatacggt gaaatatacg agacagaga aatcagggg 3066
| Residue | 1 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 | 90 | 95 | 100 | 105 | 110 | 115 | 120 | 125 | 130 | 135 | 140 | 145 | 150 | 155 | 160 | 165 | 170 | 175 | 180 | 185 | 190 | 195 | 200 | 205 | 210 | 215 | 220 | 225 | 230 | 235 | 240 | 245 | 250 | 255 | 260 | 265 | 270 | 275 | 280 | 285 | 290 | 295 | 300 | 305 | 310 | 315 | 320 | 325 | 330 | 335 |
|---------|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
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755 760 765
Leu Asn Asn Ile Val Lys Met Glu Glu His Ala Asp Ser Ile Ser Asp
770 775 780
Phe Ser Arg Ser Ser Glu Tyr Ser Thr Gly Ser Asn Gly Asp Thr Pro
785 790 795 800
Arg Arg Lys Arg Ile Leu Lys Thr Thr Asn Ala Tyr Phe Tyr Trp Val
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Thr Arg Glu Gln Gly Ser Phe Asp Trp Phe Lys Gly Val Met Asn Glu
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850 855 860
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900 905 910
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Met Lys Met Arg Gly Asp Ser Ser Asp His Glu
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Leu Gly Ile Leu Arg Gly Ala Asn Ser Asp Thr Asn Ser Asp Thr Glu
15 20 25
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Ser Ile Ala Ser Asp Arg Gly Ala Phe Ser Gly Pro Leu Gly Arg Pro
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Lys Arg Ala Ser Lys Lys Asn Ala Arg Phe Ala Asp Asp Leu Pro Lys
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Arg Ser Asn Ser Val Ala Gly Gly Arg Arg Asp Asp Glu Tyr Val
65 70 75

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Glu Ile Thr Leu Asp Ile Arg Asp Ser Val Ala Val His Ser Val
80 85 90
caa caa gca gct gga ggt gga ggc cac ctg gag gac ccg gag cta gcc
Gln Gln Ala Ala Gly Gly Gly Gly His Leu Glu Pro Glu Leu Leu Ala
95 100 105

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Leu Leu Thr Lys Lys Thr Leu Glu Ser Ser Leu Asn Asn Thr Thr Ser
110 115 120 125
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130 135 140

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Glu Leu Arg Arg Val Phe Ser Arg Arg Pro Ser Pro Ala Val Arg Arg
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Asp Lys Leu Ser Ala Asp Ser Asn Gly Leu Leu Leu Ser Ala Lys Phe
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Trp Glu Cys Leu Gly Met Asn Lys Glu Ser Lys Asp Phe Ala Asp Gln
210 215 220
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Leu Phe Arg Ala Leu Ala Arg Arg Asn Val Ser Gly Asp Ala Ile
225 230 235
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Thr Lys Glu Gln Leu Arg Ile Phe Trp Glu Gln Ile Ser Asp Glu Ser
240 245 250
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255 260 265
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270 275 280 285
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305 310 315
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320 325 330

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335 340 345

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Leu Arg Pro Ala Lys Glu Ser Asn Pro Leu Leu Arg Trp Ser Gln Lys
350 355 360 365

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Ile Lys Tyr Phe Ile Leu Asp Trp Gln Arg Leu Arg Trp Ile Met Met
370 375 380

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Leu Trp Leu Gly Ile Cys Gly Gly Leu Phe Thr Tyr Lys Phe Ile Gln
385 390 395

tac aag aac aag gct gcc tat ggt tgt aag tgt tact tgt tgt gtc
Tyr Lys Asn Ala Ala Tyr Gly Val Met Gly Tyr Cys Val Cys Val
400 405 410

gcc aag gga gcc gag act ctc aca aac aag ctc att ctc aag gcc
Ala Lys Gly Ala Glu Thr Leu Lys Phe Asn Met Ala Leu Ile Leu
415 420 425

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Leu Pro Val Cys Arg Asn Thr Ile Thr Trp Leu Arg Asn Lys Thr Lys
430 435 440 445

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Leu Gly Thr Val Pro Asp Pro Arg Leu Ala Ala Ala Asp Glu Thr Tyr
450 455 460

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Ile Ala Ser Gly Ile Val Gly Val Leu Leu His Ala Gly Ala His
465 470 475

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CA 02492784 2005-01-17

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PF 53765

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Glu Ser Pro Glu Leu Thr Leu Leu Lys Arg Asn Arg Leu Glu Lys Lys
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1968

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2016

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490
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We claim

1. A method for generating or increasing the resistance to at least one pathogen in plants, which comprises the following operating steps

   a) reduction of the protein quantity, activity or function of an NADPH oxidase in a plant or a tissue, organ, part or cell thereof, and

   b) selection of the plants in which - in contrast or in comparison with the starting plant - the resistance to at least one pathogen exists or is increased.

2. The method according to claim 1, wherein the NADPH oxidase is encoded by

   a) polypeptide sequences comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22, or

   b) polypeptide sequences of a functional equivalent of a polypeptide comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

3. The method according to claim 2, wherein the functional equivalent has at least 50% homology with one of the polypeptides as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

4. The method according to any of claims 1 to 3, wherein the reduction of the protein quantity, activity or function of an NADPH oxidase is ensured by applying a method selected from the group consisting of

   a) introducing a double-stranded NADPH oxidase RNA nucleic acid sequence or (an) expression cassette(s) ensuring its expression,

   b) introducing an NADPH oxidase antisense nucleic acid sequence or an expression cassette ensuring its expression,
71
c) introducing an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme or an expression cassette ensuring its expression,
d) introducing NADPH oxidase sense nucleic acid sequences for inducing a cosuppression or an expression cassette ensuring their expression,
e) introducing DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins or an expression cassette ensuring their expression,
f) introducing viral nucleic acid sequences and expression constructs bringing about the degradation of NADPH oxidase RNA, or an expression cassette ensuring their expression,
g) introducing constructs for inducing a homologous recombination at endogenous NADPH oxidase genes, and
h) introducing mutations into an endogenous NADPH oxidase gene.

5. The method according to any of claims 1 to 4, comprising

(i) the stable transformation of a plant cell with a recombinant expression cassette comprising, in functional linkage with a promoter which is active in plants, a nucleic acid sequence encoding

a) a double-stranded NADPH oxidase RNA ribonucleic acid sequence or

b) an NADPH oxidase antisense nucleic acid sequence or

c) an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme or

d) an NADPH oxidase sense nucleic acid sequence for inducing a cosuppression or

e) DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins

f) viral nucleic acid sequences which bring about the degradation of NADPH oxidase RNA,
(ii) regeneration of the plant from the plant cell, and

(iii) expression of said nucleic acid sequence in such a quantity and for such a time as suffices for generating or increasing a pathogen resistance in said plant.

6. The method according to any of claims 1 to 5, wherein the pathogen is selected from the group consisting of bacteria, fungi, insects, viruses and nematodes.

7. The method according to any of claims 1 to 6, wherein the pathogen is selected from the group of the fungi consisting of Plasmodiophoromycota, Oomycota, Ascomycota, Chytridiomycetes, Zygomycetes, Basidiomycota and Deuteromyceten.

8. The method according to any of claims 1 to 7, wherein the plant is selected from among the monocotyledonous and dicotyledonous plants.

9. The method according to any of claims 1 to 8, wherein the plant is selected from the group of the monocotyledonous plants consisting of wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed or sugar cane.

10. A double-stranded RNA molecule for reducing the expression of an NADPH oxidase protein comprising

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of the sense RNA transcript of a nucleic acid sequence encoding an NADPH oxidase, and

b) an antisense RNA strand which is essentially complementary to the RNA sense strand of a).

11. The double-stranded RNA molecule according to claim 10, wherein the two RNA strands of the double-stranded RNA are bonded covalently with one another.

12. The double-stranded RNA molecule according to either of claims 10 or 11, wherein one of the two RNA strands is encoded by at least a part of the nucleic acid sequence encoding an NADPH oxidase sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 or a functional equivalent thereof.
13. A transgenic expression cassette comprising, in functional linkage with a promoter which is functional in plant organisms, a nucleic acid sequence encoding a double-stranded RNA molecule according to one of Claims 10 to 12.

14. A transgenic expression cassette comprising at least a part of a nucleic acid sequence encoding an NADPH oxidase as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 or a functional equivalent thereof, where said nucleic acid sequence is linked functionally in antisense orientation with a promoter which is functional in plant organisms.

15. The transgenic expression cassette according to claim 13 or 14, wherein the promoter which is functional in plants is a pathogen-inducible promoter.

16. A transgenic vector comprising an expression cassette according to any of claims 13 to 15.

17. A transgenic organism comprising a double-stranded RNA molecule according to any of claims 10 to 12, an expression cassette according to any of claims 13 to 15 or a vector according to claim 16.

18. The transgenic organism according to claim 17, selected from the group consisting of bacteria, yeasts, animals and plants.

19. The transgenic organism according to claim 17 or 18, selected from the group of the plants consisting of wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed, sugar cane, oilseed rape, canola, cress, Arabidopsis, cabbages, soybeans, alfalfa, pea, beans, peanut, potato, tobacco, tomato, egg plant, capsicum, sunflower, Tagetes, lettuce, Calendula, melon, pumpkin/squash and zucchini.

20. A tissue, organ, part, cell, cell culture or propagation material derived from a transgenic organism according to either of claims 18 or 19.
Fig. 1