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(54) Title: STRESS RESISTANT PLANTS

(57) Abstract: Stress tolerance in plants and plant cells is achieved by using nucleotide sequences encoding enzymes involved in the NAD salvage synthesis pathway and/or the NAD de novo synthesis pathway from fungal or yeast like organisms other than Saccharomyces cereviseae e.g. for overexpression in plants.

WO 2007/107326 A1

# STRESS RESISTANT PLANTS

Methods are provided for increasing the stress resistance in plants and plant cells whereby enzymes involved in the NAD salvage synthesis pathway and/or the NAD *de novo* synthesis pathway originating from fungal organisms or yeasts, other than Saccharomyces cereviseae, are expressed in plants.

## Background art

Tolerance of plants to adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations, saline growing conditions and the like, is a very desired property for crop plants, in view of the never-ending quest to ultimately increase the actual yield of these plants.

Various ways of achieving that goal of improving what is commonly known as the stress resistance or stress tolerance of plants have been described. Since different abiotic stress conditions frequently result in the generation of harmfull reactive oxygen species ("ROS") such as superoxides or hydrogen peroxides, initial attempts to improve stress resistance in plants focused on prevention of the generation of the ROS or the removal thereof. Examples of these approaches are overexpression of ROS scavenging enzymes such as catalases, peroxidases, superoxide dismutases etc. or even increasing the amount of ROS scavenging molecules such as ascorbic acid, glutathione etc. These approaches and other attempts to engineer stress tolerant plants are reviewed e.g. in Wang et al. 2003, Planta 218:1-14.

Stress tolerance in plant cells and plants can also be achieved by reducing the activity or the level of the endogenous poly-ADP-ribose polymerases (ParP) or poly(ADP-ribose) glycohydrolases (ParG) as described in WO00/04173 and PCT/EP2004/003995, respectively. It is thought that in this way, fatal NAD and ATP depletion in plant cells subject to stress conditions, resulting in traumatic cell death, can be avoided or sufficiently postponed for the stressed cells to survive and acclimate to the stress conditions.

Uchimiya et al. (2002) et al. describe the isolation of a rice gene denoted YK1, as well as use of a chimeric YK1 gene to increase the tolerance of transgenic rice

plants harboring that gene to rice blast and several abiotic stresses such as NaCl, UV-C, submergence, and hydrogen peroxide. (Uchimiya et al., 2002, Molecular breeding 9: 25-31).

Uchimiya et al. further published a poster abstract describing that overexpression of a NAD dependent reductase gene (YK1) in rice cells also promoted the level of NAD(P)(H) through up-regulating NAD synthetase activities, and concluded that this modification in turn generated a pool of redox substances needed for ROS stress resistance (Uchimiya et al. 2003 Keystone symposium on Plant biology: Functions and control of cell death, Snowbird Utah April 10-15, 2003).

NAD synthetase from yeast has been well characterized and is the last enzyme in both the NAD *de novo* synthesis pathway and the NAD salvage. In the *de novo* pathway, quinolate is the precursor for NAD synthesis and is generated as a product of tryptophan degradation. In the salvage pathway, nicotinamide (which is a degradation product of NAD, generated through the action of various enzymes such as PARP, NAD-dependent deacetylases or other NAD glycohydrolases) is the precursor molecule. In a first step, nicotinamide is deamidated to nicotinic cid by a nicotinamidase. The nicotinic acid is transferred to 5-phosphoribosyl-1-pyrophosphate by the enzyme nicotinate phosphoribosyl transferase to yield nicotinic acid mononucleotide. This compound is shared between the *de novo* and the salvage pathway. Hence, further conversion of this compound by NAD+ pyrophosphorylase and NAD synthetase is achieved as in the *de novo* pathway.

In yeast, overexpression of PNC1 (encoding nicotinamidase) has been correlated with life span extension by calorie restriction and low-intensity stress (Anderson et al., 2003 Nature 423: p181-185; Gallo et al., 2004, Molecular and Cellular Biology 24: 1301-1312).

WO2004/016726 describes methods and compositions for modulating the life span of eukaryotic and prokaryotic cells and for protecting cells against certain stresses. One method comprises modulating the flux of the NAD+ salvage pathway in the cell, e.g. by modulating the level or activity of one or more proteins selected from the group consisting of PNC1, NMA1, NPT1 and NMA2.

Little is known about the respective enzymes of the NAD biosynthesis pathways in plants. Hunt et al., 2004 describe the use of the available genomic information from Arabidopsis to identify the plant homologues of these enzymes (Hunt et al. , 2004, New Phytologist163(1): 31-44). The identified DNA sequences have the following Accession numbers: for nicotinamidase: At5g23220; At5g23230 and At3g16190; for nicotinate phosphoribosyltransferase: At4g36940, At2g23420, for nicotinic acid mononucleotide adenyltransferase: At5g55810 and for NAD synthetase: At1g55090 (all nucleotide sequences are incorporated herein by reference).

PCT/EP 2005/010168 describes methods for increasing the stress resistance in plants and plant cells whereby enzymes involved in the NAD salvage synthesis pathway and/or the NAD *de novo* synthesis pathway are expressed in plants.

Alternative methods for increasing stress tolerance in plants are still required and the embodiments described hereinafter, including the claims, provide such methods and means.

## Summary of the invention

In one embodiment of the invention, a method is provided for obtaining a plant with increased stress resistance comprising introducing a chimeric gene into a cells of a plant to obtain transgenic cells whereby the chimeric gene comprises the following operably linked DNA fragments:

i. A plant-expressible promoter;
ii. A DNA region coding for a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase

iii. A 3'end region involved in transcription termination and polyadenylation,

followed by regenerating the transgenic cells to obtain a population of transgenic plants; and selecting a plant from the population of transgenic plants which exhibits increased stress resistance or selecting a plant which exhibits a reduced level of reactive oxygen species or maintains a high level of NADH under stress conditions when compared to a similar non-transgenic plant wherein said method is characterized in that the amino acid sequence of the plant-functional enzyme encoded by the DNA region comprises one of the following: the amino acid sequence of accession number XP_444840 (Candida glabrata), the amino acid sequence of accession number XP_456073 (Kluyveromyces lactis),,the amino acid sequence of accession number NP_986013 (Eremothecium gossypii),the amino acid sequence of accession number XP_888958 (Candida albicans),the amino acid sequence of accession number XP500320 (Yarrowia lipolytica),,the amino acid sequence of accession number XP389372 (Giberella zeae), the amino acid sequence of accession number XP_749509 (Aspergillus fumigatus), the amino acid sequence of accession number XP_712112 (Candida albicans), the amino acid sequence of accession number BAE56421 (Aspergillus oryzae), the amino acid sequence of accession number XP_567125 (Cryptococcus neofomans),the amino acid sequence of accession number XP_964547 (Neurospora crassa), the amino acid sequence of accession number XP_712135 (Candida albicans), the amino acid sequence of accession number XP_448179 (Candida glabrata), the amino acid sequence of accession number XP_453643 (Kluyveromyces lactis), the amino acid sequence of accession number NP_987024 (Eremothecium gossypii), the amino acid sequence of accession number XP_500272 (Yarrowia lipolytica), the amino acid sequence of accession number XP_722371 (Candida albicans), the amino acid sequence of accession number XP_456405 (Debaromyces hansenii), the amino acid sequence of accession number BAE61562 (Aspergillus oryzae),,the amino acid sequence of accession number XP_759702 (Ustilago maydis), the amino acid sequence of accession number EAL18079 (Cryptococcus neoformans), the amino acid

sequence of accession number NP_587771 (Schizosaccharomyces pombe), the amino acid sequence of accession number XP_681472 (Aspergillus nidulans), the amino acid sequence of accession number XP_959191 (Neurospora crassa), the amino acid sequence of accession number XP_567726 (Cryptococcus neoformans), the amino acid sequence of accession number EAQ90706 (Chaetomium globosum), the amino acid sequence of accession number XP_387574 (Giberella zeae), the amino acid sequence of accession number XP_748008 (Aspergillus fumigatus), the amino acid sequence of accession number XP_361704 (Magnaporthe grisea), the amino acid sequence of accession number Q06178, the amino acid sequence of accession number XP_444815 (Candida glabrata), the amino acid sequence of accession number NP_986687 ((Eremothecium gossypii), the amino acid sequence of accession number XP_453005 (Kluyveromyces lactis), the amino acid sequence of accession number XP_458184(Debaromyces hansenii), the amino acid sequence of accession number XP_718656 (Candida albicans), the amino acid sequence of accession number XP_504391 (Yarrowia lipolytica), the amino acid sequence of accession number NP_592856 (Schizosaccharomyces pombe), the amino acid sequence of accession number XP_762639 (Ustilago maydis), the amino acid sequence of accession number XP_571297 (Cryptococcus neoformans), the amino acid sequence of accession number BAE57070 (Aspergillus oryzae), the amino acid sequence of accession number XP_750776 (Aspergillus fumigatus), the amino acid sequence of accession number XP_659349 (Aspergillus nidulans), the amino acid sequence of accession number XP_389652 (Giberella zeae), the amino acid sequence of accession number XP_957634 (Neurospora crassa), the amino acid sequence of accession number XP_363364 (Magnaporthe grisea), the amino acid sequence of accession number XP_758179 (Ustilago maydis), the amino acid sequence of accession number EAQ85219 ((Chaetomium globosum), the amino acid sequence of accession number CAA85352 (Saccharomyces cerevisae), the amino acid sequence of accession number XP_448893 (Candida glabrata), the amino acid sequence of accession number XP_453357 (Kluyveromyces lactis),

the amino acid sequence of accession number NP_983562 (Eremothecium gossypii), the amino acid sequence of accession number XP_462577 (Debaromyces hansenii), the amino acid sequence of accession number XP_889008 (Candida albicans), the amino acid sequence of accession number XP_500338 (Yarrowia lipolytica), the amino acid sequence of accession number XP_746744 (Aspergillus fumigatus), the amino acid sequence of accession number BAE64333 (Aspergillus oryzae), the amino acid sequence of accession number XP_965789 (Neurospora crassa), the amino acid sequence of accession number EAQ93453 (Chaetomium globosum), the amino acid sequence of accession number XP_682385 (Aspergillus nidulans), the amino acid sequence of accession number AAN74808 (Gibberella moniliformis), the amino acid sequence of accession number Q9UTK3, the amino acid sequence of accession number XP_361075 (Magnaporthe grisea), the amino acid sequence of accession number EAL18922 (Cryptococcus neoformans), the amino acid sequence of accession number XP_568039 (Cryptococcus neoformans), the amino acid sequence of accession number XP_760597 (Ustilago maydis), the amino acid sequence of accession number NP_011524,the amino acid sequence of accession number XP_444815 (Candida glabrata), the amino acid sequence of accession number NP_986687 ((Eremothecium gossypii), the amino acid sequence of accession number XP_453005 (Kluyveromyces lactis), the amino acid sequence of accession number XP_458184 (Debaromyces hansenii), the amino acid sequence of accession number XP_718656 (Candida albicans), the amino acid sequence of accession number XP_504391 (Yarrowia lipolytica), the amino acid sequence of accession number NP_592856 (Schizosaccharomyces pombe), the amino acid sequence of accession number XP_762639 (Ustilago maydis), the amino acid sequence of accession number XP_571297 (Cryptococcus neoformans), the amino acid sequence of accession number BAE57070 (Aspergillus oryzae), the amino acid sequence of accession number XP_750776 (Aspergillus fumigatus), the amino acid sequence of accession number XP_659349 (Aspergillus nidulans), the amino acid sequence of accession number XP_389652 (Giberella zeae), the amino acid sequence of

accession number XP_957634 (Neurospora crassa), the amino acid sequence of accession number XP_363364 (Magnaporthe grisea), the amino acid sequence of accession number XP_758179 (Ustilago maydis) or the amino acid sequence of accession number EAQ85219 (Chaetomium globosum).

In another embodiment, the invention relates to the chimeric genes as described herein, plant cells comprising these chimeric genes, and plants consisting essentially of plant cells comprising these chimeric genes, and seeds of such plants. These plants and plant cells may be characterized in that they have a lower level of reactive oxygen species under stress conditions than a similar plant not comprising such a chimeric gene.

In yet another embodiment, the invention relates to the use of the described chimeric genes to increase the stress resistance of a plant or to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions.

The invention further provides the use of one of the mentioned DNA sequence encoding a plant functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase which are from fungal or yeast-like originto increase the stress resistance of a plant or to decrease the level of reactive oxygen species or maintain the level of NADH in a plant or a plant cell under stress conditions.

## Detailed description

The current invention is based on the finding that DNA sequences encoding plant-functional enzymes from the NAD salvage pathway in yeasts could be used to obtain transgenic plants which were more resistant to stress, particularly abiotic stress, than plants not comprising these DNA sequences. The transgenic

plants also exhibited a significantly reduced level of reactive oxygen species ("ROS") and maintained a high level of NADH, when put under stress conditions, compared to control plants

Thus in one embodiment of the invention, a method is provided to obtain a plant with increased stress resistance, whereby the method comprises the steps of

- introducing a stress resistant chimeric gene as herein described into cells of a plant to obtain cells comprising the stress resistant chimeric gene;
- regenerating these cells comprising the stress resistant chimeric gene to obtain a population of plants comprising the stress resistant chimeric gene; and
- selecting a plant from the population of these plants which exhibits increased stress resistance and/or decreased ROS level under stress conditions and/or maintains a high level of NADH, when compared to a similar non-transgenic plant.

The stress resistant chimeric gene thereby comprises a plant-expressible promoter operably linked to a DNA region coding for a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase from fungal or yeast like origin and a 3'end region involved in transcription termination and polyadenylation.

As used herein, "a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway" is an enzyme which when introduced into plants, linked to appropriate control elements such as plant expressible promoter and terminator region, can be transcribed and translated to yield a enzyme of the NAD salvage synthesis pathway functional in plant cells. Included are the enzymes (and encoding genes) from the NAD salvage synthesis, which are obtained from a yeast or fungus different from Saccharomyces cerevisae.

The latter proteins are very suitable for the methods according to the invention, since these are less likely to be subject to the enzymatic feedback regulation etc. to which similar plant-derived enzymes may be subject.

Enzymes involved in the NAD salvage synthesis pathway comprise the following

- Nicotinamidase (EC 3.5.1.19) catalyzing the hydrolysis of the amide group of nicotinamide, thereby releasing nicotinate and NH3. The enzyme is also known as nicotinamide deaminase, nicotinamide amidase, YNDase or nicotinamide amidohydrolase
- Nicotinate phophoribosyltransferase (EC 2.4.2.11) also known as niacin ribonucleotidase, nicotinic acid mononucleotide glycohydrolase; nicotinic acid mononucleotide pyrophosphorylase; nicotinic acid phosphoribosyltransferase catalyzing the following reaction

  Nicotinate-D-ribonucleotide + diphosphate = nicotinate + 5-phospho-α-D-ribose 1-diphosphate
- Nicotinate-nucleotide adenylyltransferase, (EC 2.7.7.18) also known as deamido-NAD+ pyrophosphorylase; nicotinate mononucleotide adenylyltransferase; deamindonicotinamide adenine dinucleotide pyrophsophorylase; NaMT-ATase; nicotinic acid mononucleotide adenylyltransferase catalyzing the following reaction

  ATP+nicotinate ribonucleotide = diphosphate + deamido-$NAD^+$
- NAD-synthase (EC 6.3.1.5) also known as NAD synthetase; $NAD^+$synthase; nicotinamide adenine dinucleotide synthetase; diphosphopyridine nucleotide synthetase, catalyzing the following reaction

  Deamido-$NAD^+$+ATP+NH3 = AMP+ diphosphate + $NAD^+$

In one embodiment of the invention, the coding regions encoding the different enzymes of the NAD salvage pathway comprise a nucleotide sequence encoding proteins with the amino acid sequences as set forth hereinafter.

Suitable nucleotide sequences encoding a nicotinamidase similar to PNC1 from Saccharomyces cerevisiae but from fungal or yeast-like origin include a nucleotide sequence encoding a nicotineamidase comprising an amino acid sequence selected from:

the amino acid sequence of accession number XP_444840 (Candida glabrata)

the amino acid sequence of accession number XP_456073 (Kluyveromyces lactis)

the amino acid sequence of accession number NP_986013 (Eremothecium gossypii)

the amino acid sequence of accession number XP_888958 (Candida albicans)

the amino acid sequence of accession number XP500320 (Yarrowia lipolytica)

the amino acid sequence of accession number XP389372 (Giberella zeae)

the amino acid sequence of accession number XP_749509 (Aspergillus fumigatus)

the amino acid sequence of accession number XP_712112 (Candida albicans)

the amino acid sequence of accession number BAE56421 (Aspergillus oryzae)

the amino acid sequence of accession number XP_567125 (Cryptococcus neofomans)

the amino acid sequence of accession number XP_964547 (Neurospora crassa)

the amino acid sequence of accession number XP_712135 (Candida albicans)

Suitable nucleotide sequences encoding an NAD(+) synthetase similar to Qns1 from Saccharomyces cerevisiae but from fungal origin include a nucleotide sequence encoding a NAD(+) synthetase comprising an amino acid sequence selected from:

the amino acid sequence of accession number XP_448179 (Candida glabrata)

the amino acid sequence of accession number XP_453643 (Kluyveromyces lactis)

the amino acid sequence of accession number NP_987024 (Eremothecium gossypii)

the amino acid sequence of accession number XP_500272 (Yarrowia lipolytica)

the amino acid sequence of accession number XP_722371 (Candida albicans)
the amino acid sequence of accession number XP_456405 (Debaromyces hansenii)
the amino acid sequence of accession number BAE61562 (Aspergillus oryzae)
the amino acid sequence of accession number XP_759702 (Ustilago maydis)
the amino acid sequence of accession number EAL18079 (Cryptococcus neoformans)
the amino acid sequence of accession number NP_587771 (Schizosaccharomyces pombe)
the amino acid sequence of accession number XP_681472 (Aspergillus nidulans)
the amino acid sequence of accession number XP_959191 (Neurospora crassa)
the amino acid sequence of accession number XP_567726 (Cryptococcus neoformans)
the amino acid sequence of accession number EAQ90706 (Chaetomium globosum)
the amino acid sequence of accession number XP_387574 (Giberella zeae)
the amino acid sequence of accession number XP_748008 (Aspergillus fumigatus)
the amino acid sequence of accession number XP_361704 (Magnaporthe grisea)

Suitable nucleotide sequences encoding an Nicotinic acid mononucleotide adenylyltransferase similar to NMA1 from Saccharomyces cerevisiae but from fungal origin include a nucleotide sequence encoding a acid mononucleotide adenylyltransferase comprising an amino acid sequence selected from:
the amino acid sequence of accession number Q06178
the amino acid sequence of accession number XP_444815 (Candida glabrata)
the amino acid sequence of accession number NP_986687 ((Eremothecium gossypii)
the amino acid sequence of accession number XP_453005 (Kluyveromyces lactis)

the amino acid sequence of accession number XP_458184(Debaromyces hansenii)
the amino acid sequence of accession number XP_718656 (Candida albicans)
the amino acid sequence of accession number XP_504391 (Yarrowia lipolytica)
the amino acid sequence of accession number NP_592856 (Schizosaccharomyces pombe)
the amino acid sequence of accession number XP_762639 (Ustilago maydis)
the amino acid sequence of accession number XP_571297 (Cryptococcus neoformans)
the amino acid sequence of accession number BAE57070 (Aspergillus oryzae)
the amino acid sequence of accession number XP_750776 (Aspergillus fumigatus)
the amino acid sequence of accession number XP_659349 (Aspergillus nidulans)
the amino acid sequence of accession number XP_389652 (Giberella zeae)
the amino acid sequence of accession number XP_957634 (Neurospora crassa)
the amino acid sequence of accession number XP_363364 (Magnaporthe grisea)
the amino acid sequence of accession number XP_758179 (Ustilago maydis)
the amino acid sequence of accession number EAQ85219 ((Chaetomium globosum)

Suitable nucleotide sequences encoding a nicotinate phosphoribosyltransferase similar to NPT1 from Saccharomyces cerevisiae but from fungal or yeast-like origin include a nucleotide sequence encoding nicotinate phosphoribosyltransferase comprising an amino acid sequence selected from:
the amino acid sequence of accession number CAA85352 (Saccharomyces cerevisae)
the amino acid sequence of accession number XP_448893 (Candida glabrata)
the amino acid sequence of accession number XP_453357 (Kluyveromyces lactis)
the amino acid sequence of accession number NP_983562 (Eremothecium gossypii)

the amino acid sequence of accession number XP_462577 (Debaromyces hansenii)
the amino acid sequence of accession number XP_889008 (Candida albicans)
the amino acid sequence of accession number XP_500338 (Yarrowia lipolytica)
the amino acid sequence of accession number XP_746744 (Aspergillus fumigatus)
the amino acid sequence of accession number BAE64333 (Aspergillus oryzae)
the amino acid sequence of accession number XP_965789 (Neurospora crassa)
the amino acid sequence of accession number EAQ93453 (Chaetomium globosum)
the amino acid sequence of accession number XP_682385 (Aspergillus nidulans)
the amino acid sequence of accession number AAN74808 (Gibberella moniliformis)
the amino acid sequence of accession number Q9UTK3
the amino acid sequence of accession number XP_361075 (Magnaporthe grisea)
the amino acid sequence of accession number EAL18922 (Cryptococcus neoformans)
the amino acid sequence of accession number XP_568039 (Cryptococcus neoformans)
the amino acid sequence of accession number XP_760597 (Ustilago maydis)

Suitable nucleotide sequences encoding an Nicotinic acid mononucleotide adenylyltransferase similar to NMA2 from Saccharomyces cerevisiae but from fungal or yeast like origin include a nucleotide sequence encoding a acid mononucleotide adenylyltransferase comprising an amino acid sequence selected from:
the amino acid sequence of accession number NP_011524
the amino acid sequence of accession number XP_444815 (Candida glabrata)
the amino acid sequence of accession number NP_986687 ((Eremothecium gossypii)

the amino acid sequence of accession number XP_453005 (Kluyveromyces lactis)
the amino acid sequence of accession number XP_458184 (Debaromyces hansenii)
the amino acid sequence of accession number XP_718656 (Candida albicans)
the amino acid sequence of accession number XP_504391 (Yarrowia lipolytica)
the amino acid sequence of accession number NP_592856 (Schizosaccharomyces pombe)
the amino acid sequence of accession number XP_762639 (Ustilago maydis)
the amino acid sequence of accession number XP_571297 (Cryptococcus neoformans)
the amino acid sequence of accession number BAE57070 (Aspergillus oryzae)
the amino acid sequence of accession number XP_750776 (Aspergillus fumigatus)
the amino acid sequence of accession number XP_659349 (Aspergillus nidulans)
the amino acid sequence of accession number XP_389652 (Giberella zeae)
the amino acid sequence of accession number XP_957634 (Neurospora crassa)
the amino acid sequence of accession number XP_363364 (Magnaporthe grisea)
the amino acid sequence of accession number XP_758179 (Ustilago maydis)
the amino acid sequence of accession number EAQ85219 ((Chaetomium globosum)

All amino acid sequences referred to by their accession numbers are herein incorporated by reference.

However, it will be clear that variants of these sequences, including insertions, deletions and substitutions thereof may be also be used to the same effect. Variants of the described sequenceq will have a sequence identity which is preferably at least about 80%, or 85 or 90% or 95% with identified sequences of enzymes from the NAD salvage pathway,. Preferably, these variants will be functional proteins with the same enzymatic activity as the enzymes from the

NAD salvage pathway. For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970). The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madision, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

Homologous nucleotide sequence from other fungi or yeast-like organisms may also be identified and isolated by hybridization under stringent conditions using as probes identified nucleotide sequences encoding enzymes from the NAD salvage pathway.

"Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C, preferably twice for about 10 minutes. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

The methods of the invention can be used to obtain plants tolerant to different kinds of stress-inducing conditions, particularly abiotic stress conditions including submergence, high light conditions, high UV radiation levels, increased hydrogen peroxide levels, drought conditions, high or low temperatures, increased salinity conditions. The methods of the invention can also be used to reduce the level of ROS in the cells of plants growing under adverse conditions, particularly abiotic stress conditions including submergence, high light conditions, high UV radiation levels, increased hydrogen peroxide levels, drought conditions, high or low temperatures, increased salinity conditions etc. The level of ROS or the level of NADH can be determined using the methods known in the art, including those described in Example 3.

Using the methods described herein, plants may be obtained wherein the level of ROS is equal to or lower than in control plants under non-stressed conditions, such as but not limited to low light. In these plants, under non-stressed conditions, the level of ROS may range from 50% to 100% of the level of control plants under low light conditions, more particularly from about 60% to about 85%. The level of the ROS in these plants under stress conditions is about 50% to 80% of the level of ROS in control plants under stress conditions, corresponding to about 60 to 80% of the level of ROS in control plants under non-stressed conditions. Similarly, the NADH level in these plants is equal to or higher than in control plants under non-stressed conditions, such as but not limited to low light. In these plants, under non-stressed conditions, the level of NADH may range from 100% to 160% of the level of NADH in control plants under low light conditions, more particularly from about 120% to about 140%. The level of NADH in these plants under stress conditions is about 200 to 300% of the level of NADH in control plants under stress conditions, corresponding to about 100 to 160% of the level of ROS in control plants under non-stressed conditions.

Methods to obtain transgenic plants are not deemed critical for the current invention and any transformation method and regeneration suitable for a

particular plant species can be used. Such methods are well known in the art and include *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation of intact cells, polyethyleneglycol-mediated protoplast transformation, electroporation of protoplasts, liposome-mediated transformation, silicon-whiskers mediated transformation etc. The transformed cells obtained in this way may then be regenerated into mature fertile plants.

The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene according to the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

It will be clear that the different stress resistant chimeric genes described herein, with DNA regions encoding different enzymes from the NAD salvage pathway can be combined within one plant cell or plant, to further enhance the stress tolerance of the plants comprising the chimeric genes. Thus, in one embodiment of the invention, plant cells and plants are provided which comprise at least two stress resistant chimeric genes each comprising a different coding region.

The transgenic plant cells and plant lines according to the invention may further comprise chimeric genes which will reduce the expression of endogenous PARP and/or PARG genes as described in WO 00/04173 and PCT/EP2004/003995 . These further chimeric genes may be introduced e.g. by crossing the transgenic plant lines of the current invention with transgenic plants containing PARP and/or PARG gene expression reducing chimeric genes. Transgenic plant cells or plant lines may also be obtained by introducing or transforming the chimeric genes of the invention into transgenic plant cells comprising the PARP or PARG gene expression reducing chimeric genes or vice versa.

For the purpose of the invention, the promoter is a plant-expressible promoter. As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S (Harpster et al., 1988 *Mol. Gen. Genet.* **212**, 182-190), the subterranean clover virus promoter No 4 or No 7 (WO9606932), or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An et al., 1996, *The Plant Cell* **8**, 15-30), stem-specific promoters (Keller et al., 1988, *EMBO J.* **7**, 3625-3633), leaf specific promoters (Hudspeth et al., 1989, *Plant Mol Biol* **12**, 579-589), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al.,1989, *Genes Devel.* **3**, 1639-1646), tuber-specific promoters (Keil et al., 1989, *EMBO J.* **8**, 1323-1330), vascular tissue specific promoters ( Peleman et al., 1989, *Gene* **84**, 359-369), stamen-selective promoters ( WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

The chimeric genes of the inventions may also be equipped with a nuclear localization signal ("NLS") functional in plants, operably linked to the DNA region encoding an enzyme of the NAD salvage pathway such as the SV40 NLS.

Having read this document, a person skilled in the art will immediately realize that similar effects with regard to increased stress resistance can be obtained whenever natural variants of plants are obtained wherein the endogenous genes coding for NAD salvage pathway enzymes are more active or expressed at a higher level. Such variant plants can be obtained by subjecting a population of plants to mutagenesis, such as, but not limited to EMS mutagenesis, followed by a screening for an increased activity of any one of the NAD salvage pathway enzymes, or a combination thereof.

It will also be immediately clear that a population of different varieties or cultivars can be screened for increased tolerance to the above mentioned stress conditions in general or particular selected abiotic stresses, followed by a correlation of the increased tolerance to stress conditions with the presence of a particular allele of any of the endogenous genes encoding an enzyme of the NAD salvage pathway enzyme. Such alleles can than be introduced into a plant of interest by crossing, if the species are sexually compatible, or they may be identified using conventional techniques as described herein (including hybridization or PCR amplification) and introduced using recombinant DNA technology. Introduction of particularly desired alleles using breeding techniques may be followed using molecular markers specific for the alleles of interest.

The methods and means described herein are believed to be suitable for all plant cells and plants, both dicotyledonous and monocotyledonous plant cells and plants including but not limited to cotton, Brassica vegetables, oilseed rape, wheat, corn or maize, barley, sunflowers, rice, oats, sugarcane, soybean, vegetables (including chicory, lettuce, tomato), tobacco, potato, sugarbeet, papaya, pineapple, mango, *Arabidopsis thaliana*, but also plants used in horticulture, floriculture or forestry.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

The following non-limiting Examples describe the construction of chimeric genes to increase stress resistance in plant cells and plants and the use of such genes.

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

Throughout the specification reference is made to the following entries in the Sequence listing:

SEQ ID No. 1 :XP_444840 (Candida glabrata)
SEQ ID No. 2: XP_456073 (Kluyveromyces lactis)
SEQ ID No. 3: NP_986013 (Eremothecium gossypii)
SEQ ID No. 4: XP_888958 (Candida albicans)
SEQ ID No. 5: XP500320 (Yarrowia lipolytica)
SEQ ID No. 6: XP389372 (Giberella zeae)
SEQ ID No. 7: XP_749509 (Aspergillus fumigatus)
SEQ ID No. 8: XP_712112 (Candida albicans)

SEQ ID No. 9: BAE56421 (Aspergillus oryzae)

SEQ ID No. 10: XP_567125 (Cryptococcus neofomans)

SEQ ID No. 11: XP_964547 (Neurospora crassa)

SEQ ID No. 12: XP_712135 (Candida albicans)

SEQ ID No. 13: XP_448179 (Candida glabrata)

SEQ ID No. 14: XP_453643 (Kluyveromyces lactis)

SEQ ID No. 15: NP_987024 (Eremothecium gossypii)

SEQ ID No. 16: XP_500272 (Yarrowia lipolytica)

SEQ ID No. 17: XP_722371 (Candida albicans)

SEQ ID No. 18: XP_456405 (Debaromyces hansenii)

SEQ ID No. 19: BAE61562 (Aspergillus oryzae)

SEQ ID No. 20: XP_759702 (Ustilago maydis)

SEQ ID No. 21: EAL18079 (Cryptococcus neoformans)

SEQ ID No. 22: NP_587771 (Schizosaccharomyces pombe)

SEQ ID No. 23: XP_681472 (Aspergillus nidulans)

SEQ ID No. 24: XP_959191 (Neurospora crassa)

SEQ ID No. 25: XP_567726 (Cryptococcus neoformans)

SEQ ID No. 26: EAQ90706 (Chaetomium globosum)

SEQ ID No. 27: XP_387574 (Giberella zeae)

SEQ ID No. 28: XP_748008 (Aspergillus fumigatus)

SEQ ID No. 29: XP_361704 (Magnaporthe grisea)

SEQ ID No. 30: Q06178

SEQ ID No. 31: XP_444815 (Candida glabrata)

SEQ ID No. 32: NP_986687 ((Eremothecium gossypii)

SEQ ID No. 33: XP_453005 (Kluyveromyces lactis)

SEQ ID No. 34: XP_458184(Debaromyces hansenii)

SEQ ID No. 35: XP_718656 (Candida albicans)

SEQ ID No. 36: XP_504391 (Yarrowia lipolytica)

SEQ ID No. 37: NP_592856 (Schizosaccharomyces pombe)

SEQ ID No. 38: XP_762639 (Ustilago maydis)

SEQ ID No. 39: XP_571297 (Cryptococcus neoformans)

SEQ ID No. 40: BAE57070 (Aspergillus oryzae)
SEQ ID No. 41: XP_750776 (Aspergillus fumigatus)
SEQ ID No. 42: XP_659349 (Aspergillus nidulans)
SEQ ID No. 43: XP_389652 (Giberella zeae)
SEQ ID No. 44: XP_957634 (Neurospora crassa)
SEQ ID No. 45: XP_363364 (Magnaporthe grisea)
SEQ ID No. 46: XP_758179 (Ustilago maydis)
SEQ ID No. 47: EAQ85219 ((Chaetomium globosum)
SEQ ID No. 48: CAA85352 (Saccharomyces cerevisae)
SEQ ID No. 49: XP_448893 (Candida glabrata)
SEQ ID No. 50: XP_453357 (Kluyveromyces lactis)
SEQ ID No. 51: NP_983562 (Eremothecium gossypii)
SEQ ID No. 52: XP_462577 (Debaromyces hansenii)
SEQ ID No. 53: XP_889008 (Candida albicans)
SEQ ID No. 54: XP_500338 (Yarrowia lipolytica)
SEQ ID No. 55: XP_746744 (Aspergillus fumigatus)
SEQ ID No. 56: BAE64333 (Aspergillus oryzae)
SEQ ID No. 57: XP_965789 (Neurospora crassa)
SEQ ID No. 58: EAQ93453 (Chaetomium globosum)
SEQ ID No. 59: XP_682385 (Aspergillus nidulans)
SEQ ID No. 60: AAN74808 (Gibberella moniliformis)
SEQ ID No. 61: Q9UTK3
SEQ ID No. 62: XP_361075 (Magnaporthe grisea)
SEQ ID No. 63: EAL18922 (Cryptococcus neoformans)
SEQ ID No. 64: XP_568039 (Cryptococcus neoformans)
SEQ ID No. 65: XP_760597 (Ustilago maydis)
SEQ ID No. 66 :NP_011524

All amino acid sequences referred to by their accession numbers are herein incorporated by reference.

## Examples

### Example 1: Assembly of stress resistant chimeric genes and introduction into plants.

To increase the stress resistance in plants, a chimeric gene is constructed using conventional techniques comprising the following DNA fragments in order:

- A promoter region from Cauliflower Mosaic Virus (CaMV 35S);
- A DNA fragment of about 60 bp corresponding to the untranslated leader Cab22L;
- A DNA fragment as mentioned herein elsewhere encoding a NAD salvage pathway enzyme from fungal or yeast-like origin, different from PNC1, NMA1, NMA2 or NPT1 from Saccharomyces cereviseae.
- A fragment of the 3' untranslated end from the 35 S transcript of CaMV (3' 35S)

This chimeric gene is introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene.

The T-DNA vectors are introduced into Agrobacterium strains comprising a helper Ti-plasmid using conventional methods. The chimeric genes are introduced into plants using a conventional transformation method. Transgenic plants exhibit a higher stress resistance than their counterpart plants without transgenes.

## Claims

1. A method for obtaining a plant with increased stress resistance comprising
    a. introducing a chimeric gene into a cells of a plant to obtain transgenic cells, said chimeric gene comprising the following operably linked DNA fragments:
        i. A plant-expressible promoter;
        ii. A DNA region coding for a plant-functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase;
        iii. A 3'end region involved in transcription termination and polyadenylation;
    b. regenerating said transgenic cells to obtain a population of transgenic plants; and

selecting a plant from said population of transgenic plants which exhibits increased stress resistance or selecting a plant which exhibits a reduced level of reactive oxygen species or maintains a high level of NADH under stress conditions when compared to a similar non-transgenic plant characterized in that that the amino acid sequence encoded by the DNA region comprises the amino acid sequence of any one of SEQ ID Nos. 1 to 29, 31 to 47 or 49 to 66 or an amino acid sequence having at least 90% sequence identity to said amino acid sequences of any one of SEQ ID Nos. 1 to 29, 31 to 47 or 49 to 66.

2. A chimeric gene as described in claim 1.

3. A plant cell comprising a chimeric gene as described in claim 3.

4. A plant comprising a chimeric gene as described in claim 2.

5. The plant according to claim 4, further characterized in that it has a lower level of reactive oxygen species under stress conditions than a similar plant not comprising such a chimeric gene.

6. A seed of a plant according to claim 4 or 5 comprising a chimeric gene according to claim 2.

7. Use of a chimeric gene according to claim 2 to increase the stress resistance of a plant.

8. Use of a chimeric gene according to claim 2 to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NAD in a plant or plant cell under stress conditions.

9. Use of a DNA sequence encoding a plant functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase to increase the stress resistance of a plant characterized in that said plant functional enzyme is derived from a fungal or yeast-like organism different than Saccharomyces cereviseae.

10. Use of a DNA sequence encoding a plant functional enzyme of the nicotinamide adenine dinucleotide salvage synthesis pathway selected from nicotinamidase, nicotinate phosphoribosyltransferase, nicotinic acid mononucleotide adenyl transferase or nicotinamide adenine dinucleotide synthetase to decrease the level of reactive oxygen species in a plant or a plant cell under stress conditions or to maintain the level of NAD in a plant or plant cell under stress conditions characterized in that said plant functional enzyme is derived from a fungal or yeast-like organism different than Saccharomyces cereviseae.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/002433

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 C07K14/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2004/016726 A2 (HARVARD COLLEGE [US]; SINCLAIR DAVID A [US] HARVARD COLLEGE [US]; SINC) 26 February 2004 (2004-02-26) cited in the application abstract page 5, line 14 - page 6, line 2 page 8, line 32 - page 9, line 4 page 27, line 7 - page 31, line 14; sequence 8 | 1-10 |
| X | JP 2004 261136 A (UNIV TOKYO) 24 September 2004 (2004-09-24) the whole document | 1-10 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 June 2007 | 28/06/2007 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Mundel, Christophe |

1

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# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/002433

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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| A | HUNT LEE ET AL: "NAD - new roles in signalling and gene regulation in plants" NEW PHYTOLOGIST, CAMBRIDGE UNIVERSITY PRESS, CAMBRIDGE, GB, vol. 163, no. 1, July 2004 (2004-07), pages 31-44, XP002375575 ISSN: 0028-646X | 1-10 |
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Information on patent family members

International application No
PCT/EP2007/002433

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