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Methods and means for increasing the tolerance of plants to stress conditions

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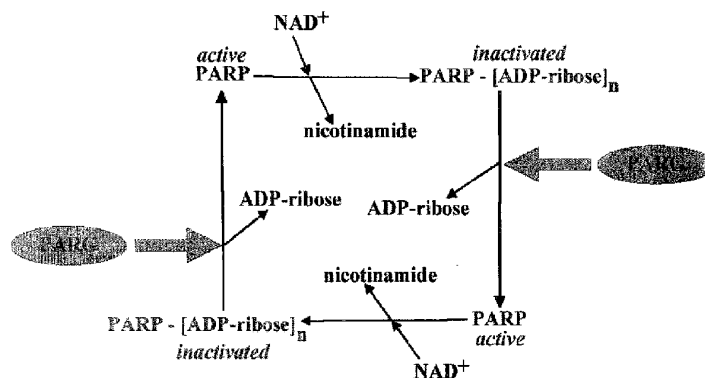
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(54) Title: METHODS AND MEANS FOR INCREASING THE TOLERANCE OF PLANTS TO STRESS CONDITIONS



(57) Abstract: Methods and means are provided to increase the tolerance of plants to abiotic stress or adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations and the like by reducing the activity of endogenous PARP proteins in plants.



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Methods and means for increasing the tolerance of plants to stress conditions.

Field of the invention.

[1] The present invention relates to the use of poly (ADP-ribose) glycohydrolases in plants to increase the tolerance of plants to adverse growing conditions, including drought, high light intensities, high temperatures, nutrient limitations and the like. Methods and means are provided to produce plants that are tolerant to abiotic stress conditions.

Background to the invention

[2] . Frequently, abiotic stress will lead either directly or indirectly to damage of the DNA of the cells of the plants exposed to the adverse conditions. Genomic damage, if left unrepaired, can lead to cell death. Tolerance to stress conditions exhibited by plants is the result of the ability of the plant cells exposed to the adverse conditions to reduce and/or repair the damage, and to survive.

[3] Plant cells, like other eukaryotic cells, have evolved an elaborate DNA repair system. The activation of poly(ADP-ribose) polymerase (PARP) by DNA strand breaks is often one of the first cellular responses to DNA damage. PARP catalyzes the post-translational modification of proteins by adding successively molecules of ADP-ribose, obtained from the conversion of nicotinamide dinucleotide (NAD), to form multibranched polymers containing up to 200 ADP-ribose residues (about 40 residues in plants). The dependence of poly(ADP-ribose) synthesis on DNA strand breaks, and the presence of PARP in multiprotein complexes further containing key effectors of DNA repair, replication and transcription reactions, strongly suggests that this posttranslational modification is involved in metabolism of nucleic acids, and DNA repair. There are also indications that poly (ADP -ribose) synthesis is involved in regulation of cell cycle and cell death.

[4] Poly (ADP-ribosylation) of proteins is transient in living cells. The poly (ADP-ribose) polymers are rapidly turned over, being converted to free ADP-ribose by the exoglycosidase and endoglycosidase activity of poly (ADP-ribose) glycohydrolase (PARG; E.C.3.2.1.143). The most proximal unit of ADP-ribose on the protein acceptor is hydrolyzed by the action of another enzyme (ADP-ribosyl protein lyase).

[5] In addition to this positive (DNA-repair associated) effect of PARP on cell survival, there is also a negative effect of PARP. The process of activating PARP upon DNA damage is associated with a rapid lowering of NAD⁺ levels, since each ADP-ribose unit transferred by PARP consumes one molecule of NAD⁺. NAD⁺ depletion in turn results in ATP depletion, because NAD⁺ resynthesis requires at least (depending on the biosynthesis pathway) three molecules of ATP per molecule of NAD⁺. Furthermore, NAD⁺ depletion block glyceraldehyde -3-phosphate dehydrogenase activity, which is required to resynthesize ATP during glycolysis. Finally, NAD⁺ is a key carrier of electrons needed to generate ATP via electron transport and oxidative phosphorylation.

[6] The physiological consequence of NAD⁺ and ATP depletion has been established in the context of DNA-damage induced cell death. It has been shown that the completion of apoptosis is absolutely dependent on the presence of ATP and that, in the absence of this nucleotide, the type of cellular demise switches from apoptosis to necrosis. Since the cellular lysis associates with necrosis generates further damage to neighboring cells it is preferable for multicellular organisms to favor apoptotic cell death rather than necrosis.

[7] It is thus very important to consider the delicate balance of positive and negative effects of the poly (ADP ribosylation) on the potential of a cell to survive DNA damage.

[8] WO 00/04173 describes methods to modulate programmed cell death (PCD) in eukaryotic cells and organisms, particularly plant cells and plants, by

introducing of "PCD modulating chimeric genes" influencing the expression and/or apparent activity of endogenous poly-(ADP-ribose) polymerase (PARP) genes. Programmed cell death may be inhibited or provoked. The invention particularly relates to the use of nucleotide sequences encoding proteins with PARP activity for modulating PCD, for enhancing growth rate or for producing stress tolerant cells and organisms.

[9] PARP encoding genes have been identified in a number of animals such as *Rattus norvegicus* (Accession numbers: NM_031339, NW_043030, AB019366,), *Mus musculus* (Accession numbers: NT_039598, NM_003631, AF079557), *Homo sapiens* (Accession numbers: NT_017696; NM_003631, AF005043), *Bos taurus* (Accession numbers: NM_174138, U78975) *Drosophila melanogaster* (Accession number: AF079556)

[10] In plants, a poly(ADP-ribose) glycohydrolase has been identified by map-based cloning of the wild-type gene inactivated in a mutant affected in clock-controlled transcription of genes in *Arabidopsis* and in photoperiod dependent transition from vegetative growth to flowering (*tef*). The nucleotide sequence of the gene can be obtained from nucleotide databases under the accession number AF394690 (Panda et al., 2002 Dev. Cell. 3, 51-61).

Summary of the invention

[11] The invention provides a method to produce a plant tolerant to stress conditions comprising the steps of providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments: a plant-expressible promoter; a DNA region, which when transcribed yields an ParG inhibitory RNA molecule; and a 3' end region involved in transcription termination and polyadenylation. A population of transgenic plant lines is regenerated from the transgenic plant cell; and a stress tolerant plant line is identified within the population of transgenic plant lines. The ParG inhibitory RNA molecule may comprise a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG

gene). The ParG inhibitory RNA molecule may also comprise a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG gene). In yet another embodiment, the parG inhibitory RNA may comprise a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell, wherein the sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides. The chimeric gene may further comprise a DNA region encoding a self-splicing ribozyme between said DNA region coding for parG inhibitory RNA molecule and the 3' end region. Stress conditions may be selected from heat, drought, nutrient depletion, oxidative stress or high light conditions.

[12] In another embodiment of the invention, a method is provided to produce a plant tolerant to stress conditions comprising the steps of: isolating a DNA fragment of at least 100 bp comprising a part of the parG encoding gene of the plant of interest; producing a chimeric gene by operably linking a plant expressible promoter region to the isolated DNA fragment comprising part of the parG encoding gene of the plant in direct orientation compared to the promoter region; and to the isolated DNA fragment comprising part of the parG encoding gene of said plant in inverted orientation compared to the promoter region, and a 3' end region involved in transcription termination and polyadenylation. These chimeric genes are then provided to plant cells to create transgenic plant cells. A population of transgenic plant lines is regenerated from the transgenic plant cells; and a stress tolerant plant line is identified within the population of transgenic plant lines. The invention also relates to stress tolerant plant cells and plants obtained by this process.

[13] In yet another embodiment of the invention, a method is provided to produce a plant tolerant to stress conditions comprising the steps of providing plant cells with a chimeric gene to create transgenic plant cells, comprising a

DNA region, which when transcribed yields an ParG inhibitory RNA molecule, whereby the DNA region comprises a nucleotide sequence of at least 21 to 100 nucleotides of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 1, 2 or 16 or at least 21 to 100 nucleotides of a nucleotide sequence of SEQ ID 3, 4, 15 or 23 operably linked to a plant-expressible promoter and a 3' end region involved in transcription termination and polyadenylation; regenerating a population of transgenic plant lines from said transgenic plant cell; and identifying a stress tolerant plant line within the population of transgenic plant lines.

[14] The invention also provides DNA molecules comprising a plant-expressible promoter, operably linked to a DNA region, which when transcribed yields an ParG inhibitory RNA molecule, and to a 3' end region involved in transcription termination and polyadenylation. The ParG inhibitory RNA molecule may comprise a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG gene). The ParG inhibitory RNA molecule may also comprise a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell (the endogenous ParG gene). In yet another embodiment, the parG inhibitory RNA may comprise a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in the plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in the plant cell, wherein the sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides. The chimeric gene may further comprise a DNA region encoding a self-splicing ribozyme between said DNA region coding for parG inhibitory RNA molecule and the 3' end region. The chimeric gene may also comprise a nucleotide sequence of at least 21 to 100 nucleotides of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 1, 2 or 16 or at least 21 to 100 nucleotides of a nucleotide sequence of SEQ ID 3, 4, 15 or 23.

[15] In yet another embodiment, the invention relates to plant cell comprising the DNA molecule of the invention and plants consisting essentially of such plant cells, as well as to processes for producing stress tolerant plants, comprising the step of further crossing such plants with another plant. Seeds and propagating material of such plants comprising the chimeric genes of the invention are also provided.

[16] The invention also relates to a method for obtaining stress tolerant plants comprising the steps of subjecting a plant cell line or a plant or plant line, to mutagenesis; identifying those plant cells or plants that have a mutation in an endogenous ParG gene; subjecting the identified plant cells or plants to stress conditions and identifying plant cells or plants that tolerate said stress conditions better than control plants. Alternatively, plant cells or plants may be selected for resistance to ParG inhibitors and further treated as described in this paragraph.

[17] The invention further relates to a stress tolerant plant cell or plant having a mutation in the endogenous ParG gene.

Brief description of the figures.

[18] Figure 1. Schematic representation of the poly-ADP ribose polymerization / depolymerization cycle by the action of PARP/PARG in a eukaryotic cell.

[19] Figure 2. Diagram of the NAD⁺ and ATP content of *Arabidopsis* lines under high light stress. Dark boxes represent NAD content under high light conditions expressed as percentage of the value for NAD content determined under low light conditions. Light boxes represent ATP content under high light conditions expressed as percentage of the value for ATP content determined under low light conditions.

[20] Figure 3. Diagram of the NAD⁺ and ATP content of corn lines under nitrogen depletion stress. Dark boxes represent NAD content while light boxes represent ATP content.

Detailed description of preferred embodiments

[21] The invention is based, on the one hand, on the demonstration that cells from stress resistant plant lines comprising a chimeric gene reducing the PARP gene expression, exhibited a higher NAD/ATP content under adverse conditions than cells from untransformed plant lines. On the other hand, it has been observed that silencing of the expression of PARG encoding gene in tobacco using a transient silencing RNA vector based on satellite viruses resulted in a similar phenotype as that observed for silencing of PARP encoding gene using the same silencing system. Furthermore, silencing the expression of PARG encoding gene in plants, such as Arabidopsis and tobacco, resulted in plants that were more resistant to stress conditions, such as e.g. those imposed by high light conditions.

[22] Although not intending to limit the invention to a specific mode of action, it is expected that silencing of PARG gene expression results in a similar phenotype as silencing of PARP gene expression for the following reasons. As can be seen from Figure 1, polymerization of ADP ribose catalyzed by PARP, consuming NAD, is followed by depolymerization of poly ADP ribose, catalyzed by PARG. Poly ADP ribosylation of the PARP protein itself results in inactivation of the PARP protein. The speed at which the ADP ribose polymerization / depolymerization cycle occurs in plant cells, leading to NAD depletion and consequently ATP depletion, can be slowed down or stopped by reduction of the PARP gene expression or of the enzymatic activity of PARP. As a result, plant cells, and plants comprising such cells are more resistant to adverse conditions. The data provided here indicate that a similar effect can be obtained through slowing down or stopping the cycle by reduction of the PARG gene expression or PARG activity.

[23] The invention relates to reduction of plant cell death in response to adverse environmental conditions, and consequently to enhanced stress resistance, by altering the level of expression of ParG genes, or by altering the activity or the apparent activity of PARG proteins in that plant cell. Conveniently, the level of expression of ParG genes may be controlled genetically by introduction of chimeric genes altering the expression of ParG genes, or by altering the endogenous PARG encoding genes, including the expression signals.

[24] In one embodiment of the invention, a method for producing plants tolerant to stress conditions or adverse growing conditions is provided comprising the steps of:

- providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments:
 - a plant-expressible promoter;
 - a DNA region, which when transcribed yields a ParG inhibitory RNA molecule;
 - a 3' end region involved in transcription termination and polyadenylation;
- regenerating a population of transgenic plant lines from said transgenic plant cell; and
- identifying a stress tolerant plant line within said population of transgenic plant lines.

[25] As used herein "a stress tolerant plant" or "a plant tolerant to stress conditions or adverse growing conditions" is a plant (particularly a plant obtained according to the methods of the invention), which, when subjected to adverse growing conditions for a period of time, such as but not limited to drought, high temperatures, limited supply of nutrients (particularly nitrogen), high light intensities, grows better than a control plant not treated according to the methods of the invention. This will usually be apparent from the general appearance of the plants and may be measured e.g., by increased biomass production, continued vegetative growth under adverse conditions or higher

seed yield. Stress tolerant plant have a broader growth spectrum, i.e. they are able to withstand a broader range of climatological and other abiotic changes, without yield penalty. Biochemically, stress tolerance may be apparent as the higher NAD⁺-NADH /ATP content and lower production of reactive oxygen species of stress tolerant plants compared to control plants under stress condition. Stress tolerance may also be apparent as the higher chlorophyll content, higher photosynthesis and lower chlorophyll fluorescence under stress conditions in stress tolerant plants compared to control plants under the same conditions.

[26] It will be clear that it is also not required that the plant be grown continuously under the adverse conditions for the stress tolerance to become apparent. Usually, the difference in stress tolerance between a plant or plant cell according to the invention and a control plant or plant cell will become apparent even when only a relatively short period of adverse conditions is encountered during growth.

[27] As used herein, a "ParG inhibitory RNA molecule" is an RNA molecule that is capable of decreasing the expression of the endogenous PARG encoding genes of a plant cell, preferably through post-transcriptional silencing. It will be clear that even when a ParG inhibitory RNA molecule decreases the expression of a PARG encoding gene through post-transcriptional silencing, such an RNA molecule may also exert other functions within a cell, such as e.g. guiding DNA methylation of the endogenous ParG gene, again ultimately leading to decreased expression of the PARG encoding gene. Also, expression of the endogenous PARG encoding genes of a plant cell may be reduced by transcriptional silencing, e.g., by using RNAi or dsRNA targeted against the promoter region of the endogenous ParG gene.

[28] As used herein, a "PARG encoding gene" or a "ParG gene" is a gene capable of encoding a PARG (poly ADP ribose glycohydrolase) protein, wherein the PARG protein catalyzes the depolymerization of poly ADP-ribose,

by releasing free ADP ribose units either by endoglycolytic or exoglycolytic action.

[29] PARG encoding genes may comprise a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 1 (*Arabidopsis thaliana*) or of SEQ ID No 2 (*Solanum tuberosum*) or of SEQ ID No 16 (*Oryza sativa*) or parts thereof, such as a DNA fragment comprising the nucleotide sequence of SEQ ID No. 3 or SEQ ID 4 or SEQ ID No 15. or SEQ ID 23 (*Zea mays*).

[30] However, it will be clear that the skilled person can isolate variant DNA sequences from other plant species, by hybridization with a probe derived from the above mentioned PARG encoding genes from plant species, or even with a probe derived from the above mentioned PARG encoding genes from animal species. To this end, the probes should preferably have a nucleotide sequence comprising at least 40 consecutive nucleotides from the coding region of those mentioned PARG encoding genes sequences, preferably from the coding region of SEQ ID No 3 or SEQ ID No 4. The probes may however comprise longer regions of nucleotide sequences derived from the ParG genes, such as about 50, 60, 75, 100, 200 or 500 consecutive nucleotides from any of the mentioned ParG genes. Preferably, the probe should comprise a nucleotide sequence coding for one of the highly conserved regions of the catalytic domain, which have been identified by aligning the different PARG proteins from animals. These regions are also present in the identified PARG protein from *Arabidopsis thaliana* and comprise the amino acid sequence LXVDFANXXXGGG (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 264; X may be any amino acid) LXVDFANXXXGGGXXXGXVQEEIRF (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 277) or LXVDFANXXXGGGXXXGXVQEEIRFXXXPE (corresponding to SEQ ID No 1 from the amino acid at position 252 to the amino acid at position 282), TGXWGCXFXGD (corresponding to SEQ ID No 1 from the amino acid at position 449 to the amino acid at position 460) or TGXWGCXFXGDXXLXXXQ (corresponding to SEQ ID No 1 from the

amino acid at position 449 to the amino acid at position 468). Other conserved regions have the amino acid sequence DXXXRXXXAIDA (corresponding to SEQ ID No 1 from the amino acid at position 335 to the amino acid at position 344) or REXXKAXXGF (corresponding to SEQ ID No 1 from the amino acid at position 360 to the amino acid at position 369) or GXXXXSXYTGY (corresponding to SEQ ID No 1 from the amino acid at position 303 to the amino acid at position 313). Hybridization should preferably be under stringent conditions.

[31] "Stringent hybridization conditions" as used herein mean 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, e.g. for about 10 min (twice). 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.

[32] Alternatively, ParG encoding genes or parts thereof may also be isolated by PCR based techniques, using as primers oligonucleotides comprising at least 20 consecutive nucleotides from a nucleotide sequence of the mentioned PARG encoding genes or the complement thereof. Such primers may comprise a nucleotide sequence encoding a conserved region, as mentioned above, or be complementary to such a nucleotide sequence. Oligonucleotides which may be used for that purpose may comprise the nucleotide sequence of either or SEQ ID No.5, SEQ ID No 6., SEQ ID No. 7 or SEQ ID No. 8. Oligonucleotides which may be used may also be degenerate, such as the oligonucleotide primers of SEQ ID No 17, SEQ ID No 18, SEQ ID No 19; SEQ ID No 20, SEQ ID No 21 or SEQ ID No 22.

[33] Specific PCR fragments from ParG genes may e.g., be obtained by using combinations of the oligonucleotides having the nucleotide sequence of SEQ ID No. 5 and SEQ ID No 6 using e.g., *Arabidopsis* genomic DNA or cDNA as a template DNA, or by using combinations of the oligonucleotides having the nucleotide sequence of SEQ ID No. 7 and SEQ ID No 8 using e.g., potato genomic DNA or cDNA as a template DNA, under stringent annealing conditions.

[34] The isolated sequences may encode a functional PARG protein or a part thereof. Preferably the isolated sequences should comprise a nucleotide sequence coding for one or more of the highly conserved regions from the catalytic domain of PARG proteins as mentioned elsewhere.

[35] However, for the purpose of the invention is not required that the isolated sequences encode a functional ParG protein nor that a complete coding region is isolated. Indeed, all that is required for the invention is that a chimeric gene can be designed or produced, based on the identified or isolated sequence of the endogenous ParG gene from a plant, which is capable of producing a ParG inhibitory RNA. Several alternative methods are available to produce such a ParG inhibitory RNA molecule.

[36] In one embodiment, the ParG inhibitory RNA molecule encoding chimeric gene is based on the so-called antisense technology. In other words, the coding region of the chimeric gene comprises a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the endogenous ParG gene of the plant cell or plant, the expression of which is targeted to be reduced. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified ParG gene, or part of such a gene, in inverse orientation, to a plant expressible promoter and 3'end formation region involved in transcription termination and polyadenylation. It will be immediately clear that there is no need to know the exact nucleotide sequence or complete nucleotide sequence of such a DNA fragment from an isolated ParG gene.

[37] In another embodiment the ParG inhibitory RNA molecule encoding chimeric gene is based on the so-called co-suppression technology. In other words, the coding region of the chimeric gene comprises a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the endogenous ParG gene of the plant cell or plant, the expression of which is targeted to be reduced. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified ParG gene, or part of such a gene, in direct orientation, to a plant expressible promoter and 3' end formation region involved in transcription termination and polyadenylation. Again it is not required to know the exact nucleotide sequence of the used DNA fragment from the isolated ParG gene.

[38] The efficiency of the above mentioned chimeric genes in reducing the expression of the endogenous ParG gene may be further enhanced by inclusion of DNA elements which result in the expression of aberrant, unpolyadenylated ParG inhibitory RNA molecules. One such DNA element suitable for that purpose is a DNA region encoding a self-splicing ribozyme, as described in WO 00/01133.

[39] The efficiency of the above mentioned chimeric genes in reducing the expression of the endogenous ParG gene of a plant cell may also be further enhanced by including into one plant cell simultaneously a chimeric gene as herein described encoding a antisense ParG inhibitory RNA molecule and a chimeric gene as herein described encoding a sense ParG inhibitory RNA molecule, wherein said antisense and sense ParG inhibitory RNA molecules are capable of forming a double stranded RNA region by base pairing between the mentioned at least 20 consecutive nucleotides, as described in WO 99/53050.

[40] As further described in WO 99/53050, the sense and antisense ParG inhibitory RNA regions, capable of forming a double stranded RNA region may be present in one RNA molecule, preferably separated by a spacer region.

The spacer region may comprise an intron sequence. Such a chimeric gene may be conveniently constructed by operably linking a DNA fragment comprising at least 20 nucleotides from the isolated or identified endogenous ParG gene, the expression of which is targeted to be reduced, in an inverted repeat, to a plant expressible promoter and 3' end formation region involved in transcription termination and polyadenylation. To achieve the construction of such a chimeric gene, use can be made of the vectors described in WO 02/059294

[41] An embodiment of the invention thus concerns a method for obtaining a stress tolerant plant line comprising the steps of

- providing plant cells with a chimeric gene to create transgenic plant cells, wherein the chimeric gene comprises the following operably linked DNA fragments:

- a plant-expressible promoter;
- a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell; or
- a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell; or
- a DNA region, which when transcribed yields a ParG inhibitory RNA molecule comprising a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell, wherein said sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides.

- a 3' end region involved in transcription termination and polyadenylation;
- regenerating a population of transgenic plant lines from said transgenic plant cell; and
- identifying a stress tolerant plant line within said population of transgenic plant lines.

[42] 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.

[43] It will thus be clear that the minimum nucleotide sequence of the antisense or sense RNA region of about 20 nt of the ParG coding region may be comprised within a larger RNA molecule, varying in size from 20 nt to a length equal to the size of the target gene.

[44] The mentioned antisense or sense nucleotide regions may thus be about from about 21 nt to about 5000 nt long, such as 21nt, 40 nt, 50 nt, 100nt, 200 nt, 300nt, 500nt, 1000 nt, 2000 nt or even about 5000 nt or larger in length.

[45] Moreover, it is not required for the purpose of the invention that the nucleotide sequence of the used inhibitory ParG RNA molecule or the encoding region of the chimeric gene, is completely identical or complementary to the endogenous ParG gene the expression of which is targeted to be reduced in the plant cell. The longer the sequence, the less stringent the requirement for the overall sequence identity is. Thus, the sense or antisense regions may have an overall sequence identity of about 40 % or

50% or 60 % or 70% or 80% or 90 % or 100% to the nucleotide sequence of the endogenous ParG gene or the complement thereof. However, as mentioned antisense or sense regions should comprise a nucleotide sequence of 20 consecutive nucleotides having about 100% sequence identity to the nucleotide sequence of the endogenous ParG gene. Preferably the stretch of about 100 % sequence identity should be about 50, 75 or 100 nt.

[46] For the purpose of this invention, the "sequence identity" of two related nucleotide 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) Computer-assisted sequence alignment, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

[47] It will be clear that whenever nucleotide sequences of RNA molecules are defined by reference to nucleotide sequence of corresponding DNA molecules, the thymine (T) in the nucleotide sequence should be replaced by uracil (U). Whether reference is made to RNA or DNA molecules will be clear from the context of the application.

[48] It will also be clear that chimeric genes capable of producing inhibitory ParG genes for a particular ParG gene in a particular plant variety or plant species, may also be used to inhibit ParG gene expression in other plant varieties or plant species. Indeed, when sufficient homology exists between the ParG inhibitory RNA region and the ParG gene, or when the ParG genes share the same stretch of 19 nucleotides, expression of those other genes will also be down-regulated.

[49] In view of the potential role of ParG in nucleic acid metabolism, it may be advantageous that the expression of the endogenous ParG gene by the ParG inhibitory RNA is not completely inhibited. Downregulating the expression of a particular gene by gene silencing through the introduction of a chimeric gene encoding ParG inhibitory RNA will result in a population of different transgenic lines, exhibiting a distribution of different degrees of silencing of the ParG gene. The population will thus contain individual transgenic plant lines, wherein the endogenous ParG gene is silenced to the required degree of silencing. A person skilled in the art can easily identify such plant lines, e.g. by subjecting the plant lines to a particular adverse condition, such as a high light intensity, oxidative stress, drought, heat etc. and selecting those plants which perform satisfactory and survive best the treatment.

[50] As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

[51] The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (i.e., regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a DNA sequence located upstream (i.e., 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (i.e., 3') of the coding sequence and which comprises suitable transcription termination (and/or regulation) signals, including one or more polyadenylation signals.

[52] In one embodiment of the invention the promoter is a constitutive promoter. In another embodiment of the invention, the promoter activity is enhanced by external or internal stimuli (inducible promoter), such as but not

limited to hormones, chemical compounds, mechanical impulses, abiotic or biotic stress conditions. The activity of the promoter may also be regulated in a temporal or spatial manner (tissue-specific promoters; developmentally regulated promoters).

[53] 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 (Hapster et al., 1988), 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), stem-specific promoters (Keller et al., 1988), leaf specific promoters (Hudspeth et al., 1989), mesophyll-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al., 1989), tuber-specific promoters (Keil et al., 1989), vascular tissue specific promoters (Peleman et al., 1989), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

[54] Methods for the introduction of chimeric genes into plants 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.

[55] The transgenic plant cells and plant lines according to the invention may further comprise chimeric genes which will reduce the expression of PARP genes as described in WO 00/04173. These further chimeric genes may be introduced e.g. by crossing the transgenic plant lines of the current

invention with transgenic plants containing PARP 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 gene expression reducing chimeric genes or vice versa. Alternatively, the PARP and PARG inhibitory RNA regions may be encoded by one chimeric gene and transcribed as one RNA molecule.

[56] The chimeric genes of the invention (or the inhibitory RNA molecules corresponding thereto) may also be introduced into plant cells in a transient manner, e.g. using the viral vectors, such as viral RNA vectors as described in WO 00/63397 or WO 02/13964.

[57] Having read this specification, it will be immediately clear to the skilled artisan, that mutant plant cells and plant lines, wherein the PARG activity is reduced may be used to the same effect as the transgenic plant cells and plant lines described herein. Mutants in ParG gene of a plant cell or plant may be easily identified using screening methods known in the art, whereby chemical mutagenesis, such as e.g., EMS mutagenesis, is combined with sensitive detection methods (such as e.g., denaturing HPLC). An example of such a technique is the so-called "Targeted Induced Local Lesions in Genomes" method as described in McCallum et al, Plant Physiology 123 439-442 or WO 01/75167. However, other methods to detect mutations in particular genome regions or even alleles, are also available and include screening of libraries of existing or newly generated insertion mutant plant lines, whereby pools of genomic DNA of these mutant plant lines are subjected to PCR amplification using primers specific for the inserted DNA fragment and primers specific for the genomic region or allele, wherein the insertion is expected (see e.g. Maes et al., 1999, Trends in Plant Science, 4, pp 90-96).

[58] Plant cell lines and plant lines may also be subjected to mutagenesis by selection for resistance to ParG inhibitors, such as gallotannines . (Ying, et al. (2001). Proc. Natl. Acad. Sci. USA 98(21), 12227-12232; Ying, W., Swanson, R.A. (2000). NeuroReport 11(7), 1385-1388.

[59] Thus, methods are available in the art to identify plant cells and plant lines comprising a mutation in the ParG gene. This population of mutant cells or plant lines can then be subjected to different abiotic stresses, and their phenotype or survival can be easily determined. Additionally, the NAD and/or the ATP content of the stressed cells can be determined and compared to results of such determinations of unstressed cells. In stress tolerant cells, the reduction of NAD content under stress conditions should when compared with unstressed cells, should be lower than for corresponding control cells.

[60] It is also an object of the invention to provide plant cells and plants containing the chimeric genes or the RNA molecules according to the invention. Gametes, seeds, embryos, either zygotic or somatic, progeny or hybrids of plants comprising the chimeric genes of the present invention, which are produced by traditional breeding methods are also included within the scope of the present invention.

[61] The plants obtained by the methods described herein may be further crossed by traditional breeding techniques with other plants to obtain stress tolerant progeny plants comprising the chimeric genes of the present invention.

[62] 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, alfalfa, peanuts, sunflowers, rice, oats, sugarcane, soybean, turf grasses, barley, rye, sorghum, sugar cane, vegetables (including chicory, lettuce, tomato, zucchini, bell pepper, eggplant, cucumber, melon, onion, leek), tobacco, potato, sugarbeet, papaya, pineapple, mango, *Arabidopsis thaliana*, but also plants used in horticulture, floriculture or forestry (poplar, fir, eucalyptus etc.).

[63] The following non-limiting Examples describe method and means for increasing stress tolerance in plants according to the invention.

[64] 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 et al. (2000) *PCR - Basics: From Background to Bench*, First Edition, Springer Verlag, Germany.

[65] Throughout the description and Examples, reference is made to the following sequences:

[66] SEQ ID N°1: amino acid sequence of the ParG protein from *Arabidopsis thaliana*.

[67] SEQ ID N°2: amino acid sequence of part of the ParG protein from *Solanum tuberosum*.

[68] SEQ ID N°3: nucleotide sequence encoding the ParG protein from *Arabidopsis thaliana*.

[69] SEQ ID N°4: nucleotide sequence encoding the part of the ParG protein from *Solanum tuberosum*.

[70] SEQ ID N°5: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

[71] SEQ ID N°6: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

- [72] SEQ ID N°7: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [73] SEQ ID N°8: nucleotide sequence of an oligonucleotide primer suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [74] SEQ ID N°9: nucleotide sequence of the T-DNA vector containing the ParG expression reducing chimeric gene based on the *Arabidopsis* ParG gene sequence.
- [75] SEQ ID N°10: amino acid sequence of conserved sequence 1 of PARG proteins.
- [76] SEQ ID N°11: amino acid sequence of conserved sequence 2 of PARG proteins.
- [77] SEQ ID N°12: amino acid sequence of conserved sequence 3 of PARG proteins.
- [78] SEQ ID N°13: amino acid sequence of conserved sequence 4 of PARG proteins.
- [79] SEQ ID N°14: amino acid sequence of conserved sequence 5 of PARG proteins.
- [80] SEQ ID N°15: nucleotide sequence of the ParG protein from *Oryza sativa*.
- [81] SEQ ID N°16: amino acid sequence of the ParG protein from *Oryza sativa*.
- [82] SEQ ID N° 17: nucleotide sequence of an oligonucleotide primer PG1 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [83] SEQ ID N° 18: nucleotide sequence of an oligonucleotide primer PG2 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [84] SEQ ID N° 19: nucleotide sequence of an oligonucleotide primer PG3 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [85] SEQ ID N° 20: nucleotide sequence of an oligonucleotide primer PG4 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.

- [86] SEQ ID N° 21: nucleotide sequence of an oligonucleotide primer PG5 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [87] SEQ ID N° 22: nucleotide sequence of an oligonucleotide primer PG6 suitable for PCR amplification of part of a ParG protein encoding DNA fragment.
- [88] SEQ ID N°23: nucleotide sequence encoding a ParG protein from *Zea mays*.
- [89] SEQ ID N°24: nucleotide sequence of a T-DNA vector comprising a chimeric gene capable of reducing PARG expression
- [90] SEQ ID N°25: nucleotide sequence of a T-DNA vector comprising a chimeric gene capable of reducing PARG expression

[91] Examples**Example 1. Analysis of the influence of stress on energy production efficiency of transgenic stress tolerant plant lines containing PARP gene expression reducing chimeric genes.**

[92] Hypocotyls of transgenic *Brassica napus* plants comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 were cultivated for 5 days on a growth medium. Explants were then transferred to liquid medium comprising 30 mg/L aspirin or acetylsalicylic acid (resulting in oxidative stress conditions) for one day. In control experiments, hypocotyls of non-transgenic *Brassica napus* plants N90-740 were cultivated on the same growth medium and then incubated for one day in liquid medium comprising 30 mg/L aspirin. In addition, hypocotyls of both the transgenic lines and the control line were cultivated on the same growth medium without aspirin.

[93] After the cultivation period, the ATP content of 125 explants was determined for each experiment. Additionally, the oxygen consumed in 3 hours by 125 explants was determined. The results are summarized in Table 1. The standard error of the mean was less than 6%. Whereas, the ratio of moles ATP per mg consumed oxygen in the control plants decreased in the control plants when oxidative stress was applied, the same ratio in the stress tolerant transgenic plant lines actually increased under stress conditions, and was considerably higher (about 24%) than in the control plants. The stress resistant transgenic lines thus maintained a constant energy production efficiency, whereas the control lines exhibited a decreased energy production efficiency. In addition, superoxide production, expressed as a percentage of superoxide production in control plants not subjected to the oxidative stress, did not increase in stress tolerant plants subjected to stress conditions.

[94] Table 1. Influence of stress on energy production efficiency of 5 days cultured *Brassica napus* hypocotyl explants.

Plant line	Stress	moles ATP per 125 explants	O ₂ mg/L consumed in 3 hrs by 125 explants	moles ATP	Superoxide production
				mg consumed O ₂	
N90-740 (control)	None	12.4×10^{-7}	2.96	4.19×10^{-7}	100%
	30mg/L aspirin	13.2×10^{-7}	4.06	3.25×10^{-7}	167%
Transgenic line	None	9.3×10^{-7}	2.33	3.99×10^{-7}	108%
	30mg/L aspirin	11.4×10^{-7}	2.82	4.04×10^{-7}	100%

[95] In another experiment, the NAD⁺ and ATP content of 4 different transgenic *Arabidopsis* lines comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 were determined under high and low light conditions, and compared to the values obtained for a non transformed control line under the same conditions. The 4 different lines exhibited different degrees of stress resistance as exhibited e.g. by their ability to withstand heat and/or drought conditions. The values obtained for the NAD and ATP contents under high light stress are expressed as a percentage of the values for the NAD and ATP contents under low light conditions, and are plotted in Figure 2.

[96] The results show that high light stress leads to a significant NAD reduction in control plant cells and in the transgenic plant line which is the least stress resistant. The more stress resistant the transgenic plant lines are, the less significant the NAD reduction is under high light stress conditions.

[97] In another experiment, the NAD⁺ and ATP content of a segregating population resulting from a cross between transgenic corn lines comprising PARP gene expression reducing chimeric genes as described in WO 00/04173 and an untransformed corn line, were determined under conditions of nutrient (nitrogen) depletion, and compared to the values obtained for a non transformed control line under the same conditions. Figure 3 is a graphic representation of the of the obtained results. Hemizygous and azygous lines were discriminated by verification for the presence of the selectable marker gene. The NAD and ATP content was significantly higher in the hemizygous, stress tolerant plants than in the untransformed control plants or the azygous plants.

Example 2. Construction of ParG gene expression reducing chimeric genes.

[98] To reduce the expression of the PARG gene e.g. in *Arabidopsis* and related plants, a chimeric gene was constructed which is capable expressing a dsRNA comprising both a sense and antisense region which can form a double stranded RNA. Such dsRNA is very effective in reducing the expression of the genes with which is shares sequence homology, by post-transcriptional silencing. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising 163 bp from the ParG gene from *Arabidopsis thaliana* in direct orientation (Genbank Accession number AF394690 from nucleotide position 973 to 1135);
- A DNA fragment encoding intron 2 from the pdk gene from *Flaveria*;
- The DNA fragment comprising 163 bp from the ParG gene from *Arabidopsis thaliana* in inverted orientation (Genbank Accession number AF394690 from nucleotide position 973 to 1135)
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*.

[99] This chimeric gene was introduced in a T-DNA vector, between the left and right border sequences from the T-DNA, together with a selectable marker gene providing resistance to the herbicide phosphinotricin.

[100] To reduce the expression of the PARG gene e.g. in potatoes and related plants, a chimeric gene is constructed which is capable expressing a dsRNA comprising both a sense and antisense region of a cDNA sequence from potato, that is capable of encoding a protein having high sequence identity with the N-terminal part of the *Arabidopsis* PARG protein. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Solanum tuberosum* in direct orientation (Genbank Accession number BE340510);
- A DNA fragment encoding intron 2 from the pdk gene from *Flaveria*;
- The DNA fragment comprising the sequence of at least 100 bp from ParG homologue from *Solanum tuberosum* in inverted orientation (Genbank Accession number BE340510);
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*

[101] 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 providing resistance to the herbicide phosphinotricin.

Example 3. Analysis of transgenic plant lines comprising ParG gene expression reducing chimeric genes.

[102] The chimeric genes of Example 2 are introduced into *Arabidopsis* or potato respectively, by *Agrobacterium* mediated transformation.

[103] The population of obtained transgenic lines is subjected to the following stress conditions, together with control plants:

- Increased heat for a period of days (greenhouse) or hours (in vitro)
- Drought for a period of days
- High light conditions for a period of days
- Nutrient depletion

[104] Individual plant lines surviving well the above mentioned stress conditions are selected.

[105] The NAD content and ATP content for the above mentioned plants is determined under control and stress conditions.

Example 4. Quantitative determination of NAD, ATP and superoxide radicals in plant cells.

[106] Quantification of ATP in plant tissues was done basically as described by Rawlyer et al. (1999), Plant Physiol. 120, 293-300. The assay was used for the determination of the ATP content of hypocotyl explants that were cultured for 4-5 days on A2S3 medium or 2 weeks old *in vitro* cultured *Arabidopsis* plants. All manipulations are performed on crushed ice unless otherwise indicated.

[107] ATP extraction

- Freeze plant material with liquid nitrogen
 - 100 hypocotyl explants
 - $\pm$ 700mg *Arabidopsis* plants (roots + shoots) (about 32-37 18-days old C24 plants)
- Put frozen hypocotyls in mortar and add 6ml of 6% perchloric acid.
- Extraction can be done at room temperature using a pestle. After extraction, put samples as soon as possible on ice.
- Centrifuge at 24,000g (Sorvall, SS34 rotor at 14,000rpm) for 10min. at

4°C.

- The supernatant is neutralized with 5M K₂CO₃ (add 350µl of 5M K₂CO₃ to 3ml of supernatant).
- KClO₄ is removed by spinning as described above.

[108] Quantitative bioluminescent determination of ATP

- The ATP bioluminescent assay kit from Sigma is used (FL-AA).
- Dilute extract 6000 x (about 6 mL extract from which 100µl is taken, that is diluted 1000 times) The dilutions are made with the 'ATP assay mix dilution buffer' (FL-AAB) of the ATP bioluminescent assay kit
- The amount of light that is produced is measured with the TD-20/20 luminometer of Turner Designs (Sunnyvale, USA).
- Standard curve: dissolve ATP standard of kit (FL-AAS) in 10ml of water (2x10⁻⁶ moles)

[109] Quantification of NAD⁺ and NADH in plant tissues was performed, essentially as described by Karp et al. (1983) or Filipovic et al. (1999) on the following plant material:

Brassica napus: 150 5-days cultured hypocotyl explants/sample

Arabidopsis: 1000mg 18-days old in vitro grown plants (shoots + roots)/sample (corresponds to ±60 C24 plants)

[110] Assay solution

- (A) For measuring NADH: 25mM potassium phosphate buffer pH7
0.1mM DTT
3µM FMN (Fluka, 83810)
30µM n-decanal (Sigma, D-7384)

(B) For measuring NAD⁺ + NADH:

idem as for measuring NADH alone
+ 2µg/mL alcohol dehydrogenase (Roche, 102 717)

[111] Extraction

- Freeze with liquid nitrogen
- Put frozen plant material in cooled mortar (cooled at -20°C) and add 5mL extraction buffer
- Grind material using a pestle
- Centrifuge at 24 000g (Sorvall, SS34 rotor at 14 000rpm) for 15 minutes at 4°C
- Take 1 mL of supernatant for analysis

[112] Assay***NADH***

- 390 μL of assay solution A
- + 10 μL extract
- + 2 μL NAD(P)H:FMN oxidoreductase
- + 100 μL luciferase solution

NAD⁺ + NADH

- 390 μL of assay solution B
- + 10 μL extract
- 2 minutes at room temperature
- + 2 μL NAD(P)H:FMN oxidoreductase
- + 100 μL luciferase solution

The amount of light that is produced is measured with the TD-20/20 luminometer of Turner Designs (Sunnyvale, USA)

NADH-standard

NADH stock solution: 1mM (7.1mg/10mL H₂O)

NADH: disodium salt, Roche, 107 735

Dilution series in 10mM potassium phosphate buffer pH7: (10^{-2}); 5×10^{-3} ; 2×10^{-3} ; 10^{-3} ; 5×10^{-4}

Add 10 μL of dilutions in 390 μL of assay solution A and perform reaction

Make standard curve

- [113]** Superoxide radicals production was measured by quantifying the reduction of XTT as described in De Block and De Brouwer (2002) Plant Physiol. Biochem. 40, 845-852

[114] BRASSICA NAPUS**[115] Media and reaction buffers**

Sowing medium (medium 201):

Half concentrated Murashige and Skoog salts

2% sucrose

pH 5.8

0.6% agar (Difco Bacto Agar)

250mg/l triacillin

Callus inducing medium A2S3:

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO₄,

0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP, 250mg/l

triacillin

Incubation medium:

25mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro) = XTT (bts, Germany, cat n° 2525)

1 drop Tween20 for 25ml buffer

[116] Sterilization of seeds - pregermination of seeds - growing of the seedlings. Seeds are soaked in 70% ethanol for 2 min, then surface-sterilized for 15 min in a sodium hypochlorite solution (with about 6% active chlorine) containing 0.1% Tween20. Finally, the seeds are rinsed with 1l of sterile tap water. Incubate seeds for at least one hour in sterile tap water (to allow diffusion from seeds of components that may inhibit germination). Seeds are put in 250ml erlenmeyer flasks containing 50ml of sterile tap water (+ 250mg/l triacillin). Shake for

about 20 hours. Seeds from which the radicle is protruded are put in Vitro Vent containers from Duchefa containing about 125ml of sowing medium (10 seeds/vessel, not too many to reduce loss of seed by contamination). The seeds are germinated at $\pm 24^{\circ}\text{C}$ and 10-30: Einstein/s⁻¹m⁻² with a daylength of 16h.

[117] Preculture of the hypocotyl explants and induction of stress

- 12-14 days after sowing, the hypocotyls are cut in about 7-10mm segments.
- The hypocotyl explants (25 hypocotyls/Optilux Petridish, Falcon S1005, Denmark) are cultured for 5 days on medium A2S3 at 25°C (at 10-30 Einstein/s⁻¹m⁻²).

[118] XTT-assay

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without XTT).
- Add 20mL reaction buffer + XTT.
(explants have to be submerged, but do not vacuum infiltrate)
- Incubate in the dark at 26°C for about 3hours
- Measure the absorption of the reaction medium at 470nm

[119] *ARABIDOPSIS THALIANA*

[120] Media and reaction buffers

Plant medium:

Half concentrated Murashige and Skoog salts
B5 vitamins
1.5% sucrose
pH 5.8
0.7% Difco agar

Incubation medium:

10mM K-phosphate buffer pH5.8
2% sucrose
1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

1mM sodium,3'-{1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) = XTT (bts, Germany, cat n° 2525)

1 drop Tween20 for 25ml buffer

[121] *Arabidopsis* plants

- *Arabidopsis* lines: control

lines to test

- Sterilization of *Arabidopsis* seeds:

2min. 70% ethanol

10 min. bleach (6% active chlorine) + 1drop Tween 20 for 20ml solution

wash 5 times with sterile tap water

- Pregermination of seeds:

In 9cm Optilux Petridishes (Falcon) containing 12ml sterile tap water.

Low light overnight to 24 hours.

- Growing of *Arabidopsis* plants

Seeds are sown in Intergrid Tissue Culture disks of Falcon (nr. 3025) containing ± 125 ml of plant medium: 1 seed/grid.

Plants are grown at 24°C

$30\mu\text{Einstein s}^{-1}\text{m}^{-2}$

16hours light - 8hours dark

for about 3 weeks (before bolting)

[122] XTT-assay

Control condition (no stress)

- Harvest shoots (roots included) from agar plates and put them directly in a 50ml Falcon tube containing reaction buffer (without XTT)

Stressed shoots

- Transfer shoots to 50ml Falcon tubes containing reaction buffer (without XTT)
- Replace reaction buffer with buffer containing XTT (40mL/tube)

- Shoots have to be submerged, but do not vacuum infiltrate
- Incubate in the dark at 26°C for about 3 hours
- Measure the absorption of the reaction medium at 470nm

[123] Quantification of respiration by measuring oxygen consumption using a Clark polarographic electrode was done in the following way:

[124] Plant material

Brassica napus

150-200* hypocotyl explants

Cultured for 5 days at 25°C

(*cfr.* protocol vigour assay)

* 150 explants error <10%; 200 explants error <6%

Arabidopsis

For $C_{24} \pm 1000\text{mg}^*$ *in vitro* plants (shoots + roots) (corresponds with ~50 18-days old plants)

Pregerminate seeds before sowing

Grow for 18 days at 24°C

(*cfr.* protocol *in vitro* growth Arabidopsis)

* for error <8%

[125] Incubation media

Brassica napus

25mM K-phosphate buffer pH5.8

2% sucrose

Tween20 (1 drop/25ml)

Arabidopsis

10mM K-phosphate buffer pH5.8

2% sucrose

Tween20 (1 drop/25ml)

$\mu\text{mol m}^{-1} \text{s}^{-1}$). After 1 day, the NAD and NADH contents were determined using the enzymatic cycling method (Karp et al. (1983) Anal. Biochem. 128, pp 175-180). A portion of the seedlings were cultivated further under high light conditions for about 3 to about days, after which the damage was scored. Damage was visible as darkening of the young leaves and shoot tip, bleaching of older leaves and growth retardation. The results are summarized in Table 1 for Arabidopsis and in Table 2 for tobacco.

[129] Table 1. Analysis of Arabidopsis (Columbia). $\pm R$ indicates that some dark pigmentation was observed. ND: not determined

	High light tolerance	NAD+NADH content in 1 gram of tissue ($10^{-3}\mu\text{M}$)	% TTC-reducing capacity vs control
Non-transgenic control	S	17.3	100
Transgenic line 9	R	28.2	ND
Transgenic line 10	R	31.7	ND
Transgenic line 11	$\pm R$	26.5	ND
Transgenic line 12	S	19.4	ND
Transgenic line 26	R	33.2	55
Transgenic line 27	S	21.3	100
Transgenic line 28	$\pm R$	26.5	75
Transgenic line 29	S	17.7	102
Transgenic line 30	R	28.3	66

[130] Table 2. Analysis of *Nicotiana tabacum* c.v. Petit Havana SR1. $\pm$ R indicates that some dark pigmentation was observed. R/S indicates that the resistance phenotype was not very clear.

	High light tolerance	% TTC-reducing capacity vs control
Non-transgenic control	S	100
Transgenic line 1	R/S	88
Transgenic line 2	$\pm$ R	79
Transgenic line 3	R	53

[131] There is a positive correlation between the resistance to high light stress in the transgenic plants and the NAD⁺/NADH content of the cells. An inverse correlation can be seen between TTC reducing capacity and high light tolerance.

Example 6. Construction of ParG gene expression reducing chimeric genes suited for use in cereal plants.

[132] To reduce the expression of the PARG gene e.g. in cereals such as rice or corn (maize) and related plants, a chimeric gene is constructed which is capable expressing a dsRNA comprising both a sense and antisense region of nucleotide sequence from rice, that is capable of encoding a protein having high sequence identity with PARG protein encoding nucleotide sequences. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Oryza sativa* (SEQ ID No 15) in direct orientation;
- A DNA fragment encoding intron 2 from the pdk gene from *Flaveria*;

- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Oryza sativa* (SEQ ID No 15) in inverted orientation;
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*.

[133] 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 providing resistance to e.g. the herbicide phosphinotricin.

[134] To reduce the expression of the PARG gene e.g. in cereals such as rice or corn (maize) and related plants, a chimeric gene is constructed which is capable expressing a dsRNA comprising both a sense and antisense region of nucleotide sequence from rice, that is capable of encoding a protein having high sequence identity with PARG protein encoding nucleotide sequences. The chimeric gene comprises the following DNA fragments:

- A promoter region from Cauliflower mosaic Virus (CaMV 35S);
- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Zea mays* (SEQ ID No 23) in direct orientation;
- A DNA fragment encoding intron 2 from the pdk gene from Flaveria;
- A DNA fragment comprising a sequence of at least 100 bp from ParG homologue from *Zea mays* (SEQ ID No 23) in inverted orientation;
- A fragment of the 3' untranslated end from the octopine synthetase gene from *Agrobacterium tumefaciens*.

[135] 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 providing resistance to e.g. the herbicide phosphinotricin. The nucleotide sequence of two examples of such T-DNA vectors

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comprising two different chimeric genes as described in the previous paragraph is represented in SEQ ID Nos. 24 and 25.

**Example 7. Analysis of transgenic plant lines comprising ParG gene expression
5 reducing chimeric genes.**

[136] The chimeric genes of Example 6 are introduced into rice or corn respectively, by Agrobacterium mediated transformation.

- 10 [137] The population of obtained transgenic lines is subjected to the following stress conditions, together with control plants:
- Increased heat for a period of days (greenhouse) or hours (in vitro)
 - Drought for a period of days
 - High light conditions for a period of days
- 15 • Nutrient depletion

[138] Individual plant lines surviving well the above mentioned stress conditions, or at least one thereof, are selected.

- 20 [139] The NAD content and ATP content for the above mentioned plants is determined under control and stress conditions.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

25

- The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.
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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method to produce a plant tolerant to stress conditions comprising the steps of
 - (a) providing plant cells with a chimeric gene to create transgenic plant cells,
5 said chimeric gene comprising the following operably linked DNA fragments
 - (i) a plant-expressible promoter;
 - (ii) a DNA region, which when transcribed yields a ParG inhibitory RNA molecule;
 - (iii) a 3' end region involved in transcription termination and
10 polyadenylation;
 - (b) regenerating a population of transgenic plant lines from said transgenic plant cell; and
 - (c) identifying a stress tolerant plant line within said population of transgenic plant lines.
15
2. The method according to claim 1, wherein said parG inhibitory RNA molecule comprises a nucleotide sequence of at least 20 consecutive nucleotides of the nucleotide sequence of the ParG gene present in said plant cell.
- 20 3. The method according to claim 1, wherein said parG inhibitory RNA molecule comprises a nucleotide sequence of at least 20 consecutive nucleotides of the complement of the nucleotide sequence of the ParG gene present in said plant cell.
4. The method according to claim 2 or 3, wherein said chimeric gene further
25 comprises a DNA region encoding a self-splicing ribozyme between said DNA region coding for said parG inhibitory RNA molecule and said 3' end region.
5. The method according to claim 1, wherein said parG inhibitory RNA comprises a sense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the
30 nucleotide sequence of the ParG gene present in said plant cell and an antisense region comprising a nucleotide sequence of at least 20 consecutive nucleotides of the complement

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of the nucleotide sequence of the ParG gene present in said plant cell, wherein said sense and antisense region are capable of forming a double stranded RNA region comprising said at least 20 consecutive nucleotides.

- 10

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10. A process for producing stress tolerant plants, comprising the step of further crossing a plant of claim 9 with another plant.
11. A plant obtained by the process of claim 10.
12. A method to produce a plant tolerant to stress conditions comprising the steps of
- (a) providing plant cells with a chimeric gene to create transgenic plant cells, said chimeric gene comprising the following operably linked DNA fragments
 - (i) a plant-expressible promoter;
 - (ii) a DNA region, which when transcribed yields a ParG inhibitory RNA molecule, said DNA region comprising a nucleotide sequence of at least 21 to 100 nucleotides of a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 1, 2 or 16 or at least 21 to 100 nucleotides of a nucleotide sequence of SEQ ID 3, 4, 15 or 23;
 - (iii) a 3' end region involved in transcription termination and polyadenylation;
 - (b) regenerating a population of transgenic plant lines from said transgenic plant cell; and
 - (c) identifying a stress tolerant plant line within said population of transgenic plant lines.
13. A method to produce a plant tolerant to stress conditions comprising the steps of
- (a) subjecting a plant cell line or a plant or plant line, to mutagenesis;
 - (b) identifying those plant cells or plants that have a mutation in an endogenous ParG gene;
 - (c) subjecting the identified plant cells or plants to stress conditions;
 - (d) identifying plant cells or plants that tolerate said stress conditions better than control plants.
14. A method to produce a plant tolerant to stress conditions comprising the steps of
- (a) selecting a plant cell line or a plant or plant line which is resistant to a ParG

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inhibitor;

(b) identifying those plant cells or plants that have a mutation in an endogenous ParG gene;

(c) subjecting the identified plant cells or plants to stress conditions;

5 (d) identifying plant cells or plants that tolerate said stress conditions better than control plants.

15. A plant cell or plant obtained by the method of any one of claims 12 to 14.

10 16. A method according to any one of claims 1, 8, 12, 13 or 14 or a plant cell or plant obtained by a said method, or a process according to claim 10 or a plant obtained by a said process, substantially as hereinbefore described with reference to the Examples and the accompanying drawings.

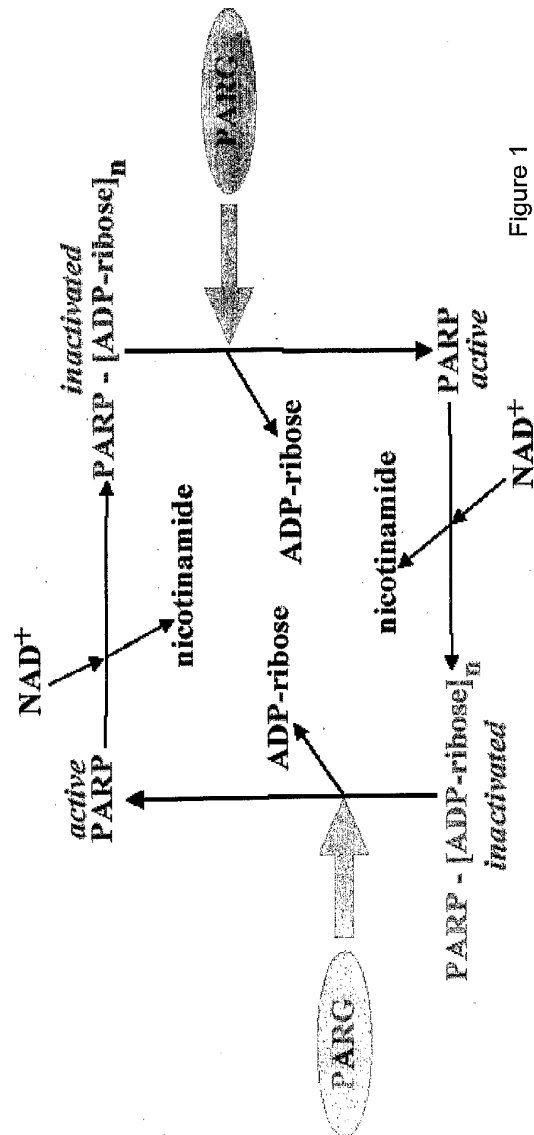


Figure 1

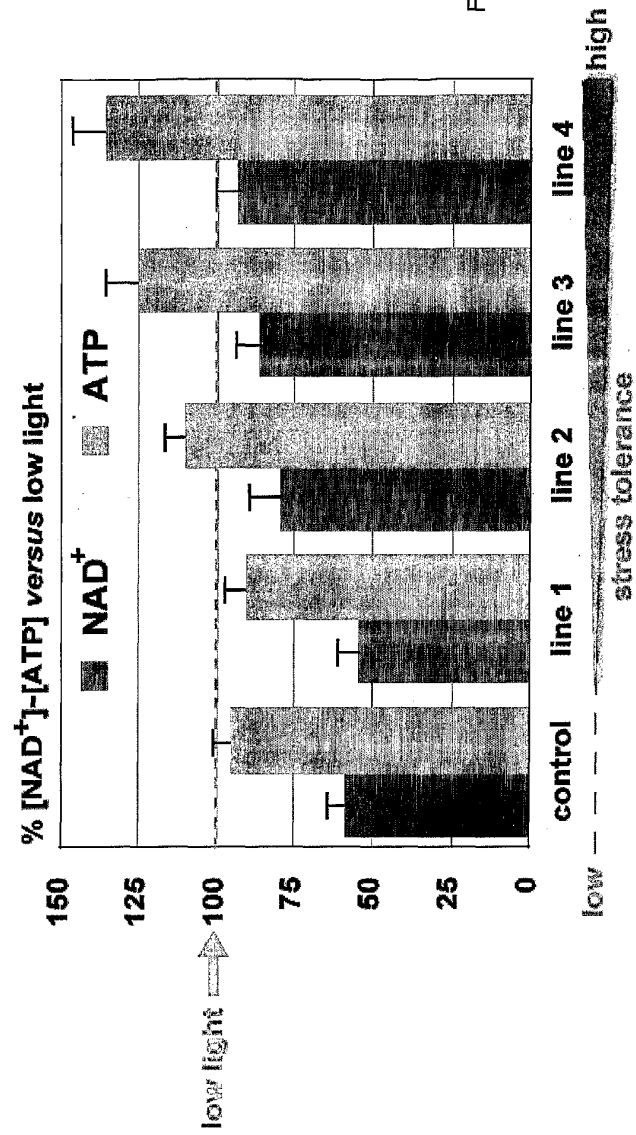


Figure 2

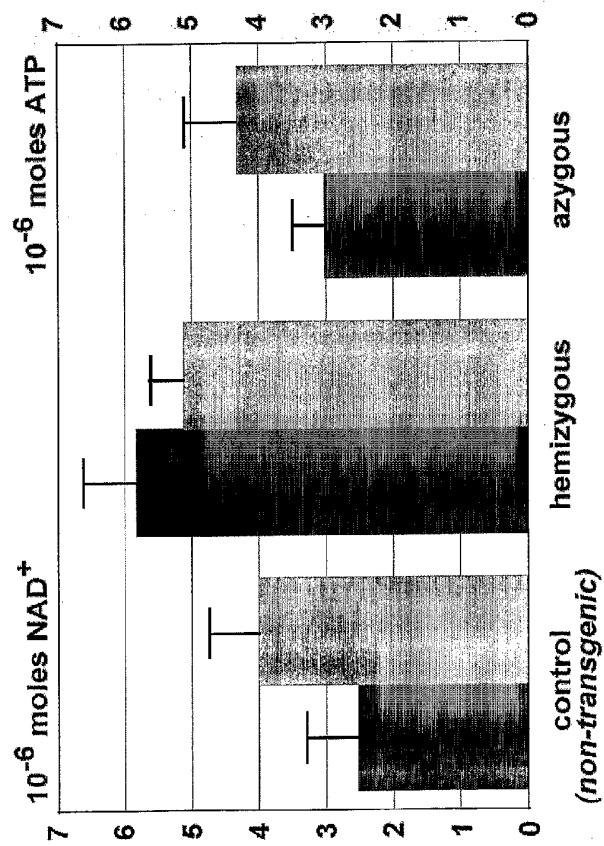


Figure 3

SEQUENCE LISTING

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De Block, Marc

<120> Methods and means for increasing the tolerance of plants to stress conditions.

<130> BCS 03 2002 WO1

<150> EP03076044.1
<151> 2003-04-09

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ctc aag gcg ctg gcg ctg ggc ccc gac gtg agc cgc gtc tcc tcc ggc      144
Leu Lys Ala Leu Ala Leu Gly Pro Asp Val Ser Arg Val Ser Ser Gly
35          40          45
gac gtc ctc gcc gac gcc ctc acc gac ctc cgc ctc gcg ctc aac ctc      192
Asp Val Leu Ala Asp Ala Leu Thr Asp Leu Arg Leu Ala Leu Asn Leu
50          55          60
gac cca ctc ccg cgc cgc gcc gcc gag ggc ttc gcg ctc ttc ttc gac      240
Asp Pro Leu Pro Arg Arg Ala Ala Glu Gly Phe Ala Leu Phe Phe Asp
65          70          75          80
gac ctc ctg tcg cgg gcg cag gcg cgg gac tgg ttc gac cac gtc gcc      288
Asp Leu Leu Ser Arg Ala Gln Ala Arg Asp Trp Phe Asp His Val Ala

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ccc tcc ctc gcc cgc etc etc etc cgc etc ccc acg ctg etc gag gcc				336
Pro Ser Leu Ala Arg Leu Leu Leu Arg Leu Pro Thr Leu Leu Glu Gly				
	100	105	110	
cac tac cgc gcc gcc ggc gac gag gct cgc ggg etc cgc atc ctg agc				384
His Tyr Arg Ala Ala Gly Asp Glu Ala Arg Gly Leu Arg Ile Leu Ser				
	115	120	125	
tcg cag gat gcc ggg etc gtg etc etc agc cag gag etc gcc gcc gcc				432
Ser Gln Asp Ala Gly Leu Val Leu Leu Ser Gln Glu Leu Ala Ala Ala				
	130	135	140	
ctg etc gcc tgc gcg etc ttc tgc ctg ttc ccc acc gcc gat agg gcc				480
Leu Leu Ala Cys Ala Leu Phe Cys Leu Phe Pro Thr Ala Asp Arg Ala				
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gag gcg tgc etc ccg gcg atc aat ttc gat agc cta ttt gcg gca ctg				528
Glu Ala Cys Leu Pro Ala Ile Asn Phe Asp Ser Leu Phe Ala Ala Leu				
	165	170	175	
tgt tat aat tcg agg caa agc cag gag cag aag gtg agg tgc ctt gtt				576
Cys Tyr Asn Ser Arg Gln Ser Gln Glu Gln Lys Val Arg Cys Leu Val				
	180	185	190	
cac tat ttt gac agg gtg acc gct tct aca cct act ggt tcc gtt tcg				624
His Tyr Phe Asp Arg Val Thr Ala Ser Thr Pro Thr Gly Ser Val Ser				
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ttt gag cgt aag gtt ctt cct cgc cgt cct gaa tct gat gcc att acg				672
Phe Glu Arg Lys Val Leu Pro Arg Arg Pro Glu Ser Asp Gly Ile Thr				
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Tyr Pro Asp Met Asp Thr Trp Met Lys Ser Gly Val Pro Leu Cys Thr				
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Phe Arg Val Phe Ser Ser Gly Leu Ile Glu Asp Glu Glu Gln Glu Ala				
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ott gaa gtt gac ttt gca aat aga tat ttg gga ggt ggc gca ctt tcc				816
Leu Glu Val Asp Phe Ala Asn Arg Tyr Leu Gly Gly Gly Ala Leu Ser				
	260	265	270	
aga gcc tgc gtg cag gaa gaa atc cgg ttc atg ata aac cca gaa ttg				864
Arg Gly Cys Val Gln Glu Glu Ile Arg Phe Met Ile Asn Pro Glu Leu				
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atc gtg gcc atg etc ttc atg gtt tca atg gaa gat aat gaa gct ata				912
Ile Val Gly Met Leu Phe Met Val Ser Met Glu Asp Asn Glu Ala Ile				
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gaa att gtt ggt gca gaa agg ttc tca cag tac atg ggg tat ggt tcc				960
Glu Ile Val Gly Ala Glu Arg Phe Ser Gln Tyr Met Gly Tyr Gly Ser				
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tca ttc cgt ttt act ggt gac tac tta gat agc aaa ccc ttt gat gcg				1008
Ser Phe Arg Phe Thr Gly Asp Tyr Leu Asp Ser Lys Pro Phe Asp Ala				
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Met Gly Arg Arg Lys Thr Arg Ile Val Ala Ile Asp Ala Leu Asp Cys				
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cca act agg tta cag ttt gaa tct agt ggt ctt cta agg gaa gtg aac				1104
Pro Thr Arg Leu Gln Phe Glu Ser Ser Gly Leu Leu Arg Glu Val Asn				
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Lys Ala Phe Cys Gly Phe Leu Asp Gln Ser Asn His Gln Leu Cys Ala				
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Lys Leu Val Gln Asp Leu Asn Thr Lys Asp Asn Cys Pro Ser Val Ile				
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cct gat gaa tgc ata gga gtt tca act gga aac tgg ggt tgc ggg gct				1248
Pro Asp Glu Cys Ile Gly Val Ser Thr Gly Asn Trp Gly Cys Gly Ala				
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Phe Gly Gly Asn Pro Glu Ile Lys Ser Met Ile Gln Trp Ile Ala Ala
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Ser Gln Ala Leu Arg Ser Phe Ile Asn Tyr Tyr Thr Phe Glu Ser Glu
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tca ctg aaa aga tta gaa gag gtg acc cag tgg ata ttg cgc cat agg      1392
Ser Leu Lys Arg Leu Glu Glu Val Thr Gln Trp Ile Leu Arg His Arg
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Trp Thr Val Gly Glu Leu Trp Asp Met Leu Val Glu Tyr Ser Ser Gln
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Arg Leu Arg Gly Asp Thr Asn Glu Gly Phe Leu Thr Trp Leu Leu Pro
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      35      40      45
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      50      55      60
Asp Pro Leu Pro Arg Arg Ala Ala Glu Gly Phe Ala Leu Phe Phe Asp
      65      70      75      80
Asp Leu Leu Ser Arg Ala Gln Ala Arg Asp Trp Phe Asp His Val Ala
      85      90      95
Pro Ser Leu Ala Arg Leu Leu Leu Arg Leu Pro Thr Leu Leu Glu Gly
      100      105      110
His Tyr Arg Ala Ala Gly Asp Glu Ala Arg Gly Leu Arg Ile Leu Ser
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Ser Gln Asp Ala Gly Leu Val Leu Leu Ser Gln Glu Leu Ala Ala Ala
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Leu Leu Ala Cys Ala Leu Phe Cys Leu Phe Pro Thr Ala Asp Arg Ala
      145      150      155      160
Glu Ala Cys Leu Pro Ala Ile Asn Phe Asp Ser Leu Phe Ala Ala Leu
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Cys Tyr Asn Ser Arg Gln Ser Gln Glu Gln Lys Val Arg Cys Leu Val
      180      185      190

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His Tyr Phe Asp Arg Val Thr Ala Ser Thr Pro Thr Gly Ser Val Ser
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 Tyr Pro Asp Met Asp Thr Trp Met Lys Ser Gly Val Pro Leu Cys Thr
 225 230 235 240
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 Leu Glu Val Asp Phe Ala Asn Arg Tyr Leu Gly Gly Gly Ala Leu Ser
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