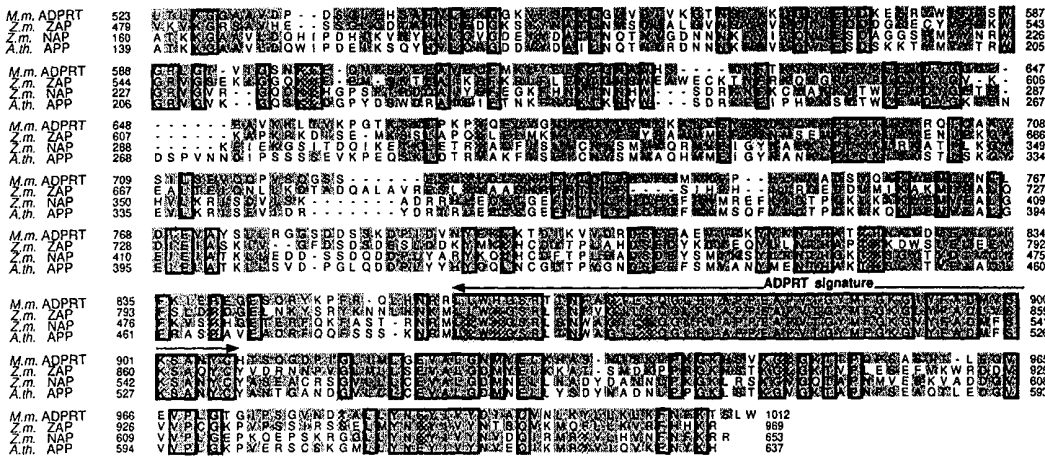


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<b>(54) Title:</b> METHODS AND MEANS TO MODULATE PROGRAMMED CELL DEATH IN EUKARYOTIC CELLS				
				
<b>(57) Abstract</b> <p>Means and methods are provided to modulate programmed cell death (PCD) in eukaryotic cells and organisms, particularly plant 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 of for producing stress tolerant cells and organisms.</p>				

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## **Methods And Means To Modulate Programmed Cell Death In Eukaryotic Cells.**

### **Field of the invention**

The invention relates to the use of poly (ADP-ribose) polymerase (PARP) proteins, particularly mutant PARP proteins or parts thereof, and genes encoding the same, to produce eukaryotic cells and organisms, particularly plant cells and plants, with modified programmed cell death. Eukaryotic cells and organisms, particularly plant cells and plants, are provided wherein either in at least part of the cells, preferably selected cells, the programmed cell death (PCD) is provoked, or wherein, on the contrary, PCD of the cells or of at least part of the cells in an organism is inhibited, by modulation of the level or activity of PARP proteins in those cells. The invention also relates to eukaryotic cells and organisms, particularly plant cells and plants, expressing such genes.

### **Description of related art**

Programmed cell death (PCD) is a physiological cell death process involved in the elimination of selected cells both in animals and in plants during developmental processes or in response to environmental cues (for a review see Ellis *et al.* 1991; Pennell and Lamb, 1997). The disassembly of cells undergoing PCD is morphologically accompanied by condensation, shrinkage and fragmentation of the cytoplasm and nucleus, often into small sealed packets (Cohen 1993, Wang *et al.* 1996). Biochemically, PCD is characterized by fragmentation of the nuclear DNA into generally about 50 kb fragments representing oligonucleosomes, as well as the induction of cysteine proteinases and endonucleases. The fragmentation of the DNA can be detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of DNA 3'-OH groups in sections of cells. (Gavrieli *et al.* 1992). Cell death by PCD is clearly distinct from cell death by necrosis, the latter involving cell swelling, lysis and leakage of the cell contents.

In animals, PCD is involved in the elimination or death of unwanted cells such as tadpole tail cells at metamorphosis, cells between developing digits in vertebrates,

overproduced vertebrate neurons, cells during cell specialization such as keratocytes etc. Damaged cells, which are no longer able to function properly, can also be eliminated by PCD, preventing them from multiplying and/or spreading. PCD, or the lack thereof, has also been involved in a number of pathological conditions in humans (AIDS, Alzheimer's disease, Huntington's disease, Lou Gehring's disease, cancers).

In plants, PCD has been demonstrated or is believed to be involved in a number of developmental processes such as e.g., removal of the suspensor cells during the development of an embryo, the elimination of aleurone cells after germination of monocotyledonous seeds; the elimination of the root cap cells after seed germination and seedling growth; cell death during cell specialization as seen in development of xylem tracheary element or trichomes, or floral organ aborting in unisexual flowers. Also the formation of aerenchyma in roots under hypoxic conditions and the formation of leaf lobes or perforations in some plants seem to involve PCD. Large scale cell death in plants occurs during upon senescence of leaves or other organs. The hypersensitive response in plants, in other words the rapid cell death occurring at the site of entry of an avirulent pathogen leading to a restricted lesion, is another example of PCD in response to an environmental cue.

Animal or plant cells dying in suspension cultures, particularly in low-density cell suspension cultures, also demonstrate the characteristics of PCD.

An enzyme which has been implied to be involved in PCD or apoptosis is poly(ADP-ribose) polymerase. Poly(ADP-ribose) polymerase (PARP), also known as poly(ADP-ribose) transferase (ADPRT) (EC 2.4.2.30), is a nuclear enzyme found in most eukaryotes, including vertebrates, arthropods, molluscs, slime moulds, dinoflagellates, fungi and other low eukaryotes with the exception of yeast. The enzymatic activity has also been demonstrated in a number of plants (Payne *et al.*, 1976; Willmitzer and Wagner, 1982; Chen *et al.*, 1994; O'Farrell, 1995).

PARP catalyzes the transfer of an ADP-ribose moiety derived from NAD<sup>+</sup>, mainly to the carboxyl group of a glutamic acid residue in the target protein, and subsequent ADP-ribose polymerization. The major target protein is PARP itself, but also histones,

high mobility group chromosomal proteins, a topoisomerase, endonucleases and DNA polymerases have been shown to be subject to this modification.

The PARP protein from animals is a nuclear protein of 113-120 kDa, abundant in most cell types, that consist of three major functional domains: an amino-terminal DNA-binding domain containing two Zn-finger domains, a carboxy-terminal catalytic domain, and an internal domain which is automodified (de Murcia and Ménissier de Murcia, 1994; Kameshita *et al.*, 1984; Lindahl *et al.*, 1995). The enzymatic activity *in vitro* is greatly increased upon binding to single-strand breaks in DNA. The *in vivo* activity is induced by conditions that eventually result in DNA breaks (Alvarez-Gonzalez and Althaus, 1989; Ikejima *et al.*, 1990). Automodification of the central domain apparently serves as a negative feedback regulation of PARP.

PARP activity in plant cells was first demonstrated by examining the incorporation of  $^3\text{H}$  from labelled  $\text{NAD}^+$  into the nuclei of root tip cells (Payne *et al.*, 1976; Willmitzer and Wagner, 1982). The enzymatic activity was also partially purified from maize seedlings and found to be associated with a protein of an apparent molecular mass of 113 kDa, suggesting that the plant PARP might be similar to the enzyme from animals (Chen *et al.*, 1994; O'Farrell, 1995).

cDNAs corresponding to PARP proteins have isolated from several species including mammals, chicken, *Xenopus*, insects and *Caenorhabditis elegans*.

Chen *et al.* (1994) have reported PARP activity in maize nuclei and associated this enzymatic activity with the presence of an approximately 114 kDa protein present in an extract of maize nuclei. O' Farrel (1995) reported that RT-PCR-amplification on RNA isolated from maize (using degenerate primers based on the most highly conserved sequences) resulted in a 300 bp fragment, showing 60% identity at the amino acid level with the human PARP protein. Lepinieć *et al.* (1995) have isolated and cloned a full length cDNA from *Arabidopsis thaliana* encoding a 72 kDa protein with high similarity to the catalytic domain of vertebrate PARP. The N-terminal domain of the protein does not reveal any sequence similarity with the corresponding domain of PARP from vertebrates but is composed of four stretches of amino acids (named A1, A2, B and C) showing similarity to the N-terminus of a number of nuclear and

DNA binding proteins. The predicted secondary structure of A1 and A2 was a helix-loop-helix structure.

The Genbank database contains the sequences of two cDNAs from *Zea mays* for which the amino acid sequence of the translation products has either homology to the conventional PARP proteins (AJ222589) or to the non-conventional PARP proteins, as identified in *Arabidopsis* (AJ222588)

The function(s) of PARP and poly-ADP ribosylation in eukaryotic cells is (are) not completely clear. PARP is involved or believed to be involved either directly or indirectly in a number of cellular processes such as DNA repair, replication and recombination, in cell division and cell differentiation or in the signalling pathways that sense alterations in the integrity of the genome. As PARP activity may significantly reduce the cellular NAD<sup>+</sup> pool, it has also been suggested that the enzyme may play a critical role in programmed cell death (Heller *et al.*, 1995; Zhang *et al.*, 1994). Further, it has been suggested that nicotinamide resulting from NAD<sup>+</sup> hydrolysis or the products of the turn-over of poly-ADP-ribose by poly-ADP-ribose glycohydrolase may be stress response signals in eukaryotes.

The information currently available on the biological function of plant PARP has come from experiments involving PARP inhibitors suggesting an *in vivo* role in the prevention of homologous recombination at sites of DNA damage as rates of homologous intrachromosomal recombination in tobacco are increased after application of 3-aminobenzamide (3ABA) (Puchta *et al.*, 1995). Furthermore, application of PARP inhibitors, such as 3ABA, nicotinamide, and 6(5H)-phenasthridinone, to differentiating cells of *Zinnia* or of *Helianthus tuberosum* has been shown to prevent development of tracheary elements (Hawkins and Phillips, 1983; Phillips and Hawkins, 1985; Shoji *et al.*, 1997; Sugiyama *et al.*, 1995), which is considered to be an example of programmed cell death in plants.

PCT application WO97/06267 describes the use of PARP inhibitors to improve the transformation (qualitatively or quantitatively) of eukaryotic cells, particularly plant cells.

Lazebnik *et al.* (1994) identified a protease with properties similar to the interleukin 1- $\beta$ -converting enzyme capable of cleaving PARP, which is an early event in apoptosis of animal cells.

Kuepper *et al.* (1990) and Molinette *et al.* (1993) have described the overproduction of the 46 kDa human PARP DNA-binding domain and various mutant forms thereof, in transfected CV-1 monkey cells or human fibroblasts and have demonstrated the trans-dominant inhibition of resident PARP activity and the consequent block of base excision DNA repair in these cells.

Ding *et al.* (1992), and Smulson *et al.* (1995) have described depletion of PARP by antisense RNA expression in mammalian cells and observed a delay in DNA strand break joining, and inhibition of differentiation of 3T3-L1 preadipocytes.

Ménissier de Murcia *et al.*, (1997) and Wang *et al.* (1995, 1997) have generated transgenic "knock-out" mice mutated in the PARP gene, indicating that PARP is not an essential protein. Cells of PARP-deficient mice are, however, more sensitive to DNA damage and differ from normal cells of animals in some aspects of induced cell death (Heller *et al.*, 1995).

### **Summary and objects of the invention.**

The invention provides a method for modulating programmed cell death in a eukaryotic cell, comprising reducing the functional level of the total PARP activity in a eukaryotic cell using the nucleotide sequence of a PARP gene of the ZAP class, and the nucleotide sequence of a PARP gene of the NAP class, preferably to reduce expression of the endogenous PARP genes, to reduce the apparent activity of the proteins encoded by the endogenous PARP genes or to alter the nucleotide sequence of the endogenous PARP genes.

The invention also provides a method for modulating programmed cell death in a eukaryotic cell, comprising introducing a first and a second PCD modulating chimeric gene in a eukaryotic cell, preferably a plant cell, wherein the first PCD modulating

chimeric gene comprises the following operably linked DNA regions: a promoter, operative in a eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a Zn-finger containing PARP protein of the ZAP class; or is capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of ZAP class and a DNA region involved in transcription termination and polyadenylation

and wherein the second PCD modulating chimeric gene comprises the following operably linked DNA regions :a promoter, operative in the eukaryotic cell; a DNA region, which when transcribed yields a RNA molecule which is either capable of reducing the functional level of a PARP protein of the NAP class; or capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of the NAP class, and a DNA region involved in transcription termination and polyadenylation; and wherein the total apparent PARP activity in the eukaryotic cell is reduced significantly, (preferably the total apparent PARP activity is reduced from about 75% to about 90% of the normal apparent PARP activity in the eukaryotic cell, and the eukaryotic cell is protected against programmed cell death) or almost completely (preferably the total apparent PARP activity is reduced from about 90% to about 100% of the normal apparent PARP activity in the eukaryotic cell, and the cell is killed by programmed cell death).

Preferably the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode a sense RNA molecule which is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In an alternative method for modulating programmed cell death, provided by the invention, the first transcribed DNA region or the second transcribed DNA region or both, comprise a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the ZAP or the NAP class, and encode an RNA molecule which is capable of reducing the expression of said endogenous PARP gene of the ZAP or the NAP class.



In yet an alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP or the NAP class and the RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the mRNA resulting from transcription of the endogenous PARP gene of the ZAP or the NAP class, wherein the sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein that RNA molecule is capable of reducing the expression of the endogenous PARP gene of the ZAP or the NAP class.

In a further alternative method for modulating programmed cell death, provided by the invention, the first and/ or second transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP or the NAP class, preferably comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138 or comprising an amino acid sequence selected from the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370, the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11.

The promoter of the first and second chimeric PCD modulating genes, or both, may be a tissue specific or inducible promoter such as a promoter is selected from a fungus-responsive promoter, a nematode-responsive promoter, an anther-selective promoter, a stigma-selective promoter, a dehiscence-zone selective promoter.

The invention also provides a method for modulating programmed cell death in a plant cell, comprising introduction of a PCD modulating chimeric gene in said plant cell, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter, a DNA region, which when

transcribed yields a RNA molecule, which is either capable of reducing the expression of endogenous PARP genes; or is capable of being translated into a peptide or protein which when expressed reduces the apparent PARP activity in the plant cell, and a DNA region involved in transcription termination and polyadenylation, wherein the total apparent PARP activity in the plant cell is reduced from about 75% to about 100% of the normal apparent PARP activity in the plant cell.

It is another objective of the invention to provide the first and second chimeric PCD modulating gene as well as a eucaryotic cell, particularly a plant cell comprising the first and second chimeric PCD modulating gene and non-human eukaryotic organisms, particularly plants comprising such cells.

It is yet another objective of the invention to provide a method for modulating programmed cell death in cells of a plant, comprising introducing a PCD modulating chimeric gene in the cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and a DNA region involved in transcription termination and polyadenylation.

The invention also relates to a method for increasing the growth rate of a plant, comprising introducing a PCD modulating chimeric gene in cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule, being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and a DNA region involved in transcription termination and polyadenylation.

It is another objective of the invention to provide a method for producing stress tolerant cells of a plant comprising introducing a PCD modulating chimeric gene into cells of a plant, wherein the PCD modulating chimeric gene comprises the following operably linked DNA regions: a plant-expressible promoter; a DNA region, which when transcribed yields a RNA molecule, RNA molecule being capable of reducing

the expression of an endogenous PARP gene of the ZAP class; and a DNA region involved in transcription termination and polyadenylation.

The invention also relates to the use of a nucleotide sequence encoding a protein with PARP activity, preferably a PARP protein of the ZAP class, to modulate programmed cell death in a plant cell or plant or to produce a stress tolerant plant cell or plant or to increase the growth rate of a plant cell or plant.

### Brief description of the drawings

**Figure 1.** The deduced N-terminal amino acid sequences of plant poly(ADP-ribose) polymerases.

- (A) Alignment of the sequences upstream of the NAD<sup>+</sup>-binding domain found in *Arabidopsis thaliana* APP (*A.th.* APP; EMBL accession number Z48243; SEQ ID No 6) and the maize homolog NAP (*Z.m.* NAP; EMBL accession number AJ222588; SEQ ID No 4). The domain division shown is as previously proposed (Lepiniec *et al.*, 1995). The nuclear localization signal (NLS) located in the B domain is indicated by the bracket. The sequence of the B domain is not very well conserved between dicotyledonous and monocotyledonous plants. The C domain is probably comparable in function to the automodification domain of PARP from animals. The imperfect repeats, A1 and A2, are also present in maize NAP. To illustrate the internally imperfect two-fold symmetry within the repeat sequence, the properties of amino acid residues are highlighted below the sequences as follows: filled-in circles, hydrophobic residue; open circle, glycine; (+), positively charged residue; (-), negatively charged residue; wavy line, any residue. The axis of symmetry is indicated by the vertical arrowhead and arrowhead lines mark the regions with the inverted repetition of amino acid side chain properties.
- (B) Alignment of the DNA-binding and auto-catalytic domains of mouse PARP and maize ZAP. Zn-finger-containing maize ZAP1 and ZAP2 (partial cDNA found by the 5'RACE PCR analysis) are indicated as *Z.m.* ZAP (EMBL accession number AJ222589; SEQ ID No 2) and *Z.m.* ZAP(race) (SEQ ID No 11 from amino acid at position 1 to amino acid at position 98), respectively, and the mouse PARP, *M.m.* ADPRT (Swissprot accession number P11103). The Zn-fingers and bipartite NLS of the mouse enzyme are indicated by brackets, the Caspase 3 cleavage site by

the asterisk, and the putative NLS in the ZAP protein by the bracket in bold below the maize sequence. The amino acid residues that are conserved in all sequences are boxed; amino acid residues with similar physico-chemical properties are shaded with the uppermost sequence as a reference.

**Figure 2.** Comparison of the NAD<sup>+</sup>-binding domain of mouse PARP and plant PARP proteins. The range of the "PARP signature" is indicated above the sequences. Names and sequence alignment are as in Figure 1.

**Figure 3.** Estimation of the gene copy number and transcript size for the *nap* and *zap* genes.

(A) and (B) Maize genomic DNA of variety LG2080 digested with the indicated restriction endonucleases, resolved by agarose gel electrophoresis, blotted, and hybridized with radioactively labelled DNA probes prepared from the 5' domains of the *nap* and *zap* cDNA, which do not encode the NAD<sup>+</sup>-binding domain. The hybridization pattern obtained with the *nap* probe (A) is simple and indicates a single *nap* gene in the maize genome. As can be seen from the hybridization pattern (B), there might be at least two *zap* genes. To determine the size of the transcripts encoded by the *zap* and *nap* genes, approximately 1 µg of poly(A)<sup>+</sup> RNA extracted from roots (lane 1) and shoots (lane 2) of 6-day-old seedlings were resolved on an agarose gel after denaturation with glyoxal, blotted, and hybridized with *nap* (C) and *zap* (D) <sup>32</sup>P-labelled cDNA. <sup>33</sup>P 5' end-labelled *Bst*II fragments of λDNA were used as a molecular weight markers in both DNA and RNA gel blot experiments; their positions are indicated in kb to the left of each panel.

**Figure 4.** Analysis of APP expression in yeast.

(A) Schematic drawing of the expression cassette in pV8SPA. The expression of the *app* cDNA is driven by a chimeric yeast promoter, which consists of the minimal TATA box-containing promoter region of the *cycl* gene (*CYC1*) and an upstream activating promoter region of the *ga110* gene (*GAL10*), the latter providing promoter activation by galactose. Downstream regulatory sequences are derived from the gene encoding phosphoglycerol kinase (3PGK) (Kuge and Jones, 1994). The *app*-coding region is drawn with a division in putative domains as proposed earlier (Lepiniec *et al.*, 1995): A1 and A2 correspond to imperfect 27-

amino acid repeats, in between which there is a sequence (B domain), rich in positively charged amino acids and resembling the DNA-binding domains of a number of DNA-binding proteins. The amino acid sequence of the B domain is shown below the map and the stretch of arginine and lysine residues, which may function as an NLS is drawn in bold. Methionine residues (M<sup>1</sup>, M<sup>72</sup>), which may function as translation initiation codons, are indicated above the map. The C domain is rich in glutamic acid residues, resembling in its composition, but not in its sequence, the auto-modification domain of PARP from animals.

- (B) Immunoblot (Western blot) and Northern blot analyses of the DY (pYeDP1/8-2) and DY(pV8SPA) strains, indicated as (vector) and (app), respectively. Strains were grown in SDC medium supplemented with glucose (GLU), galactose (GAL), galactose and 3mM of 3ABA (GAL+3ABA), or galactose and 5 mM nicotinamide (GAL+NIC). Total RNA or total protein were extracted from the same cultures. Ten micrograms of total protein were fractionated by electrophoresis on 10% SDS-PAGE, electroblotted, and probed with anti-APP antisera. Five micrograms of total RNA were resolved by electrophoresis on an 1.5% agarose gel, blotted onto nylon membranes, and hybridized with <sup>32</sup>P-labeled DNA fragments derived from the app cDNA. Positions of the molecular weight marker bands are indicated to the left in kilobases (kb) and kilodalton (kDa).

**Figure 5.** Poly(ADP-ribose) polymerase activity of the APP protein.

- (A) The total protein extracts were prepared from DY(pYeDP1/8-2) grown on SDC with 2% galactose (vector GAL) and DY(pV8SPA) grown either on SDC with 2% glucose (app GLU), on SDC with 2% galactose (app GAL), or on SDC with 2% galactose and 3 mM 3ABA (app GAL+3ABA). To detect the synthesis of the poly(ADP-ribose) in these extracts, samples were incubated with <sup>32</sup>P-NAD<sup>+</sup> for 40 min at room temperature. Two control reactions were performed: 100 ng of the purified human PARP were incubated either in a reaction buffer alone (PARP) (lane 5), or with protein extract made from DY(pYeDP1/8-2) culture grown on glucose (vector GLU+PARP) (lane 6). The autoradiograph obtained after exposure of the dried gel to X-Omat Kodak film is shown. ORi corresponds to the beginning of the sequencing gel.
- (B) Stimulation of poly(ADP-ribose) synthesis by DNA in protein extracts from DY(pV8SPA). Amounts of sonicated salmon sperm DNA added to the nucleic acid

depleted yeast extracts are indicated in  $\mu\text{g ml}^{-1}$ . The synthesis of the poly(ADP-ribose) is blocked by 3ABA, which was added in one of the reactions at a concentration of 3 mM (lane 5). To ensure the maximal recovery of the poly(ADP-ribose), 20  $\mu\text{g}$  of glycogen were included as a carrier during precipitation steps; this, as can be seen, however resulted in high carry-over of the unincorporated label.

**Figure 6.** Schematic representation of the T-DNA vectors comprising the PCD modulating chimeric genes of the invention. P35S: CaMV35S promoter; L: cab22 leader; ZAP; coding region of a PARP gene of the ZAP class; 5'ZAP: N-terminal part of the coding region of a PARP gene of the ZAP class in inverted orientation; 3' 35S: CaMV35S 3' end transcription termination signal and polyadenylation signal; pACT2: promoter region of the actin gene; pNOS; nopaline synthase gene promoter; gat: gentamycin acetyl transferase; bar: phosphinotricin acetyl transferase; 3'NOS: 3' end transcription termination signal and polyadenylation signal of nopaline synthase gene; APP: coding region of a PARP gene of the NAP class; 5'APP: N-terminal part of the coding region of a PARP gene of the NAP class in inverted orientation ; LB: left T-DNA border; RB: right T-DNA border; pTA29: tapetum specific promoter, pNTP303: pollen specific promoter.

### Detailed description of preferred embodiments

For the purpose of the invention, the term "plant-expressible promoter" means a promoter which is capable of driving 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, e.g., certain promoters of viral or bacterial origin such as the CaMV35S or the T-DNA gene promoters.

The term "expression of a gene" refers to the process wherein a DNA region under control of regulatory regions, particularly the promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another

nucleic acid or protein or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA or a ribozyme. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide.

The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (e.g., a mRNA) in a cell under control of suitable regulatory regions, e.g., a plant-expressible promoter. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. An endogenous plant gene is a gene which is naturally found in a plant species. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory regions of the gene.

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 invention is based on the one hand on the finding that eukaryotic cells, particularly plant cells, quite particularly *Zea mays* cells contain simultaneously at least two functional major PARP protein isoforms(classes) which differ in size and amino-acid sequence, yet are both capable of binding DNA, particularly DNA with single stranded breaks, and both have poly-ADP ribosylation activity. On the other hand, the inventors have realized that programmed cell death in eukaryotes, particularly in plants, can be modulated by altering the expression level of the PARP genes or by altering the activity of the encoded proteins genetically, and that in order

to achieve this goal, the expression of both genes needs to be altered or in the alternative both classes of proteins need to be altered in their activity.

It is clear that the failure of the art to show that eukaryotic cells, particularly plant cells, comprise two functional isoforms of PARP proteins, encoded by different classes of genes, has hampered efficient modulation of PARP activity in those cells by recombinant DNA methods. Various embodiments of the methods and means are represented by the description, the Examples and the claims.

Thus, the invention relates to modulation -i.e. the enhancement or the inhibition- of programmed cell death or apoptosis in eukaryotic cells, preferably plant cells, by altering the level of expression of PARP genes, or by altering the activity or apparent activity of PARP proteins in that eukaryotic cell. Conveniently, the level of expression of PARP genes or the activity of PARP proteins is controlled genetically by introduction of PCD modulating chimeric genes altering the expression of PARP genes and/or by introduction of PCD modulating chimeric genes altering the apparent activity of the PARP proteins and/or by alteration of the endogenous PARP encoding genes.

As used herein, "enhanced PCD" with regard to specified cells, refers to the death of those cells, provoked by the methods of the invention, whereby the killed cells were not destined to undergo PCD when compared to similar cells of a normal plant not modified by the methods of the invention, under similar conditions.

"Inhibited PCD" with regard to specified cells is to be understood as the process whereby a larger fraction of those cells or groups of cells, which would normally (without the intervention by the methods of this invention) undergo programmed cell death under particular conditions, remain alive under those conditions.

The expression of the introduced PCD modulating chimeric genes or of the modified endogenous genes will thus influence the functional level of PARP protein, and indirectly interfere with programmed cell death. A moderate decrease in the functional level of PARP proteins leads to an inhibition of programmed cell death,



particularly to prevention of programmed cell death, while a severe decrease in the functional level of the PARP proteins leads to induction of programmed cell death.

In accordance with the invention, it is preferred that in order to inhibit or prevent programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased significantly, however avoiding that DNA repair (governed directly or indirectly by PARP) is inhibited in such a way that the cells wherein the function of the PARP proteins is inhibited cannot recover from DNA damage or cannot maintain their genome integrity. Preferably, the level and/or activity of the PARP proteins in the target cells, should be decreased about 75 %, preferably about 80%, particularly about 90% of the normal level and/or activity in the target cells so that about 25%, preferably about 20%, particularly about 10% of the normal level and/or activity of PARP is retained in the target cells . It is further thought that the decrease in level and/or activity of the PARP proteins should not exceed 95%, preferably not exceed 90% of the normal activity and/or level in the target cells. Methods to determine the content of a specific protein such as the PARP proteins are well known to the person skilled in the art and include, but are not limited to (histochemical) quantification of such proteins using specific antibodies. Methods to quantify PARP activity are also available in the art and include the above-mentioned TUNEL assay (*in vivo*) or the *in vitro* assay described Collinge and Althaus (1994) for synthesis of poly (ADP-ribose) (see Examples).

Also in accordance with the invention, it is preferred that in order to trigger programmed cell death in a eukaryotic cell, particularly in a plant cell, the combined level of both PARP proteins and/or their activity or apparent activity is decreased substantially, preferably reduced almost completely such that the DNA repair and maintenance of the genome integrity are no longer possible. Preferably, the combined level and/or activity of the PARP proteins in the target cells, should be decreased at least about 90%, preferably about 95%, more preferably about 99%, of the normal level and/or activity in the target cells, particularly the PARP activity should be inhibited completely. It is particularly preferred that the functional levels of both classes of PARP proteins separately are reduced to the mentioned levels.

For the purpose of the invention, PARP proteins are defined as proteins having poly (ADP-ribose) polymerase activity, preferably comprising the so-called "PARP signature". The PARP signature is an amino acid sequence which is highly conserved between PARP proteins, defined by de Murcia and Menussier de Murcia (1994) as extending from amino acid at position 858 to the amino acid at position 906 from the *Mus musculus* PARP protein. This domain corresponds to the amino acid sequence from position 817 to 865 of the conventional PARP protein of *Zea mays* (ZAP1; SEQ ID No 2) or to the amino acid sequence from position 827 to 875 of the conventional PARP protein of *Zea mays* (ZAP2; SEQ ID No 11) or to the amino acid sequence from position 500 to 547 of the non-conventional PARP protein of *Zea mays* (SEQ ID No 4) or to the amino acid sequence from position 485 to 532 of the non-conventional PARP protein of *Arabidopsis thaliana* (SEQ ID No 6). This amino sequence is highly conserved between the different PARP proteins (having about 90% to 100% sequence identity). Particularly conserved is the lysine at position 891 (corresponding to position 850 of SEQ ID No 2, position 861 of SEQ ID No 11, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6) of the PARP protein from *Mus musculus*, which is considered to be involved in the catalytic activity of PARP proteins. Particularly the amino acids at position 865, 866, 893, 898 and 899 of the PARP protein of *Mus musculus* or the corresponding positions for the other sequences are variable. PARP proteins may further comprise an N-terminal DNA binding domain and/or a nuclear localization signal (NLS).

Currently, two classes of PARP proteins have been described. The first class, as defined herein, comprises the so-called classical Zn-finger containing PARP proteins (ZAP). These proteins range in size from 113-120 kDA and are further characterized by the presence of at least one, preferably two Zn-finger domains located in the N-terminal domain of the protein, particularly located within the about 355 to about 375 first amino acids of the protein. The Zn-fingers are defined as peptide sequences having the sequence CxxCx<sub>n</sub>HxxC (whereby n may vary from 26 to 30) capable of complexing a Zn atom. Examples of amino acid sequences for PARP proteins from the ZAP class include the sequences which can be found in the PIR protein database with accession number P18493 (*Bos taurus*), P26466 (*Gallus gallus*), P35875 (*Drosophila melanogaster*), P09874 (*Homo sapiens*), P11103 (*Mus musculus*), Q08824 (*Oncorhynchus masou*), P27008 (*Rattus norvegicus*), Q11208

(*Sarcophaga peregrina*), P31669 (*Xenopus laevis*) and the currently identified sequences of the ZAP1 and ZAP2 protein from *Zea mays* (SEQ ID No 2 / SEQ ID No 11).

The nucleotide sequence of the corresponding cDNAs can be found in the EMBL database under accession numbers D90073 (*Bos taurus*), X52690 (*Gallus gallus*), D13806 (*Drosophila melanogaster*), M32721 (*Homo sapiens*), X14206 (*Mus musculus*), D13809 (*Oncorhynchus masou*), X65496 (*Rattus norvegicus*), D16482 (*Sarcophaga peregrina*), D14667 (*Xenopus laevis*) and in SEQ ID No 1 and 10 (*Zea mays*).

The second class as defined herein, comprises the so-called non-classical PARP proteins (NAP). These proteins are smaller (72-73 kDa) and are further characterized by the absence of a Zn-finger domain at the N-terminus of the protein, and by the presence of an N-terminal domain comprising stretches of amino acids having similarity with DNA binding proteins. Preferably, PARP protein of these class comprise at least one amino acid sequence of about 30 to 32 amino acids which comprise the sequence R G x x x x G x K x x x x R L (amino acids are represented in the standard one-letter code, whereby x stands for any amino acid; SEQ ID No 7). Even more preferably these PARP proteins comprise at least 1 amino acid sequence of about 32 amino acids having the sequence x L x V x x x R x x L x x R G L x x x G V K x x L V x R L x x A I (SEQ ID No 8) (the so-called A1 domain) or at least 1 amino acid sequence of about 32 amino acids having the sequence G M x x x E L x x x A x x R G x x x x G x K K D x x R L x x (SEQ ID No 9) (the so-called A2 domain) or both. Particularly, the A1 and A2 domain are capable of forming a helix-loop-helix structure. These PARP proteins may further comprise a basic "B" domain (K/R rich amino acid sequence of about 35 to about 56 amino acids, involved in targeting the protein to the nucleus) and/or a an acid "C" domain (D/E rich amino acid sequence of about 36 amino acids). Examples of protein sequences from the NAP class include the APP protein from *Arabidopsis thaliana* (accessible from PIR protein database under accession number Q11207; SEQ ID No 6) and the NAP protein from *Zea mays* (SEQ ID No 4). The sequence of the corresponding cDNAs can be found in the EMBL database under accession number Z48243 (SEQ ID No 5) and in SEQ ID No 3. That the second class of PARP proteins are indeed functional PARP proteins, i.e.

are capable of catalyzing DNA dependent poly(ADP-ribose) polymerization has been demonstrated by the inventors (see Example 2).

The inventors have further demonstrated that eukaryotic cells, particularly plant cells, express simultaneously genes encoding PARP proteins from both classes.

It is clear that for the purpose of the invention, other genes or cDNAs encoding PARP proteins from both classes as defined, or parts thereof, can be isolated from other eukaryotic species or varieties, particularly from other plant species or varieties. These PARP genes or cDNAs can be isolated e.g. by Southern hybridization (either low-stringency or high-stringency hybridization depending on the relation between the species from which one intends to isolate the PARP gene and the species from which the probe was ultimately derived) using as probes DNA fragments with the nucleotide sequence of the above mentioned PARP genes or cDNAs, or parts thereof, preferably parts which are conserved such as a gene fragment comprising the nucleotide sequence encoding the PARP signature mentioned supra. The nucleotide sequences corresponding to the PARP signature from the PARP proteins encoded by plant genes are the nucleotide sequence of SEQ ID No 1 from nucleotide 2558 to 2704 or the nucleotide sequence of SEQ ID No 3 from nucleotide 1595 to 1747 or the nucleotide sequence of SEQ ID No 5 from nucleotide 1575 to 1724. If a discrimination is to be made between the classes of PARP genes, parts of the PARP genes which are specific for the class, such as the N-terminal domains preceding the catalytic domain or parts thereof, should preferably be used.

Alternatively, the genes or cDNAs encoding PARP proteins or parts thereof, can also be isolated by PCR-amplification using appropriate primers such as the degenerated primers with the nucleotide sequence corresponding to the sequences indicated in SEQ ID No 13, SEQ ID No 14, or primers with the nucleotide sequence corresponding to the sequences indicated in SEQ ID No 15 to 20. However, it is clear that the person skilled in the art can design alternative oligonucleotides for use in PCR or can use oligonucleotides comprising a nucleotide sequence of at least 20, preferably at least about 30, particularly at least about 50, consecutive nucleotides of any of the PARP genes to isolate the genes or part thereof by PCR amplification.

It is clear that a combination of these techniques, or other techniques (including e.g. RACE-PCR), available to the skilled artisan to isolate genes or cDNAs on the basis of partial fragments and their nucleotide sequence, e.g. obtained by PCR amplification, can be used to isolate PARP genes, or parts thereof, suitable for use in the methods of the invention.

Moreover, PARP genes, encoding PARP proteins wherein some of the amino acids have been exchanged for other, chemically similar, amino acids (so-called conservative substitutions), or synthetic PARP genes (which encode similar proteins as natural PARP genes but with a different nucleotide sequence, based on the degeneracy of the genetic code) and parts thereof are also suited for the methods of the invention.

In one aspect of the invention, PCD in eukaryotic cells, particularly in plant cells, is inhibited by a moderate decrease in the functional level of PARP in those eukaryotic cells.

In one embodiment of this first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is moderately decreased.

In a particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of expression of the endogenous PARP genes. To this end, the transcribed DNA region encodes a biologically active RNA which decreases the mRNAs encoding NAP and ZAP class PARP proteins, that is available for translation. This can be achieved through techniques such as antisense RNA, co-suppression or ribozyme action.

As used herein, "co-suppression" refers to the process of transcriptional and/or post-transcriptional suppression of RNA accumulation in a sequence specific manner, resulting in the suppression of expression of homologous endogenous genes or transgenes.

Suppressing the expression of the endogenous PARP genes can thus be achieved by introduction of a transgene comprising a strong promoter operably linked to a DNA region whereby the resulting transcribed RNA is a sense RNA or an antisense RNA comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% sequence identity with or is identical to the coding or transcribed DNA sequence (sense) or to the complement (antisense) of part of the PARP gene whose expression is to be suppressed. Preferably, the transcribed DNA region does not code for a functional protein. Particularly, the transcribed region does not code for a protein. Further, the nucleotide sequence of the sense or antisense region should preferably be at least about 100 nucleotides in length, more preferably at least about 250 nucleotides, particularly at least about 500 nucleotides but may extend to the full length of the coding region of the gene whose expression is to be reduced.

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 Wilbur and Lipmann algorithm (Wilbur and Lipmann, 1983) using a window-size of 20 nucleotides or amino acids, a word length of 2 amino acids, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can be conveniently performed using commercially available software packages such as the programs of the Intelligenetics<sup>TM</sup> Suite (Intelligenetics Inc., CA).

It will be clear to a skilled artisan that one or more sense or antisense PCD modulating chimeric genes can be used to achieve the goals of the first aspect of the invention. When one sense or antisense PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes. This can e.g. be achieved by choosing the transcribed region of the chimeric gene in such a way that expression of both classes of genes can be regulated by one sense or antisense RNA, i.e. by choosing target regions corresponding to the highest homology DNA region of the PARP genes of both classes and incorporating a sense or antisense transcribed DNA region corresponding to both target regions, conform to the conditions described above for sense and antisense RNA. Alternatively, different sense or antisense RNA regions, each specific for regulating the expression of one class of PARP genes, can be combined into one RNA molecule, encoded by one transcribed region of one PCD modulating chimeric gene. Obviously, the different sense or antisense RNA regions specific for regulating the expression of one class of PARP genes can be introduced as separate PCD modulating chimeric genes.

Preferred sense and antisense encoding transcribed regions comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the N-terminal domains of the PARP genes, preferably corresponding to a sequence of at least about 100 consecutive nucleotides selected from the sequence of SEQ ID No 1 from nucleotide position 113 to 1189, the sequence of SEQ ID No 3 from nucleotide position 107 to 583, the sequence of SEQ ID No 5 from nucleotide position 131 to 542 or the sequence of SEQ ID No 10 from nucleotide position 81 to 1180. However, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the N-terminal

domain of the PARP genes, or even to complete sequence of the PARP genes, particularly the protein-encoding region thereof. Further preferred are sense and antisense encoding transcribed regions which comprise a nucleotide sequence corresponding (with sequence identity constraints as indicated above) to a sequence of at least about 100 consecutive nucleotides selected from the C-terminal catalytic domains of the PARP genes, preferably a sequence of at least 100 nucleotides encompassing the PARP-signature encoding nucleotide sequences, particularly the PARP-signature encoding nucleotide sequences indicated *supra*. Again, it is clear that sense or antisense encoding transcribed regions can be used comprising a sequence corresponding to the complete sequence of the C-terminal domain of the PARP genes.

In another particularly preferred embodiment, the PCD modulating chimeric genes decrease the functional level of the endogenous PARP activity by reducing the level of apparent activity of the endogenous PARPs of both classes. To this end, the transcribed DNA region encodes a biologically active RNA which is translated into a protein or peptide inhibiting NAP or ZAP class PARP proteins or both, such as inactivating antibodies or dominant negative PARP mutants.

"Inactivating antibodies of PARP proteins" are antibodies or parts thereof which specifically bind at least to some epitopes of PARP proteins, such as the epitope covering part of the ZN finger II from position 111-118 in ZAP1 or a corresponding peptide in ZAP2, and which inhibit the activity of the target protein.

"Dominant negative PARP mutants" as used herein, are proteins or peptides comprising at least part of a PARP protein (or a variant thereof), preferably a PARP protein endogenous to the eukaryotic target host cell, which have no PARP activity, and which have an inhibitory effect on the activity of the endogenous PARP proteins when expressed in that host cell. Preferred dominant negative PARP mutants are proteins comprising or consisting of a functional DNA binding domain (or a variant thereof) without a catalytic domain (such as the N-terminal Zn-finger containing domain of about 355 to about 375 amino acids of a PARP of the ZAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 or a DNA binding protein domain comprising



the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11 from amino acid at position 1 to the amino acid at position 98, or such as the N-terminal DNA binding protein domain of about 135 to 160 amino acids of a PARP of the NAP class, particularly a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or a DNA binding protein domain comprising the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138) or without a functional catalytic domain (such as inactive PARP mutants, mutated in the so-called PARP signature, particularly mutated at the conserved lysine of position 850 of SEQ ID No 2, position 532 of SEQ ID No 4, position 517 of SEQ ID No 6). Preferably, dominant negative PARP mutants should retain their DNA binding activity. Dominant negative PARP mutants can be fused to a carrier protein, such as a  $\beta$ -glucuronidase (SEQ ID No 12).

Again, one or more PCD modulating genes encoding one or more dominant negative PARP mutants can be used to achieve the goals of the first aspect of the invention. When one PCD modulating chimeric gene is used, this gene must be capable of simultaneously reducing the expression of PARP genes of both classes.

In another embodiment of the first aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced by modification of the nucleotide sequence of the endogenous PARP genes in those cells so that the encoded mutant PARP proteins retain about 10% of their activity. Methods to achieve such a modification of endogenous PARP genes include homologous recombination to exchange the endogenous PARP genes for mutant PARP genes e.g. by the methods described in US patent 5,527,695. In a preferred embodiment such site-directed modification of the nucleotide sequence of the endogenous PARP genes is achieved by introduction of chimeric DNA/RNA oligonucleotides as described in WO 96/22364 or US patent 5,565,350.

For plant cells, it has however been found that introduction of one PCD modulating chimeric gene, preferably encoding biologically active RNA active in reducing the expression of one class of the PARP genes, particularly of PARP genes of the ZAP class, may be sufficient for reduction of the total PARP activity in those plant cells in accordance with the first aspect of the invention, i.e. for inhibiting or preventing programmed cell death in those plant cells.

In this embodiment of the invention, the PCD modulating chimeric gene preferably comprises a transcribed region which codes for a biologically active RNA which comprises at least one RNA region, preferably of at least 100 nucleotides in length, classifying according to the herein mentioned criteria as a sense RNA for one of the endogenous PARP genes, and which comprises at least one other RNA region, preferably of at least 100 nucleotides in length, classifying according to the herein mentioned criteria as an antisense RNA for one of the endogenous PARP genes, whereby the antisense and sense RNA region are capable of combining into a double stranded region, preferably over a distance of at least about 100 nucleotides.

It is expected that introduction of one PCD modulating chimeric gene, which can decrease the functional or apparent level of one class of PARP proteins, particularly a PARP protein of the ZAP class, as herein described, may likewise be sufficient for reduction of the total PARP activity in plant cells in accordance with the first aspect of the invention.

The reduced or inhibited programmed cell death in plant cells comprising at least one PCD modulating chimeric gene in accordance with the first aspect of the invention can result in enhanced resistance to adversary conditions, such as resistance to stress imposed by treatment with chemicals, cold stress resistance, resistance to stress imposed by pathogens and pests, drought resistance, heat stress resistance etc.

In another aspect of the invention, programmed death of eukaryotic cells, preferably selected cells, particularly selected plant cells is enhanced by a severe decrease in the functional level of PARP, preferably reduced almost completely, such that the DNA repair and maintenance of the genome integrity are no longer possible.

In one embodiment of this aspect of the invention, the functional level of PARP in eukaryotic cells, particularly in plant cells is reduced severely, particularly abolished almost completely, by introduction of at least one PCD modulating chimeric gene in those cells, comprising a promoter capable of directing transcription in these cells, preferably a plant-expressible promoter, and a functional 3' transcription termination and polyadenylation region, operably linked to a DNA region which when transcribed yields a biologically active RNA molecule which is capable of decreasing the functional level of the endogenous PARP activity encoded by both classes of PARP genes.

In a preferred embodiment of the second aspect of the invention, at least two such PCD modulating chimeric genes are introduced in the cells, whereby the biologically active RNA encoded by the first PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the NAP class, and whereby the biologically active RNA encoded by the second PCD modulating chimeric gene decreases the functional level of the endogenous PARP activity encoded by the genes of the ZAP class, so that the combined PARP activity is severely decreased, particularly almost completely eliminated.

As mentioned for the first aspect of this invention, the transcribed regions of the PCD modulating chimeric genes encode biologically active RNA, which can interfere with the expression of the endogenous PARP genes (e.g. through antisense action, co-suppression or ribozyme action) or the biologically active RNA can be further translated into a peptide or protein, capable of inhibiting the PARP proteins of the NAP and ZAP class, such as inactivating antibodies or dominant negative PARP mutants.

In a particularly preferred embodiment of the second aspect of the invention, the transcribed region of the PCD modulating chimeric genes (PCD enhancing chimeric genes) codes for a biologically active RNA which comprises at least one RNA region (preferably of at least about 100 nucleotides in length) classifying according to the above mentioned criteria as a sense RNA for at least one of the endogenous PARP genes, and at least one other RNA region (preferably of at least about 100 nucleotides in length), classifying according to the above mentioned criteria as an

antisense RNA for at least one of the endogenous PARP genes, whereby the antisense and sense RNA region are capable of combining into a double stranded RNA region (preferably over a distance of at least about 100 nucleotides). In an especially preferred embodiment, two such PCD modulating genes, one targeted to reduce the functional level of a PARP protein of the NAP class, and the other targeted to reduce the functional level of a PARP protein of the ZAP class are introduced into an eukaryotic cell or organism, preferably a plant cell or plant.

It is clear that the different embodiments for the transcribed DNA regions of the chimeric PCD modulating genes of the invention can be used in various combinations to arrive at the goals of the invention. E.g. a first chimeric PCD modulating gene may encode a sense RNA designed to reduce the expression of an endogenous PARP gene of the ZAP class, while the second chimeric PCD modulating gene may encode a dominant negative PARP mutant designed to reduce the expression of an endogenous PARP gene of the NAP class.

Whether the introduction of PCD modulating chimeric genes into eukaryotic cells will ultimately result in a moderately reduced or a severely reduced functional level of combined PARP in those cells -i.e. in inhibited PCD or enhanced PCD- will usually be determined by the expression level (either on transcriptional level or combined transcriptional/translational level) of those PCD modulating genes. A major contributing factor to the expression level of the PCD modulating gene is the choice of the promoter region, although other factors (such as, but not limited to, the choice of the 3'end, the presence of introns, codon usage of the transcribed region, mRNA stability, presence of consensus sequence around translation initiation site, choice of 5' and 3' untranslated RNA regions, presence of PEST sequences, the influence of chromatin structure surrounding the insertion site of a stable integrated PCD modulating gene, copy number of the introduced PCD modulating genes, etc.) or combinations thereof will also contribute to the ultimate expression level of the PCD modulating gene. In general, it can be assumed that moderate reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively weak promoter, while severe reduction of functional levels of combined PARP can be achieved by PCD modulating genes comprising a relatively strong promoter. However, the expression level of a PCD modulating gene comprising a

specific promoter and eventually its effect on PCD, can vary as a function of the other contributing factors, as already mentioned.

For the purpose of particular embodiments of the invention, the PCD modulating chimeric genes may comprise a constitutive promoter, or a promoter which is expressed in all or the majority of the cell types throughout the organism, particularly throughout the plant, such as the promoter regions derived from the T-DNA genes, particularly the opine synthase genes of *Agrobacterium* Ti- or Ri-plasmids (e.g. nos, ocs promoters), or the promoter regions of viral genes (such as CaMV35S promoters, or variants thereof).

It may be further be advantageous to control the expression of the PCD modulating gene at will or in response to environmental cues, e.g. by inclusion of an inducible promoter which can be activated by an external stimuli, such as, but not limited to application of chemical compounds (e.g. safeners, herbicides, glucocorticoids), light conditions, exposure to abiotic stress (e.g. wounding, heavy metals, extreme temperatures, salinity or drought) or biotic stress (e.g. pathogen or pest infection including infection by fungi, viruses, bacteria, insects, nematodes, mycoplasmas and mycoplasma like organisms etc.). Examples of plant-expressible inducible promoters suitable for the invention are: nematode inducible promoters (such as disclosed in WO 92/21757), fungus inducible promoters (WO 93/19188, WO 96/28561), promoters inducible after application of glucocorticoids such as dexamethasone ( ), or promoters repressed or activated after application of tetracyclin (Gatz *et al.* 1988 ; Weimann *et al.* 1994)

In several embodiments of the invention, particularly for the second aspect of the invention (i.e. enhanced PCD), it may be convenient or required to restrict the effect on programmed cell death to a particular subset of the cells of the organism, particularly of the plant, hence the PCD modulating genes may include tissue-specific or cell type-specific promoters. Examples of suitable plant-expressible promoters selectively expressed in particular tissues or cell types are well known in the art and include but are 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), meristem specific promoters (such as the promoter of the *SHOOTMERISTEMLESS* (*STM*) gene, Long *et al.*, 1996), primordia specific promoter (such as the promoter of the *Antirrhinum* CycD3a gene, Doonan *et al.*, 1998), anther specific promoters (WO 89/10396, WO9213956, WO9213957) stigma-specific promoters (WO 91/02068), dehiscence-zone specific promoters (WO 97/13865), seed-specific promoters (WO 89/03887) etc.

Preferably the chimeric PCD modulating genes of the invention are accompanied by a marker gene, preferably a chimeric marker gene comprising a marker DNA that is operably linked at its 5' end to a plant-expressible promoter, preferably a constitutive promoter, such as the CaMV 35S promoter, or a light inducible promoter such as the promoter of the gene encoding the small subunit of Rubisco; and operably linked at its 3' end to suitable plant transcription 3' end formation and polyadenylation signals. It is expected that the choice of the marker DNA is not critical, and any suitable marker DNA can be used. For example, a marker DNA can encode a protein that provides a distinguishable "color" to the transformed plant cell, such as the A1 gene (Meyer *et al.*, 1987) or Green Fluorescent Protein (Sheen *et al.*, 1995), can provide herbicide resistance to the transformed plant cell, such as the *bar* gene, encoding resistance to phosphinothricin (EP 0,242,246), or can provide antibiotic resistance to the transformed cells, such as the *aac(6')* gene, encoding resistance to gentamycin (WO94/01560).

Methods to introduce PCD modulating chimeric genes into eukaryotic cells, particularly methods to transform plant cells are well known in the art, and are believed not to be critical for the methods of the invention. Transformation results in either transient or stably transformed cells (whereby the PCD modulating chimeric genes are stably inserted in the genome of the cell, particularly in the nuclear genome of the cell).

It is clear that the methods and means described in this invention to alter the programmed cell death in eukaryotic cells and organisms, particularly in plant cells and plants, has several important application possibilities. Inhibition of PCD by the

methods and means of the invention, can be used to relieve the stress imposed upon the cells, particularly the plant cells, during transformation and thus to increase transformation efficiency, as described in WO 97/06267. Inhibition of PCD can also be used to improve cell culture of eukaryotic cells, particularly of plant cells. Triggering of PCD in particular cell types using the means and methods of the invention, can be used for methods which call upon the use of a cytotoxin. Since PCD is the "natural" way for cells to die, the use of PCD enhancing chimeric genes of the invention constitutes an improvement over the use of other cytotoxic genes such as RNase or diphtheria toxin genes which lead to cell lysis. Moreover, low-level expression of PCD enhancing genes in cells different than the targeted cells, will lead to a moderate reduction instead of a severe reduction of PARP activity in those cells, thus actually inhibiting PCD in non-target cells.

For plants, preferred applications of PCD enhancing chimeric genes include, but are not limited to:

1. the generation of plants protected against fungus infection, whereby the PCD enhancing chimeric gene or genes comprise a fungus-responsive promoter as described in WO 93/19188 or WO 96/28561.
2. the generation of nematode resistant plants, whereby the PCD enhancing chimeric gene or genes comprise a nematode inducible promoters such as disclosed in WO 92/21757
3. the generation of male or female sterile plants, whereby the PCD enhancing chimeric gene or genes comprise anther-specific promoters (such as disclosed in WO 89/10396, WO9213956, WO9213957) or stigma-specific promoters (such as disclosed in WO 91/02068)
4. the generation of plants with improved seed shatter characteristics whereby the PCD enhancing chimeric gene or genes comprise dehiscence zone-specific promoters (such as disclosed in WO 97/13865).

Unexpectedly, it has been found that upon introduction of a PCD modulating chimeric gene according to the first aspect of the invention, preferably a chimeric gene modulating the expression of a PARP gene of the ZAP class, particularly a chimeric gene modulating the expression of a PARP gene of the ZAP class wherein the transcribed region codes for a biologically active RNA comprising simultaneously

a sense and antisense RNA as herein described, the transformed plant cells, plant calli and plants exhibited an enhanced growth.

Although not intending to limit the invention to a particular mode of action, it is believed that the enhanced growth is a consequence of the reduced number of cells which undergo programmed cell death, probably by increasing the threshold for a signal inhibiting cell division, thus leading to more vigorously growing plants. These plants are also more stress resistant as explained elsewhere in this application.

Therefore, in a third aspect, the invention also relates to a method for enhancing growth, preferably vegetative growth, of plant cells, plant tissues and plants comprising at least one PCD modulating chimeric gene according the first aspect of the invention preferably a chimeric gene modulating the expression of a PARP gene of the ZAP class, particularly a chimeric gene modulating the expression of a PARP gene of the ZAP class wherein the transcribed region codes for a biologically active RNA comprising simultaneously a sense and antisense RNA.

Although it is clear that the invention can be applied essentially to all plant species and varieties, the invention will be especially suited to alter programmed cell death in plants with a commercial value. Particularly preferred plants to which the invention can be applied are corn, oil seed rape, linseed, wheat, grasses, alfalfa, legumes, a brassica vegetable, tomato, lettuce, cotton, rice, barley, potato, tobacco, sugar beet, sunflower, and ornamental plants such as carnation, chrysanthemum, roses, tulips and the like.

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 cell-division controlling gene of the invention in other varieties of the same or related plant species. Seeds obtained from the transformed plants contain the PCD modulating gene of the invention as a stable genomic insert.

The following non-limiting Examples describe the construction of chimeric apoptosis controlling genes and the use of such genes for the modulation of the programmed cell death in eukaryotic cells and organisms. 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.

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

- SEQ ID No 1: DNA sequence of the ZAP gene of *Zea mays* (*zap1*)
- SEQ ID No 2: protein sequence of the ZAP protein of *Zea mays* (ZAP1)
- SEQ ID No 3: DNA sequence of the NAP gene of *Zea mays* (*nap*)
- SEQ ID No 4: protein sequence of the NAP protein of *Zea mays* (NAP)
- SEQ ID No 5: DNA sequence of the NAP gene of *Arabidopsis thaliana* (*app*)
- SEQ ID No 6: protein sequence of the NAP protein of *Arabidopsis thaliana* (APP)
- SEQ ID No 7: consensus sequence for the A domain of non-conventional PARP proteins
- SEQ ID No 8: consensus sequence for the A1 domain of non-conventional PARP proteins
- SEQ ID No 9: consensus sequence for the A2 domain of non-conventional PARP proteins
- SEQ ID No 10: DNA sequence of the second ZAP gene of *Zea mays* (*Zap2*)
- SEQ ID No 11: protein sequence of the ZAP protein of *Zea mays* (ZAP2)
- SEQ ID No 12: amino acid sequence of a fusion protein between the DNA binding domain of APP and the GUS protein
- SEQ ID No 13: degenerated PCR primer
- SEQ ID No 14: degenerated PCR primer
- SEQ ID No 15: PCR primer
- SEQ ID No 16: PCR primer
- SEQ ID No 17: PCR primer
- SEQ ID No 18: PCR primer
- SEQ ID No 19: PCR primer

SEQ ID No 20: PCR primer

SEQ ID No 21: app promoter-gus translational fusion

Sequence listing free text

The following free text has been used in the Sequence Listing part of this application

<223> Description of Artificial Sequence:A domain of

"

non-conventional PARP proteins

<223> Description of Artificial Sequence:A1 domain on

non conventional PARP protein

<223> Description of Artificial Sequence: A2 domain of

"

non-conventional PARP protein

"

<223> Description of Artificial Sequence: fusion protein

between APP N-terminal domain and GUS protein

<223> Description of Artificial Sequence: degenerated

"

PCR primer

<223> Description of Artificial Sequence:oligonucleotide

"

for use as PCR primer

"

<223> Description of Artificial Sequence: APP promoter

fusion with beta-glucuronidase gene

<223> translation initiation codon

## Examples

### Experimental procedures

#### *Yeast and bacterial strains*

*Saccharomyces cerevisiae* strain DY (MATa *his3 can1-10 ade2 leu2 trp1 ura3::*(3xSV40 AP1-lacZ) (Kuge and Jones, 1994) was used for the expression of the APP protein. Yeast transformation was carried out according to Dohmen *et al.* (1991). Strains were grown on a minimal SDC medium (0.67% yeast nitrogen base, 0.37% casamino acids, 2% glucose, 50 mg l<sup>-1</sup> of adenine and 40 mg l<sup>-1</sup> of tryptophan). For the induction of the APP expression, glucose in SDC was substituted with 2% galactose.

*Escherichia coli* strain XL-1 (Stratagene, La Jolla, CA) was used for the plasmid manipulations and library screenings, which were carried out according to standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). *E. coli* BL21 (Studier and Moffat, 1986) was used for the APP protein expression and *Agrobacterium tumefaciens* C58C1Rif<sup>R</sup>(pGV2260) (Deblaere *et al.*, 1985) for the stable transformation of plants.

#### *Poly(ADP-ribose)polymerase activity assay*

Enzymatic activity of the APP was assayed in total protein extracts of yeast strains prepared as follows. DY(pV8SPA) or DY(pYeDP1/8-2) were grown in 50 ml of SDC medium overnight at 30°C on a gyratory shaker at 150 rpm. Yeast cells were harvested by centrifugation at 1,000×g, washed three times with 150 ml of 0.1 M potassium phosphate buffer (pH 6.5), and resuspended in 5 ml of sorbitol buffer (1.2 M sorbitol, 0.12 M K<sub>2</sub>HPO<sub>4</sub>, 0.033 M citric acid, pH 5.9). Lyticase (Boehringer, Mannheim, Germany) was added to the cell suspension to a final concentration of 30 U ml<sup>-1</sup> and cells were incubated at 30°C for 1 h. Yeast spheroplasts were then washed three times with sorbitol buffer and resuspended in 2 ml of ice-cold lysis buffer (100 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT). After sonication, the lysate was centrifuged at 20,000×g for 20 min at 4°C and the

supernatant was desalted on a Econo-Pack™ 10 DG column (Bio-Rad, Richmond, CA) equilibrated with reaction buffer (100 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 1 mM DTT). To reduce proteolytic degradation of proteins, the lysis and reaction buffers were supplemented with a protease inhibitor cocktail (Boehringer), one tablet per 50 ml. Nucleic acids were removed from the total extracts by adding NaCl and protamine sulfate to a final concentration of 600 mM and 10 mg ml<sup>-1</sup>, respectively. After incubation at room temperature for 10 min, the precipitate was removed by centrifugation at 20,000×g for 15 min at 4°C. The buffer of the supernatant was exchanged for the reaction buffer by gel filtration on an Econo-Pack™ 10 DG column.

The assay for the synthesis of poly(ADP-ribose) was adapted from Collinge and Althaus (1994). Approximately 500 µg of total yeast protein were incubated in a reaction buffer supplemented with 30 µCi of <sup>32</sup>P-NAD<sup>+</sup> (500 Ci mmol<sup>-1</sup>), unlabeled NAD<sup>+</sup> to a final concentration of 60 µM, and 10 µg ml<sup>-1</sup> sonicated salmon sperm DNA. After incubation for 40 min at room temperature, 500 µl of the stop buffer (200 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 5 mM EDTA, 1% Na<sup>+</sup>-N-lauroyl-sarcosine, and 20 µg ml<sup>-1</sup> proteinase K) were added and reactions incubated at 37°C overnight. After phenol and phenol/chloroform extractions, polymers were precipitated with 2.5 volumes of ethanol with 0.1 M NaAc (pH 5.2). The pellet was washed with 70% ethanol, dried, and dissolved in 70% formamide, 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol. Samples were heated at 80°C for 10 min and then loaded onto a 12% polyacrylamide/6 M urea sequencing gel. Gels were dried on 3MM paper (Whatman International, Maidstone, UK) and exposed either to Kodak X-Omat X-ray film (Eastman Kodak, Richmond, NY) or scanned using a PhosphorImager™ 445SI (Molecular Dynamics, Sunnyvale, CA).

#### *Immunological techniques*

A truncated *app* cDNA encoding an APP polypeptide from amino acids Met<sup>310</sup> to His<sup>637</sup> was expressed as a translation fusion with six histidine residues at the N terminus after induction of a 500-ml culture of the *E. coli* BL21(pETΔNdeSPA) with 1 mM isopropyl-β-D-thiogalactopyranoside. The APP polypeptide was purified to near homogeneity by affinity chromatography under denaturing conditions (in the presence

of 6 M guanidinium hydrochloride) on a  $\text{Ni}^{2+}$ -NTA-agarose column, according to the manufacturer's protocol (Qiagen, Chatsworth, CA). After dialysis against PBS, a mixture of the soluble and insoluble APP polypeptides was used to immunize two New Zealand White rabbits following a standard immunization protocol (Harlow and Lane, 1988). For the Western blot analysis, proteins were resolved by denaturing SDS-PAGE (Sambrook *et al.*, 1989; Harlow and Lane, 1988) and transferred onto nitrocellulose membranes (Hybond-C; Amersham), using a Semi-Dry Blotter II (Kem-En-Tec, Copenhagen, Denmark).

*In situ* antigen localization in yeast cells was carried out as described (Harlow and Lane, 1988). For the localization of the APP protein in yeast spheroplasts, anti-APP serum was diluted 1:3,000 to 1:5,000 in Tris-buffered saline-BSA buffer. 10H monoclonal antibody, which specifically recognizes poly(ADP-ribose) polymer (Ikajima *et al.*, 1990) was used in a 1:100 dilution in PBS buffer. The mouse antibody were detected with the sheep anti-mouse IgG F(ab')<sub>2</sub> fragment conjugated to fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:200. Rabbit IgG was detected with CY-3 conjugated sheep anti-rabbit IgG sheep F(ab')<sub>2</sub> fragment (Sigma), at a dilution of 1:200. For the visualization of DNA, slides were incubated for 1 min in PBS with 10  $\mu\text{g ml}^{-1}$  of 4',6-diamidino-2-phenylindole (DAPI; Sigma). Fluorescence imaging was performed on an Axioskop epifluorescence microscope (Zeiss, Jena, Germany). For observation of FITC and CY-3 fluorochromes, 23 and 15 filter cubes were used, respectively. Cells were photographed with Fuji Color-100 super plus film.

#### *Plant material and histochemical analysis*

*Nicotiana tabacum* SR1 (Maliga *et al.*, 1975) was used for the generation of stable transformants following the procedure of leaf disc cocultivation (De Block *et al.*, 1987) with *A. tumefaciens* C58C1Rif<sup>R</sup>(pGV2260; pGCNSPAGUS). *N. tabacum* SR1 line transformed with authentic GUS under the control of the 35S CaMV was used as a control. *Arabidopsis thaliana* ecotype Columbia was used for the transformation of the *app*-promoter-GUS fusion following the *in situ* infiltration procedure.

For *in situ* histochemical staining of the GUS activity, plant samples were fixed in ice-cold 90% acetone for 30 min, washed in 0.1 M  $\text{K}_2\text{HPO}_4$  (pH 7.8), and then

incubated in staining buffer (0.1 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.8, 2 mM X-Gluc, 20 mM Fe<sup>3+</sup>-EDTA) at 37°C. Stained plant tissues were stored in 70% ethanol at 4°C. When necessary, browning of tissues due to phenolic oxidation was reduced by incubation with lactophenol (Beeckman and Engler, 1994). The GUS staining was examined under a Jenalumar light microscope (Zeiss). Plant tissues were photographed with Fuji Color-100 super plus film.

#### *Miscellaneous methods*

The plasmid construction steps were routinely verified by DNA sequencing carried out according to protocols provided by USB Biochemicals (Cleveland, OH). <sup>32</sup>P-labeled DNA probes for nucleic acid hybridization were synthesized by the Ready-Prime DNA labelling kit (Amersham). For DNA and RNA hybridization experiments, the buffer system of Church and Gilbert (1984) was used (0.25 M sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA). For Western blot analysis, yeast total proteins were extracted with phenol essentially as described for plant tissues (Hurkman and Tanaka, 1986). For Northern blot analysis, total yeast RNA was extracted with hot phenol as described (Ausubel *et al.*, 1987). RNA was resolved on 1.5% agarose gels after denaturation with glyoxal (Sambrook *et al.*, 1989). Hybond-N nylon filters (Amersham) were used for the nucleic acid blotting.

#### **Example 1: Isolation of genes encoding PARP homologues from *Zea mays*.**

With the purpose of isolating maize cDNA encoding PARP homologue(s) two approaches were followed. First, a maize cDNA library was screened under low-stringency DNA—DNA hybridization conditions using a DNA probe prepared from the *Arabidopsis app* cDNA. Secondly, PCR amplification of part of the maize PARP was performed, using the first-strand cDNA as a template and two degenerate primers, designed on the basis of the sequence of the "PARP signature", the most conserved amino acid sequence between all known PARP proteins.

A λZAP (Stratagene) cDNA library from leaves of maize (*Zea mays* L.), inbred line B734 . Plaques (500,000) were screened according to standard procedures (Sambrook *et al.*, 1989). After screening with the *Arabidopsis app* probe, one

non-full-length cDNA of 1.4 kbp was purified. After the initial cDNA library screening with the *app* probe and a subsequent 5' rapid amplification of cDNA ends (RACE) PCR analysis, the *nap* gene, a maize homologue of the *Arabidopsis app*, was identified. For the 5'RACE PCR, the template was prepared with the Marathon kit (Clontech, Palo Alto, CA) and 0.5 µg of maize poly(A)<sup>+</sup> RNA isolated from inner sheath, outer sheath, and leaves of 1-week-old maize seedlings. The gene-specific, nested primers for PCR amplification were 5'-GGGACCATGTAGTTTATCTTGACCT-3' (SEQ ID No 15) and 5'-GACCTCGTACCCCAACTCTTCCCAT-3' (SEQ ID No 16) for *nap* primers. The amplified PCR products were subcloned and sequenced. A fragment of 800 bp was amplified with *nap*-specific primers which allowed to reconstruct the 2295-bp-long sequence of *nap* cDNA (SEQ ID No 3).

The NAP protein was 653 amino acids long (molecular mass ~73 kDa; SEQ ID No 4) and highly similar (61% sequence identity and 69% similarity) to the APP. Most importantly, NAP had an organization of the N-terminus congruent to APP (Figure 1A), suggesting a rather strict selection pressure on the structure of APP-like proteins in plants. The *nap* gene was unique in the maize genome (Figure 2A) and encoded a transcript of 2.4 kb (Figure 2C).

Using degenerate primers based on very highly conserved regions in the "PARP signature" and first-strand cDNA from *Zea mays* as a template, a 310-bp fragment was amplified. For the PCR with degenerate primers 5'-CCGAATTCGGNTAYATGTTYGGNAA-3' (SEQ ID No 13) and 5'-CCGAATTCACNATRTAYTCRTTRTA-3' (SEQ ID No 14) with Y=C/T; R=A/G; N=A/G/C/T), the first strand cDNA was used as a template and was synthesized using 5 µg of poly(A)<sup>+</sup> RNA from young maize leaves and MuMLV reverse transcriptase. PCR amplifications were performed with *Taq* DNA polymerase in 100 µl volume using the following conditions: 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, followed by 38 cycles of 1 min at 95°C, 2 min at 45°C, 3 min at 72°C, with a final incubation for 10 min at 72°C.

The sequence of the 310 bp fragment showed 55% sequence identity and 64% sequence similarity with human PARP over the same region, but was, however, different from the sequence of the *nap* cDNA. Three *zap* cDNAs were identified after screening with the 310-bp fragment, which was obtained by PCR with degenerate primers. These three purified cDNA were all derived from the same transcript because they had identical 3' non-coding regions; the longest clone (#9) was sequenced on both strands (SEQ ID No 1). This cDNA encoded a PARP-homologous polypeptide of 689 amino acids (SEQ ID No 2; molecular mass ~109 kDa), which we designated as ZAP1 (Figure 1B). The first Zn-finger of ZAP1 was probably nonfunctional because it had the sequence CKSCxxxHASV, which included no third cysteine residue.

5'RACE PCR analysis of *zap* transcripts from the maize line LG2080 (the screened cDNA library was made from the inbred line B734) was performed as described above using the following *zap* specific primers 5'-AAGTCGACGCGGCCGCCACACCTAGTGCCAGGTCAG-3' (SEQ ID No 17) and 5'-ATCTCAATTGTACATTTCTCAGGA-3' (SEQ ID No 18). A 450-bp PCR product was obtained after PCR with *zap*-specific primers. Eight independent, because of their slight differences in lengths at their 5' ends, 5'RACE PCR fragments generated with *zap*-specific primers were sequenced. In all the transcripts from the LG2080 maize plants, there was an insertion of additional sequence in the coding region, which made the ZAP protein longer by 11 amino acids (980 amino acids, molecular mass ~110.4 kDa). The Zn-finger I of ZAP2 was standard and read CKSCxxxHARC (Figure 1B; SEQ ID No 11). The sequence difference may be due either to differences between maize varieties, to the expression of two homologous genes, or to alternative splicing. In fact, maize may have at least two *zap* genes (Figure 2B), which encode a transcript of 3.4-3.5 kb (Figure 2D). The DNA gel blot experiment with a probe prepared from the *zap* cDNA showed that homologous genes were present in *Arabidopsis*.

Structurally ZAP was very similar to PARP from animals. It had a well conserved DNA-binding domain composed of two Zn-fingers (36% identity and 45% similarity to the DNA-binding domain of mouse PARP). Even higher homology was shown by comparing only the sequences of the Zn-fingers, Ala<sup>1</sup>-Phe<sup>162</sup> in the mouse enzyme (44% identity and 54% similarity), or a subdomain downstream from the nuclear



localization signal (NLS), Leu<sup>237</sup>-Ser<sup>360</sup> in mouse PARP (40% identity and 50% similarity). Whereas the bipartite nuclear localization signal characteristic of mammalian PARP could not be identified in ZAP, the sequence KRKK fitted a monopartite NLS (Figure 1B). The putative automodification domain was poorly conserved and was shorter in ZAP than in mouse PARP. The compilation of the homology of the catalytic domains between ZAP, NAP, APP and mouse PARP is shown in Figure 2. It should be noted that the NAD<sup>+</sup>-binding domain of ZAP was more similar to the mammalian enzyme (48% identity) than to that of APP and NAP (40% and 42% sequence identity, respectively), whereas APP and NAP were 68% identical and 76% similar in their catalytic domain.

**Example 2 Demonstration that non-conventional PARP protein has a DNA-dependent poly(ADP-ribose) polymerase activity.**

*APP is a DNA-dependent poly(ADP-ribose) polymerase*

A more detailed study of the APP protein (expressed in yeast) was performed to understand the activity of PARP-like proteins from the NAP class. The choice of yeast as the organism for the expression and enzymatic analysis of the *Arabidopsis* APP protein was made for a number of reasons. As an eukaryote, *Saccharomyces cerevisiae* is better suited for the expression of native proteins from other eukaryotic organisms, and unlike most other eukaryotic cells, it does not possess endogenous PARP activity (Lindahl *et al.*, 1995).

The full-length *app* cDNA was placed in pYeDP1/8-2 under the control of a galactose-inducible yeast promoter in the following way. the full-length *app* cDNA was excised from pC3 (Lepiniec *et al.*, 1995) as an *XhoI-EcoRI* fragment. The ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was subcloned into the *SmaI* site of the yeast expression vector pYeDP1/8-2 (Cullin and Pompon, 1988). The resulting expression vector pV8SPA (Figure 4A) was transformed into *S. cerevisiae* strain DY.

For APP expression in *E. coli*, the complete coding region of the *app* cDNA was PCR amplified with *Pfu* DNA polymerase (Stratagene), using the primers

5'-AGGATCCCATGGCGAACAAGCTCAAAGTGAC-3' (SEQ ID No 19) and 5'-AGGATCCTTAGTGCTTGTAGTTGAAT-3' (SEQ ID No 20), and subcloned as a *Bam*HI fragment into pET19b (Novagene, Madison, WI), resulting in pETSPA. The expression of the full-length APP in *E. coli* BL21 from pETSPA was very poor. To obtain better expression, pETSPA was digested with *Nco*I and *Nde*I or with *Sma*I, the ends were filled in by the Klenow fragment of DNA polymerase I, and the plasmids were then self-ligated. Of the resulting plasmids pETΔNdeSPA and pETΔSmaSPA, only pETΔNdeSPA gave satisfactory expression of the truncated APP polypeptide (Met<sup>310</sup> to His<sup>637</sup>) in *E. coli* BL21.

The expression of the APP in yeast was verified by Northern and Western blot analysis. (Fig 4) As the promoter in pV8SPA is inactive when cells are grown on glucose and derepressed on galactose-containing media, the expression was expected to be tightly regulated by the carbon source. However, Northern blot analysis of RNA and immunoblot analysis of proteins in DY(pV8SPA) as compared to the control DY strain containing the empty vector, showed that app mRNA and APP protein were expressed in yeast even when grown on glucose-containing media (Figure 4B, lane 2). The peculiarity of the expression observed on glucose-containing medium was that both app mRNA and APP protein were shorter than the ones detected after induction with galactose (compare lanes 2 and 4 in Figure 4B). The APP polypeptide with the higher molecular weight, (apparently a full-length protein) was only detected on galactose-containing medium, although such cells also expressed the truncated mRNA and protein. The most probable explanation for this finding is that when the DY(pV8SPA) strain is grown on glucose, there is a leaky expression from the expression cassette, with transcription beginning 200-300 bp downstream from the transcription start observed after galactose induction. This shorter mRNA probably does not code for the first methionine (Met<sup>1</sup>) of APP and, therefore, translation is initiated at Met<sup>72</sup>. This would explain the observed difference of -5 kDa (calculated difference being 7.5 kDa) in the molecular masses of the APP polypeptides from strains grown on glucose or on galactose. The possibility that the differences in molecular masses may be attributed to self-modification through poly(ADP-ribos)ylation was ruled out by growing strains in the presence of PARP

inhibitors, such as 3ABA and nicotinamide (Figure 4B, compare lanes 6 and 8 to lane 4).

To detect the synthesis of poly(ADP-ribose), total proteins were extracted from yeast strains grown under different conditions and incubated in the presence of radioactively labeled  $\text{NAD}^+$ . To prevent synthesis of poly(ADP-ribose) and possible automodification of the APP *in vivo*, strains were also grown in the presence of 3ABA, a reversible inhibitor of PARP, which was subsequently removed from the protein extracts during desalting. Figure 5 shows that poly(ADP-ribose) is synthesized by protein extracts of DY(pV8SPA) grown on galactose (Figure 5A, lanes 1 and 2), but not by a strain containing the empty vector (Figure 5A, lane 4). It can also be seen that *Arabidopsis* APP could synthesize polymers up to 40 residues in length (Figure 5A, lane 1) with the majority of the radioactivity being incorporated into 10-15-mer. This observation is consistent with the polymer sizes detected by other authors (Chen *et al.*, 1994). More radioactivity was incorporated into polymer when the yeast strain was grown with 3ABA than without (Figure 5A, lane 1 compared to lane 2); the reason might be that either the APP extracted from inhibited cultures was less automodified (it is believed that automodification inhibits the activity of PARP) or the labeled  $\text{NAD}^+$  was used by the enzyme from the uninhibited culture for the extension of existing polymer, resulting in a lower specific activity overall. Under the same reaction conditions poly(ADP-ribose) synthesized by human PARP, either in reaction buffer alone or in the presence of a yeast total protein extract from DY(pYeDP1/8-2) (Figure 5A, lanes 5 and 6, respectively), showed much longer chains, possibly up to 400-mer (de Murcia and Ménissier de Murcia, 1994).

The stimulation of enzymatic activity by nicked DNA is a well known property of PARP from animals (Alvarez-Gonzalez and Althaus, 1989). We therefore tested whether the activity of the APP protein was DNA dependent. After removal of yeast nucleic acids (DNA, RNA) and some basic proteins from the galactose-grown DY(pV8SPA) protein extract the synthesis of poly(ADP-ribose) was analyzed in the presence of increasing concentrations of sonicated salmon sperm DNA. As can be seen in Figure 5B, there was a direct correlation between the amount of DNA present in the reaction and the incorporation of  $^{32}\text{P}$ - $\text{NAD}^+$ . Scanning of the phosphor-images indicated that ~6-fold more radioactivity was incorporated into poly(ADP-ribose) in the reaction mixture

containing 40  $\mu\text{g ml}^{-1}$  of DNA than into that with 2  $\mu\text{g ml}^{-1}$  of DNA (Figure 5B, lanes 4 and 2, respectively). The synthesis of the polymer was sensitive to 3ABA in the reaction mix (Figure 5B, lane 5).

#### *APP is a nuclear protein*

In animal cells PARP activity is localized in the nucleus (Schreiber *et al.*, 1992). The intracellular localization, if nuclear, of APP could provide an important additional indication that APP is a *bona fide* plant PARP. To this end, the localization of the APP polypeptides in yeast cells was analyzed using anti-APP antisera. The APP polypeptide synthesized in yeast grown on galactose was found mainly in the nucleus. This localization was unaffected by the presence in the media of the PARP inhibitors.

In addition, we tested whether APP was constitutively active in yeast cells, as has been reported for the human PARP (Collinge and Althaus, 1994). Here, fixed yeast spheroplasts were incubated with monoclonal 10H antibody, which specifically recognizes poly(ADP-ribose) polymers (Kawamitsu *et al.*, 1984). A positive yellowish-green fluorescence signal with 10H antibody was localized in the nucleus and was observed only in DY(pV8SPA) cells grown on galactose. Positive staining was greatly reduced in cells grown in the presence of the PARP inhibitors, 3ABA and nicotinamide.

To identify the intracellular localization of APP in plant cells, a widely adopted approach in plant studies was used, *i.e.*, the examination of the subcellular location of a fusion protein formed between the protein in question and a reporter gene, once the protein fusion was produced in transgenic plants or transfected cells (Citovsky *et al.*, 1994; Sakamoto and Nagatani, 1996; Terzaghi *et al.*, 1997; von Arnim and Deng, 1994). An N-terminal translational fusion of GUS with the part of the APP polypeptide extending from the Met<sup>1</sup> to Pro<sup>407</sup> was made. The translational fusion of APP with bacterial GUS was constructed as follows. Plasmid pETSPA was cut with *Sma*I, treated with alkaline phosphatase, and ligated to a blunted *Nco*I-*Xba*I fragment from pGUS1 (Plant Genetic Systems N.V., Gent, Belgium). The ligation mix was transformed into *E. coli* XL-I and cells were plated onto LB medium supplemented with 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside, 40  $\mu\text{g ml}^{-1}$

5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide, and 100  $\mu\text{g ml}^{-1}$  of ampicillin. In this way, pETSPAGUS was selected as blue colonies. The expression in *E. coli* of the ~110-kDa fusion protein was confirmed by *in situ* GUS activity gels (Lee *et al.*, 1995). The APP-GUS fusion was placed under the control of the 35S promoter of the CaMV (the Klenow-blunted *Bam*HI fragment from pETSPAGUS was subcloned into *Sma*I-digested pJD330; Gallie and Walbot, 1992) and the resulting expression cassette was subcloned as an *Xba*I fragment into the *Xba*I site of the pCGN1547 binary vector (McBride and Summerfelt, 1990) to give pGCNSPAGUS. The pGCNSPAGUS was finally introduced into *A. tumefaciens* C58C1Rif<sup>R</sup>(pGV2260) by the freezing-thawing transformation procedure.

Expression of the fusion protein was verified in *E. coli*. The chimeric cDNA under the control of the 35S CaMV promoter was stably integrated into the tobacco genome. Progeny from four independent transgenic tobacco plants were analyzed for the subcellular distribution of the GUS activity after *in situ* histochemical staining (Jefferson *et al.*, 1987). In 2-day-old seedlings GUS activity could be detected in cotyledons and in roots, but not in hypocotyls or root tips. Because of the transparency of root tissues, GUS staining was clearly localized in the nuclei of root hairs and epidermal cells. Additionally, some diffuse, non-localized staining of other root cells was seen, in particular along the vascular cylinders. This non-nuclear GUS staining was more pronounced in leaf tissues. Whereas young true leaves or cotyledons displayed intense blue staining of the nuclei, there was also some diffuse staining of the cytoplasm. In fully expanded leaves, however, GUS staining became homogenous and similar to the staining of control plants transformed with GUS under the control of the CaMV 35S promoter, in which GUS was expressed in the cytoplasm. Eventually, older leaves or cotyledons exhibited practically no histochemically detectable GUS activity, with the exception of the vascular bundles, where the GUS staining could not be confined to any particular cell compartment.

#### *Deficiency in DNA ligase I induces expression of the app gene*

PARP in animal cells is one of the most abundant nuclear proteins and its activity is regulated by allosteric changes in the protein upon binding to damaged DNA. We found that the *app* gene in *Arabidopsis* had a rather low level of expression,

suggesting that transcriptional activation of this gene might be essential for APP function *in vivo*. To test this hypothesis, the expression of the *app* gene was studied during *in vivo* genome destabilization caused by a DNA ligase I deficiency. A T-DNA insertion mutation, line SK1B2, in the *Arabidopsis* DNA ligase I gene was isolated previously (Babiychuk *et al.*, 1997). The mutation is lethal in the homozygous state, but the mutant allele shows normal transmission through the gametes. We therefore expected that cells homozygous for the mutation would die due to incomplete DNA synthesis during the S phase of the cell cycle, soon after the fertilization of the mutant embryo sac with mutant pollen.

An *app* promoter-GUS translational fusion, in which the coding region of GUS was fused in-frame with the first five amino acids of APP and 2 kb of *app* 5' flanking sequences was constructed (SEQ ID No 21). The gene encoding the fusion protein was transformed into *Arabidopsis*. After two back-crosses to a wild type, heterozygous plants transformed with *app* promoter-GUS were crossed with *Arabidopsis* line SK1B2. The inflorescences of the control plants and plants heterozygous for the ligase mutation were stained for the activity of GUS. The GUS staining pattern mostly detected in aging tissues probably reflects the expression of the *app* gene, although we have no firm evidence that all of the regulatory sequences were present in the constructs used. This pattern was the same both in the inflorescences of control plants, not carrying the mutant ligase gene and plants heterozygous for a mutation. Approximately one-fourth of the ovules in the mutant plants with the fusion protein are GUS positive. Closer microscopical examination showed that in the GUS-positive ovules only the gametophyte was stained. The only difference between the control plants and the mutant plant was a mutation in a DNA ligase gene. We therefore conclude that the *app* gene is induced because of either the accumulation of DNA breaks, or the death of the mutant embryo sacs fertilized with mutant pollen. GUS staining of embryo sacs was found to appear within 24 h after pollination, or therefore very soon after fertilization.

**Example 3. Construction of PCD modulating chimeric genes and introduction of the T-DNA vectors comprising such PCD modulating genes in an *Agrobacterium* strain.**

**3.1. Construction of the p35S:(dsRNA-APP) and p35S:(dsRNA-ZAP) genes**

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 1 and 5):

For the p35S:(dsRNA-ZAP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the p35S:(dsRNA-APP) chimeric gene

- a CaMV 35S promoter region (Odell *et al.*, 1985)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.2. Construction of the pNOS:(dsRNA-APP) and pNOS:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 2 and 6):

For the pNOS:(dsRNA-ZAP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pNOS:(dsRNA-APP) chimeric gene

- a NOS promoter region (Herrera-Estrella *et al.*, 1983)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.3. Construction of the pTA29:(dsRNA-APP) and pTA29:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 3 and 7):

For the pTA29:(dsRNA-ZAP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pTA29:(dsRNA-APP) chimeric gene

- a TA29 promoter region (WO 89/10396)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.4. Construction of the pNTP303:(dsRNA-APP) and pNTP303:(dsRNA-ZAP) genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6 (constructs 4 and 8):

For the pNTP303:(dsRNA-ZAP) chimeric gene

- a NTP303 promoter region (Wetering 1994)



- a Cab22 leader region (Harpster *et al.*, 1988)
- a ZAP encoding DNA region (about complete) (the *Arabidopsis thaliana* homologue to SEQ ID No 10, isolated by hybridization)
- about 500 bp of the 5' end of the ZAP2 encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

For the pNTP303:(dsRNA-APP) chimeric gene

- a NTP303 promoter region (Wetering, 1994)
- a Cab22 leader region (Harpster *et al.*, 1988)
- an APP encoding DNA region (about complete) (SEQ ID No 5)
- about 500 bp of the 5' end of the APP encoding DNA region in inverse orientation
- a CaMV35S 3' end region (Mogen *et al.*, 1990)

### 3.5 Construction of the chimeric marker genes

Using standard recombinant DNA procedures, the following DNA regions are operably linked, as schematically outlined in Figure 6:

For the *gat* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a aminoglycoside 6'-acetyltransferase encoding DNA (WO 94/26913)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

For the *bar* marker gene

- an Act2 promoter region (An *et al.*, 1996)
- a phosphinotricin acetyltransferase encoding DNA (US 5,646,024)
- a 3' end region of a nopaline synthase gene (Depicker *et al.*, 1982)

### 3.6. Construction of the T-DNA vectors comprising the PCD modulating chimeric genes

Using appropriate restriction enzymes, the chimeric PCD modulating genes described under 3.1 to 3.5 are excised and introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 ( WO 97/13865) together

with either the *gat* marker gene or the *bar* marker gene. The resulting T-DNA vectors are schematically represented in Figure 6.

### 3.7. Introduction of the T-DNA vectors in *Agrobacterium*

The T-DNA vectors are introduced in *Agrobacterium tumefaciens* C58C1Rif(pGV4000) by electroporation as described by Walkerpeach and Velten (1995) and transformants are selected using spectinomycin and streptomycin.

#### **Example 4. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* with the T-DNA vectors of Example 3.**

The *Agrobacterium* strains are used to transform *Arabidopsis thaliana* var. C24 applying the root transformation method as described by Valvekens et al. (1992). The explants are coinfectd with the *Agrobacteria* strains containing the dsRNA-APP respectively the dsRNA-ZAP constructs. The dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transformants are selected for phosphinothricin resistance. The regenerated rooted transgenic lines are tested for the presence of the other T-DNA by screening for kanamycin resistance. Transgenic lines containing both T-DNA's are transfered to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

#### **Example 5. *Agrobacterium*-mediated transformation of *Brassica napus* with the T-DNA vectors of Example 3.**

The *Agrobacterium* strains are used to transform the *Brassica napus* var. N90-740 applying the hypocotyl transformation method essentially as described by De Block et al. (1989), except for the following modifications:

- hypocotyl explants are precultured for 1 day on A2 medium [MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.5% agarose, 1 mg/l 2,4-D, 0.25 mg/l naphthalene acetic acid (NAA) and 1 mg/l 6-benzylaminopurine (BAP)].

- infection medium A3 is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 0.1 mg/l NAA, 0.75 mg/l BAP and 0.01 mg/l gibberellinic acid (GA3).
  - selection medium A5G is MS, 0.5 g/l Mes (pH5.7), 1.2% glucose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l polyvinylpyrrolidone (PVP), 0.5% agarose, 0.1 mg/l NAA, 0.75 mg/l BAP, 0.01 mg/l GA3, 250 mg/l carbenicillin, 250 mg/l triacillin, 5 mg/l AgNO<sub>3</sub> for three weeks. After this period selection is continued on A5J medium (similar a A5G but with 3% sucrose)
  - regeneration medium A6 is MS, 0.5 g/l Mes (pH5.7), 2% sucrose, 40 mg/l adenine.SO<sub>4</sub>, 0.5 g/l PVP, 0.5% agarose, 0.0025mg/l BAP and 250 mg/l triacillin.
  - healthy shoots are transferred to rooting medium which was A9: half concentrated MS, 1,5% sucrose (pH5.8), 100 mg/l triacillin, 0.6 % agar in 1 liter vessels.
- MS stands for Murashige and Skoog medium (Murashige and Skoog, 1962)

For introducing both the dsRNA-APP and the dsRNA-ZAP T-DNA constructs into a same plant cell the co-transformation method is applied, essentially as described by De Block and Debrouwer (1991). Transformed plant lines are selected on phosphinothricin containing medium after which the presence of the second T-DNA is screened by testing the regenerated rooted shoots for kanamycin resistance. In the co-transformation experiments, the dsRNA-APP constructs are used in combination with the *pact:bar* gene. The dsRNA-ZAP constructs are used in combination with the *pact:gat* gene. Transgenic lines containing both T-DNA's are transferred to the greenhouse. The phenotype of the T0-transgenic lines is scored and the T1-generations are studied further in more detail.

## **Example 6. In vitro assay to test vigor of plant lines**

### 6.1. Fitness assay for *Brassica napus*

#### *Media and reaction buffers*

Sowing medium:

Half concentrated Murashige and Skoog salts

2% sucrose

pH 5.8

0.6% agar

Callus inducing medium: A2S

MS medium, 0.5g/l Mes (pH 5.8), 3% sucrose, 40mg/l adenine-SO<sub>4</sub>, 0.5% agarose, 1mg/l 2,4-D, 0.25mg/l NAA, 1mg/l BAP

Incubation medium:

25mM K-phosphate buffer pH5.8

2% sucrose

1 drop Tween20 for 25ml medium

Reaction buffer:

50mM K-phosphate buffer pH7.4

10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)

1 drop Tween20 for 25ml buffer

#### *Sterilization of seeds and 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 distilled water. Put 7 seeds/1l vessel (Weck) containing about 75ml of sowing medium. The seeds are germinated at 23°C and 30  $\mu\text{Einstein/s}^{-1}\text{m}^{-2}$  with a daylength of 16h.

The line N90-740 is always included for standardization between experiments.

#### *Preculture of the hypocotyl explants*

- 12-14 days after sowing, the hypocotyls are cut in about 7mm segments.  
25 hypocotyls/Optilux Petridisch (Falcon S1005)
- The hypocotyl explants are cultured for 4 days on medium A2S at 23-25°C (at 30  $\mu\text{Einstein/s}^{-1}\text{m}^{-2}$ ).  
☐ P.S.: about 150-300 hypocotyl explants/line are needed to carry out the assay
- Transfer the hypocotyl explants to Optilux Petridishes (Falcon S1005) containing 30ml of incubation medium.
- Incubate for about 20hours at 24°C in the dark.

#### *TTC-assay*

- Transfer 150 hypocotyl explants to a 50ml Falcon tube.
- Wash with reaction buffer (without TTC).

- Add 25ml-30ml of reaction buffer/tube.

tube 1 □ no TTC added

\* for measuring background absorption

\* one line/experiment is sufficient

tube 2 □ +10mM TTC

(explants have to be submerged, but do not vacuum infiltrate!)

- turn tubes upside down
- Incubate for about 1 hour in the dark at 26°C (no end reaction!)
- Wash hypocotyls with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room°t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1 hour
- Measure absorptions of extracts at 485nm

P.S.: reduced TTC-H is not stable □ keep in the dark and measure O.D.<sub>485</sub> as soon as possible

$$\text{O.D.}_{485} (\text{TTC-H}) = (\text{O.D.}_{485} + \text{TTC}) - (\text{O.D.}_{485} - \text{TTC})$$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of N90-740 in the different experiment at 100%.
- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.

## 6.2. Fitness assay *Arabidopsis*

### *Media and reaction buffers*

Plant medium: Half concentrated Murashige and Skoog salts

1.5% sucrose

pH 5.8

0.6% agar

→ autoclave 15min.

add filter sterilized -100mg/l myo-inositol

- 0.5mg/l pyridoxine
  - 0.5mg/l nicotinic acid
  - 1mg/l thiamine
- Incubation medium: 10mM K-phosphate buffer pH5.8
- 2% sucrose
- 1 drop Tween20 for 25ml medium
- Reaction buffer: 50mM K-phosphate buffer pH7.4
- 10mM 2,3,5-triphenyltetrazoliumchloride (TTC) (= 3.35mg/ml)
- 1 drop Tween20 for 25ml buffer

### *Arabidopsis plants*

- Sterilization of *Arabidopsis* seeds
  - 2min. 70% ethanol
  - 10 min. bleach (6% active chlorine) + 1 drop Tween 20 for 20ml solution
  - wash 5 times with sterile water
  - P.S.: sterilization is done in 2ml eppendorf tubes
  - Arabidopsis* seeds sink to the bottom of the tube, allowing removal of the liquids by means of a 1ml pipetman
- Growing of *Arabidopsis* plants
  - Seeds are sown in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025) containing  $\pm 100\mu\text{l}$  of plant medium: 1 seed/grid.
  - Plants are grown at 23°C
  - $40\mu\text{Einstein s}^{-1}\text{m}^{-2}$
  - 16hours light - 8hours dark
  - for about 3 weeks (plants start to form flower buds)
  - P.S.: \*about 90-110 plants/line are needed to carry out the assay
  - \* include control line (C24; Columbia; ...) for calibration

### *Pre-incubation*

- Harvest *Arabidopsis* shoots by cutting of roots (by means of scissors)
- Put each shoot immediately in incubation medium (shoots have to be submerged, but do not vacuum infiltrate)

Incubation medium:  $\pm 150$ ml in 'Intergrid Tissue Culture disks of Falcon' (nr. 3025)

- a) incubation medium: for quantification of background absorption (see TTC-assay)
- b) incubation medium
- c) incubation medium + 2mM niacinamide

30 - 35 shoots/petridish (but same amount of shoots for all lines and for each condition)

- Incubate at 24°C in the dark for  $\pm 20$ hours

#### *TTC-assay*

- Transfer shoots to 50ml Falcon tubes
- Wash with reaction buffer (without TTC)
- Add 30-35ml of reaction buffer/tube
  - a) no TTC added (for measuring background absorption)
  - b and c) +10mM TTC
- (Shoots have to be submerged, but do not vacuum infiltrate!)
- Incubate for about 2hours in the dark at 26°C (**no** end reaction!)
- Wash shoots with deionized water
- Remove water
- Freeze at -70°C for 30min.
- Thaw at room<sup>o</sup>t (in the dark)
- Add 50ml ethanol (technical)
- Extract reduced TTC-H by shaking for 1hour
- Measure absorptions of extracts at 485nm

P.S.: reduced TTC-H is not stable → keep in the dark and  
measure O.D.<sub>485</sub> as soon as possible

- Compare reducing profiles of tested lines *versus* control line (for population of 30 to 35 plants)

$$\text{O.D.}_{485}(\text{TTC-H}) = (\text{O.D.}_{485} + \text{TTC}) - (\text{O.D.}_{485} - \text{TTC})$$

- Comparison of the TTC-reducing capacities between samples of different independent experiments can be done by setting the TTC-reducing capacity of control line (C24; Columbia; ...) in the different experiments at 100%.

- Lines with a high TTC-reducing capacity are vigorous, while lines with a low TTC-reducing capacity are weak.
- If the addition of niacinamide to the incubation medium results in a higher TTC-reducing capacity indicates to a lower fitness (as shown for C24 and Columbia).

**Example 7. Phenotypic analyses of the transgenic lines containing both dsRNA-APP and dsRNA-ZAP constructs.**

The flower phenotype and pollen viability (Alexander staining (Alexander, 1969) and germination assay) of the T0-lines containing dsRNA-APP and dsRNA-ZAP under the control of tapetum or pollen specific promoters were scored. For *Arabidopsis*, the T1-generation is obtained by selfing or if the plants are male sterile by backcrossing using pollen of non-transformed wild type plants. For *Brassica napus*, the T1-generation is always obtained by backcrossing using pollen of non-transformed plants.

T1-seed is germinated on kanamycin containing medium after which the resistant plants are scored by means of the ammonium-multiwell assay for phosphinothricine resistance (De Block et al., 1995). One half of the plants that contains both T-DNA's is transferred to the greenhouse to score the male fertility of the plants, while the other half is used to quantify the vigor of the plants by means of the fitness assay.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of 35S or NOS promoter, a high vigor is observed in a number of the transgenic lines.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of TA29 male sterility is observed in a number of the transgenic lines.

For plants comprising combinations (APP/ZAP) of PCD modulating genes under control of NTP303 sterile pollen is observed in a number the transgenic lines.



**Example 8. Phenotypic analysis of plants comprising a PCD modulating chimeric gene.**

Another example of a p35S::(dsRNA-ZAP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a CaMV35S2 promoter region (Odell et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a ZAP2 encoding DNA region of Zea Mays from the HincII site to the SnaBI site having the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 1728
- the 5' end of the ZAP2 encoding region from the HincII site to the EcoRV site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 792
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG33, which was introduced in *Agrobacterium* C58C1Rif(pGV4000) by electroporation as described.

Another example of a pNos::(dsRNA-ZAP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a nopaline synthase promoter region (Herrera-Estrella et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a ZAP2 encoding DNA region of Zea Mays from the HincII site to the SnaBI site having the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 1728
- the 5' end of the ZAP2 encoding region from the HincII site to the EcoRV site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 10 from the nucleotide at position 279 to the nucleotide at position 792
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG34, which was introduced in *Agrobacterium* C58C1Rif(pGV4000) by electroporation as described.

Another example of a p35S::(dsRNA-APP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a CaMV35S2 promoter region (Odell et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a APP encoding DNA region of *Arabidopsis thaliana* from the Scal site to the SmaI site having the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 1349
- the 5' end of the ZAP2 encoding region from the Scal site to the HaeIII site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 784)
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG29, which was introduced in *Agrobacterium* C58C1Rif(pGV4000) by electroporation as described.

Another example of a pNos::(dsRNA-APP) chimeric gene was constructed using standard recombinant DNA procedures, by operably linking the following DNA regions:

- a nopaline synthase promoter region (Herrera-Estrella et al., 1985)
- a Cab 22 leader region encoding DNA (Harpster et al. 1988)
- a APP encoding DNA region of *Arabidopsis thaliana* from the Scal site to the SmaI site having the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 1349
- the 5' end of the ZAP2 encoding region from the Scal site to the HaeIII site in inverse orientation (having the complement of the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 189 to the nucleotide at position 784)
- a CaMV35S 3'end region (Mogen et al., 1990).

This chimeric gene was introduced in the polylinker between the T-DNA borders of a T-DNA vector derived from pGSV5 (described in WO 97/13865) together with the bar marker gene, and yield T-DNA vector pTYG30, which was introduced in *Agrobacterium* C58C1Rif(pGV4000) by electroporation as described.

The resulting *Agrobacterium* strains were used to introduce the different PCD modulating genes separately into *Brassica napus* and *Arabidopsis thaliana* (Columbia and C24) plants as described in Examples 4 and 5.

Transgenic *Arabidopsis thaliana* plants obtained by selfing of the T0 generation (T1 generation) were germinated on medium containing phosphinotricin. The resistant transgenic plants were further cultivated.

Growth of transgenic T1 plants (both derived from Columbia or C24) comprising a pNOS::(dsRNA-ZAP) construct as in pTYG33 or a p35S::(dsRNA-ZAP) construct as in pTYG34, was significantly faster than control transgenic plants transformed by the T-DNA of the T-DNA vector without PCD modulating chimeric gene (see Table 1).

Stress tolerance of the *Arabidopsis* T1 transgenic plants (derived from Columbia) was evaluated by floating small plants on a salicylic acid solution of either 10 or 50 mg/L or for control just on H<sub>2</sub>O. Stress sensitive plants developed bleached and curled leaves after 1 to 2 days incubation, while stress tolerant plants remained intact for at least five days. Again transgenic plants comprising a pNOS::(dsRNA-ZAP) construct as in pTYG33 or a p35S::(dsRNA-ZAP) construct as in pTYG34, were significantly more stress-tolerant than control transgenic plants (see Table 1).

PPT-resistant transgenic callus obtained from *Brassica napus* transformed by the dsRNA-ZAP or dsRNA-APP constructs of pTYG29, pTYG30, pTYG33 or pTYG34, was incubated on a medium containing 50 mg/L aspirine for 2 days. After 2 days, the weight of the calli was determined and the calli were transferred on a medium without aspirine and further incubated for 5 days. At the end of the 5 days period, the weight of the calli was determined, and the increase in weight was expressed as a percentage of the weight after the two days period incubation. As a control, transgenic callus transformed by a T-DNA without a PCD modulating chimeric gene

was taken through the same procedure with the exception that no aspirine was added during the 2 day incubation. The results are summarized in Table II and indicate that transgenic *Brassica napus* cells comprising a PCD modulating chimeric gene are more stress resistant than the control cells.

Table 1. Evaluation of transgenic *Arabidopsis* plants (T1 generation)

Chimeric PCD modulating gene	Growth (Columbia and C24)	Stress tolerance (Columbia)
pNOS::(dsRNA-ZAP)	+++	++
p35S::(dsRNA-ZAP)	++	+
pNOS::(dsRNA-APP)	+	+/-
p35S::(dsRNA-APP)	+	-
Control	+	+/- (**)

\*\* *A. thaliana* Columbia has a certain degree of natural tolerance to aspirin.

Table 2. Regrowth of the transgenic *Brassica* calli after incubation on aspirine.

Chimeric PCD modulating gene	Increase in weight (%)
pNOS::(dsRNA-ZAP)	80
p35S::(dsRNA-ZAP)	90
pNOS::(dsRNA-APP)	75
p35S::(dsRNA-APP)	85
Control	70

Standard error of the mean is < 5%.

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We claim:

1. A method for modulating programmed cell death in a eukaryotic cell, said method comprising using (I) a nucleotide sequence of a poly(ADP-ribose) polymerase (PARP) gene of the ZAP class, and (II) a nucleotide sequence of a PARP gene of the NAP class to reduce the functional level of the total PARP activity in said eukaryotic cell.
2. The method of claim 1, further comprising reducing expression of PARP genes endogenous to said eukaryotic cell by using said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.
3. The method of claim 1, further comprising reducing the apparent activity of the proteins encoded by the endogenous PARP genes by using said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.
4. The method of claim 1, further comprising altering the nucleotide sequence of the endogenous PARP genes with said nucleotide sequence of said PARP gene of the ZAP class, and the nucleotide sequence of said PARP gene of the NAP class.
5. A method for modulating programmed cell death (PCD) in a eukaryotic cell, comprising introducing a first and a second PCD modulating chimeric gene in said eukaryotic cell, wherein said first PCD modulating chimeric gene comprises the following operably linked DNA regions:
  - a) a first promoter, operative in said eukaryotic cell;
  - b) a first DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either
    - i) capable of reducing the functional level of a Zn-finger containing PARP protein of the ZAP class; or



- ii) capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of ZAP class.
- c) a DNA region involved in transcription termination and polyadenylation

and wherein said second PCD modulating chimeric gene comprises the following operably linked DNA regions:

- a) a second promoter, operative in said eukaryotic cell;
- b) a second DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either
  - i) capable of reducing the functional level of a PARP protein of the NAP class; or
  - ii) capable of being translated into a peptide or protein which when expressed reduces the functional level of a PARP protein of the NAP class
- c) a DNA region involved in transcription termination and polyadenylation;

wherein the total apparent PARP activity in said eukaryotic cell is reduced significantly or almost completely.

6. The method of claim 5, wherein said first transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

7. The method of claim 5, wherein said second transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the NAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

8. The method of claim 7, wherein said first transcribed DNA region encodes a sense RNA molecule, said DNA region comprising a nucleotide sequence of at least about

100 nucleotides with 75% identity to the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said sense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

9. The method of claim 5, wherein said first transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the DNA strand of an endogenous PARP gene of the ZAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

10. The method of claim 5, wherein said second transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the NAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

11. The method of claim 10, wherein said first transcribed DNA region encodes an antisense RNA molecule, said DNA region comprising a nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of the sense DNA strand of an endogenous PARP gene of the ZAP class, and wherein said antisense RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

12. The method of claim 5, wherein said first transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the ZAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule

is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

13. The method of claim 5, wherein said second transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the NAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the NAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule is capable of reducing the expression of said endogenous PARP gene of the NAP class.

14. The method of claim 10, wherein said first transcribed DNA region encodes a RNA molecule comprising a sense nucleotide sequence of at least about 100 nucleotides with 75% identity to the mRNA resulting from transcription of an endogenous PARP gene of the ZAP class, said RNA molecule further comprising an antisense nucleotide sequence of at least about 100 nucleotides with 75% identity to the complement of said mRNA resulting from transcription of said endogenous PARP gene of the ZAP class, wherein said sense and antisense nucleotide sequence are capable of forming a double stranded RNA region, and wherein said RNA molecule is capable of reducing the expression of said endogenous PARP gene of the ZAP class.

15. The method of claim 5, wherein said first transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP class.

16. The method of claim 5, wherein said second transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the NAP class.

17. The method of claim 16, wherein said first transcribed DNA region encodes a dominant negative PARP mutant capable of reducing the apparent activity of the PARP protein encoded by an endogenous PARP gene of the ZAP class.

18. The method of claim 16, wherein said dominant negative PARP mutant comprises an amino acid sequence selected from the amino acid sequence of SEQ ID No 4 from amino acid 1 to 159 or the amino acid sequence of SEQ ID No 6 from amino acid 1 to 138.

19. The method of claim 17, wherein said dominant negative PARP mutant comprises an amino acid sequence selected from the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370, the amino acid sequence of SEQ ID No 11 from amino acid 1 to 98, or the amino acid sequence of SEQ ID No 2 from amino acid 1 to 370 wherein the amino acid sequence from amino acid 1 to 88 is replaced by the amino acid sequence of SEQ ID No 11

20. The method of claim 5, wherein said first or said second promoter is a tissue specific or inducible promoter.

21. The method of claim 20, wherein said first or said second promoter is selected from a fungus-responsive promoter, a nematode-responsive promoter, an anther-selective promoter, a stigma-selective promoter, a dehiscence-zone selective promoter.

22. The method of any one of claim 5 to claim 21, wherein said total apparent PARP activity is reduced from about 75% to about 90% of the normal apparent PARP activity in said eukaryotic cell, and wherein said eukaryotic cell is protected against programmed cell death.

23. The method of any one of claim 5 to claim 21, wherein said total apparent PARP activity is reduced from about 90% to about 100% of the normal apparent PARP activity in said eukaryotic cell, and wherein said eukaryotic cell is killed by programmed cell death.

24. The method claim 22, wherein said eukaryotic cell is a plant cell.
25. The method of claim 23, wherein said eukaryotic cell is a plant cell.
26. A method for modulating programmed cell death in a plant cell, comprising introducing a PCD modulating chimeric gene in said plant cell, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:
- a) a plant-expressible promoter;
  - b) DNA region, which when transcribed yields a RNA molecule, said RNA molecule being either
    - i) capable of reducing the expression of endogenous PARP genes; or
    - ii) capable of being translated into a peptide or protein which when expressed reduces the apparent PARP activity in said plant cell; and
  - c) a DNA region involved in transcription termination and polyadenylation;
- wherein the total apparent PARP activity in said plant cell is reduced from about 75% to about 100% of the normal apparent PARP activity in said plant cell.
27. A first and second chimeric PCD modulating gene as claimed in any one of claims 5 to 21.
28. A eukaryotic cell comprising a first and second chimeric PCD modulating gene of claim 27.
29. The eukaryotic cell of claim 28, which is a plant cell
30. An non-human eukaryotic organism which comprises the eucaryotic cell of claim 28.
31. A plant comprising the plant cell of claim 29.
32. A seed of the plant of claim 31, comprising the first and second chimeric PCD modulating gene of claim 27.

33. A method for modulating programmed cell death in cells of a plant, said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

- (a) a plant-expressible promoter;
- (b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and
- (c) a DNA region involved in transcription termination and polyadenylation

34. A method for increasing the growth rate of a plant, said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

- (a) a plant-expressible promoter;
- (b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and
- (c) a DNA region involved in transcription termination and polyadenylation

35. A method for producing stress tolerant cells of a plant said method comprising introducing a PCD modulating chimeric gene in said cells of a plant, wherein said PCD modulating chimeric gene comprises the following operably linked DNA regions:

- (a) a plant-expressible promoter;
- (b) a DNA region, which when transcribed yields a RNA molecule, said RNA molecule being capable of reducing the expression of an endogenous PARP gene of the ZAP class; and
- (c) a DNA region involved in transcription termination and polyadenylation

36. Use of a nucleotide sequence encoding a protein with PARP activity to modulate programmed cell death in a plant cell or plant.

37. The use according to claim 36, wherein said protein with PARP activity is a PARP protein of the ZAP class.

38. Use of a nucleotide sequence encoding a protein with PARP activity to produce a stress tolerant plant cell or plant.

39. The use according to claim 38, wherein said protein with PARP activity is a PARP protein of the ZAP class.

40. Use of a nucleotide sequence encoding a protein with PARP activity to increase the growth rate of a plant cell or plant.

41. The use according to claim 40, wherein said protein with PARP activity is a PARP protein of the ZAP class.

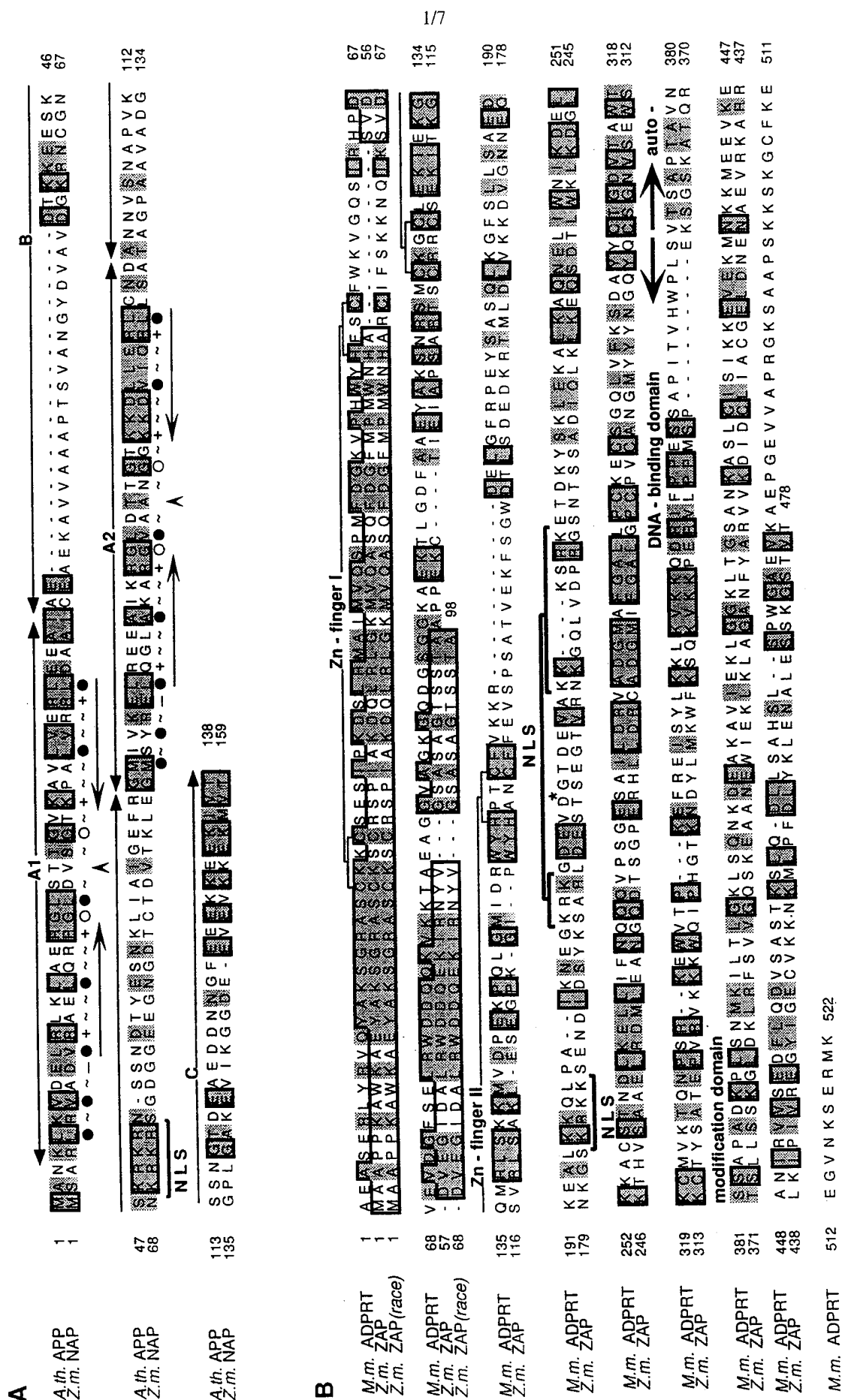


Figure 1



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A.th. APP	139	205	526
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Z.m. ZAP	544	606	925
NAP	227	287	608
A.th. APP	206	267	593
M.m. ADPRT	648	708	
Z.m. ZAP	607	666	
NAP	288	349	
A.th. APP	268	334	
M.m. ADPRT	709	767	
Z.m. ZAP	667	727	
NAP	350	409	
A.th. APP	335	394	
M.m. ADPRT	768	834	
Z.m. ZAP	728	792	
NAP	410	475	
A.th. APP	395	460	
M.m. ADPRT	835		
Z.m. ZAP	793		
NAP	476		
A.th. APP	461		
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Figure 2

## SUBSTITUTE SHEET (RULE 26)

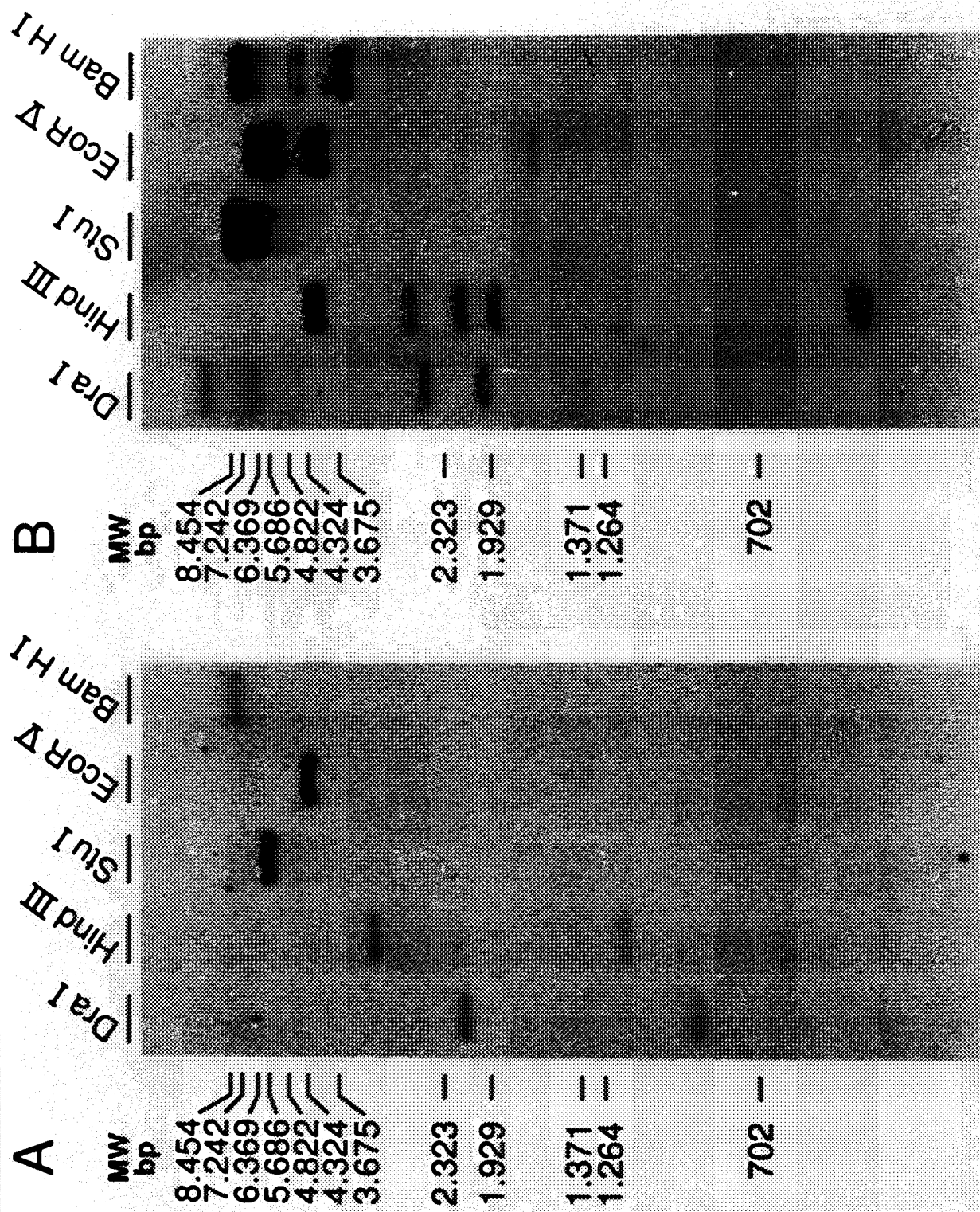


Figure 3

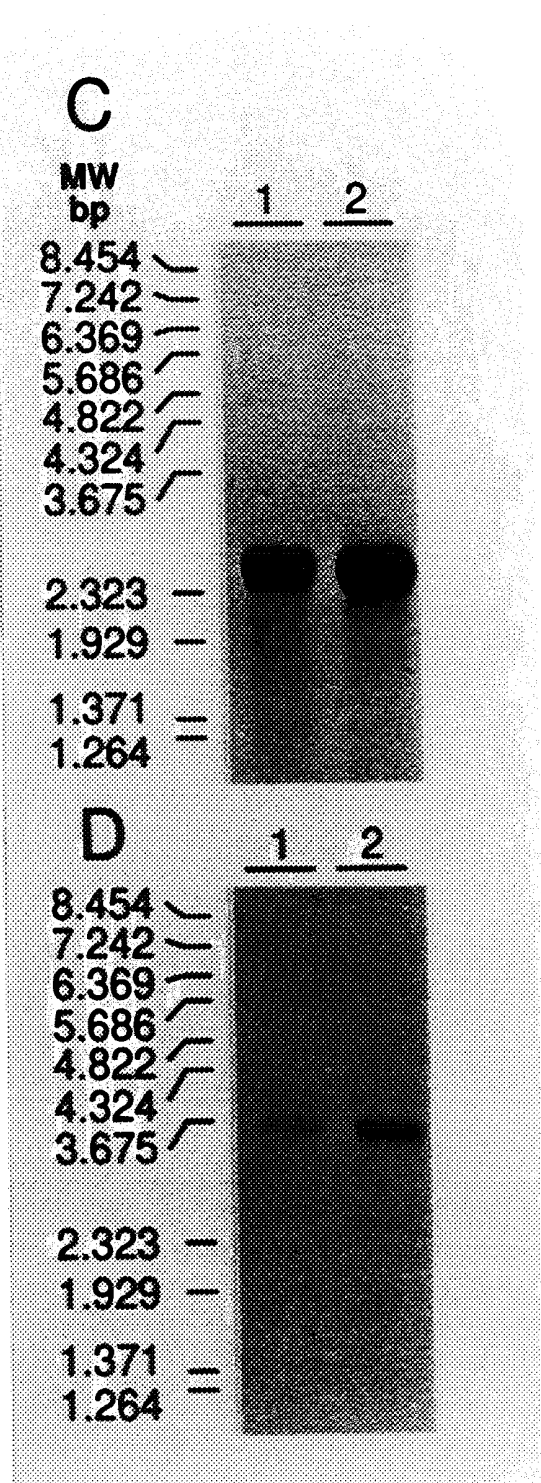


Figure 3 continued

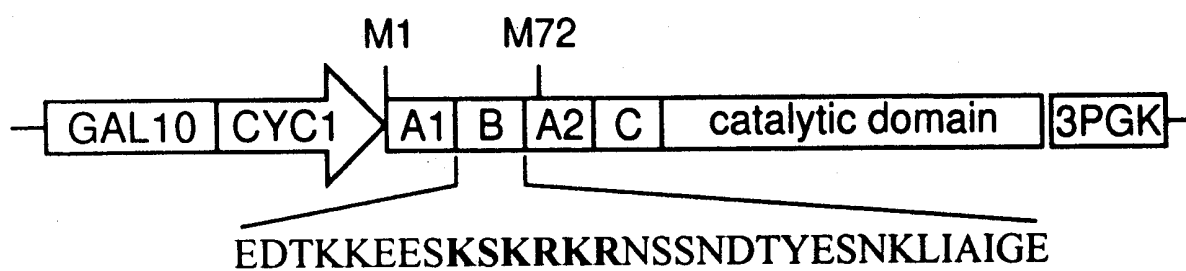
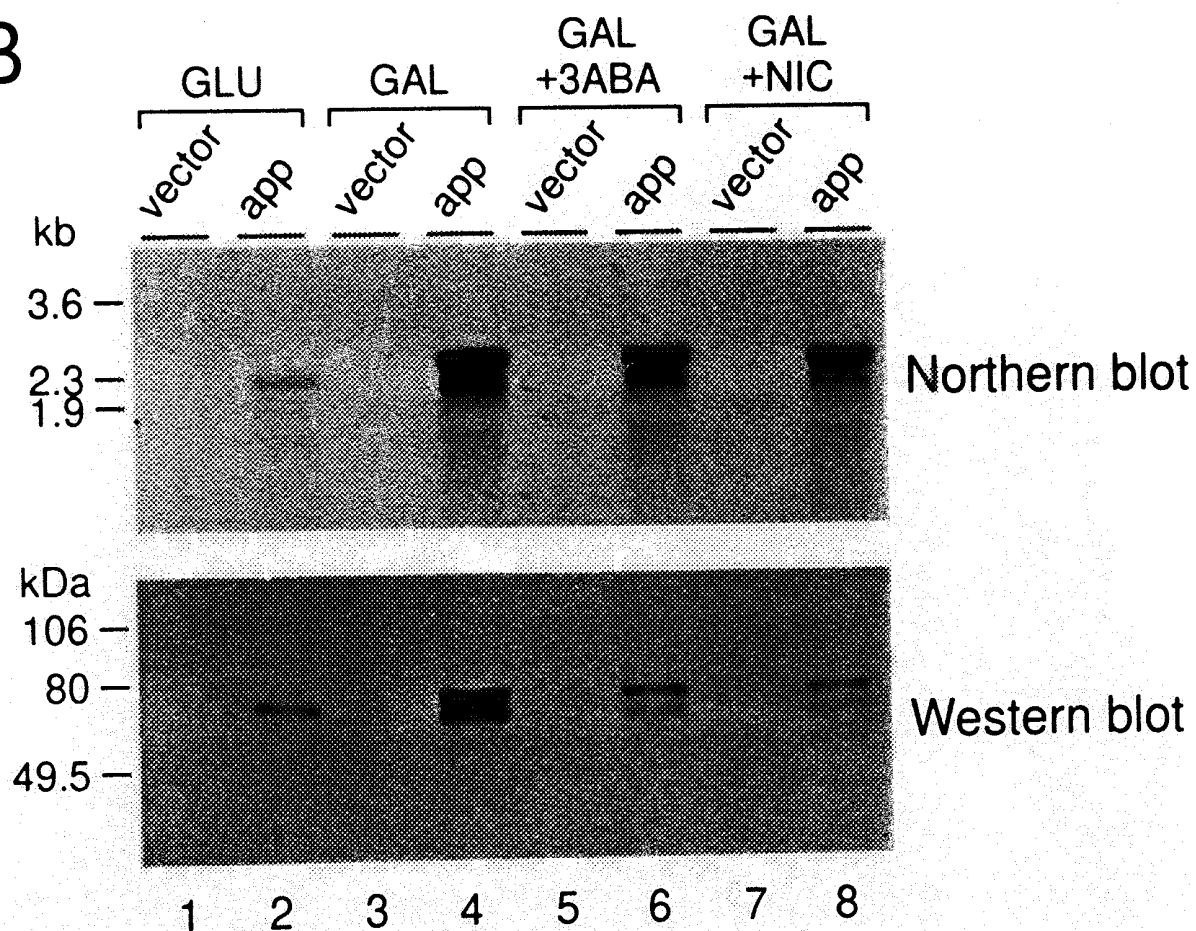
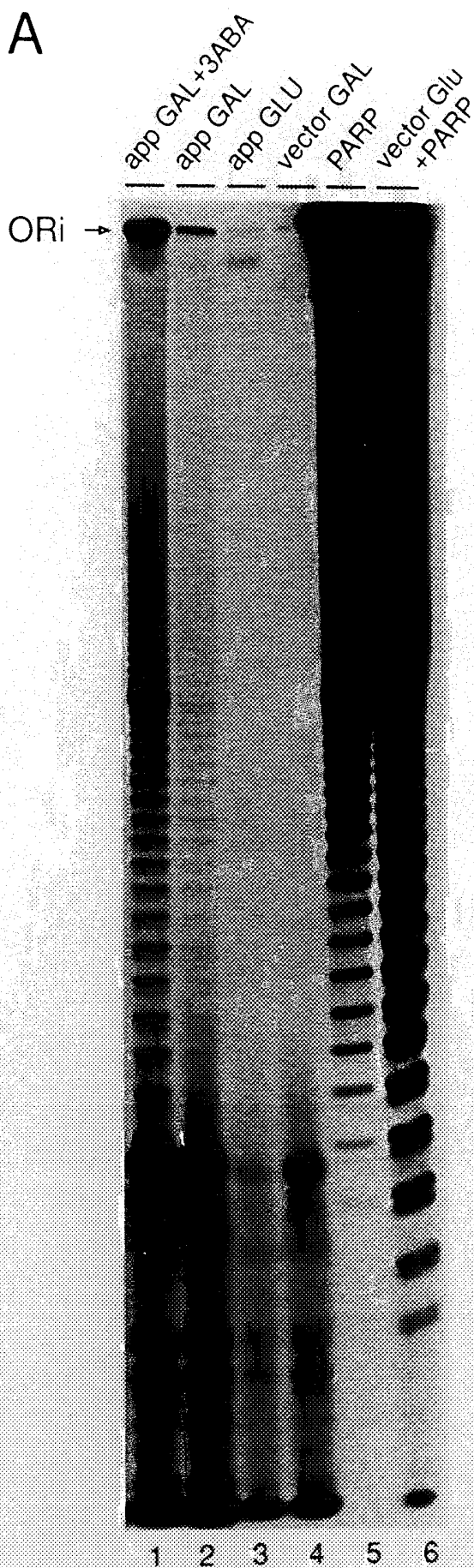
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Figure 4

A



B

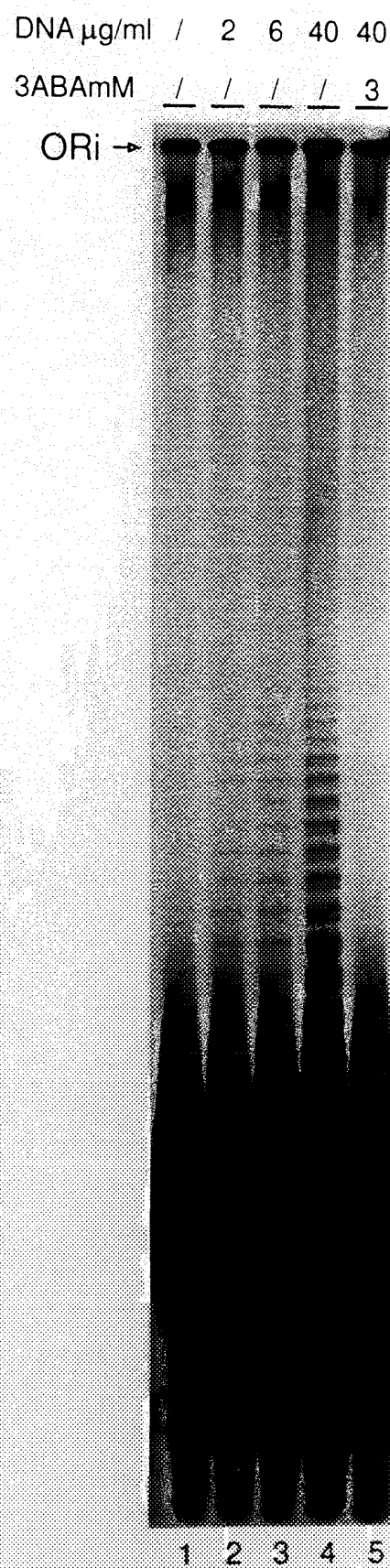


Figure 5

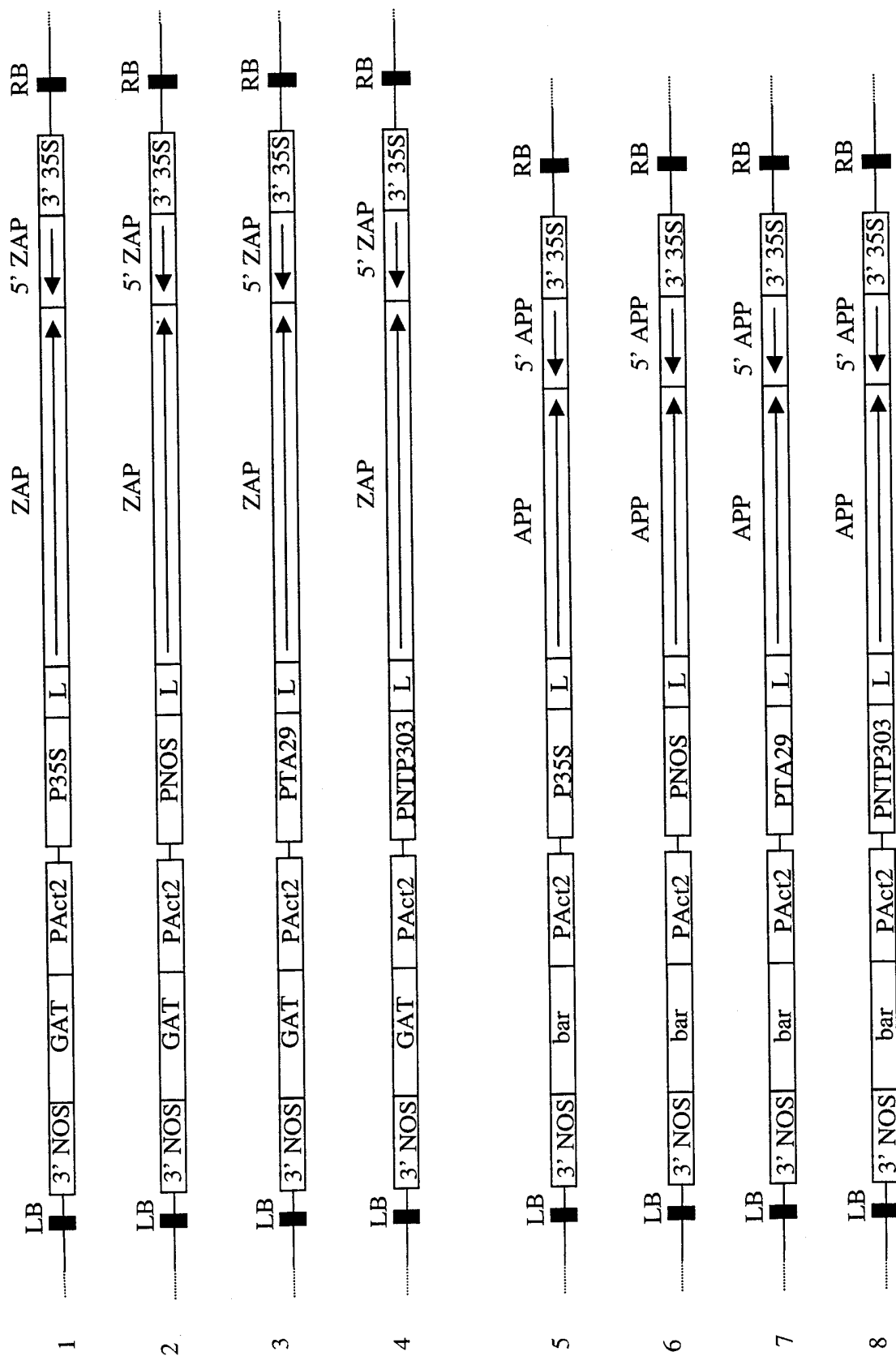


Figure 6

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Met Ala

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 Met Glu Phe Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys Leu Ser  
 645 650 655

aag gaa aat att gag aaa gga ttt gaa gca tta act gag ata cag aat 2134  
 Lys Glu Asn Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile Gln Asn  
 660 665 670

tta ttg aag gac acc gct gat caa gca ctg gct gtt aga gaa agc tta 2182  
 Leu Leu Lys Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu Ser Leu  
 675 680 685 690

att gtt gct gcg agc aat cgc ttt ttc act ctt atc cct tct att cat 2230  
 Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser Ile His  
 695 700 705

cct cat att ata cgg gat gag gat gat ttg atg atc aaa gcg aaa atg 2278  
 Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala Lys Met  
 710 715 720

ctt gaa gct ctg cag gat att gaa att gct tca aag ata gtt ggc ttc 2326  
 Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val Gly Phe  
 725 730 735

gat agc gac agt gat gaa tct ctt gat gat aaa tat atg aaa ctt cac 2374  
 Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys Leu His  
 740 745 750

tgt gac atc acc ccg ctg gct cac gat agt gaa gat tac aag cta att 2422  
 Cys Asp Ile Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys Leu Ile  
 755 760 765 770

gag cag tat ctc ctc aac aca cat gct cct act cac aag gac tgg tcg 2470  
 Glu Gln Tyr Leu Leu Asn Thr His Ala Pro Thr His Lys Asp Trp Ser  
 775 780 785

ctg gaa ctg gag gaa gtt ttt tca ctt gat cga gat gga gaa ctt aat 2518  
 Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu Leu Asn  
 790 795 800

aag tac tca aga tat aaa aat aat ctg cat aac aag atg cta cta tgg 2566  
 Lys Tyr Ser Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu Leu Trp  
 805 810 815

cac ggt tca agg ttg acg aat ttt gtg gga att ctt agt caa ggg cta 2614

His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln Gly Leu  
 820 825 830

aga att gca cct cct gag gca cct gtt act ggc tat atg ttc ggc aaa 2662  
 Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly Lys  
 835 840 845 850

ggc ctc tac ttt gca gat cta gta agc aag agc gca caa tac tgt tat 2710  
 Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr Cys Tyr  
 855 860 865

gtg gat agg aat aat cct gta ggt ttg atg ctt ctt tct gag gtt gct 2758  
 Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu Val Ala  
 870 875 880

tta gga gac atg tat gaa cta aag aaa gcc acg tcc atg gac aaa cct 2806  
 Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp Lys Pro  
 885 890 895

cca aga ggg aag cat tcg acc aag gga tta ggc aaa acc gtg cca ctg 2854  
 Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val Pro Leu  
 900 905 910

gag tca gag ttt gtg aag tgg agg gat gat gtc gta gtt ccc tgc ggc 2902  
 Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro Cys Gly  
 915 920 925 930

aag ccg gtg cca tca tca att agg agc tct gaa ctc atg tac aat gag 2950  
 Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr Asn Glu  
 935 940 945

tac atc gtc tac aac aca tcc cag gtg aag atg cag ttc ttg ctg aag 2998  
 Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu Leu Lys  
 950 955 960

gtg cgt ttc cat cac aag agg tag ctgggagact aggcaagtag agttggaagg 3052  
 Val Arg Phe His His Lys Arg  
 965 970

tagagaagca gagttaggcg atgcctcttt tgggtattatt agtaagcctg gcatgtat 3112

atgggtgctc gcgcttgatc cattttggta agtggttgctt gggcatcagc gcgaatagca 3172

ccaatcacac acttttacct aatgacgttt tactgtata 3211

<210> 2

<211> 969

&lt;212&gt; PRT

&lt;213&gt; Zea mays

&lt;400&gt; 2

Met Ala Ala Pro Pro Lys Ala Trp Lys Ala Glu Tyr Ala Lys Ser Gly

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10

15

Arg Ala Ser Cys Lys Ser Cys Arg Ser Pro Ile Ala Lys Asp Gln Leu

20

25

30

Arg Leu Gly Lys Met Val Gln Ala Ser Gln Phe Asp Gly Phe Met Pro

35

40

45

Met Trp Asn His Ala Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu

50

55

60

Arg Trp Asp Asp Gln Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser

65

70

75

80

Ala Gly Thr Ser Ser Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu

85

90

95

Ile Ala Pro Ser Ala Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile

100

105

110

Thr Lys Gly Ser Val Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro

115

120

125

Lys Gly Ile Pro Trp Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro

130

135

140

Ser Ala Thr Val Glu Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu

145

150

155

160

Asp Lys Arg Thr Met Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn

165

170

175

Glu Gln Asn Lys Gly Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp

180

185

190

Ser Tyr Lys Ser Ala Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val

195

200

205

Arg Asn Lys Gly Gln Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser

210

215

220

Ala Asp Ile Gln Leu Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys

225

230

235

240

Leu Lys Asp Gly Leu Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp  
 245 250 255

Met Leu Glu Ala Asn Gly Gln Asp Thr Ser Gly Pro Glu Arg His Leu  
 260 265 270

Leu Asp Arg Cys Ala Asp Gly Met Ile Phe Gly Ala Leu Gly Pro Cys  
 275 280 285

Pro Val Cys Ala Asn Gly Met Tyr Tyr Tyr Asn Gly Gln Tyr Gln Cys  
 290 295 300

Ser Gly Asn Val Ser Glu Trp Ser Lys Cys Thr Tyr Ser Ala Thr Glu  
 305 310 315 320

Pro Val Arg Val Lys Lys Lys Trp Gln Ile Pro His Gly Thr Lys Asn  
 325 330 335

Asp Tyr Leu Met Lys Trp Phe Lys Ser Gln Lys Val Lys Lys Pro Glu  
 340 345 350

Arg Val Leu Pro Pro Met Ser Pro Glu Lys Ser Gly Ser Lys Ala Thr  
 355 360 365

Gln Arg Thr Ser Leu Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe  
 370 375 380

Ser Val Val Gly Gln Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys  
 385 390 395 400

Leu Lys Leu Ala Gly Ala Asn Phe Tyr Ala Arg Val Val Lys Asp Ile  
 405 410 415

Asp Cys Leu Ile Ala Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val  
 420 425 430

Arg Lys Ala Arg Arg Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile  
 435 440 445

Gly Glu Cys Val Lys Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys  
 450 455 460

Leu Glu Asn Ala Leu Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys  
 465 470 475 480

Val Lys Gly Arg Ser Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr  
 485 490 495

Ala His Ile Leu Glu Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn  
 500 505 510

Met Ser Asp Leu Ala Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile  
 515 520 525

Ile Glu Gln Asp Asp Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly  
 530 535 540

Arg Val Gly Ser Glu Lys Ile Gly Gly Gln Lys Leu Glu Glu Met Ser  
 545 550 555 560

Lys Thr Glu Ala Ile Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr  
 565 570 575

Gly Asn Ser Trp Glu Ala Trp Glu Cys Lys Thr Asn Phe Arg Lys Gln  
 580 585 590

Pro Gly Arg Phe Tyr Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala  
 595 600 605

Pro Lys Arg Lys Asp Ile Ser Glu Met Lys Ser Ser Leu Ala Pro Gln  
 610 615 620

Leu Leu Glu Leu Met Lys Met Leu Phe Asn Val Glu Thr Tyr Arg Ala  
 625 630 635 640

Ala Met Met Glu Phe Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys  
 645 650 655

Leu Ser Lys Glu Asn Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile  
 660 665 670

Gln Asn Leu Leu Lys Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu  
 675 680 685

Ser Leu Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser  
 690 695 700

Ile His Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala  
 705 710 715 720

Lys Met Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val  
 725 730 735

Gly Phe Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys  
 740 745 750

Leu His Cys Asp Ile Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys  
 755 760 765  
 Leu Ile Glu Gln Tyr Leu Leu Asn Thr His Ala Pro Thr His Lys Asp  
 770 775 780  
 Trp Ser Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu  
 785 790 795 800  
 Leu Asn Lys Tyr Ser Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu  
 805 810 815  
 Leu Trp His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln  
 820 825 830  
 Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe  
 835 840 845  
 Gly Lys Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr  
 850 855 860  
 Cys Tyr Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu  
 865 870 875 880  
 Val Ala Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp  
 885 890 895  
 Lys Pro Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val  
 900 905 910  
 Pro Leu Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro  
 915 920 925  
 Cys Gly Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr  
 930 935 940  
 Asn Glu Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu  
 945 950 955 960  
 Leu Lys Val Arg Phe His His Lys Arg  
 965

&lt;210&gt; 3

&lt;211&gt; 2295

&lt;212&gt; DNA

&lt;213&gt; Zea mays



&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (107)..(2068)

&lt;400&gt; 3

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cccgagcctt ctgcaatctt gcgagaacct caggggagag gagcag atg tcg gcg 115  
 Met Ser Ala  
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agg cta cgg gtg gcg gac gtc cgc gcg gag ctt cag cgc cgc ggc ctc 163  
 Arg Leu Arg Val Ala Asp Val Arg Ala Glu Leu Gln Arg Arg Gly Leu  
 5 10 15

gat gta tcc ggc acc aag cct gct ctc gtg cgg agg ctg gac gcc gca 211  
 Asp Val Ser Gly Thr Lys Pro Ala Leu Val Arg Arg Leu Asp Ala Ala  
 20 25 30 35

att tgc gag gcg gag aag gcc gtg gtg gct gct gcg cca acc agt gtg 259  
 Ile Cys Glu Ala Glu Lys Ala Val Val Ala Ala Ala Pro Thr Ser Val  
 40 45 50

gca aat ggg tat gac gta gcc gta gat ggc aaa agg aac tgc ggg aat 307  
 Ala Asn Gly Tyr Asp Val Ala Val Asp Gly Lys Arg Asn Cys Gly Asn  
 55 60 65

aat aag agg aaa agg tcc ggg gat ggg ggt gaa gag gga aac ggc gat 355  
 Asn Lys Arg Lys Arg Ser Gly Asp Gly Gly Glu Glu Gly Asn Gly Asp  
 70 75 80

acg tgt aca gat gtg aca aaa cta gag ggc atg agc tat cgt gag ctg 403  
 Thr Cys Thr Asp Val Thr Lys Leu Glu Gly Met Ser Tyr Arg Glu Leu  
 85 90 95

cag gga ttg gcc aag gca cgt gga gtt gcg gca aat ggg ggc aag aaa 451  
 Gln Gly Leu Ala Lys Ala Arg Gly Val Ala Ala Asn Gly Gly Lys Lys  
 100 105 110 115

gat gtt atc cag agg ttg ctc tcg gcg act gct ggt cct gct gca gtt 499  
 Asp Val Ile Gln Arg Leu Leu Ser Ala Thr Ala Gly Pro Ala Ala Val  
 120 125 130

gca gat ggt ggt cct ctg ggc gcc aag gaa gtc ata aaa ggt ggt gat 547  
 Ala Asp Gly Gly Pro Leu Gly Ala Lys Glu Val Ile Lys Gly Gly Asp  
 135 140 145

gag gag gtt gag gtg aaa aag gag aag atg gtt act gcc acg aag aag 595  
 Glu Glu Val Glu Val Lys Lys Glu Lys Met Val Thr Ala Thr Lys Lys  
 150 155 160

gga gct gca gtg ctg gat cag cac att ccc gat cac ata aaa gtg aac 643  
 Gly Ala Ala Val Leu Asp Gln His Ile Pro Asp His Ile Lys Val Asn  
 165 170 175

tat cat gtc ttg caa gtg ggc gat gaa atc tat gat gcc acc ttg aac 691  
 Tyr His Val Leu Gln Val Gly Asp Glu Ile Tyr Asp Ala Thr Leu Asn  
 180 185 190 195

cag act aat gtt gga gac aac aac aat aag ttc tat atc att caa gtt 739  
 Gln Thr Asn Val Gly Asp Asn Asn Asn Lys Phe Tyr Ile Ile Gln Val  
 200 205 210

tta gaa tct gat gct ggt gga agc ttt atg gtt tac aat aga tgg gga 787  
 Leu Glu Ser Asp Ala Gly Gly Ser Phe Met Val Tyr Asn Arg Trp Gly  
 215 220 225

aga gtt ggg gta cga ggt caa gat aaa cta cat ggt ccc tcc cca aca 835  
 Arg Val Gly Val Arg Gly Gln Asp Lys Leu His Gly Pro Ser Pro Thr  
 230 235 240

cga gac caa gca ata tat gaa ttt gag ggg aag ttc cac aac aaa acc 883  
 Arg Asp Gln Ala Ile Tyr Glu Phe Glu Gly Lys Phe His Asn Lys Thr  
 245 250 255

aat aat cat tgg tct gat cgc aag aac ttc aaa tgt tat gca aag aaa 931  
 Asn Asn His Trp Ser Asp Arg Lys Asn Phe Lys Cys Tyr Ala Lys Lys  
 260 265 270 275

tac act tgg ctt gaa atg gat tat ggt gaa act gag aaa gaa ata gag 979  
 Tyr Thr Trp Leu Glu Met Asp Tyr Gly Glu Thr Glu Lys Glu Ile Glu  
 280 285 290

aaa ggt tcc att act gat cag ata aaa gag aca aaa ctt gaa act aga 1027  
 Lys Gly Ser Ile Thr Asp Gln Ile Lys Glu Thr Lys Leu Glu Thr Arg  
 295 300 305

att gcg cag ttc ata tcc ctg atc tgc aat att agc atg atg aag caa 1075  
 Ile Ala Gln Phe Ile Ser Leu Ile Cys Asn Ile Ser Met Met Lys Gln  
 310 315 320

aga atg gtg gaa ata ggt tat aat gct gaa aag ctt ccc ctt gga aag 1123  
 Arg Met Val Glu Ile Gly Tyr Asn Ala Glu Lys Leu Pro Leu Gly Lys  
 325 330 335

cta agg aaa gct aca ata ctt aag ggt tat cat gtt ttg aaa agg ata	1171
Leu Arg Lys Ala Thr Ile Leu Lys Gly Tyr His Val Leu Lys Arg Ile	
340 345 350 355	
tcc gat gtt att tca aag gcg gac agg aga cat ctt gag caa ttg act	1219
Ser Asp Val Ile Ser Lys Ala Asp Arg Arg His Leu Glu Gln Leu Thr	
360 365 370	
ggg gaa ttc tac acc gtg att cct cat gac ttt ggt ttc aga aag atg	1267
Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly Phe Arg Lys Met	
375 380 385	
cgt gaa ttt att atc gat act cct cag aaa cta aaa gct aag ctg gag	1315
Arg Glu Phe Ile Ile Asp Thr Pro Gln Lys Leu Lys Ala Lys Leu Glu	
390 395 400	
atg gtt gaa gcc ctt ggt gag att gaa att gca act aaa ctt ttg gag	1363
Met Val Glu Ala Leu Gly Glu Ile Glu Ile Ala Thr Lys Leu Leu Glu	
405 410 415	
gat gat tca agt gac cag gat gat ccg ttg tat gct cga tac aag caa	1411
Asp Asp Ser Ser Asp Gln Asp Asp Pro Leu Tyr Ala Arg Tyr Lys Gln	
420 425 430 435	
ctt cat tgt gat ttc aca cct ctt gaa gct gat tca gat gag tac tct	1459
Leu His Cys Asp Phe Thr Pro Leu Glu Ala Asp Ser Asp Glu Tyr Ser	
440 445 450	
atg ata aaa tca tat ttg aga aat aca cat gga aaa aca cac tct ggt	1507
Met Ile Lys Ser Tyr Leu Arg Asn Thr His Gly Lys Thr His Ser Gly	
455 460 465	
tat acg gtg gac ata gtg caa ata ttt aag gtt tca agg cat ggt gaa	1555
Tyr Thr Val Asp Ile Val Gln Ile Phe Lys Val Ser Arg His Gly Glu	
470 475 480	
aca gag cga ttt caa aaa ttt gct agt aca aga aat agg atg ctt ttg	1603
Thr Glu Arg Phe Gln Lys Phe Ala Ser Thr Arg Asn Arg Met Leu Leu	
485 490 495	
tgg cat ggt tct cgg ttg agc aac tgg gct ggg atc ctt tct cag ggt	1651
Trp His Gly Ser Arg Leu Ser Asn Trp Ala Gly Ile Leu Ser Gln Gly	
500 505 510 515	
ctg cga atc gct cct cct gaa gca cct gtt act ggt tac atg ttt ggc	1699
Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly	
520 525 530	

aag ggt gtt tac ttt gct gac atg ttt tca aag agt gca aac tat tgc 1747  
 Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser Ala Asn Tyr Cys  
                   535                                  540                                  545

tac gcc tct gaa gca tgt aga tct gga gta ctg ctt tta tgt gag gtt 1795  
 Tyr Ala Ser Glu Ala Cys Arg Ser Gly Val Leu Leu Leu Cys Glu Val  
                   550                                  555                                  560

gca ttg ggc gat atg aat gag cta ctg aat gca gat tac gat gct aat 1843  
 Ala Leu Gly Asp Met Asn Glu Leu Leu Asn Ala Asp Tyr Asp Ala Asn  
                   565                                  570                                  575

aac ctg ccc aaa gga aaa tta aga tcc aag gga gtt ggt caa aca gca 1891  
 Asn Leu Pro Lys Gly Lys Leu Arg Ser Lys Gly Val Gly Gln Thr Ala  
 580                                  585                                  590                                  595

cct aac atg gtc gag tct aag gtc gct gac gat ggt gtt gtt gtt ccc 1939  
 Pro Asn Met Val Glu Ser Lys Val Ala Asp Asp Gly Val Val Val Pro  
                   600                                  605                                  610

ctt ggc gaa ccc aaa cag gaa cct tcc aaa agg ggt ggc ttg ctt tat 1987  
 Leu Gly Glu Pro Lys Gln Glu Pro Ser Lys Arg Gly Gly Leu Leu Tyr  
                   615                                  620                                  625

aat gag tac ata gtg tac aac gta gac cag ata aga atg cgg tat gtc 2035  
 Asn Glu Tyr Ile Val Tyr Asn Val Asp Gln Ile Arg Met Arg Tyr Val  
                   630                                  635                                  640

tta cat gtt aac ttc aat ttc aag aga cgg tag atgttgcaaa gagctgaaac 2088  
 Leu His Val Asn Phe Asn Phe Lys Arg Arg  
                   645                                  650

tgttgctgag atcttagcag aacatatgtg gacttatagc accaggtgcc ctgagcctca 2148

ttttctgagc aaatttggtg gcctttgcat ttcgattttg gtttcagctt ctagcccat 2208

tgatgattga tactgagtgt atatatgaac cattgatatc caccttccat gtacttaagt 2268

ttttttaaca tgtcccatgc ataataa 2295

<210> 4

<211> 653

<212> PRT

<213> Zea mays

<400> 4

Met Ser Ala Arg Leu Arg Val Ala Asp Val Arg Ala Glu Leu Gln Arg

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Asp Ala Ala Ile Cys Glu Ala Glu Lys Ala Val Val Ala Ala Ala Pro			
35	40	45	
Thr Ser Val Ala Asn Gly Tyr Asp Val Ala Val Asp Gly Lys Arg Asn			
50	55	60	
Cys Gly Asn Asn Lys Arg Lys Arg Ser Gly Asp Gly Gly Glu Glu Gly			
65	70	75	80
Asn Gly Asp Thr Cys Thr Asp Val Thr Lys Leu Glu Gly Met Ser Tyr			
85	90	95	
Arg Glu Leu Gln Gly Leu Ala Lys Ala Arg Gly Val Ala Ala Asn Gly			
100	105	110	
Gly Lys Lys Asp Val Ile Gln Arg Leu Leu Ser Ala Thr Ala Gly Pro			
115	120	125	
Ala Ala Val Ala Asp Gly Gly Pro Leu Gly Ala Lys Glu Val Ile Lys			
130	135	140	
Gly Gly Asp Glu Glu Val Glu Val Lys Lys Glu Lys Met Val Thr Ala			
145	150	155	160
Thr Lys Lys Gly Ala Ala Val Leu Asp Gln His Ile Pro Asp His Ile			
165	170	175	
Lys Val Asn Tyr His Val Leu Gln Val Gly Asp Glu Ile Tyr Asp Ala			
180	185	190	
Thr Leu Asn Gln Thr Asn Val Gly Asp Asn Asn Asn Lys Phe Tyr Ile			
195	200	205	
Ile Gln Val Leu Glu Ser Asp Ala Gly Gly Ser Phe Met Val Tyr Asn			
210	215	220	
Arg Trp Gly Arg Val Gly Val Arg Gly Gln Asp Lys Leu His Gly Pro			
225	230	235	240
Ser Pro Thr Arg Asp Gln Ala Ile Tyr Glu Phe Glu Gly Lys Phe His			
245	250	255	
Asn Lys Thr Asn Asn His Trp Ser Asp Arg Lys Asn Phe Lys Cys Tyr			

260	265	270
Ala Lys Lys Tyr Thr Trp Leu Glu Met Asp Tyr Gly Glu Thr Glu Lys		
275	280	285
Glu Ile Glu Lys Gly Ser Ile Thr Asp Gln Ile Lys Glu Thr Lys Leu		
290	295	300
Glu Thr Arg Ile Ala Gln Phe Ile Ser Leu Ile Cys Asn Ile Ser Met		
305	310	315 320
Met Lys Gln Arg Met Val Glu Ile Gly Tyr Asn Ala Glu Lys Leu Pro		
325	330	335
Leu Gly Lys Leu Arg Lys Ala Thr Ile Leu Lys Gly Tyr His Val Leu		
340	345	350
Lys Arg Ile Ser Asp Val Ile Ser Lys Ala Asp Arg Arg His Leu Glu		
355	360	365
Gln Leu Thr Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly Phe		
370	375	380
Arg Lys Met Arg Glu Phe Ile Ile Asp Thr Pro Gln Lys Leu Lys Ala		
385	390	395 400
Lys Leu Glu Met Val Glu Ala Leu Gly Glu Ile Glu Ile Ala Thr Lys		
405	410	415
Leu Leu Glu Asp Asp Ser Ser Asp Gln Asp Asp Pro Leu Tyr Ala Arg		
420	425	430
Tyr Lys Gln Leu His Cys Asp Phe Thr Pro Leu Glu Ala Asp Ser Asp		
435	440	445
Glu Tyr Ser Met Ile Lys Ser Tyr Leu Arg Asn Thr His Gly Lys Thr		
450	455	460
His Ser Gly Tyr Thr Val Asp Ile Val Gln Ile Phe Lys Val Ser Arg		
465	470	475 480
His Gly Glu Thr Glu Arg Phe Gln Lys Phe Ala Ser Thr Arg Asn Arg		
485	490	495
Met Leu Leu Trp His Gly Ser Arg Leu Ser Asn Trp Ala Gly Ile Leu		
500	505	510
Ser Gln Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr		

515                                      520                                      525  
 Met Phe Gly Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser Ala  
 530                                      535                                      540  
 Asn Tyr Cys Tyr Ala Ser Glu Ala Cys Arg Ser Gly Val Leu Leu Leu  
 545                                      550                                      555                                      560  
 Cys Glu Val Ala Leu Gly Asp Met Asn Glu Leu Leu Asn Ala Asp Tyr  
 565                                      570                                      575  
 Asp Ala Asn Asn Leu Pro Lys Gly Lys Leu Arg Ser Lys Gly Val Gly  
 580                                      585                                      590  
 Gln Thr Ala Pro Asn Met Val Glu Ser Lys Val Ala Asp Asp Gly Val  
 595                                      600                                      605  
 Val Val Pro Leu Gly Glu Pro Lys Gln Glu Pro Ser Lys Arg Gly Gly  
 610                                      615                                      620  
 Leu Leu Tyr Asn Glu Tyr Ile Val Tyr Asn Val Asp Gln Ile Arg Met  
 625                                      630                                      635                                      640  
 Arg Tyr Val Leu His Val Asn Phe Asn Phe Lys Arg Arg  
 645                                      650

<210> 5  
 <211> 2147  
 <212> DNA  
 <213> Arabidopsis thaliana

<220>  
 <221> CDS  
 <222> (129)..(2042)

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 acgaaaacca tacttcctca gtctcattcc ctttccgacg aactattctc ctgaagaaga 120  
 agacgaaa atg gcg aac aag ctc aaa gtc gac gaa ctc cgt tta aaa ctc 170  
 Met Ala Asn Lys Leu Lys Val Asp Glu Leu Arg Leu Lys Leu  
 1                                      5                                      10  
 gcc gag cgt gga ctc agt act act gga gtc aaa gcc gtt ctg gtg gag 218  
 Ala Glu Arg Gly Leu Ser Thr Thr Gly Val Lys Ala Val Leu Val Glu  
 15                                      20                                      25                                      30

agg ctt gaa gag gct atc gca gaa gac act aag aag gaa gaa tca aag 266  
Arg Leu Glu Glu Ala Ile Ala Glu Asp Thr Lys Lys Glu Glu Ser Lys  
35 40 45

agc aag agg aaa aga aat tct tct aat gat act tat gaa tcg aac aaa 314  
Ser Lys Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys  
50 55 60

ttg att gca att ggc gaa ttt cgt ggg atg att gtg aag gaa ttg cgt 362  
Leu Ile Ala Ile Gly Glu Phe Arg Gly Met Ile Val Lys Glu Leu Arg  
65 70 75

gag gaa gct att aag aga ggc tta gat aca aca gga acc aaa aag gat 410  
Glu Glu Ala Ile Lys Arg Gly Leu Asp Thr Thr Gly Thr Lys Lys Asp  
80 85 90

ctt ctt gag agg ctt tgc aat gat gct aat aac gtt tcc aat gca cca 458  
Leu Leu Glu Arg Leu Cys Asn Asp Ala Asn Asn Val Ser Asn Ala Pro  
95 100 105 110

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Val Lys Ser Ser Asn Gly Thr Asp Glu Ala Glu Asp Asp Asn Asn Gly  
115 120 125

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Phe Glu Glu Glu Lys Lys Glu Glu Lys Ile Val Thr Ala Thr Lys Lys  
130 135 140

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Gly Ala Ala Val Leu Asp Gln Trp Ile Pro Asp Glu Ile Lys Ser Gln  
145 150 155

tac cat gtt cta caa agg ggt gat gat gtt tat gat gct atc tta aat 650  
Tyr His Val Leu Gln Arg Gly Asp Asp Val Tyr Asp Ala Ile Leu Asn  
160 165 170

cag aca aat gtc agg gat aat aat aac aag ttc ttt gtc cta caa gtc 698  
Gln Thr Asn Val Arg Asp Asn Asn Asn Lys Phe Phe Val Leu Gln Val  
175 180 185 190

cta gag tcg gat agt aaa aag aca tac atg gtt tac act aga tgg gga 746  
Leu Glu Ser Asp Ser Lys Lys Thr Tyr Met Val Tyr Thr Arg Trp Gly  
195 200 205

aga gtt ggt gtg aaa gga caa agt aag cta gat ggg cct tat gac tca 794  
Arg Val Gly Val Lys Gly Gln Ser Lys Leu Asp Gly Pro Tyr Asp Ser  
210 215 220



tgg gat cgt gcg ata gag ata ttt acc aat aag ttc aat gac aag aca	842
Trp Asp Arg Ala Ile Glu Ile Phe Thr Asn Lys Phe Asn Asp Lys Thr	
225 230 235	
aag aat tat tgg tct gac aga aag gag ttt atc cca cat ccc aag tcc	890
Lys Asn Tyr Trp Ser Asp Arg Lys Glu Phe Ile Pro His Pro Lys Ser	
240 245 250	
tat aca tgg ctc gaa atg gat tac gga aaa gag gaa aat gat tca ccg	938
Tyr Thr Trp Leu Glu Met Asp Tyr Gly Lys Glu Glu Asn Asp Ser Pro	
255 260 265 270	
gtc aat aat gat att ccg agt tca tct tcc gaa gtt aaa cct gaa caa	986
Val Asn Asn Asp Ile Pro Ser Ser Ser Ser Glu Val Lys Pro Glu Gln	
275 280 285	
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Ser Lys Leu Asp Thr Arg Val Ala Lys Phe Ile Ser Leu Ile Cys Asn	
290 295 300	
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Val Ser Met Met Ala Gln His Met Met Glu Ile Gly Tyr Asn Ala Asn	
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Lys Leu Pro Leu Gly Lys Ile Ser Lys Ser Thr Ile Ser Lys Gly Tyr	
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Arg Leu Glu Glu Leu Ser Gly Glu Phe Tyr Thr Val Ile Pro His Asp	
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Ala Thr Lys Leu Leu Ser Val Asp Pro Gly Leu Gln Asp Asp Pro Leu	
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Tyr	Tyr	His	Tyr	Gln	Gln	Leu	Asn	Cys	Gly	Leu	Thr	Pro	Val	Gly	Asn	
415				420				425					430			
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Asp	Ser	Glu	Glu	Phe	Ser	Met	Val	Ala	Asn	Tyr	Met	Glu	Asn	Thr	His	
				435				440					445			
gca	aag	acg	cat	tcg	gga	tat	acg	gtt	gag	att	gcc	caa	cta	ttt	aga	1514
Ala	Lys	Thr	His	Ser	Gly	Tyr	Thr	Val	Glu	Ile	Ala	Gln	Leu	Phe	Arg	
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gct	tcg	aga	gct	gtt	gaa	gct	gat	cga	ttc	caa	cag	ttt	tca	agt	tcg	1562
Ala	Ser	Arg	Ala	Val	Glu	Ala	Asp	Arg	Phe	Gln	Gln	Phe	Ser	Ser	Ser	
		465					470					475				
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Lys	Asn	Arg	Met	Leu	Leu	Trp	His	Gly	Ser	Arg	Leu	Thr	Asn	Trp	Ala	
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Gly	Ile	Leu	Ser	Gln	Gly	Leu	Arg	Ile	Ala	Pro	Pro	Glu	Ala	Pro	Val	
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act	ggg	tac	atg	ttt	gga	aaa	ggg	gtt	tac	ttt	gcg	gat	atg	ttc	tcc	1706
Thr	Gly	Tyr	Met	Phe	Gly	Lys	Gly	Val	Tyr	Phe	Ala	Asp	Met	Phe	Ser	
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Lys	Ser	Ala	Asn	Tyr	Cys	Tyr	Ala	Asn	Thr	Gly	Ala	Asn	Asp	Gly	Val	
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ctg	ctc	ctc	tgc	gag	gtt	gct	ttg	gga	gac	atg	aat	gaa	ctt	ctg	tat	1802
Leu	Leu	Leu	Cys	Glu	Val	Ala	Leu	Gly	Asp	Met	Asn	Glu	Leu	Leu	Tyr	
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Ser	Asp	Tyr	Asn	Ala	Asp	Asn	Leu	Pro	Pro	Gly	Lys	Leu	Ser	Thr	Lys	
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Gly	Val	Gly	Lys	Thr	Ala	Pro	Asn	Pro	Ser	Glu	Ala	Gln	Thr	Leu	Glu	
575				580				585						590		
gac	ggg	gtt	gtt	gtt	cca	ctt	ggc	aaa	cca	gtg	gaa	cgt	tca	tgc	tcc	1946
Asp	Gly	Val	Val	Val	Pro	Leu	Gly	Lys	Pro	Val	Glu	Arg	Ser	Cys	Ser	
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 625 630 635

aacttatgta tattagcttt tgaacatcaa ctaattatcc aaaaatcagc gttttattgt 2102

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Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys Leu Ile  
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Ala Ile Gly Glu Phe Arg Gly Met Ile Val Lys Glu Leu Arg Glu Glu  
 65 70 75 80

Ala Ile Lys Arg Gly Leu Asp Thr Thr Gly Thr Lys Lys Asp Leu Leu  
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Glu Arg Leu Cys Asn Asp Ala Asn Asn Val Ser Asn Ala Pro Val Lys  
 100 105 110

Ser Ser Asn Gly Thr Asp Glu Ala Glu Asp Asp Asn Asn Gly Phe Glu  
 115 120 125

Glu Glu Lys Lys Glu Glu Lys Ile Val Thr Ala Thr Lys Lys Gly Ala  
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Ala Val Leu Asp Gln Trp Ile Pro Asp Glu Ile Lys Ser Gln Tyr His

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				165					170					175	
Asn	Val	Arg	Asp	Asn	Asn	Asn	Lys	Phe	Phe	Val	Leu	Gln	Val	Leu	Glu
			180					185					190		
Ser	Asp	Ser	Lys	Lys	Thr	Tyr	Met	Val	Tyr	Thr	Arg	Trp	Gly	Arg	Val
	195						200					205			
Gly	Val	Lys	Gly	Gln	Ser	Lys	Leu	Asp	Gly	Pro	Tyr	Asp	Ser	Trp	Asp
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Arg	Ala	Ile	Glu	Ile	Phe	Thr	Asn	Lys	Phe	Asn	Asp	Lys	Thr	Lys	Asn
225					230					235				240	
Tyr	Trp	Ser	Asp	Arg	Lys	Glu	Phe	Ile	Pro	His	Pro	Lys	Ser	Tyr	Thr
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Asn	Asp	Ile	Pro	Ser	Ser	Ser	Ser	Glu	Val	Lys	Pro	Glu	Gln	Ser	Lys
	275						280					285			
Leu	Asp	Thr	Arg	Val	Ala	Lys	Phe	Ile	Ser	Leu	Ile	Cys	Asn	Val	Ser
	290					295					300				
Met	Met	Ala	Gln	His	Met	Met	Glu	Ile	Gly	Tyr	Asn	Ala	Asn	Lys	Leu
305					310					315				320	
Pro	Leu	Gly	Lys	Ile	Ser	Lys	Ser	Thr	Ile	Ser	Lys	Gly	Tyr	Glu	Val
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Leu	Lys	Arg	Ile	Ser	Glu	Val	Ile	Asp	Arg	Tyr	Asp	Arg	Thr	Arg	Leu
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Glu	Glu	Leu	Ser	Gly	Glu	Phe	Tyr	Thr	Val	Ile	Pro	His	Asp	Phe	Gly
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Phe	Lys	Lys	Met	Ser	Gln	Phe	Val	Ile	Asp	Thr	Pro	Gln	Lys	Leu	Lys
	370					375					380				
Gln	Lys	Ile	Glu	Met	Val	Glu	Ala	Leu	Gly	Glu	Ile	Glu	Leu	Ala	Thr
385				390						395				400	
Lys	Leu	Leu	Ser	Val	Asp	Pro	Gly	Leu	Gln	Asp	Asp	Pro	Leu	Tyr	Tyr

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His Tyr Gln Gln Leu Asn Cys Gly Leu Thr Pro Val Gly Asn Asp Ser					
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Glu Glu Phe Ser Met Val Ala Asn Tyr Met Glu Asn Thr His Ala Lys					
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Thr His Ser Gly Tyr Thr Val Glu Ile Ala Gln Leu Phe Arg Ala Ser					
	450		455		460
Arg Ala Val Glu Ala Asp Arg Phe Gln Gln Phe Ser Ser Ser Lys Asn					
	465		470		475
					480
Arg Met Leu Leu Trp His Gly Ser Arg Leu Thr Asn Trp Ala Gly Ile					
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Leu Ser Gln Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly					
	500		505		510
Tyr Met Phe Gly Lys Gly Val Tyr Phe Ala Asp Met Phe Ser Lys Ser					
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Ala Asn Tyr Cys Tyr Ala Asn Thr Gly Ala Asn Asp Gly Val Leu Leu					
	530		535		540
Leu Cys Glu Val Ala Leu Gly Asp Met Asn Glu Leu Leu Tyr Ser Asp					
	545		550		555
					560
Tyr Asn Ala Asp Asn Leu Pro Pro Gly Lys Leu Ser Thr Lys Gly Val					
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Gly Lys Thr Ala Pro Asn Pro Ser Glu Ala Gln Thr Leu Glu Asp Gly					
	580		585		590
Val Val Val Pro Leu Gly Lys Pro Val Glu Arg Ser Cys Ser Lys Gly					
	595		600		605
Met Leu Leu Tyr Asn Glu Tyr Ile Val Tyr Asn Val Glu Gln Ile Lys					
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&lt;211&gt; 16

&lt;212&gt; PRT

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<220>

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<210> 8

<211> 33

<212> PRT

<213> Artificial Sequence

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Ile

<210> 9

<211> 30

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 Met Ala Ala Pro Pro Lys Ala Trp Lys Ala Glu  
 1 5 10

tat gcc aag tct ggg cgg gcc tcg tgc aag tca tgc cgg tcc cct atc 161  
 Tyr Ala Lys Ser Gly Arg Ala Ser Cys Lys Ser Cys Arg Ser Pro Ile  
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gcc aag gac cag ctc cgt ctt ggc aag atg gtt cag gcg tca cag ttc 209  
 Ala Lys Asp Gln Leu Arg Leu Gly Lys Met Val Gln Ala Ser Gln Phe  
 30 35 40

gac ggc ttc atg ccg atg tgg aac cat gcc agg tgc atc ttc agc aag 257  
 Asp Gly Phe Met Pro Met Trp Asn His Ala Arg Cys Ile Phe Ser Lys  
 45 50 55

aag aac cag ata aaa tcc gtt gac gat gtt gaa ggg ata gat gca ctt 305  
 Lys Asn Gln Ile Lys Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu  
 60 65 70 75

aga tgg gat gat caa gag aag ata cga aac tac gtt ggg agt gcc tca 353  
 Arg Trp Asp Asp Gln Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser  
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gct ggt aca agt tct aca gct gct cct cct gag aaa tgt aca att gag 401  
 Ala Gly Thr Ser Ser Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu  
 95 100 105

att gct cca tct gcc cgt act tca tgt aga cga tgc agt gaa aag att 449  
 Ile Ala Pro Ser Ala Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile  
 110 115 120

aca aaa gga tcg gtc cgt ctt tca gct aag ctt gag agt gaa ggt ccc 497  
 Thr Lys Gly Ser Val Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro  
 125 130 135

aag ggt ata cca tgg tat cat gcc aac tgt ttc ttt gag gta tcc ccg	545
Lys Gly Ile Pro Trp Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro	
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Ser Ala Thr Val Glu Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu	
160 165 170	
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Asp Lys Arg Thr Met Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn	
175 180 185	
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Glu Gln Asn Lys Gly Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp	
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Ser Tyr Lys Ser Ala Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val	
205 210 215	
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Arg Asn Lys Gly Gln Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser	
220 225 230 235	
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Ala Asp Ile Gln Leu Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys	
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Leu Lys Asp Gly Leu Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp	
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270 275 280	
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285 290 295	
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320 325 330	



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365 370 375	
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Gln Arg Thr Ser Leu Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe	
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Ser Val Val Gly Gln Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys	
400 405 410	
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415 420 425	
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Asp Cys Leu Ile Ala Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val	
430 435 440	
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Arg Lys Ala Arg Arg Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile	
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Gly Glu Cys Val Lys Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys	
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Leu Glu Asn Ala Leu Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys	
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Val Lys Gly Arg Ser Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr	
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Ala His Ile Leu Glu Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn	
510 515 520	

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Met Ser Asp Leu Ala Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile	
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Ile Glu Gln Asp Asp Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly	
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Arg Val Gly Ser Glu Lys Ile Gly Gly Gln Lys Leu Glu Glu Met Ser	
560 565 570	
aaa act gag gca atc aag gaa ttc aaa aga tta ttt ctt gag aag act	1841
Lys Thr Glu Ala Ile Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr	
575 580 585	
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Gly Asn Ser Trp Glu Ala Trp Glu Cys Lys Thr Asn Phe Arg Lys Gln	
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Pro Gly Arg Phe Tyr Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala	
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670 675 680	
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Gln Asn Leu Leu Lys Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu	
685 690 695	
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Ser Leu Ile Val Ala Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser	
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Ile His Pro His Ile Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala	
720 725 730	
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Lys Met Leu Glu Ala Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val	
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Gly Phe Asp Ser Asp Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys	
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765 770 775	
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780 785 790 795	
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Trp Ser Leu Glu Leu Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu	
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Leu Trp His Gly Ser Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln	
830 835 840	
ggg cta aga att gca cct cct gag gca cct gtt act ggc tat atg ttc	2657
Gly Leu Arg Ile Ala Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe	
845 850 855	
ggc aaa ggc ctc tac ttt gca gat cta gta agc aag agc gca caa tac	2705
Gly Lys Gly Leu Tyr Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr	
860 865 870 875	
tgt tat gtg gat agg aat aat cct gta ggt ttg atg ctt ctt tct gag	2753
Cys Tyr Val Asp Arg Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu	
880 885 890	
gtt gct tta gga gac atg tat gaa cta aag aaa gcc acg tcc atg gac	2801
Val Ala Leu Gly Asp Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp	
895 900 905	

aaa cct cca aga ggg aag cat tcg acc aag gga tta ggc aaa acc gtg 2849  
 Lys Pro Pro Arg Gly Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val  
 910 915 920

cca ctg gag tca gag ttt gtg aag tgg agg gat gat gtc gta gtt ccc 2897  
 Pro Leu Glu Ser Glu Phe Val Lys Trp Arg Asp Asp Val Val Val Pro  
 925 930 935

tgc ggc aag ccg gtg cca tca tca att agg agc tct gaa ctc atg tac 2945  
 Cys Gly Lys Pro Val Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr  
 940 945 950 955

aat gag tac atc gtc tac aac aca tcc cag gtg aag atg cag ttc ttg 2993  
 Asn Glu Tyr Ile Val Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu  
 960 965 970

ctg aag gtg cgt ttc cat cac aag agg tagctgggag actaggcaag 3040  
 Leu Lys Val Arg Phe His His Lys Arg  
 975 980

tagagttgga aggtagagaa gcagagttag gcgatgcctc ttttggtatt attagtaagc 3100

ctggcatgta tttatgggtg ctgcgccttg atccattttg gtaagtgttg cttgggcatc 3160

agcgcgaata gcaccaatca cacactttta cctaatagacg ttttactgta ta 3212

<210> 11

<211> 980

<212> PRT

<213> Zea mays

<400> 11

Met Ala Ala Pro Pro Lys Ala Trp Lys Ala Glu Tyr Ala Lys Ser Gly  
 1 5 10 15

Arg Ala Ser Cys Lys Ser Cys Arg Ser Pro Ile Ala Lys Asp Gln Leu  
 20 25 30

Arg Leu Gly Lys Met Val Gln Ala Ser Gln Phe Asp Gly Phe Met Pro  
 35 40 45

Met Trp Asn His Ala Arg Cys Ile Phe Ser Lys Lys Asn Gln Ile Lys  
 50 55 60

Ser Val Asp Asp Val Glu Gly Ile Asp Ala Leu Arg Trp Asp Asp Gln  
 65 70 75 80

Glu Lys Ile Arg Asn Tyr Val Gly Ser Ala Ser Ala Gly Thr Ser Ser  
                             85                            90                            95

Thr Ala Ala Pro Pro Glu Lys Cys Thr Ile Glu Ile Ala Pro Ser Ala  
                             100                            105                            110

Arg Thr Ser Cys Arg Arg Cys Ser Glu Lys Ile Thr Lys Gly Ser Val  
                             115                            120                            125

Arg Leu Ser Ala Lys Leu Glu Ser Glu Gly Pro Lys Gly Ile Pro Trp  
                             130                            135                            140

Tyr His Ala Asn Cys Phe Phe Glu Val Ser Pro Ser Ala Thr Val Glu  
 145                            150                            155                            160

Lys Phe Ser Gly Trp Asp Thr Leu Ser Asp Glu Asp Lys Arg Thr Met  
                             165                            170                            175

Leu Asp Leu Val Lys Lys Asp Val Gly Asn Asn Glu Gln Asn Lys Gly  
                             180                            185                            190

Ser Lys Arg Lys Lys Ser Glu Asn Asp Ile Asp Ser Tyr Lys Ser Ala  
                             195                            200                            205

Arg Leu Asp Glu Ser Thr Ser Glu Gly Thr Val Arg Asn Lys Gly Gln  
                             210                            215                            220

Leu Val Asp Pro Arg Gly Ser Asn Thr Ser Ser Ala Asp Ile Gln Leu  
 225                            230                            235                            240

Lys Leu Lys Glu Gln Ser Asp Thr Leu Trp Lys Leu Lys Asp Gly Leu  
                             245                            250                            255

Lys Thr His Val Ser Ala Ala Glu Leu Arg Asp Met Leu Glu Ala Asn  
                             260                            265                            270

Gly Gln Asp Thr Ser Gly Pro Glu Arg His Leu Leu Asp Arg Cys Ala  
                             275                            280                            285

Asp Gly Met Ile Phe Gly Ala Leu Gly Pro Cys Pro Val Cys Ala Asn  
                             290                            295                            300

Gly Met Tyr Tyr Tyr Asn Gly Gln Tyr Gln Cys Ser Gly Asn Val Ser  
 305                            310                            315                            320

Glu Trp Ser Lys Cys Thr Tyr Ser Ala Thr Glu Pro Val Arg Val Lys  
                             325                            330                            335

Lys Lys Trp Gln Ile Pro His Gly Thr Lys Asn Asp Tyr Leu Met Lys  
 340 345 350  
 Trp Phe Lys Ser Gln Lys Val Lys Lys Pro Glu Arg Val Leu Pro Pro  
 355 360 365  
 Met Ser Pro Glu Lys Ser Gly Ser Lys Ala Thr Gln Arg Thr Ser Leu  
 370 375 380  
 Leu Ser Ser Lys Gly Leu Asp Lys Leu Arg Phe Ser Val Val Gly Gln  
 385 390 395 400  
 Ser Lys Glu Ala Ala Asn Glu Trp Ile Glu Lys Leu Lys Leu Ala Gly  
 405 410 415  
 Ala Asn Phe Tyr Ala Arg Val Val Lys Asp Ile Asp Cys Leu Ile Ala  
 420 425 430  
 Cys Gly Glu Leu Asp Asn Glu Asn Ala Glu Val Arg Lys Ala Arg Arg  
 435 440 445  
 Leu Lys Ile Pro Ile Val Arg Glu Gly Tyr Ile Gly Glu Cys Val Lys  
 450 455 460  
 Lys Asn Lys Met Leu Pro Phe Asp Leu Tyr Lys Leu Glu Asn Ala Leu  
 465 470 475 480  
 Glu Ser Ser Lys Gly Ser Thr Val Thr Val Lys Val Lys Gly Arg Ser  
 485 490 495  
 Ala Val His Glu Ser Ser Gly Leu Gln Asp Thr Ala His Ile Leu Glu  
 500 505 510  
 Asp Gly Lys Ser Ile Tyr Asn Ala Thr Leu Asn Met Ser Asp Leu Ala  
 515 520 525  
 Leu Gly Val Asn Ser Tyr Tyr Val Leu Gln Ile Ile Glu Gln Asp Asp  
 530 535 540  
 Gly Ser Glu Cys Tyr Val Phe Arg Lys Trp Gly Arg Val Gly Ser Glu  
 545 550 555 560  
 Lys Ile Gly Gly Gln Lys Leu Glu Glu Met Ser Lys Thr Glu Ala Ile  
 565 570 575  
 Lys Glu Phe Lys Arg Leu Phe Leu Glu Lys Thr Gly Asn Ser Trp Glu  
 580 585 590

Ala Trp Glu Cys Lys Thr Asn Phe Arg Lys Gln Pro Gly Arg Phe Tyr  
 595 600 605  
 Pro Leu Asp Val Asp Tyr Gly Val Lys Lys Ala Pro Lys Arg Lys Asp  
 610 615 620  
 Ile Ser Glu Met Lys Ser Ser Leu Ala Pro Gln Leu Leu Glu Leu Met  
 625 630 635 640  
 Lys Met Leu Phe Asn Val Glu Thr Tyr Arg Ala Ala Met Met Glu Phe  
 645 650 655  
 Glu Ile Asn Met Ser Glu Met Pro Leu Gly Lys Leu Ser Lys Glu Asn  
 660 665 670  
 Ile Glu Lys Gly Phe Glu Ala Leu Thr Glu Ile Gln Asn Leu Leu Lys  
 675 680 685  
 Asp Thr Ala Asp Gln Ala Leu Ala Val Arg Glu Ser Leu Ile Val Ala  
 690 695 700  
 Ala Ser Asn Arg Phe Phe Thr Leu Ile Pro Ser Ile His Pro His Ile  
 705 710 715 720  
 Ile Arg Asp Glu Asp Asp Leu Met Ile Lys Ala Lys Met Leu Glu Ala  
 725 730 735  
 Leu Gln Asp Ile Glu Ile Ala Ser Lys Ile Val Gly Phe Asp Ser Asp  
 740 745 750  
 Ser Asp Glu Ser Leu Asp Asp Lys Tyr Met Lys Leu His Cys Asp Ile  
 755 760 765  
 Thr Pro Leu Ala His Asp Ser Glu Asp Tyr Lys Leu Ile Glu Gln Tyr  
 770 775 780  
 Leu Leu Asn Thr His Ala Pro Thr His Lys Asp Trp Ser Leu Glu Leu  
 785 790 795 800  
 Glu Glu Val Phe Ser Leu Asp Arg Asp Gly Glu Leu Asn Lys Tyr Ser  
 805 810 815  
 Arg Tyr Lys Asn Asn Leu His Asn Lys Met Leu Leu Trp His Gly Ser  
 820 825 830  
 Arg Leu Thr Asn Phe Val Gly Ile Leu Ser Gln Gly Leu Arg Ile Ala  
 835 840 845

Pro Pro Glu Ala Pro Val Thr Gly Tyr Met Phe Gly Lys Gly Leu Tyr  
 850 855 860

Phe Ala Asp Leu Val Ser Lys Ser Ala Gln Tyr Cys Tyr Val Asp Arg  
 865 870 875 880

Asn Asn Pro Val Gly Leu Met Leu Leu Ser Glu Val Ala Leu Gly Asp  
 885 890 895

Met Tyr Glu Leu Lys Lys Ala Thr Ser Met Asp Lys Pro Pro Arg Gly  
 900 905 910

Lys His Ser Thr Lys Gly Leu Gly Lys Thr Val Pro Leu Glu Ser Glu  
 915 920 925

Phe Val Lys Trp Arg Asp Asp Val Val Val Pro Cys Gly Lys Pro Val  
 930 935 940

Pro Ser Ser Ile Arg Ser Ser Glu Leu Met Tyr Asn Glu Tyr Ile Val  
 945 950 955 960

Tyr Asn Thr Ser Gln Val Lys Met Gln Phe Leu Leu Lys Val Arg Phe  
 965 970 975

His His Lys Arg  
 980

<210> 12

<211> 1010

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: fusion protein  
 between APP N-terminal domain and GUS protein

<400> 12

Met Ala Asn Lys Leu Lys Val Asp Glu Leu Arg Leu Lys Leu Ala Glu  
 1 5 10 15

Arg Gly Leu Ser Thr Thr Gly Val Lys Ala Val Leu Val Glu Arg Leu  
 20 25 30

Glu Glu Ala Ile Ala Glu Asp Thr Lys Lys Glu Glu Ser Lys Ser Lys  
 35 40 45

Arg Lys Arg Asn Ser Ser Asn Asp Thr Tyr Glu Ser Asn Lys Leu Ile



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

305	310	315	320
Pro Leu Gly Lys Ile Ser Lys Ser Thr Ile Ser Lys Gly Tyr Glu Val			
325	330	335	
Leu Lys Arg Ile Ser Glu Val Ile Asp Arg Tyr Asp Arg Thr Arg Leu			
340	345	350	
Glu Glu Leu Ser Gly Glu Phe Tyr Thr Val Ile Pro His Asp Phe Gly			
355	360	365	
Phe Lys Lys Met Ser Gln Phe Val Ile Asp Thr Pro Gln Lys Leu Lys			
370	375	380	
Gln Lys Ile Glu Met Val Glu Ala Leu Gly Glu Ile Glu Leu Ala Thr			
385	390	395	400
Lys Leu Leu Ser Val Asp Pro Met Val Arg Pro Val Glu Thr Pro Thr			
405	410	415	
Arg Glu Ile Lys Lys Leu Asp Gly Leu Trp Ala Phe Ser Leu Asp Arg			
420	425	430	
Glu Asn Cys Gly Ile Asp Gln Arg Trp Trp Glu Ser Ala Leu Gln Glu			
435	440	445	
Ser Arg Ala Ile Ala Val Pro Gly Ser Phe Asn Asp Gln Phe Ala Asp			
450	455	460	
Ala Asp Ile Arg Asn Tyr Ala Gly Asn Val Trp Tyr Gln Arg Glu Val			
465	470	475	480
Phe Ile Pro Lys Gly Trp Ala Gly Gln Arg Ile Val Leu Arg Phe Asp			
485	490	495	
Ala Val Thr His Tyr Gly Lys Val Trp Val Asn Asn Gln Glu Val Met			
500	505	510	
Glu His Gln Gly Gly Tyr Thr Pro Phe Glu Ala Asp Val Thr Pro Tyr			
515	520	525	
Val Ile Ala Gly Lys Ser Val Arg Ile Thr Val Cys Val Asn Asn Glu			
530	535	540	
Leu Asn Trp Gln Thr Ile Pro Pro Gly Met Val Ile Thr Asp Glu Asn			
545	550	555	560
Gly Lys Lys Lys Gln Ser Tyr Phe His Asp Phe Phe Asn Tyr Ala Gly			

	565		570		575
Ile His Arg Ser Val Met Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp					
	580		585		590
Asp Ile Thr Val Val Thr His Val Ala Gln Asp Cys Asn His Ala Ser					
	595		600		605
Val Asp Trp Gln Val Val Ala Asn Gly Asp Val Ser Val Glu Leu Arg					
	610		615		620
Asp Ala Asp Gln Gln Val Val Ala Thr Gly Gln Gly Thr Ser Gly Thr					
625		630		635	640
Leu Gln Val Val Asn Pro His Leu Trp Gln Pro Gly Glu Gly Tyr Leu					
	645		650		655
Tyr Glu Leu Cys Val Thr Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr					
	660		665		670
Pro Leu Arg Val Gly Ile Arg Ser Val Ala Val Lys Gly Glu Gln Phe					
	675		680		685
Leu Ile Asn His Lys Pro Phe Tyr Phe Thr Gly Phe Gly Arg His Glu					
	690		695		700
Asp Ala Asp Leu Arg Gly Lys Gly Phe Asp Asn Val Leu Met Val His					
705		710		715	720
Asp His Ala Leu Met Asp Trp Ile Gly Ala Asn Ser Tyr Arg Thr Ser					
	725		730		735
His Tyr Pro Tyr Ala Glu Glu Met Leu Asp Trp Ala Asp Glu His Gly					
	740		745		750
Ile Val Val Ile Asp Glu Thr Ala Ala Val Gly Phe Asn Leu Ser Leu					
	755		760		765
Gly Ile Gly Phe Glu Ala Gly Asn Lys Pro Lys Glu Leu Tyr Ser Glu					
	770		775		780
Glu Ala Val Asn Gly Glu Thr Gln Gln Ala His Leu Gln Ala Ile Lys					
785		790		795	800
Glu Leu Ile Ala Arg Asp Lys Asn His Pro Ser Val Val Met Trp Ser					
	805		810		815
Ile Ala Asn Glu Pro Asp Thr Arg Pro Gln Gly Ala Arg Glu Tyr Phe					

820	825	830
Ala Pro Leu Ala Glu Ala Thr Arg Lys Leu Asp Pro Thr Arg Pro Ile		
835	840	845
Thr Cys Val Asn Val Met Phe Cys Asp Ala His Thr Asp Thr Ile Ser		
850	855	860
Asp Leu Phe Asp Val Leu Cys Leu Asn Arg Tyr Tyr Gly Trp Tyr Val		
865	870	875
Gln Ser Gly Asp Leu Glu Thr Ala Glu Lys Val Leu Glu Lys Glu Leu		
885	890	895
Leu Ala Trp Gln Glu Lys Leu His Gln Pro Ile Ile Ile Thr Glu Tyr		
900	905	910
Gly Val Asp Thr Leu Ala Gly Leu His Ser Met Tyr Thr Asp Met Trp		
915	920	925
Ser Glu Glu Tyr Gln Cys Ala Trp Leu Asp Met Tyr His Arg Val Phe		
930	935	940
Asp Arg Val Ser Ala Val Val Gly Glu Gln Val Trp Asn Phe Ala Asp		
945	950	955
Phe Ala Thr Ser Gln Gly Ile Leu Arg Val Gly Gly Asn Lys Lys Gly		
965	970	975
Ile Phe Thr Arg Asp Arg Lys Pro Lys Ser Ala Ala Phe Leu Leu Gln		
980	985	990
Lys Arg Trp Thr Gly Met Asn Phe Gly Glu Lys Pro Gln Gln Gly Gly		
995	1000	1005
Lys Gln		
1010		

&lt;210&gt; 13

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: degenerated  
PCR primer

&lt;400&gt; 13

ccgaattcgg ntayatgtty ggnaa

25

&lt;210&gt; 14

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: degenerated  
PCR primer

&lt;400&gt; 14

ccgaattcac natrtaytcr ttrta

25

&lt;210&gt; 15

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: oligonucleotide  
for use as PCR primer

&lt;400&gt; 15

gggaccatgt agtttatctt gacct

25

&lt;210&gt; 16

&lt;211&gt; 26

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
oligonucleotide for use in PCR

&lt;400&gt; 16

gacctcgtag cccaactctt ccccat

26

&lt;210&gt; 17

&lt;211&gt; 36

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide for use in PCR

<400> 17

aagtcgacgc ggccgccaca cctagtgccg ggtag

36

<210> 18

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide for use in PCR

<400> 18

atctcaattg tacattcttc agga

24

<210> 19

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide for use in PCR

<400> 19

aggatcccat ggcgaacaag ctcaaagtga c

31

<210> 20

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
oligonucleotide for use in PCR

<400> 20

aggatcctta gtgctcttag ttgaat

26

<210> 21

<211> 4947

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: APP promoter  
fusion with beta-glucuronidase gene

<220>

<221> promoter

<222> (1)..(1961)

<220>

<221> misc\_signal

<222> (1962)..(1964)

<223> translation initiation codon

<400> 21

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tagtTTtaagt atgtatTTTT ggattacaag tgtggttggc atcaagacaa ggatggtgat 180  
agcctttctc tgtaatttgg tTTaagaaaa gTTTTtgcT tttatgtata aacgtgTTTT 240  
TTTTTTataa tttcaaattt caacaaaaaa caattTTTTt taataatgat tgaccactat 300  
agacaattta aatgataaaa aaaaggggga atTTTTcaca atgtTTtgga gattagtcta 360  
gattTTTTgt ccaaatttTc cgattgtaag aattaagaag caatgaacat ttgtgttaag 420  
cttaatgatt tgtactcaca atatctTTta aattTaaaat tgTTaaccaa aatatcctat 480  
atattgtact tgtaatagaa atataaacta tTaaaaacaa cactttattc atataatata 540  
agTtaaaaca tatgtTTTTt ttagtatgtt ctaatcacac ctattaaaaa aagttgaagc 600  
taaTgagcc aaaaagaaaa ataaagatag gggatgggga caggctgtaa tgTTaggcgg 660  
ttggTatatg aactgagaac atgtctgttg gttcggtcca tctacgccac tcaaccattt 720  
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tcaataatca ggaagtgatg gagcatcagg gcggctatac gccatttgaa gccgatgtca 2340



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tcccggcatc cgcttacaga caagctgtga ccgtctccgg gagctgcatg tgtcagagg 4920  
tttcaccgtc atcaccgaaa cgcgcga 4947

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 99/04940

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N15/54 C12N9/10 C12N5/10 A01H5/00

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)

IPC 7 C12N

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)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEPINIEC, L., ET AL. : "characterization of an Arabidopsis thaliana cDNA homologue to animal poly (ADP ribose) polymerase" FEBS LETTERS, vol. 364, 1995, pages 103-108, XP002102933 the whole document	1-41
A	MÉNISSIER DE MURCIA, J., ET AL. : "requirement of poly (ADP ribose) polymerase in recovery from DNA damage in mice and in cells" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 94, July 1997 (1997-07), pages 7303-7307, XP002120253 page 7306-7307 abstract	1-41

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search

26 October 1999

Date of mailing of the international search report

10/11/1999

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 99/04940

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUEPPER J ET AL: "MOLECULAR GENETIC SYSTEMS TO STUDY THE ROLE OF POLY(ADP-RIBOSYL)ATION IN THE CELLULAR RESPONSE TO DNA DAMAGE" BIOCHIMIE, vol. 77, no. 6, 1995, pages 450-455, XP000789938 ISSN: 0300-9084 the whole document ---	1-41
A	WO 97 06267 A (PLANT GENETIC SYSTEMS NV ;BLOCK MARC DE (BE)) 20 February 1997 (1997-02-20) abstract, page 4 + 5 ---	1-41
A	LAUTIER D ET AL: "MOLECULAR AND BIOCHEMICAL FEATURES OF POLY (ADP-RIBOSE) METABOLISM" MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 122, no. 2, 26 May 1993 (1993-05-26), pages 171-193, XP000560778 ISSN: 0300-8177 the whole document ---	1-41
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Inter-      nal Application No

PCT/EP 99/04940

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>BABIYCHUK, E., ET AL. : "higher plants possess two structurally different poly (ADP ribose) polymerases"</p> <p>THE PLANT JOURNAL,</p> <p>vol. 15, no. 5, September 1998 (1998-09),</p> <p>pages 635-645, XP002102932</p> <p>the whole document</p> <p style="text-align: center;">-----</p>	1-41

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Information on patent family members

International Application No

PCT/EP 99/04940

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