(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 July 2004 (22.07.2004)

PCT

(10) International Publication Number
WO 2004/061112 A2

(51) International Patent Classification: C12N 15/82

(21) International Application Number: PCT/IL2003/001119

(22) International Filing Date: 31 December 2003 (31.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/437,985 6 January 2003 (06.01.2003) US

(71) Applicant (*for all designated States except US*): **RAMOT AT TEL AVIV UNIVERSITY LTD.** [IL/IL]; 32 Haim Levanon Street, 69 975 Tel Aviv (IL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **STEIN, Hanan** [IL/IL]; 10 Hatavor Street, 74 019 Nes ziona (IL). **ZILBERSTEIN, Aviah** [IL/IL]; Mikve Israel Agricultural School, 58 910 Holon (IL). **MILLER, Gadi** [IL/IL]; 117 Weizmann Street, 62 262 Tel Aviv (IL).

(74) Agent: **G. E. EHRLICH (1995) LTD.**; 11 Menachem Begin Street, 52 521 Ramat Gan (IL).

(81) Designated States (*national*): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, EG, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF IDENTIFYING ORGANISMS HAVING A MUTATED SIGNAL TRANSDUCTION PATHWAY

(57) Abstract: A method of identifying an organism having a mutated signal transduction pathway which comprises: (i) exposing a plurality of organisms to a compound capable of negatively affecting physiology and/or morphology of an organism having a normal signal transduction pathway; while at the same time not substantially affecting the physiology and/or morphology of the organism having the mutated signal transduction pathway; and (ii) identifying an organism of the plurality of organisms not substantially effected by the compound, thereby identifying the organism having the mutated signal transduction pathway.

WO 2004/061112 A2

METHOD OF IDENTIFYING ORGANISMS HAVING A MUTATED SIGNAL TRANSDUCTION PATHWAY

FIELD AND BACKGROUND OF THE INVENTION

5 The present invention relates to a method of identifying organisms having a mutated signal transduction pathways, and more particularly, to methods of identifying mutants of stress-induced signal transduction pathways. In addition, the present invention relates to methods of identifying genes and promoters associated with signal transduction pathways and identifying agents capable of protecting 10 organisms from stress conditions.

Living organisms, such as plants, may be exposed to an extremely wide range of biotic and abiotic stresses. A biotic stress may be inflicted by pathogenic microorganisms (e.g., viruses, bacteria fungi, or nematodes) or pests (e.g., insects, mites, or grazing animals), while an abiotic stress may be, for example, a condition 15 of drought, salinity, frost, high or low temperature, flooding, nutrient deficiency, toxic chemicals, and mechanical injury.

An exposure of an organism to a stress condition can trigger complex signal transduction pathways leading to resistance or tolerance to the stress condition. In plants, several stress-induced signal transduction pathways which respond to a wide 20 range of stress conditions have been described.

The "systemic induced resistance" (SIR) signal transduction pathway exists in a wide range of plants. The SIR can be induced by herbivore insects attack or by mechanical injury and is characterized by accumulation of jasmonic acid (JA; Creelman and Mullet, Annual Review of Plant Physiology and Plant Molecular 25 Biology 48: 355–381, 1997; Wasternack and Parthier, Trends in Plant Science 2: 302–307, 1997).

Another broad spectrum stress-induced signal transduction pathway which exists in a wide range of plants is the "systemic acquired resistance" (SAR). The SAR promotes disease resistance by inducible response to a broad spectrum of viral, 30 bacterial, and fungal pathogens. The SAR is characterized by accumulation of salicylic acid (SA; Ryals *et al.*, Plant Cell 8:1809-1819, 1996; Hunt *et al.*, Critical Reviews in Plant Sciences 15: 583-606, 1996).

An additional signal transduction pathway which exists in a wide range of plants is characterized by free proline accumulation. This pathway promotes an inducible stress tolerance to abiotic stresses such as water deprivation, salinity, low temperature, high temperature, heavy metal toxicity, anaerobiosis, nutrient deficiency, atmospheric pollution and UV irradiation (Hare and Cress, 1997).

Despite much research and the use of sophisticated and intensive crop-protection measures, including genetic transformation of plants, losses due to stress conditions remain in the billions of dollars annually. While disease and pest resistance genes have been cloned, the transgenic plants transformed with these genes are typically resistant only to specific strains of a particular pathogen or pest species. Similarly, transgenic plants which have been transformed with genes conferring higher tolerance to abiotic stresses such as frost, drought or salinity (Kasuga *et al.*, 1999; Holmström *et al.*, Nature 379: 683-684, 1996; Xu *et al.*, Plant Physiol 110: 249-257, 1996; Pilon-Smits and Ebskamp, Plant Physiol 107: 125-130, 1995; Tarczynski *et al.*, Science 259: 508-510, 1993) are also characterized by partial tolerance.

Genes associated with stress-induced signal transduction pathways may also be utilized to improve plants resistance or tolerance to stress conditions. For example, U.S. Pat. No. 6,091,004 describes an isolated gene (designated N1M1) encoding a protein which is involved in the signal transduction pathway of SAR. Plants which have been transformed with N1M1 exhibited broad spectrum disease resistance.

Similarly, stress-induced gene promoters, associated with stress-induced signal transduction pathways, may be utilized for research or commercial plant genetic engineering. For example, stress-inducible promoters can be utilized to regulate a timely expression of desired traits (Winicov and Bastola, Plant Physiol. 120: 473-480, 1999; Su *et al.*, Plant Physiol. 117: 913-922, 1998).

Stress-induced signal transduction pathways are complicated and not fully understood and, therefore, the identification of useful genes involved in the pathways is a daunting task. Furthermore, the state of the art for identifying useful genes which participate in signal transduction pathways typically involves generating random mutants followed by a selection for signal transduction pathway mutants by their inability to grow normally during stress conditions. Such a

negative selection procedure requires individual and precise identification of each mutant in the experiment, or duplications of every mutant under normal as well as under stress conditions. As a result, such negative selection methods of identifying mutant signal transduction pathways are extremely laborious and time consuming.

5 There is thus a widely recognized need for, and it would be highly advantageous to have, an effective and simple method of identifying mutants of stress-induced signal transduction pathways, which method is suitable for high throughput screening and devoid of the above limitations.

10 SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of identifying an organism having a mutated signal transduction pathway, including (i) exposing a plurality of organisms to a compound capable of negatively affecting physiology and/or morphology of an organism having a normal signal transduction 15 pathway, while at the same time not substantially affecting physiology and/or morphology of the organism having the mutated signal transduction pathway; and (ii) identifying an organism of the plurality of organisms not substantially effected by the compound, thereby identifying the organism having the mutated signal transduction pathway.

20 According to another aspect of the present invention there is provided a method of identifying a stress inducible gene promoter, including: (i) genetically modifying a plurality of organisms to include a randomly inserted reporter gene; (ii) exposing the plurality of organisms resulting from step (i) to a stress condition; (iii) exposing the organisms resulting from step (ii) to a compound capable of negatively 25 affecting physiology and/or morphology of an organism having a normal signal transduction pathway; while at the same time not substantially affecting physiology and/or morphology of an organism having a mutated signal transduction pathway generated by insertion of the reporter gene; (iv) selecting organisms not being substantially affected by the compound; (v) selecting from the organisms resulting 30 from step (iv) at least one organism expressing the reporter gene; and (vi) identifying from the at least one organism resulting from step (v) a polynucleotide sequence positioned upstream of the reporter gene, thereby identifying the stress inducible gene promoter.

According to yet another aspect of the present invention there is provided a method of identifying a stress signal transduction gene, including: (i) mutating a plurality of organisms; (ii) exposing the mutated organisms resulting from step (i) to a stress condition; (iii) exposing the mutated organisms resulting from step (ii) to a compound capable of negatively affecting physiology and/or morphology of an organism having a normal signal transduction pathway, while at the same time not substantially affecting physiology and/or morphology of an organism having a mutated signal transduction pathway; (iv) selecting mutated organisms not being substantially affected by the compound; and (v) identifying a mutated gene from the mutated organisms resulting from step (iv), thereby identifying the stress signal transduction gene.

According to still another aspect of the present invention there is provided a stress indicator organism having a mutated signal transduction pathway responsive to a stress condition.

According to an additional aspect of the present invention there is provided a method of detecting a stress condition in an agricultural environment, including: (i) cultivating a stress indicator plant having a mutated signal transduction pathway responsive to the stress condition in the agricultural environment; and (ii) observing an abnormal physiology and/or morphology of the stress indicator plant thereby detecting the stress condition in the agricultural environment.

According to yet an additional aspect of the present invention there is provided a method of identifying an agent capable of protecting an organism from a stress condition, comprising: (i) generating an organism exhibiting high sensitivity to the stress condition; (ii) exposing the organism to a plurality of molecules; (iii) exposing the organism to the stress condition; and (iv) identifying a molecule from the molecules capable of conferring tolerance to the stress condition to the organism, thereby identifying the agent capable of protecting the organism from the stress condition.

According to further features in preferred embodiments of the invention described below, the organism is a plant.

According to still further features in the described preferred embodiments exposing a plurality of organisms to a compound further includes exposing the plurality of organisms to a stress condition.

According to still further features in the described preferred embodiments the stress condition is an abiotic stress condition.

According to still further features in the described preferred embodiments the abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

According to still further features in the described preferred embodiments the stress condition is a biotic stress condition.

According to still further features in the described preferred embodiments the biotic stress condition is an exposure to a pathogen.

According to still further features in the described preferred embodiments the pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

According to still further features in the described preferred embodiments the biotic stress condition is an exposure to a pest.

According to still further features in the described preferred embodiments the pest is an insect or a mite.

According to still further features in the described preferred embodiments the normal signal transduction pathway downregulates an activity or expression of an enzyme in the organism.

According to still further features in the described preferred embodiments the enzyme is proline dehydrogenase.

According to still further features in the described preferred embodiments the compound is a proline analogue.

According to still further features in the described preferred embodiments the proline analogue is L-Thiazolidine-4-carboxylic acid.

According to still further features in the described preferred embodiments the proline analogue is L-Azetidine-2-carboxylic acid.

According to still further features in the described preferred embodiments the normal signal transduction pathway upregulates an activity or expression of an enzyme in the organism.

According to still further features in the described preferred embodiments the reporter gene is selected from the group consisting of genes encoding for β-

galactosidase, β-glucuronidase, luciferase, chloramphenicol acetyltransferase, nopaline synthase, green fluorescent protein, red fluorescent protein and blue fluorescent protein.

According to still further features in the described preferred embodiments the mutation is effected by a random insertion of T-DNA into a genome of the organisms.

According to still further features in the described preferred embodiments the mutation is effected by a random insertion of a transposable element into a genome of the organisms.

According to still further features in the described preferred embodiments the mutation is effected by exposing the organisms to ethylmethane sulfonate.

According to still further features in the described preferred embodiments the agricultural environment is a commercial field.

According to still further features in the described preferred embodiments cultivating a stress indicator plant includes cultivating a plurality of normal plants.

According to still further features in the described preferred embodiments the abnormal physiology and/or morphology is selected from the group consisting of a reduced growth, chlorosis, necrosis and wilt.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a simple and practical method of identifying mutants of stress-induced signal transduction pathways, which is suitable for high throughput screening.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail,

it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 schematically illustrates the proline biosynthesis pathway in bacteria and plants indicating the genes and enzymes participating in the pathway. Abbreviations: AcGSA, N-acetyl glutamyl semialdehyde; GSA, glutamic gamma-semialdehyde; GP, glutamyl phosphate; P5C, D1-pyrroline-5-carboxylate. Enzymes: ARG, Arginase; gamma-GK, gamma-Glutamyl Kinase; GSD, Glutamic gamma-Semialdehyde Dehydrogenase (also known as GPR); OAT, ornithine amino transferase; P5CS, P5C synthetase; P5CR, P5C reductase; PDh, Proline dehydrogenase; P5C-Dh, P5C dehydrogenase.

FIGs. 2a-c are images depicting PDh mRNA levels and free proline levels in roots and leaves of alfalfa seedlings during salt stress (170 mM NaCl) imposition and recovery. Figure 2a illustrates Northern blot analysis of PDh mRNA levels in roots; alfalfa PDh cDNA and ribosomal DNA (18S) fragments were used as probes. Figure 2b illustrates the proline content of alfalfa roots. Bars represent the standard error of three replicates. Figure 2c illustrates Northern blot analysis of PDh mRNA levels in leaves. Sampling at identical times was practiced in order to eliminate differences associated with circadian rhythm. The images illustrate enhanced levels of alfalfa PDh mRNA and of free proline accumulation during salt stress.

FIG. 3 illustrates the molecular structures of proline and the proline analogs L-thiazolidine-4-carboxylic acid (T4C) and L-azetidine-2-carboxylic acid (AZC).

FIG. 4 is an image depicting Northern blot analysis of alfalfa PDh mRNA levels in leaves of transgenic tobacco expressing alfalfa PDh, under normal conditions and during salt stress (170 mM NaCl). Alfalfa PDh cDNA fragment was used as a probe. Wild type alfalfa plants (alfalfa) served as a positive control and wild type tobacco plants (w.t.) served as a negative control. The image illustrates an

enhanced level of alfalfa PDh mRNA in transgenic tobacco plants being under salt stress.

FIG. 5 is a group of images depicting salt dependent T4C sensitivity in wild type and transgenic tobacco expressing alfalfa PDh. Transgenic two week-old tobacco seedlings carrying the coding fragment from alfalfa PDh driven by CaMV-35S promoter (35S-PDh10) were transferred to solid MS media supplemented with different combinations of T4C (3 mM) and NaCl (50 mM). Wild type tobacco plants (w.t.) served as controls. The plants were photographed five weeks following transfer to the T4C and NaCl supplemented media. The images illustrate that the alfalfa PDh - transformed tobacco plants are more tolerant to T4C than the wild type plants used as control. This increased tolerance exhibited by the transgenic plants of the present invention was more pronounced under exposure to NaCl at a concentration of 50 mM.

FIGs. 6a-b are images depicting the hypersensitivity to AZC exhibited by transgenic tobacco plants carrying an alfalfa PDh anti-sense fragment (p7-24PDh). Two week old seedlings were transferred to a solid MS medium supplemented with 1.5 mM AZC and 0.5 mM proline, without NaCl (Figure 6a) or with 50 mM NaCl (Figure 6b) and photographed three weeks later. Wild-type tobacco plants (w.t.) served as controls. The images illustrate hypersensitivity of the alfalfa PDh anti-sense transgenic tobacco plants to AZC.

FIGs. 7a-d are images depicting salt dependent AZC sensitivity exhibited by wild-type *Arabidopsis thaliana* plants. Plant seeds were surface sterilized and germinated as described in Example 5 in the Examples section hereinbelow. Eighteen day old seedlings were treated with four different combinations of NaCl (0 or 150 mM) and AZC (0 or 1 mM) and photographed 6 days following treatment. Figure 7a illustrates untreated seedlings. Figure 7b illustrates NaCl-only treated seedlings exhibiting no visible toxicity. Figure 7c illustrates AZC-only treated seedlings exhibiting moderate chlorosis and growth inhibition. Figure 7d illustrates seedling treated with AZC combined with NaCl exhibiting complete chlorotic and drastically reduced growth.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of methods of identifying mutants of stress-induced signal transduction pathways. Mutants identified according to the methods of the present invention, can be utilized to identify novel stress-inducible promoters and genes which participate in stress-induced signal transduction pathways. The present invention also provides a stress indicator plant, a method of using such plant for detecting stress conditions and a method of identifying agents capable of protecting organisms from stress conditions.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing the present invention to practice, the present inventors uncovered that transgenic tobacco plants expressing alfalfa proline dehydrogenase (PDh) exhibited higher tolerance to the toxic proline analog T4C. This increased tolerance of these transgenic plants to T4C was more pronounced under salt stress (see Example 3 in the Examples section hereinbelow). Conversely, transgenic tobacco plants carrying an alfalfa PDh anti-sense fragment were hypersensitive to the proline analog AZC, thus indicating that PDh participates in AZC catabolism (see Example 4 in the Examples section hereinbelow). The present inventors also uncovered that *Arabidopsis thaliana* sensitivity to AZC increased substantially under salt stress conditions (see Example 5 in the Examples section hereinbelow), thus indicating that the sensitivity of normal plants grown under stress condition to toxic proline analogues is due to downregulation of PDh which results from stress signaling. Consequently, stress-induced signal transduction pathway mutants incapable of downregulating PDh would be tolerant to toxic proline analogs such as T4C or AZC.

These finding that signal transduction pathway mutants are capable of tolerating compounds which are otherwise toxic to normal organisms, have led the present inventors to formulate a novel approach which can be utilized for positive selection of signal transduction pathway mutants.

5 Thus, according to one aspect of the present invention, there is provided a method of identifying an organism, such as a plant, which has a mutated signal transduction pathway.

The method is effected by exposing a plurality of organisms to a compound capable of (i) negatively affecting physiology and/or morphology of an organism 10 having a normal signal transduction pathway; and (ii) not substantially affecting the physiology and/or the morphology of the organism having the mutated signal transduction pathway. This step is followed by identifying an organism which is not substantially affected (as far as physiology and/or morphology) by the compound, thereby identifying the organism having the mutated signal transduction pathway.

15 As used herein, the phrase "an organism having mutated signal transduction pathway" refers to an organism having an alteration in one or more genes which are involved in the signal transduction pathway. The mutant can be naturally occurring or artificially generated. The latter can be generated by chemical mutagenesis or by random insertion of a polynucleotide, such as a transposable element or a tagged 20 DNA sequence, in the genome of the organism.

As used herein, the phrase "affecting physiology and/or morphology refers to altering the normal appearance, formation (e.g., growth), structure and/or function of the organism. Accordingly, a negative effect on the physiology and/or morphology of an organism may manifest through, for example, growth inhibition, chlorosis 25 (chlorophyll destruction), necrosis (cells death) or wilting (loss of rigidity).

The signal transduction pathway can be activated by a stress condition (e.g., a biotic or an abiotic stress) which causes, in normally responsive plants (w.t. plants), upregulation or downregulation of one or more pathway components, which may be, for example, enzymes. Examples of signal transduction pathway 30 components which are upregulated or downregulated, in normally responsive plants, are listed in Table 1 below.

Table 1
Up- and down-regulated components of stress-induced signal transduction pathways

Pathway	Regulation	Component	Precursor	Product	Reference
Proline	down	PDh	proline	P5C	Yoshiba *et al.*, 1997
Proline	up	P5CS	glutamate	GSA	Yoshiba *et al.*, 1997
salicylic acid	up	PAL	phenylalanine	t-CA	Ryan et al., Plant Cell 8:1809-1819, 1996
salicylic acid	up	benzoic acid 2-hydroxylase	benzoic acid	salicylic acid	Ryan et al., Plant Cell 8:1809-1819, 1996
jasmonic acid	up	phospholipase (PLD)	systemin	α-linolenic acid	Devoto and Turner, Ann. Bot. 92:329-337, 2003
jasmonic acid	up	lipoxygenase (LOX)	α-linolenic acid	13(S)-HPOT	Devoto and Turner, Ann. Bot. 92:329-337, 2003
jasmonic acid	up	allene oxide synthase (AOS)	13(S)-HPOT	allene oxide	Devoto and Turner, Ann. Bot. 92:329-337, 2003
jasmonic acid	up	allene oxide cyclase (AOC)	allene oxide	12-OXO-PDA	Devoto and Turner, Ann. Bot. 92:329-337, 2003
jasmonic acid	up	proteinase inhibitor I, II	phytodienoic acid	jasmonic acid	O'Donnel et al., Science 274:1914-1918, 1996
jasmonic acid	up	ACS and ACO	S-adenosyl-methionine	ethylene	O'Donnel et al., Science 274:1914-1918, 1996

Abbreviation: ACS, 1-aminocylopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-carboxylate oxidase; PAL, phenylalanine ammonia lyase; PDh, proline dehydrogenase; P5CS, delta-pyrroline-5-carboxylase-synthase; 13(S)-HPOT, (9Z, 11E,15Z,13S)-13-hydroperoxy-9,11,15-octadecatrieonic acid; 12-OXO-PDA, 12-oxo-10,15(Z)- octadecatrieonic acid;P5C, D1-pyrroline-5-carboxylate; GSA, glutamic γ-semialdehyde; t-CA, trabs-cinnamic acid.

The section which follows describes applications of the present methodology in identification of mutants of stress-induced signal transduction pathways which do not exhibit upregulation or downregulation of the components listed above due to a mutation in one or more genes or promoters.

One example of a signal transduction pathway which involves the downregulation of a pathway component is the stress-induced pathway which leads to proline accumulation in plants.

Proline accumulation in plants may result in response to an abiotic stress condition such as water deficit or salinity (Hare and Cress, 1997). During an exposure to stresses proline is synthesized from glutamate by the sequential action of the enzymes delta-pyrroline-5-carboxylate synthase (P5CS) and P5C reductase (P5CR; Figure 1; Roosens *et al.*, 1998). Proline oxidation to glutamate is carried out

in the mitochondria by the sequential action of the enzymes proline dehydrogenase (PDh) and P5C-dehydrogenase (P5C-Dh; Figure 1).

A substantial body of literature indicates that the accumulation of free proline under stress results from an upregulation of proline biosynthesis combined with a downregulation of proline degradation. For example, Yoshiba *et al.* (1997) reported that P5CS gene expression under dehydration condition increases, while the PDh gene expression decreases. Analyses of transcription during osmotic stress and recovery periods revealed that P5CS transcription is elevated during stress and gradually diminished during the post-stress period (Ginzberg *et al.,* 1998; Peng *et al.*, 1996; Strizhov *et al.*, 1997; Yoshiba *et al.*, 1997). Conversely, PDh transcription is gradually reduced within several hours of stress, and rapidly increased upon relief from stress (Peng *et al.*, 1996; Yoshiba *et al.*, 1997). Similarly, PDh activity decreases while free proline accumulates during cold-stress in greenbean plants (Ruiz *et al.*, 2002).

Since this pathway is characterized by downregulation of PDh in wild-type plants, identification of mutants of this pathway which result in a lack of PDh downregulation can be facilitated by utilizing a compound which is toxic to plant cell when such cells express low levels of PDh. As a result, such a compound negatively affects physiology and/or morphology of an organism having a normal signal transduction pathway while it does not substantially affect the physiology and/or the morphology of an organism which has the mutated signal transduction pathway, since the latter organism would not exhibit downregulated PDh expression.

Examples of such a compound include, but are not limited to proline analogues, such as, 3,4-dehydro-L-proline (Dhp), the proline analog available from Sigma-Aldrich (http://www.sigmaaldrich.com/) L-Thiazolidine-4-carboxylic acid (T4C) and L-Azetidine-2-carboxylic acid (AZC).

Proline analogs, such as L-Azetidine-2-carboxylic acid (AZC; see Figure 3), L-Thiazolidine-4-carboxylic acid (T4C; see Figure 3) and 3,4-dehydro-L-proline (Dhp), resemble proline with respect to molecular weight, steric conformation, charge and PDh substrate recognition. If not catabolized by PDh, such analogs are toxic to plant cells through several possible mechanisms, including incorporation into proteins followed by misfolding and abnormality, inactivation of P5CS, or inactivation of prolyl hydroxylase. For examples, Dhp is a selective

inhibitor of prolyl hydroxylase in tobacco cells (Bucher *el al.*, 1997). AZC, on the other hand, is transported into the cells via proline transporters. It is then incorporated into cellular proteins competitively with L proline which results in the synthesis of abnormal misfolded proteins, thereby inhibiting cell growth (Shichiri *et al.*, 2001).

The above described compound can be used by the present invention to identify mutants of the proline pathway as follows.

Mutated and non-mutated (normal) plants are exposed to an abiotic stress condition, such as water depravation, suboptimal temperature, nutrient deficiency, or preferably a salt stress condition. Salt stress can be effected in many ways such as, for example, by irrigating the plants with a hyperosmotic solution, by cultivating the plants hydroponically in a hyperosmotic growth solution (e.g., Hoagland solution), or by culturing the plants in a hyperosmotic growth medium (e.g., MS medium). Since different plants vary considerably in their tolerance to salinity, the salt concentration in the irrigation water, growth solution, or growth medium is preferably adjusted according to the specific characteristics of the specific plant cultivar or variety, so as to inflict a mild or moderate effect on the physiology and/or morphology of the plants. Preferably, a salt stress is induced to alfalfa, tobacco or *Arabidopsis thaliana* plants via culturing seedlings in MS media (Duchefa) which include 170, 50 or 150 mM NaCl, respectively (see Examples 1-4 of the Examples section which follows).

Stressed-induced plants are further exposed to a toxic proline analog compound, preferably AZC or T4C, which can be administered to the plants by way of spraying, dusting, drenching, irrigating, root dipping, seed soaking, or by supplementing to growth solution or growth medium. Preferably, the compound is administered at a minimum dosage that is capable of substantially affecting the physiology and/or morphology of normal plants grown under the stress condition. Tobacco plants can be exposed to T4C-supplemented MS growth medium at a concentration of 3 mM (see Example 2 of the Examples section which follows), while tobacco or *Arabidopsis thaliana* plants can be exposed to AZC-supplemented MS growth medium at a concentration of 1.5 or 1 mM, respectively (see Examples 3-4 of the Examples section which follows).

Following exposure to the compound, plants are frequently monitored until substantial physiological and/or morphological effects appear in normal plants. Mutated plants are subsequently examined and plants not exhibiting substantial physiological and/or morphological effects are identified as signal transduction pathway mutants.

Organisms having mutated signal transduction pathways may also be identified by utilizing the activity of upregulated enzymes. In this case, the enzymes which are upregulated in normal plants would be downregulated or unaffected in the mutated plants. Examples of upregulated enzymes which may be utilized by the present invention are provided in Table 1.

The enzyme delta-pyrroline-5-carboxylase-synthase (P5CS) is upregulated during an abiotic stress activation of the signal transduction pathway in plants, which is characterized by proline accumulation (Yoshiba *et al.*, 1997; Ginzberg *et al.,* 1998; Peng *et al.*, 1996; Strizhov *et al.*, 1997; Yoshiba *et al.*, 1997).

The pathogen induced systemic acquired resistance (SAR) signal transduction pathway in plants is characterized by accumulation of salicylic acid. Upregulated enzymes of this pathway, which may be utilized by the present invention include, for example, phenylalanine ammonia lyase (PAL), and benzoic acid 2-hydroxylase (Ryan et al., Plant Cell 8:1809-1819, 1996; Hunt and Ryals, Crit. Rev. in Plant Sci. 15: 583-606, 1996; Delaney *et al.*., Science 266: 1247-1250, 1994; Delaney *et al.*, Proc. Natl. Acad. Sci. USA 92: 6602-6606, 1995; Delaney, Plant Phys. 113: 5-12, 1997; Bi *et al.*, Plant J. 8: 235-245, 1995; and Mauch-Mani and Slusarenko, Plant Cell 8: 203-212, 1996 and U.S. Pat. No. 6,037,490).

The wound or pest systemic induced-resistance (SIR) signal transduction pathway is characterized by accumulation of jasmonic acid. Several upregulated enzymes of this pathway can be utilized by the present invention such as, for example, phospholipase (PLD), lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), proteinase inhibitor I and II, ACS, 1-aminocylopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-carboxylate oxidase (ACO; Devoto and Turner, Ann. Bot. 92:329-337, 2003; O'Donnel *et al.*, Science 274:1914-1918, 1996; Constabel *et al.*, Proc. Natl. Acad. Sci. USA 92, 407-411, 1995)

Identification of signal transduction pathway mutants, which are characterized by a lack of enzyme upregulation, can be effected by utilizing a compound which is toxic to plant cells when such cells express high levels of the enzyme, but is nontoxic to plant cells when such cells express low levels of the enzyme.

As a result, such a compound negatively affects the physiology and/or morphology of an organism having a normal signal transduction pathway, while it does not substantially affect the physiology and/or the morphology of an organism which has the mutated signal transduction pathway, since such an organism would not exhibit upregulated enzyme expression.

A suitable compound may be any non-toxic precursor molecule which can be converted by the enzyme into a toxic molecule. Suitable compounds may be generated using various molecular design techniques known in the art such as described, for example, in U.S. Pat. No. 6,549,900. Preferably, substrate analogs can be synthesized so as to be capable of releasing, upon the enzyme's activity, a toxic metabolite such as, for example, cyanide (e.g., phenylacetonitrile which is capable of releasing toxic cyanide upon cytochrome p450 activity; U.S. Pat. No. 6,643,591).

The compound may be administered to plants in a variety of ways such as, for example, by spraying, dusting, irrigating, drenching, soaking, dipping, digesting or injecting. The compound's dosage is preferably adjusted as the minimum dosage capable of substantially effecting the physiology and/or morphology of normal plants when exposed to a stress condition.

Prior to, or concomitantly with the compound exposure, all plants are exposed to a stress condition capable of inducing the signal transduction pathway in normal plants. Accordingly, a stress condition capable of inducing the proline synthesis pathway may be an abiotic stress such as, for example salinity, water depravation, suboptimal temperature, or nutrient deficiency. Preferably, the stress condition is salinity stress condition effected as described hereinabove.

A stress condition capable of inducing the SAR pathway can be effected by exposure to a pathogenic virus, bacterium, fungus or a nematode using various inoculation techniques well known in the art such as described, for example, in U.S. Pat. Nos. 6,057,490 and 6,495,737.

A stress condition capable of inducing the SIR pathway can be effected by wounding plant tissues using procedures such as described, for example, by Weber *et al.* (Proc. Natl. Acad. Sci. USA 94:10473-10478, 1997) and Titarenko *et al.* (Plant Physiol. 115:817-826, 1997).

5 Following plants exposure to the compound and stress condition, the plants are frequently monitored until substantial physiological and/or morphological effects appear in the normal plants. Then, all plants are carefully examined and the plants not exhibiting substantial physiological and/or morphological effects are identified as mutants of the signal transduction pathway.

10 Random mutation of stress-induced signal transduction pathways may generate mutants in which the chromosomal positions of stress-inducible genes and their promoters are tagged and thus identifiable. Accordingly, mutants of stress-induced signal transduction pathways, identified according to the teachings of the present invention, may be utilized for isolating novel stress-inducible promoters.

15 Thus, according to another aspect of the present invention, there is provided a method of identifying a stress inducible gene promoter.

The method according to this aspect of the present invention is effected by genetically modifying a plurality of organisms by randomly inserting a reporter gene and identifying from the plurality of organisms modified, one or more signal 20 transduction pathway mutants which display reporter activity. Such mutants are then further genetically analyzed in order to identify and isolate the stress inducible gene promoter which lies upstream of the reporter gene.

As used herein, the phrase “promoter” refers to a region of DNA which lies upstream of the transcriptional initiation site of a gene to which RNA polymerase 25 binds to initiate transcription of RNA. The promoter controls where (e.g., which portion of a plant, which organ within an animal, etc.) and when (e.g., which stage in the lifetime of an organism) the gene is expressed.

The phrase “stress inducible promoter” used herein refers to a particular promoter which initiates a gene expression in response to a stress condition.

30 The phrase “reporter gene” used herein refers to a polynucleotide sequence which encodes a detectable polypeptide. Examples of suitable detectable polypeptides include, but not limited to, green fluorescent protein (GFP), red fluorescent protein, blue fluorescent protein, ß-glucoronidase (GUS), ß-galactosidase

(LAC), chloramphenicol acetyltransferase (CAT), nopaline synthase (NOS), firefly luciferase (LUC) and bacterial luciferase (LUX) (Daunert *et al.*, Chem. Rev. 100: 2705-2738, 2000).

A suitable reporter gene which does not include a promoter (promoter-less reporter gene) can be obtained from readily available commercial sources such as Clonthech, Quantum Biotechnologies, Packard instruments, and Pharmingen.

There are various methods of introducing foreign genes into both monocotyledonous and dicotyledonous plants (Potrykus, I., Annu. Rev. Plant. Physiol., Plant. Mol. Biol. 42:205-225, 1991; Shimamoto *et al.*, Nature 338:274-276, 1989).

The principle methods of causing random and stable integration of exogenous DNA into plant genomic DNA include two main approaches:

(i) *Agrobacterium*-mediated gene transfer: Klee *et al.*, Annu. Rev. Plant Physiol. 38:467-486, 1987; Klee and Rogers in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes, eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 2-25; Gatenby, in Plant Biotechnology, eds. Kung, S. and Arntzen, C. J., Butterworth Publishers, Boston, Mass. (1989) p. 93-112.

(ii) direct DNA uptake: Paszkowski *et al.*, in Cell Culture and Somatic Cell Genetics of Plants, Vol. 6, Molecular Biology of Plant Nuclear Genes eds. Schell, J., and Vasil, L. K., Academic Publishers, San Diego, Calif. (1989) p. 52-68; including methods for direct uptake of DNA into protoplasts, Toriyama, K. *et al.*, Bio/Technology 6:1072-1074, 1988. DNA uptake induced by brief electric shock of plant cells: Zhang *et al.* Plant Cell Rep. 7:379-384, 1988. Fromm *et al.* Nature 319:791-793, 1986. DNA injection into plant cells or tissues by particle bombardment, Klein *et al.* Bio/Technology 6:559-563, 1988; McCabe *et al.* Bio/Technology 6:923-926, 1988; Sanford, Physiol. Plant. 79:206-209, 1990; by the use of micropipette systems: Neuhaus *et al.*, Theor. Appl. Genet. 75:30-36, 1987; Neuhaus and Spangenberg, Physiol. Plant. 79:213-217, 1990; glass fibers or silicon carbide whisker transformation of cell cultures, embryos or callus tissue, U.S. Pat. No. 5,464,765 or by the direct incubation of DNA with germinating pollen, DeWet *et al.* in Experimental Manipulation of Ovule Tissue, eds. Chapman, G. P. and Mantell, S.

H. and Daniels, W. Longman, London, (1985) p. 197-209; and Ohta, Proc. Natl. Acad. Sci. USA 83:715-719, 1986.

The *Agrobacterium* system includes the use of plasmid vectors that contain defined DNA segments that randomly integrates into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the *Agrobacterium* delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. Horsch *et al.* in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the *Agrobacterium* delivery system in combination with vacuum infiltration. The *Agrobacterium* system is especially viable in the creation of transgenic dicotyledenous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Once transgenic plants carrying promoter-less reporter genes are generated, stress-induced signal transduction pathway mutants can be selected as described hereinabove. The mutants are further selected for a expressing the reporter proteins using, for example, a fluorescent microscope to detect GFP expression in a plant tissue. Stress-inducible promoters that are positioned in functional relationship to the reporter gene, can be isolated by using the reporter polynucleotide sequence as a basis for cloning the upstream chromosomal DNA sequence. The isolation of the inducible promoter sequence can be accomplished using standard cloning techniques well known in the art, such as described in Sambrook *et al.*, "Molecular Cloning: A laboratory Manual, 2nd Edition" (1989); Ausubel *et al.*., "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); and Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988);

Organisms characterized by mutated stress-induced signal transduction pathway may also be utilized for isolating genes which are involved in stress-induced signal transduction pathways.

Thus, according to another aspect of the present invention, there is provided a method of identifying stress signal transduction genes.

The method according to this aspect of the present invention is effected by mutating a plurality of organisms and identifying from the plurality of organisms mutated, one or more signal transduction pathway mutants. Such mutants are then genetically analyzed and the mutated genes are identified and isolated.

As mentioned hereinabove, plant mutation can be effected by chemical mutagenesis or by randomly inserting a tagged DNA in the chromosome. Chemical mutagenesis can be effected by exposing the organism to a mutagen such as, for example, ethylmethane sulfonate (EMS; Feldman, *et al.*, "Mutagenesis in Arabidopsis, in Arabidopsis, ed by Meyerwitz et al. " 1994, Cold Spring Harbor Press, pp. 137-172, 1994). Chemical-induced mutagenesis is technically straightforward and simple to perform, however identifying mutated genes is difficult.

Preferably, plant mutation is effected by randomly inserting a “tagged DNA” sequence, such as a transposable element or an *Agrobacterium* transforming DNA (T-DNA) into the chromosomal sequence. Methods of T-DNA and transposon-insertion mutagenesis are well known in the art (see, for example, Koncz *et al.*, Proc Natl Acad Sci USA 86: 8467-4871, 1989; Feldman *et al.* Science 243: 1351-1354, 1989; Marks and Feldman Plant Cell 1: 1053-1050, 1989; Honma *et al.* Proc. Natl. Acad. Sci. USA 90: 6242-6246, 1993; Aarts *et al.* Nature 363: 715-717, 1993; Walbot V., Ann. Rev. Plant Phys. Mol. Biol. 43:49-82, 1992; and U.S. Pat. No. 6,420,524).

Once random mutants are generated, one or more stress-induced signal transduction pathway mutants can be selected using the method described hereinabove. Genes tagged by T-DNA can be isolated by first cloning the T-DNA tagged gene, and then using mutated sequences that flank the T-DNA sequence as probes in the cloning of the wild-type gene, using methods such as described by Feldman *et al.* (Science 243: 1351-1354, 1989), and Marks and Feldman (Plant Cell 1: 1053-1050, 1989).

Genes tagged by transposable elements can be cloned using similar techniques such as described by Honma et al. (Proc. Natl. Acad. Sci. USA 90: 6242-6246, 1993) and Aarts *et al.* (Nature 363: 715-717, 1993).

Since mutated stress-induced signal transduction pathway organisms generated and selected as described hereinabove, may be highly sensitive to s stress

condition, they can be utilized as stress indicators. Preferably, the stress indicator organism is a plant which can be utilized to detect a stress condition in an agricultural environment.

Thus, according to another aspect of the present invention there is provided a method of detecting a stress condition in an agricultural environment, such as a commercial field.

The method according to this aspect of the present invention is effected by cultivating the stress indicator plant in the commercial field. Preferably the indicator plant is cultivated concomitantly with a commercial crop, such that, under stress condition, the indicator plant exhibits visible and easily recognized abnormal physiological and/or morphological symptoms such as substantial reduction of growth, chlorosis, necrosis or wilt. Since stress induced symptoms may be observed in the stress-indicator plant long before the commercial crop is damaged, control measures may be undertaken on time to avoid damage to the commercial crop.

Mutant stress-induced signal transduction pathway organisms may also be utilized to identify agents capable of protecting organisms from stress conditions.

Thus, according to another aspect of the present invention, there is provided a method of identifying an agent capable of protecting an organism from a stress condition.

The method includes exposing mutated organisms, such as plants exhibiting high sensitivity to a stress condition identified according to the teachings of the present invention, to a plurality of molecules such as, for example, putative anti-respirants, anti-desicants, anti-freezants, growth-regulators, pesticides, fungicides, bacteriocides or biological agents. The mutants' exposure to the molecules can be effected by spraying, dusting, irrigating, drenching, soaking, dipping, digesting or injecting. The treated mutants are then exposed to a stress condition sufficient to substantially affect the physiology and/or morphology of untreated mutants. Following exposure to the stress condition, the treated mutants exhibiting tolerance to the stress condition are selected, thereby identifying the agent capable of protecting the organism from the stress condition

Hence, the invention provides a novel, simple and effective method of identifying stress-induced signal transduction pathway mutants. The method is based on a positive selection and is therefore particularly suitable for high

throughput screenings. In addition, mutants, which are selected according to the teaching of the present invention, can be utilized to identify novel stress-induced promoters; to identify novel genes involved in signal transduction pathways; to detect stress conditions in an agricultural field environment; and to identify agents capable of protecting organisms from stress conditions.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752;

3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984); "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996) and Parfitt *et al.* (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J. Bone Miner Res 2 (6), 595-610; all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Osmotic stress induces transcription of proline dehydrogenase and accumulation of free proline in alfalfa seedlings

Materials and Methods:

Culturing: alfalfa seedlings were grown in 5 liter containers in liquid MS medium (Duchefa) and exposed to salt stress by replacing the isotonic medium with a medium containing 170 mM NaCl. Tissue samples were taken from roots and leaves of 3 week old seedlings for proline dehydrogenase (PDh) mRNA and free proline analyses.

Northern blot analysis: Total RNA was extracted from alfalfa tissue using the Trizol-Reagent kit (GIBCO, BRL, USA). The extracted RNA (20 µg) was dissolved in glyoxal and DMSO, then loaded onto 1 % agarose, blotted onto a nylon membrane (Hybond N, Amersham, UK) and fixed using a UV cross-linker. Following fixation, the glyoxal was washed out from the membrane with boiled solution of 20 mM Tris buffer (pH 8). Fragments of PDh cDNA (SEQ ID NO:1) and

ribosomal DNA (18S) were labeled with [32P]α-dCTP (Amersham Pharmacia, UK) using Klenow fragment DNA polymerase (MBI Fermentas, USA). The labeled fragments were hybridized overnight at 65 °C. Following hybridization, the blots were washed with 2 X SSC, 0.1 % SDS at 65 °C for 20 min, followed by another wash with 1 X SSC, 0.1 % SDS at 65 °C for 20 min, then exposed to an X-ray film (Fujifilm, Fuji, Japan). Quantification of transcript levels was performed on scanned images using Image-gouge software (Fujifilm, Fuji, Japan).

Estimation of free proline content in plants: Proline content was measured in root or shoot tissue samples using the Ninhydrin procedure as described by Bates *et al.* (Plant and Soil 39:205-207, 1973).

Results:

As illustrated in Figures 2a and 2b, three forms of PDh mRNA were observed, all revealing a similar transcription regulation pattern. PDh transcription in roots (Figure 2a) and in leaves (Figure 2c) sharply decreased in response to salt stress. These low levels of expression persisted when the plants were maintained under salt stress conditions. However, once salt stress conditions were removed, PDh expression strongly increased during the recovery period.

Free proline levels in the plant tissue inversely correlated with changes in PDh mRNA levels. During the seven days of salt stress, proline level in roots increased up to twenty-fold compared with the normal level, while a gradual decrease in proline levels was observed during the recovery period (Figure 2b).

EXAMPLE 2

Proline analogT4C is catabolized in transgenic tobacco plants expressing alfalfa PDh

Materials and Methods:

Plants transformation: The alfalfa PDh coding region (SEQ ID NO:2) was cloned in the *Agrobacterium*-plant shuttle vector pPCV702 downstream to a CaMV 35S as described by Koncz *et al.* (Proc Natl Acad Sci U S A 86: 8467-4871, 1989).

Agrobacterium tumefaciens strain GV3101-pMP90RK harboring the pPCV702-PDh plasmid was incubated in 50 ml LB supplemented with 100 mg/L rifampicin, 25 mg/L gentamycin and 100 mg/L carbenicillin for 72 hr at 28 °C. The

stationary phase culture was centrifuged for 15 min at 6,000 x *g* and the pellet was resuspended in MS medium (SIGMA, ST. LOUIS, USA) to a final density of 0.5 A_{600}.

Tissue discs (1 cm i.d.) were excised from young leaves of axenically grown
5 tobacco (*Nicotiana tabacum*, NN) and immersed for 1 hour in a suspension of *A. tumefaciens* harboring the pPCV702-PDh plasmid. The inoculated leaf disks were then incubated in MS medium supplemented with 0.8 % agar, 30 g/L sucrose, 2 mg/L kinetin and 0.8 mg/L IAA for 2 days at 25 °C under light conditions of 16 hour fluorescent light illumination period followed by an 8 hour dark period. Following
10 incubation, the discs were transferred to a fresh MS medium supplemented with 0.8 % agar, 30 g/L sucrose, 2 mg/L kinetin, 0.8 mg/L IAA, 500 mg/L claforan and 100 mg/L Kanamycin and incubated until regenerated shoots could be observed. The shoots were then excised and transferred to MS medium supplemented with 0.8% agar, 500 mg/L claforan and 100 mg/L Kanamycin and incubated until roots developed. The
15 formation of roots by the excised shoots in the presence of kanamycin was an indication that the plants have been successfully transformed with the desired nucleic acid construct.

Plants exposure to NaCl and T4C: two week old seedlings were transferred to solid MS media supplemented with NaCl and T4C, as described in Table 2 below.
20 Following a 5 week growth period, the plants were comparatively analyzed for treatment-induced expression of alfalfa PDh and for toxicity.

Table 2
NaCl and T4C combinations

NaCl concentration (mM)	T4C concentration (mM)
0	0
0	3
50	0
50	3

25

Northern blot analysis: PDh mRNA levels in tobacco leaf samples were determined by Northern blot analysis performed using the procedure described in Example 1 hereinabove and by using alfalfa PDh cDNA (SEQ ID NO: 1) as a probe.

Results:

Figures 4 and 5 illustrate that transgenic tobacco plants expressing alfalfa PDh exhibited a moderate tolerance to T4C, as compared with the wild type plants grown under normal conditions. Yet, when plants were exposed to a salt-induced stress condition (which induces downregulation of endogenic PDh), the transgenic plants exhibited a substantially higher tolerance to T4C, as compared with the wild type plants.

The results indicate that the PDh enzyme catabolizes toxic proline analog T4C in plants and that this synthetic substrate can be utilized to identify plants which do not respond to salt stress with downregulation of PDh.

EXAMPLE 3

Transgenic tobacco plants carrying alfalfa PDh anti-sense are hypersensitive to proline analog T4C

Materials and Methods:

Plants: transgenic tobacco plants carrying an anti-sense fragment from alfalfa PDh operably linked to a CaMV-35S promoter (p7-24PDh a.s.) were generated as described by Stein *et al.* (2000).

Culturing: transgenic tobacco-seedlings (2 wk old) were transferred to solid MS media supplemented with NaCl, AZC and proline, as described in Table 3 below. Following a 5 week growth period, the plants were comparatively observed for treatment-induced toxicity.

Table 3
NaCl, AZC and proline combinations

NaCl concentration (mM)	AZC concentration (mM)	Proline concentration (mM)
0	1.5	0.5
50	1.5	0.5

Results:

As is illustrated in Figures 6a-b, the transgenic tobacco plants carrying an alfalfa PDh anti-sense were hypersensitive to AZC-induced toxicity under normal growth conditions.

The results indicate that the PDh enzyme is involved in AZC catabolism in plants.

EXAMPLE 4

Arabidopsis thaliana sensitivity to the proline analog AZC is stress dependent

Materials and Methods:

Wild type *Arabidopsis thaliana* seeds (2-3 mg) were surface sterilized via a 3
5 hour exposure to chlorine gas (Cl_2; generated by mixing 100 ml sodium hypochlorite with 5 ml HCl) in a closed chamber. About 100 surface sterilized seeds were germinated on 50 ml liquid MS medium (Duchefa) supplemented with Gamborg B5 vitamins mixture x 1 (Duchefa) and 1% Sucrose contained in 200 ml flasks. Following 48 hours of vernalization at 4^0C, the flasks where transferred to a growth
10 room providing 16 hours light / 8 hours dark diurnal conditions and incubated for 18 days in an orbit shaker at 20^0C and 80 rpm. Following incubation, the seedlings were treated with four different combinations of NaCl (Merck) AZC (L-Azetidine-2-carboxylic acid; Sigma) through supplementation of their growth media as described in Table 4 below. The treated seedlings were incubated for additional six days under
15 same conditions then comparatively evaluated for treatment induced toxicity.

Table 4
NaCl and AZC combinations

NaCl concentration (mM)	AZC concentration (mM)
0	0
0	1
150	0
150	1

20 ***Results:***

As illustrated in Figures 7a-d, no visible toxicity was observed in the seedlings treated with salt only (150 mM NaCl, no AZC) or in the untreated control (no NaCl, no AZC). Treatment with AZC only (1 mM AZC, no NaCl) resulted in a moderate chlorosis and growth inhibition, as compared to treatments lacking AZC
25 (no NaCl, no AZC; and 150 mM NaCl, no AZC). The combined treatment of AZC and NaCl (1 mM AZC, 150 mM NaCl) resulted in a complete chlorosis and a substantial growth inhibition, as compared with AZC only treatment.

Overall, the results shown in the Examples hereinabove clearly demonstrate that proline analogs, such as AZC and T4C, are toxic to plants having
30 downregulated PDh, such as the case with normal plants grown under stress. On the

other hand, mutants of the signal induced pathway are incapable of downregulating PDh and therefore would not be sensitive to the toxic proline analogs.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications and GenBank Accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application or GenBank Accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

REFERENCES

(Additional references are cited hereinabove)

1. Bucher M, Schroeer B, Willmitzer L, Riesmeier JW 1997 Two genes encoding extension-like proteins are predominantly expressed in tomato root hair cells. Plant Mol Biol. 35:497-508.

2. Forlani G, Scainelli D, Nielsen E 1997 Delta1-pyrroline-5-carboxylate dehydrogenase from cultured cells of potato. Plant Physiol. 113: 1413-1418.

3. Ginzberg I, Stein H, Kapulnik Y, Szabados L, Strizhov N, Schell J, Koncz C, Zilberstein A 1998 Isolation and characterization of two different cDNAs of DELTA1-pyrroline-5-carboxylate synthase in alfalfa, transcriptionally induced upon salt stress. Plant Molecular Biology 38: 755-764.

4. Hare PD, Cress WA 1997 Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation 21: 79-102.

5. Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K 1999 Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol. 17: 287-91.

6. Kiyosue T, Yoshiba Y, Yamaguchi-Shinozaki K, Shinozaki K 1996 A nuclear gene, encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *Arabidopsis*. The Plant Cell 8: 1323-1335.

7. Peng Z, Lu Q, Verma DP 1996 Reciprocal regulation of delta1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes control proline level during and after osmotic stress in plants. Mol. Gen. Genet. 253: 334-341.

8. Roosens NHCJ, Thu TT, Iskander HM, Jacobs M 1998 Isolation of the ornithine-delta-aminotransferase cDNA and effect of salt stress on its expression in *Arabidopsis thaliana*. Plant Physiol. 117: 263-271.

9. Ruiz JM, Sanchez E, Garcia PC, Lopez-Lefebre LR, Rivero RM, Romero L 2002 Proline metabolism and NAD kinase activity in greenbean plants subjected to cold-shock. Phytochemistry. 59:473-478.

10. Shichiri M, Hoshikawa C, Nakamori S, Takagi H 2001 A novel acetyltransferase found in Saccharomyces cerevisiae Sigma1278b that detoxifies a proline analogue, azetidine-2-carboxylic acid. J Biol Chem. 276: 41998-2002.

11. Stein, H., Zilberstein, A., Miller, G., and Kapulnik, Y 2000 Plants tolerant of environmental stress conditions, methods of generating same and novel polynucleotide sequence utilized thereby. Patent application No. 09/490,454 submitted to the United States patent office.

12. Strizhov N, Abraham E, Okresz L, Blickling S, Zilberstein A, Schell J, Koncz C, Szabados L 1997 Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis*. Plant J. 12: 557-569.

13. Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K 1997 Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol. 38: 1095-1102.

WHAT IS CLAIMED IS:

1. A method of identifying an organism having a mutated signal transduction pathway, comprising:

(a) exposing a plurality of organisms to a compound capable of:

(i) negatively affecting physiology and/or morphology of an organism having a normal signal transduction pathway; and

(ii) not substantially affecting physiology and/or morphology of the organism having the mutated signal transduction pathway; and

(b) identifying an organism of said plurality of organisms not substantially effected by said compound, thereby identifying the organism having the mutated signal transduction pathway.

2. The method of claim 1, wherein the organism is a plant.

3. The method of claim 1, wherein step (a) further includes exposing said plurality of organisms to a stress condition.

4. The method of claim 3, wherein said stress condition is an abiotic stress condition.

5. The method of claim 4, wherein said abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

6. The method of claim 3, wherein said stress condition is a biotic stress condition.

7. The method of claim 6, wherein said biotic stress condition is an exposure to a pathogen.

8. The method of claim 7, wherein said pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

9. The method of claim 6, wherein said biotic stress condition is an exposure to a pest.

10. The method of claim 9, wherein said pest is an insect or a mite.

11. The method of claim 1, wherein said normal signal transduction pathway downregulates an activity or expression of an enzyme in the organism.

12. The method of claim 11, wherein said enzyme is proline dehydrogenase.

13. The method of claim 1, wherein said compound is a proline analogue.

14. The method of claim 13, wherein said proline analogue is L-Thiazolidine-4-carboxylic acid.

15. The method of claim 13, wherein said proline analogue is, L-Azetidine-2-carboxylic acid.

16. The method of claim 1, wherein said normal signal transduction pathway upregulates an activity or expression of an enzyme in the organism.

17. A method of identifying a stress inducible gene promoter, comprising:

(a) genetically modifying a plurality of organisms to include a randomly inserted reporter gene;

(b) exposing said plurality of organisms resulting from step (a) to a stress condition;

(c) exposing the organisms resulting from step (b) to a compound capable of:

(i) negatively affecting physiology and/or morphology of an organism having a normal signal transduction pathway; and

(ii) not substantially affecting physiology and/or morphology of an organism having a mutated signal transduction pathway generated by insertion of said reporter gene;

(d) selecting organisms not being substantially affected by said compound;

(e) selecting from the organisms resulting from step (d) at least one organism expressing said reporter gene; and

(f) identifying from the at least one organism resulting from step (e) a polynucleotide sequence positioned upstream of said reporter gene, thereby identifying the stress inducible gene promoter.

18. The method of claim 17, wherein said reporter gene is selected from the group consisting of genes encoding for β-galactosidase, β-glucuronidase, luciferase, chloramphenicol acetyltransferase, nopaline synthase, green fluorescent protein, red fluorescent protein and blue fluorescent protein.

19. The method of claim 17, wherein said organisms are plants.

20. The method of claim 17, wherein said stress condition is an abiotic stress condition.

21. The method of claim 20, wherein said abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

22. The method of claim 17, wherein said stress condition is a biotic stress condition.

23. The method of claim 22, wherein said biotic stress condition is an exposure to a pathogen.

24. The method of claim 23, wherein said pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

25. The method of claim 22, wherein said biotic stress condition is an exposure to a pest.

26. The method of claim 25, wherein said pest is an insect or a mite.

27. The method of claim 17, wherein said compound is a proline analogue.

28. The method of claim 27, wherein said proline analogue is L-Thiazolidine-4-carboxylic acid.

29. The method of claim 27, wherein said proline analogue is, L-Azetidine-2-carboxylic acid.

30. A method of identifying a stress signal transduction gene, comprising:

(a) mutating a plurality of organisms;

(b) exposing the mutated organisms resulting from step (a) to a stress condition;

(c) exposing the mutated organisms resulting from step (b) to a compound capable of:

(i) negatively affecting physiology and/or morphology of an organism having a normal signal transduction pathway; and

(ii) not substantially affecting physiology and/or morphology of an organism having a mutated signal transduction pathway;

(d) selecting mutated organisms not being substantially affected by said compound; and

(e) identifying a mutated gene from the mutated organisms resulting from step (d), thereby identifying said stress signal transduction gene.

31. The method of claim 30, wherein said mutation is effected by a random insertion of T-DNA into a genome of said organisms.

32. The method of claim 30, wherein said mutation is effected by a random insertion of a transposable element into a genome of said organisms.

33. The method of claim 30, wherein said mutation is effected by exposing said organisms to ethylmethane sulfonate.

34. The method of claim 30, wherein said organisms are plants.

35. The method of claim 30, wherein said stress condition is an abiotic stress condition.

36. The method of claim 35, wherein said abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

37. The method of claim 30, wherein said stress condition is a biotic stress condition.

38. The method of claim 37, wherein said biotic stress condition is an exposure to a pathogen.

39. The method of claim 38, wherein said pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

40. The method of claim 37, wherein said biotic stress condition is an exposure to a pest.

41. The method of claim 40, wherein said pest is an insect or a mite.

42. The method of claim 30, wherein said ›mpound is a proline analogue.

43. The method of claim 42, wherein said proline analogue is L-Thiazolidine-4-carboxylic acid.

44. The method of claim 42, wherein said proline analogue is, L-Azetidine-2-carboxylic acid.

45. A stress indicator organism having a mutated signal transduction pathway responsive to a stress condition.

46. The stress indicator organism of claim 45, wherein said organism is a plant.

47. The stress indicator organism of claim 46, wherein a T-DNA is inserted in a genome of said plant.

48. The stress indicator organism of claim 46, wherein a transposable element is inserted in a genome of said plant.

49. A method of detecting a stress condition in an agricultural environment, comprising:

(a) cultivating a stress indicator plant having a mutated signal transduction pathway responsive to the stress condition in the agricultural environment; and

(b) observing an abnormal physiology and/or morphology of said stress indicator plant thereby detecting said stress condition in said agricultural environment.

50. The method of claim 49, wherein said agricultural environment is a commercial field.

51. The method of claim 49, wherein step (a) includes cultivating a plurality of normal plants.

52. The method of claim 49, wherein said abnormal physiology and/or morphology is selected from the group consisting of a reduced growth, chlorosis, necrosis and wilt.

53. The method of claim 49, wherein said stress condition is an abiotic stress condition.

54. The method of claim 53, wherein said abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

55. The method of claim 49, wherein said stress condition is a biotic stress condition.

56. The method of claim 55, wherein said biotic stress condition is an exposure to a pathogen.

57. The method of claim 56, wherein said pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

58. The method of claim 55, wherein said biotic stress condition is an exposure to a pest.

59. The method of claim 58, wherein said pest is an insect or a mite.

60. The method of claim 49, wherein said compound is a proline analogue.

61. The method of claim 60, wherein said proline analogue is L-Thiazolidine-4-carboxylic acid.

62. The method of claim 60, wherein said proline analogue is, L-Azetidine-2-carboxylic acid.

63. A method of identifying an agent capable of protecting an organism from a stress condition, comprising:

(a) generating an organism exhibiting high sensitivity to the stress condition;

(b) exposing said organism to a plurality of molecules;

(c) exposing said organism to the stress condition; and

(d) identifying a molecule from said molecules capable of conferring tolerance to the stress condition to said organism, thereby identifying the agent capable of protecting the organism from said stress condition.

64. The method of claim 63, wherein step (a) is effected by a random insertion of T-DNA into a genome of said organisms.

65. The method of claim 63, wherein step (a) is effected by a random insertion of a transposable element into a genome of said organisms.

66. The method of claim 63, wherein step (a) is effected by exposing said organisms to ethylmethane sulfonate.

67. The method of claim 63, wherein said stress condition is an abiotic stress condition.

68. The method of claim 67, wherein said abiotic stress condition is selected from the group consisting of a salinity, a drought, a flood, a frost, a suboptimal temperature, a suboptimal nutrition, a toxic pollution, a UV irradiation and a mechanical injury.

69. The method of claim 63, wherein said stress condition is a biotic stress condition.

70. The method of claim 69, wherein said biotic stress condition is an exposure to a pathogen.

71. The method of claim 70, wherein said pathogen is selected from the group consisting of a virus, a bacterium, a fungus and a nematode.

72. The method of claim 69, wherein said biotic stress condition is an exposure to a pest.

73. The method of claim 72, wherein said pest is an insect or a mite.

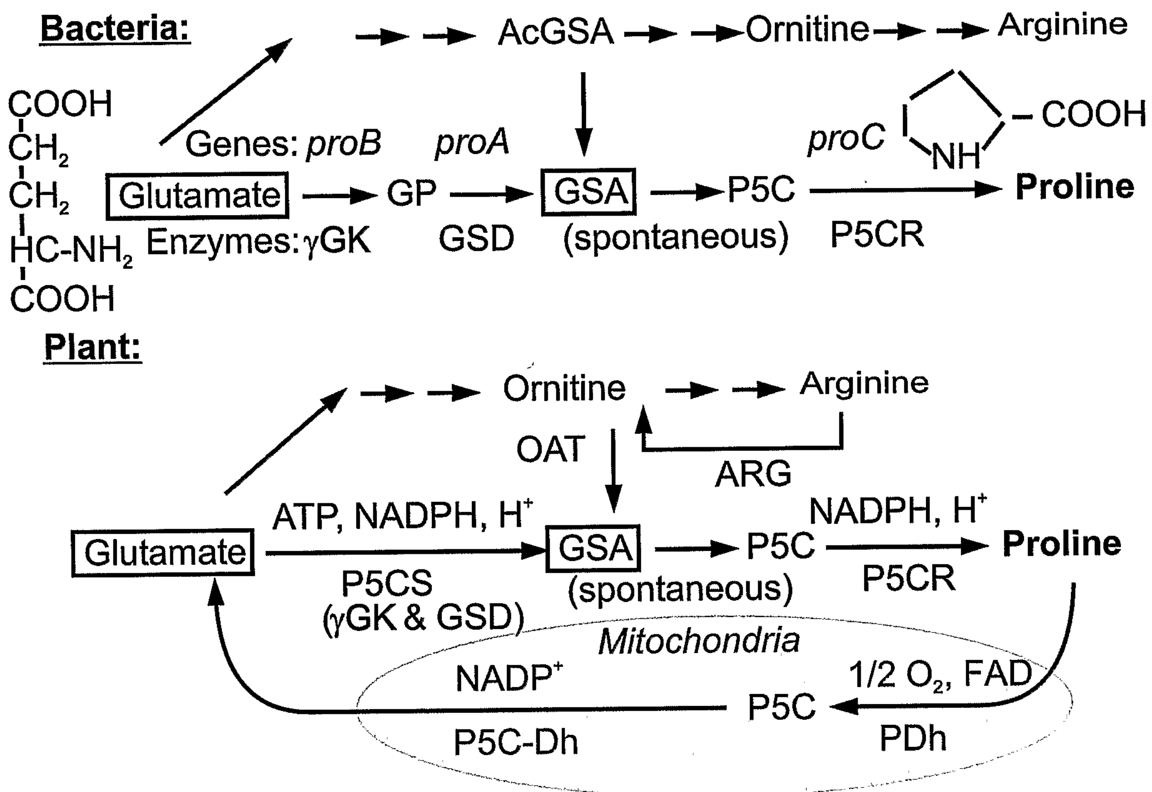

Fig. 1

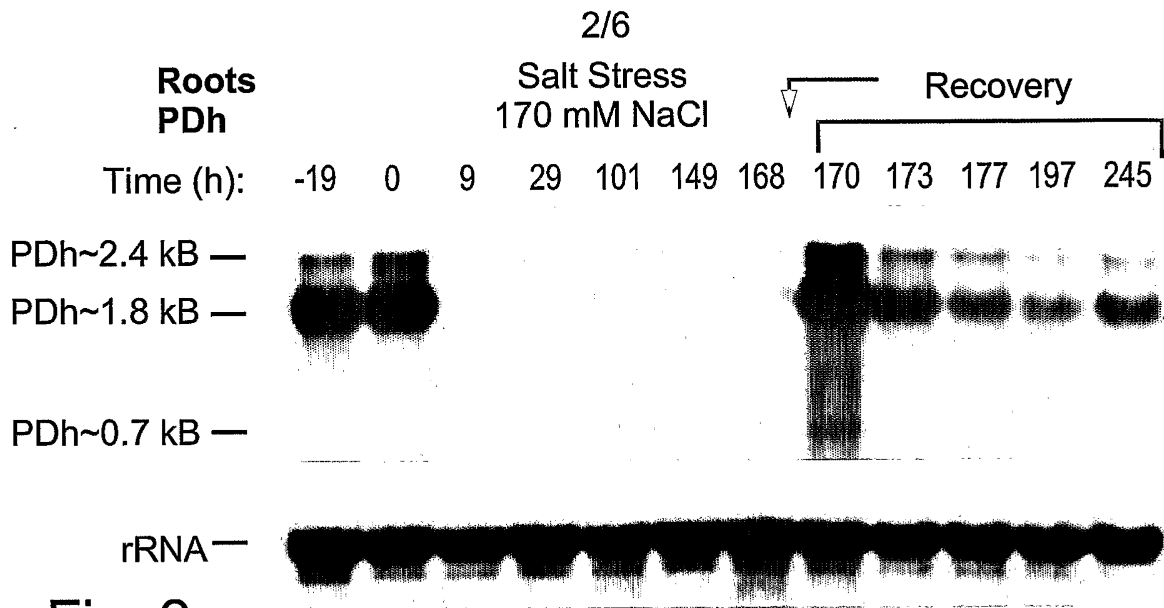

Fig. 2a

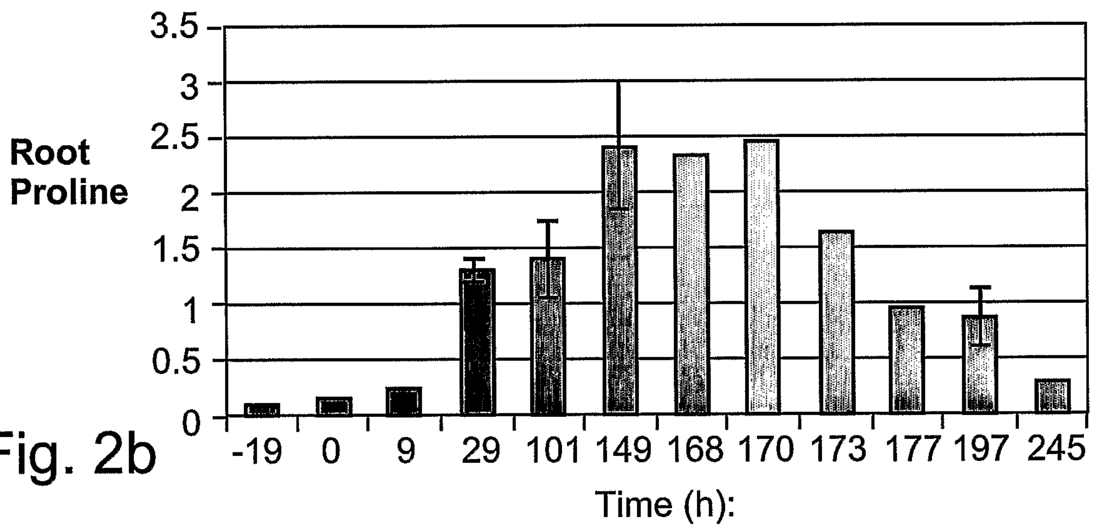

Fig. 2b

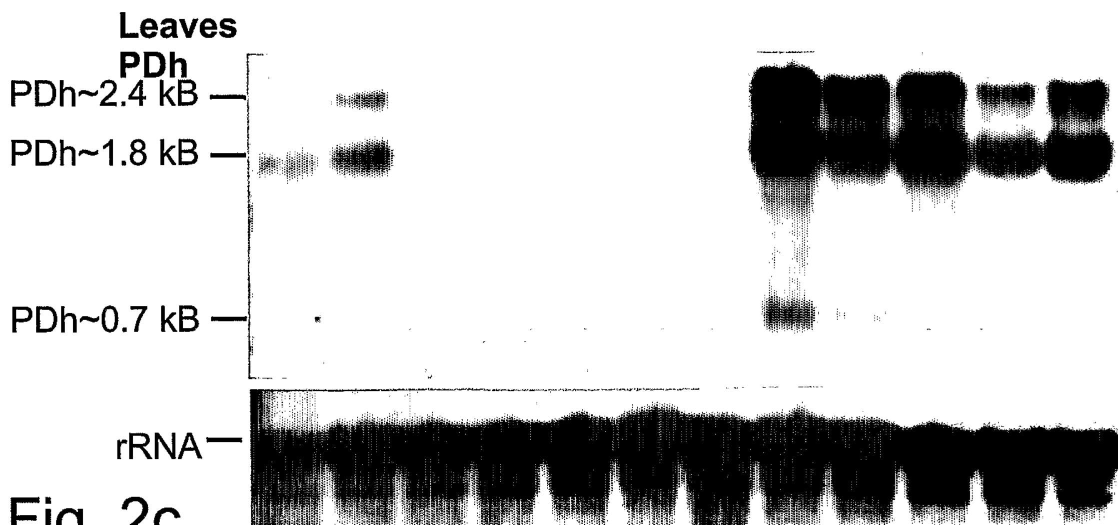

Fig. 2c

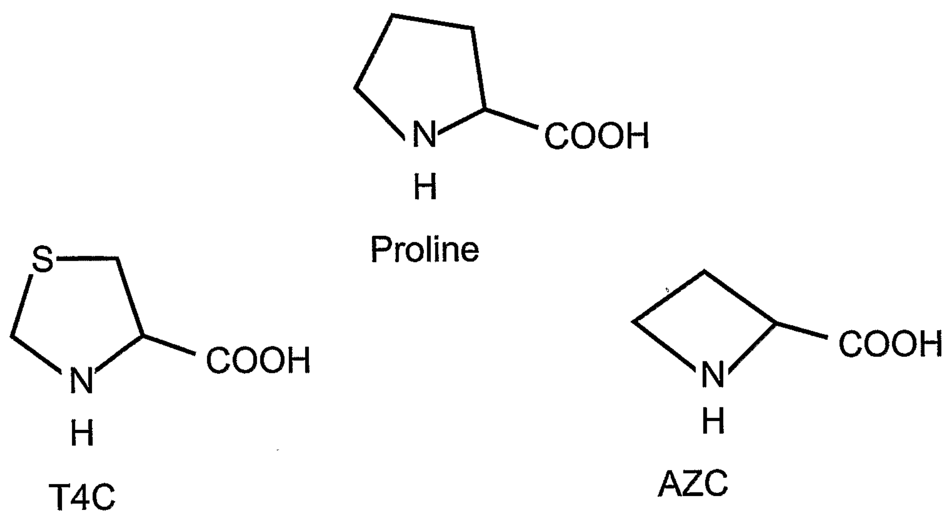

Fig. 3

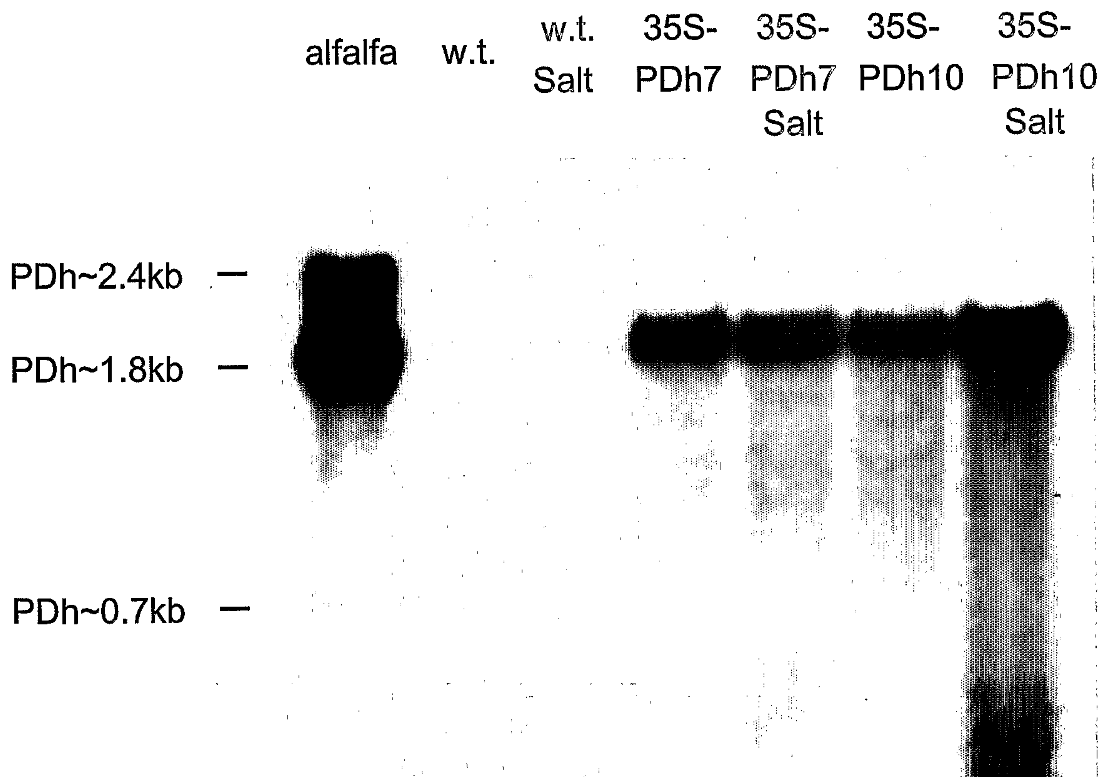

Fig. 4

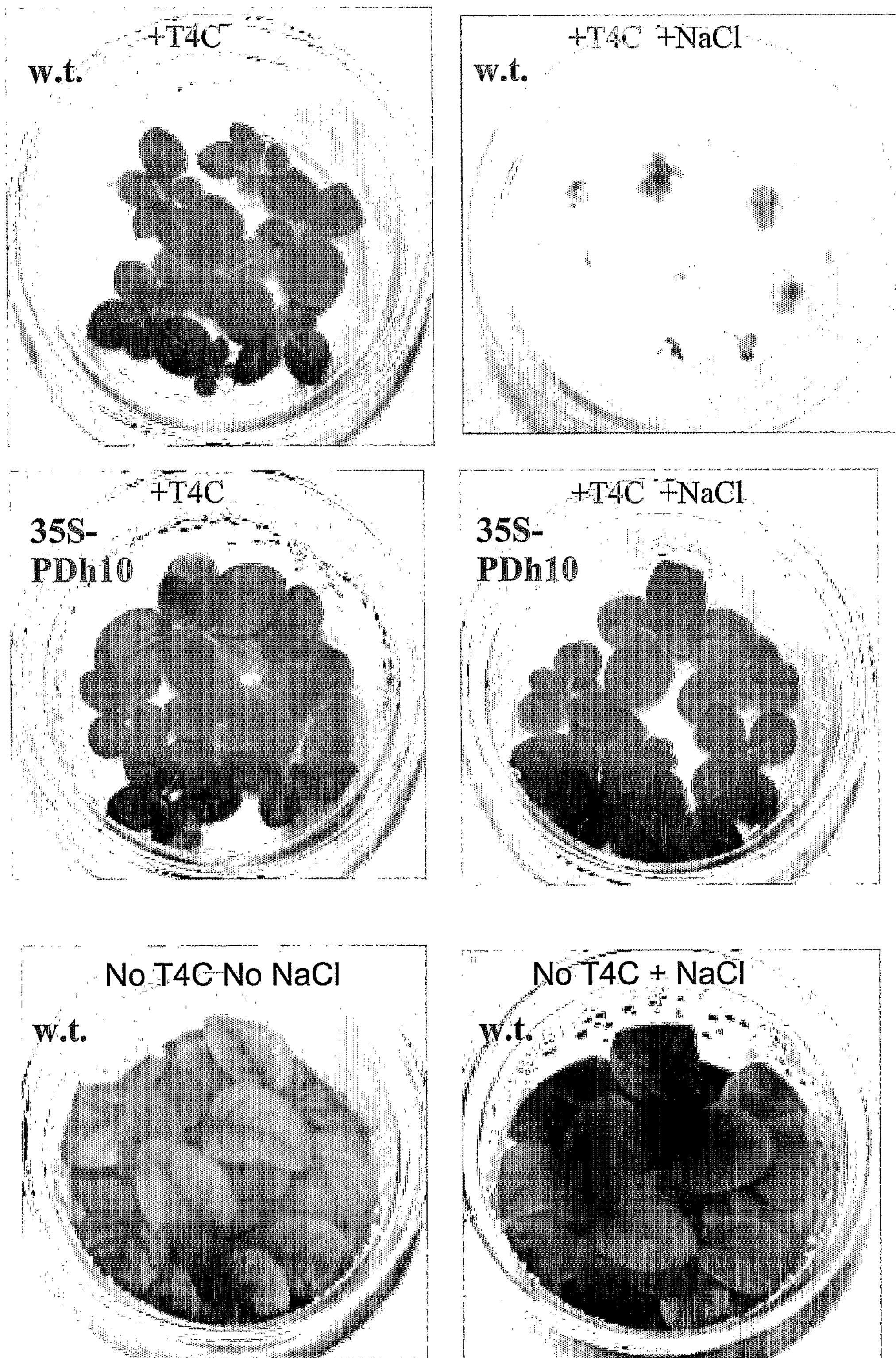

Fig. 5

M.S. + 1.5mM AZC + 0.5mM proline

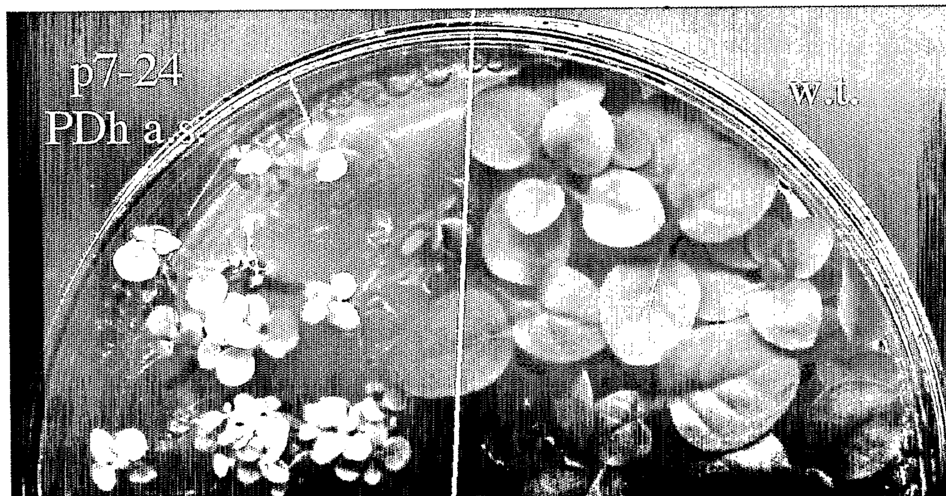

Fig. 6a

M.S. + 1.5mM AZC + 0.5mM proline + 50 mM Na Cl

Fig. 6b

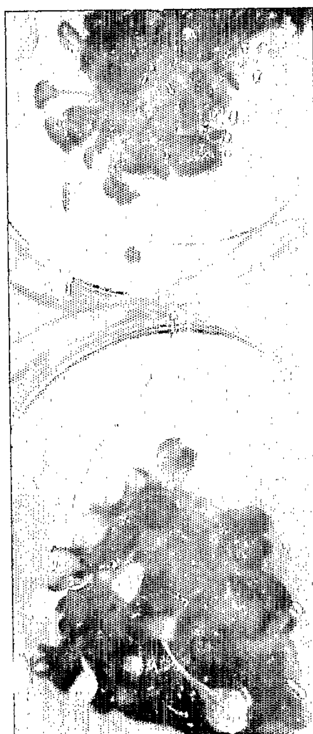

Fig. 7a
No NaCl , No AZC

No NaCl , AZC 1mM
Fig. 7c

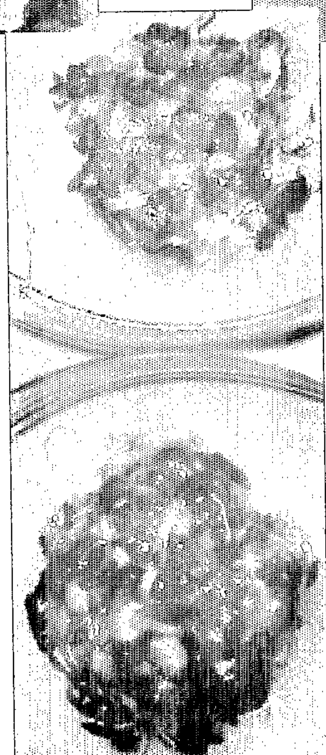

Fig. 7b
NaCl 150 mM, No AZC

NaCl 150 mM, AZC 1mM
Fig. 7d

SEQUENCE LISTING

<110> Stein, Hanan
Zilberstein, Aviah
Miller, Gadi

<120> A METHOD OF IDENTIFYING ORGANISMS HAVING A MUTATED SIGNAL TRANSDUCTION PATHWAY

<130> 27166

<160> 2

<170> PatentIn version 3.2

<210> 1
<211> 2596
<212> DNA
<213> Medicago sativa

<220>
<221> misc_feature
<223> A 2.6kb fragment representing the full length of alfalfa PDh gene starting from the predicted transcription start site, ending in the polyadenylation site

<400> 1
```
aggtcaagaa gaaaaaaaaa taatcaaagg aaactggttt tttattcatc atatcaaaac      60

atatatcttc gtgtttatat catatagatc atctttgacc ggttgatggc caccagagta     120

atcccaccaa gaattctaag gaaactccga tacaacaccg ccacaaaacc cttccaacct     180

gcccttacct cgcccgctct cgctcctacg tctaatattt tagaccaaaa accgccatca     240

tcgacaacaa ccctcctccc tcccgacgcc gagctcaact tccacgacgt cgaaaaactc     300

ttttcgcacg tcccaactac caaacttctc aaatcaactg ccatcctcca tgctaccgcg     360

gtcgagccaa tggttgacct cggtacatgg atgttgaggt ctgatctcat gcagaccaat     420

aatcctttac gtaacattgc tatggctacc acacgtgcca ctttttcga tcatttttgt      480

gccggagaag atgctatcac cgctggaaaa agtattgccg ggttgaatga agccggttta     540

cgtggaatgc tggtttacgg cgttgaagat gctcatgata acgctggctg tgatcgcaat     600

ctcaaaggtt ttcttcacac cgttgatgtc agcagatcgc ttcctccatc ttcggtaatc     660

tcttctttat ttcatctctt tatttcaaaa tgtaactgtt ttcttttttt gctttttttgg    720

ttcggtttct tcattgtcac agaagagaga tgcaatctgc tttaatggat ccaaattcaa     780

ttgagaataa ccgttttcac ggtgccgagt tcggcattcg tgactgcaac cgcaatttaa     840

tacagcacta ctgatgacgt tgttgctgct gatgtgatgt gacgtgacgt atctggtgtc     900

cgtgtctgtg tctgattcaa tggctcaagg agggaatgga aaagaaaact aagcatggaa     960

caatggaaat tggaatttga ttcatgaaaa tcaatcaaag catggattgg aacaatggaa    1020

atgggaatgg gaattggaac aaacaaacaa ttgtttttgg taaaagattc tcaagcacta    1080

ataagccaca cagaaaaaaa aactgtggtt gctggtgcat ttttaatact tttttaaaa     1140
```

```
ttatgtaaaa aaaattaggt tatgaatttg agtggttgtt ttaattttaa gtttaatatt   1200

atcattatga atgttatgta ctaatgtttt caaatttaaa ctttttctg tgtataggta    1260

agctttgtga ttgtgaagat tactgcaata tgtccaatga gtttgcttga aagaatgagt   1320

gatttgctga gatggcagaa gaaagaccct tcatttgttt taccatggaa gcaagattca   1380

ttgccaattt tctctgagtc aagtcctttg taccatacaa ggaagagacc agagccatta   1440

acagcagaag aagagagtga tcttgatctt gctaacaaga gattccttga gctttgtcag   1500

aaatgtgtgc aagccaatat tccattattg gttgatgctg aacatacttc agttcaacct   1560

gctattgatt actttactta ctcttctgct attatgcata acaaaggtga aaaccctatt   1620

gtgtttggaa cccttcagac ttatttgaaa gatgctaagg agagaatgtt gttggcatca   1680

aaggctgctg agaaaatggg gataccaatg ggatttaagt tggttagagg tgcttatatg   1740

tctagtgaaa gaaaattggc tgctgatttg ggttatgctt ctccaattca taacactatt   1800

aaggatacac ataagtgttt caatgattgt tcaaattaca tgcttgagaa gattgctaat   1860

ggtcctggtg gagttgttct tgcaactcat aacattgaat caggtatata ttatatgata   1920

ttatgataat gatttatatt aatgtatgca attgaggggt atgagtttga tttaattaat   1980

gatttttgta taggaaaatt ggctgctgca aaagcacatg aattagggat tggaaaggtg   2040

aaccataaga tggaatttgc acaactatat ggaatgtctg aggcactatc ttttggttta   2100

agcaatgcag ggtttcaagt tagcaagtat atgccatttg gtcctgtgga gactgttatg   2160

ccatacctct tgagaagggc tgaggagaat agaggagtgt tggctgcatc aggctttgac   2220

aggcaactga tgaggtaaaa atttcaaaaa aatttaccat ttcatattta ctcgttaatt   2280

ttgtgtgatt ctgtttctga cattgaattt gttttgtgta acaggaagga gttggtcagg   2340

agagtaaaag cttctgtgct ttaaatttgt tggatgagtt gatgggatgt aataatgtag   2400

gcaacagggt tcaccattct gtgtacaaat tagagagaat cctgtaattt gcttaaattt   2460

tgtgcattca agttaatgaa atgtgcattt gtcttaagtg tattgatcca catttccact   2520

tgtttgtaca ttaatgccat tgaattgttt aagaaattgt tattcataaa gtgtcttatg   2580

tgtttgaggt gattcc                                                   2596
```

<210> 2
<211> 1476
<212> DNA
<213> Medicago sativa

<400> 2

```
atggccacca gagtaatccc accaagaatt ctaaggaaac tccgatacaa caccgccaca     60

aaacccttcc aacctgccct tacctcgccc gctctcgctc ctacgtctaa tattttagac    120

caaaaaccgc catcatcgac aacaaccctc ctccctcccg acgccgagct caacttccac    180

gacgtcgaaa aactcttttc gcacgtccca actaccaaac ttctcaaatc aactgccatc    240

ctccatgcta ccgcggtcga gccaatggtt gacctcggta catggatgtt gaggtctgat    300
```

```
ctcatgcaga ccaataatcc tttacgtaac attgctatgg ctaccacacg tgccactttt    360
ttcgatcatt tttgtgccgg agaagatgct atcaccgctg gaaaaagtat tgccgggttg    420
aatgaagccg gtttacgtgg aatgctggtt tacggcgttg aagatgctca tgataacgct    480
ggctgtgatc gcaatctcaa aggttttctt cacaccgttg atgtcagcag atcgcttcct    540
ccatcttcgg taagctttgt gattgtgaag attactgcaa tatgtccaat gagtttgctt    600
gaaagaatga gtgatttgct gagatggcag aagaaagacc cttcatttgt tttaccatgg    660
aagcaagatt cattgccaat tttctctgag tcaagtcctt tgtaccatac aaggaagaga    720
ccagagccat taacagcaga agaagagagt gatcttgatc ttgctaacaa gagattcctt    780
gagctttgtc agaaatgtgt gcaagccaat attccattat tggttgatgc tgaacatact    840
tcagttcaac ctgctattga ttactttact tactcttctg ctattatgca taacaaaggt    900
gaaaacccta ttgtgtttgg aacccttcag acttatttga aagatgctaa ggagagaatg    960
ttgttggcat caaaggctgc tgagaaaatg ggataccaa tgggatttaa gttggttaga    1020
ggtgcttata tgtctagtga aagaaaattg gctgctgatt tgggttatgc ttctccaatt    1080
cataacacta ttaaggatac acataagtgt ttcaatgatt gttcaaatta catgcttgag    1140
aagattgcta atggtcctgg tggagttgtt cttgcaactc ataacattga atcaggaaaa    1200
ttggctgctg caaaagcaca tgaattaggg attggaaagg tgaaccataa gatggaattt    1260
gcacaactat atggaatgtc tgaggcacta tcttttggtt taagcaatgc agggtttcaa    1320
gttagcaagt atatgccatt tggtcctgtg gagactgtta tgccatacct cttgagaagg    1380
gctgaggaga atagaggagt gttggctgca tcaggctttg acaggcaact gatgaggaag    1440
gagttggtca ggagagtaaa agcttctgtg ctttaa                              1476
```