

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
15 March 2007 (15.03.2007)

PCT

(10) International Publication Number
WO 2007/029955 A1

(51) International Patent Classification:
C07K 14/415 (2006.01)

(21) International Application Number:
PCT/KR2006/003514

(22) International Filing Date:
5 September 2006 (05.09.2006)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
10-2005-0082044
5 September 2005 (05.09.2005) KR

(71) Applicant (for all designated States except US): **INDUSTRY-ACADEMIC COOPERATION FOUNDATION GYEONGSANG NATIONAL UNIVERSITY** [KR/KR]; 900, Gajwa-dong, Jinju-si, Gyeongsangnam-do 660-701 (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **HAN, Chang-Deok** [KR/KR]; 109-803 Kumho Seokryumaeul Apt., Juyak-dong, Jinju-si, Gyeongsangnam-do 660-770 (KR). **EUN, Moo Young** [KR/KR]; 111-303 Daewoo Apt., Mae-sanno 2-ga, Paldal-gu, Suwon-si, Gyeonggi-do 442-783

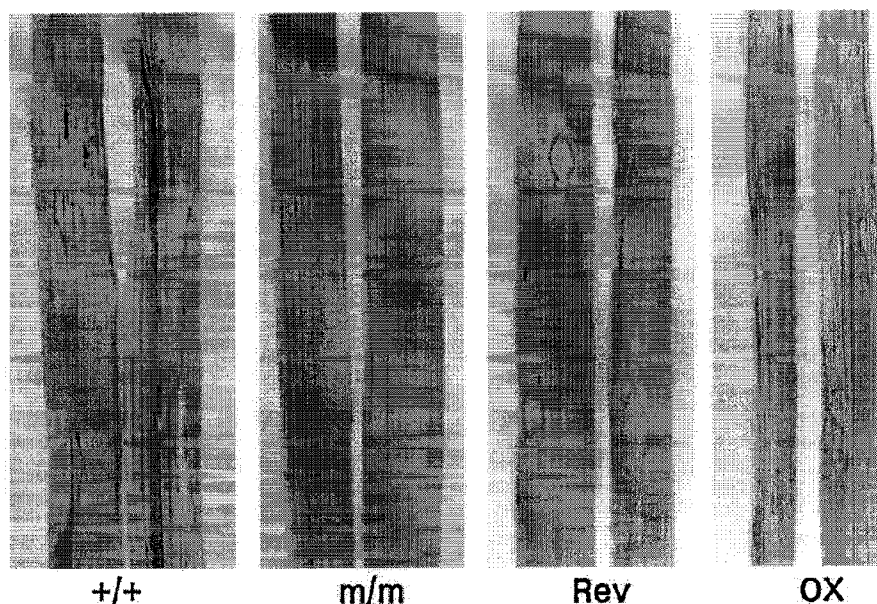
(KR). **YUN, Doh Won** [KR/KR]; 127-304 Hanil Town, Jowon-dong, Jangan-gu, Suwon-si, Gyeonggi-do 440-709 (KR). **NAM, Min-Hee** [KR/KR]; 101-1503 Nobyeon Dae-baek Apt., Nobyeon-dong, Suseong-gu, Daegu 706-751 (KR). **YI, Gihwan** [KR/KR]; 304-203 Daewoo Apts., Sammun-dong, Miryang-si, Gyeongsangnam-do 627-750 (KR). **HAN, Seong Sook** [KR/KR]; 104-502 LD Kolon Apt., Guun-dong, Gwonseon-gu, Suwon-si, Gyeonggi-do 441-734 (KR). **LEE, Jeung Joo** [KR/KR]; 113-1404 DeulmalHeunghan Town, Pyeonggeo-dong, Jinju-si, Gyeongsangnam-do 660-776 (KR). **PIAO, Hai Long** [CN/KR]; 302-705 Jugong Apt., Gajwa-dong, Jinju-si, Gyeongsangnam-do 660-789 (KR). **PARK, Soo Hyun** [KR/KR]; 1-1 Jungan-dong, Jinju-si, Gyeongsangnam-do 660-010 (KR).

(74) Agent: **KIM, Won Joon**; 205, Gold Venture Tower, 386, Mannyeon-dong, Seo-gu, Daejeon 302-834 (KR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU,

[Continued on next page]

(54) Title: A NEW GENE RELATED TO STRESSES AND A METHOD FOR PRODUCING A STRESS-RESISTANT PLANT USING THE SAME



(57) Abstract: The present invention relates to a protein associated with stress resistance, a gene encoding the same, a method of imparting stress resistance to a plant using the gene, and a plant constructed by the method. The invention provides a protein, which is involved in the deposition of callose (1→3 β glucan) and has an amino acid sequence showing a homology of at least 90% to SEQ ID NO: 1. Also, it provides a gene encoding the protein, a plant in which the gene was inhibited, and a transformed plant in which the gene was over expressed. According to the invention, it is possible to produce a plant having reduced callose deposition and improved stress resistance by inhibiting the expression of an OsCIPK gene. Also, it is possible to improve the stress resistance of plants by inducing the mutation of OsCIPK through various mutagens.

WO 2007/029955 A1



LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

- (84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,

RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

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.

【DESCRIPTION】**【Invention Title】**

A NEW GENE RELATED TO STRESSES AND A METHOD FOR PRODUCING A STRESS-RESISTANT PLANT USING THE SAME

【Technical Field】

The present invention relates to a protein involved in stress resistance, a gene encoding the same, a method for imparting stress resistance to a plant using the gene, and a plant constructed by the method.

【Background Art】

Plants undergo various environmental stresses during their life cycle, and the environmental stresses adversely affect the growth of plants. Plants induce positive resistant responses to cope with a changing environment, the most interesting of which is the induction of the expression of various defense-related genes.

It was found that, if plants are, for example, wounded, undergo low-and high-temperature stresses, are attacked by pathogens such as viruses or microorganisms, are exposed to heavy metals, particularly aluminum, (1→3) β -glucan (called "callose" will be deposited on the cell wall (Stone, B. A. and Clarke, A. E., 1992, "The Chemistry and Biology of (1,3)-[β -Glucans]" La Trobe University Press, Australia; Samuels, A. L., Giddings, T.H. Jr. and Staehelin. L.A., 1995, J. Cell Biol. 130: 1345-1357; Brown, R.C., Lemmon, B.E., Stone, B.A. and Olsen, O.A., 1997, Planta 202: 414-426). Such phenomena occur because callose synthase is activated due to an increase in intracellular Ca^{++} concentrations, and it is known that the produced callose is an important substance rendering plants resistant to stresses, diseases or heavy metals (Stone, B. A. and Clarke, A. E., 1992, "The Chemistry and Biology of (1,3)-[β -Glucans]" La Trobe University Press, Australia; Granados G, Pandey S and Ceballos H., 1993, Crop Sci., 33: 936-940; Staß A and Horst W.J., 1995, Plant Soil, 171: 89-103; Taylor G, 1995, J. Plant Soi,

171, 111-118). However, how the activity of callose synthase is regulated by such internal and external stimulations to produce and deposit callose is not yet found.

About 31 similar *OsCIPK* (*Oryza sativa* Calcineurin B-like protein interacting protein kinase) genes are present in a rice plant, and are phosphoprotein genes which bind to calcinurin B-like genes in cells according to a change in intracellular Ca^{++} concentrations (Uner Kolukisaoglu et al., 2004, Plant Physiology, 134: 43-58; Albrecht et al., 2001, EMBO J 20: 1051-1063). However, the function of these genes, whether these genes are associated with stress resistance, the signal transduction pathway of these genes in connection with the expression of stress resistance, if any, and precise mechanisms by which these genes confer resistance, are largely unknown.

The present inventors have conducted studies to investigate genes associated with the environmental stress resistance of plants and, as a result, found that *OsCIPK9* among *OsCIPK* genes present in a rice plant has a function related to the production or deposition of callose in cells, thereby completing the present invention.

【Disclosure】

【Technical Problem】

It is an object of the present invention to provide a gene involved in the environmental stress of plants, and a protein encoding the gene.

Another object of the present invention is to provide a stress-resistant plant in which a gene involved in plant stress resistance was mutated.

【Technical Solution】

To achieve the above objects, the present invention provides a protein, which functions is related to the accumulation of callose in a plant and has an amino acid sequence showing a homology of at least 90% to SEQ ID NO: 1,

and a gene encoding the protein. The gene and protein according to the present invention may be an *OsCIPK* gene derived from a rice plant, and an *OsCIPK9* gene of SEQ ID NO: 2 or an *OsCIPK9* gene cDNA of SEQ ID NO: 4.

Also, the present invention provides an expression vector for plants, comprising the gene. For example, the expression vector may be expression vector pCAMBIA-CIPK9.

Furthermore, the present invention provides a method for inducing the production and deposition of callose in a plant by transforming the plant with said expression vector, and a stress-resistant gene constructed by the method.

As described above, the present invention has been made by first finding that a rice-derived *OsCIPK9* gene, the function or role of which has not been found in the prior art, regulates the production and deposition of callose. Specifically, the present inventors obtained a plant mutant through the *Ac/Ds* tagging system (Chin HG et al., 1999 Plant J 19, 615-623; Kim et al., 2004 Plant J 39: 252-263), which is an insertional mutation system in rice, developed by the present inventors. Also, the present inventors analyzed the characteristic of each of the obtained plant mutant. The *Ac/Ds* tagging system was constructed by genetically manipulating *As* and *Ds*, transposable elements of maize, and then inserting the genetically manipulated elements into the genome of rice by transformation. This system was constructed, considering the fact that *Ds* undergoes transposition when *Ac* exists in the same genome. Also, said system was so designed that a reporter gene is placed in *Ds*, such that, when *Ds* is inserted around or into a gene on a chromosome, the reporter gene is expressed by a host gene. The tagging system is a highly potent research method, which can identify the function of genes through mutation and, at the same time, observe gene expression through a reporter gene. Although GUS was used as the reporter gene in said paper and the present invention, any gene can be used as the reporter gene, as long as it is a gene having a suitable marker function.

The inventive mutant (*OsCIPK9::Ds*) obtained using the *Ac/Ds* tagging

system comprise *Ds* inserted into the *OsCIPK9* gene. Through studies on said mutant, the present inventors have found that the *OsCIPK9* gene is a gene involved in the accumulation of callose. Then, the present inventors have found that the mutant has resistance to a disease (rice blight) and a herbicide (Paraquat (Gramoxone)), and a plant transformed with an overexpression vector containing the *OsCIPK9* gene deposits a large amount of callose, but does not obtain resistance to disease and herbicide, thereby completing the present invention.

Hereinafter, each step of the present invention will be described in detail.

(1) Selection of specific mutant, and analysis of expression of mutated gene and mutant

Large amounts of rice mutants were generated using the *Ac/Ds* tagging system, an insertional mutation system developed by the present inventors, and mutants showing a specific phenotype were analyzed. A GUS coding region is present in *Ds* of the *Ac/Ds* tagging system. Thus, in the mutants obtained using said system, GUS can be expressed as a fusion transcript with a host gene in which a *Ds* element is inserted. GUS expression can be determined through GUS staining. Also, because the use of said system enables *Ds* flanking DNA to be obtained through TAIL-PCR, a *Ds*-inserted region (gene) on a chromosome can be easily isolated.

In the present invention, among various rice mutants having transposable element *Ds* inserted therein, a mutant showing GUS expression in the vascular system was selected. Then, DNA of the mutant was extracted according to a conventional method, and information on rice genomic DNA having *Ds* located therein was obtained using a TAIL-PCR method. In the TAIL-PCR method used in the present invention, the extracted DNA as a template was subjected to PCR three times using three primers complementary to the terminus of *Ds*, and one AD primer, thereby obtaining rice genomic DNA (*Ds* flanking DNA). On the basis of the *Ds* flanking DNA, SEQ ID NO: 2 for the gene, which was not modified with *Ds*, was obtained through bioinformatics

search and cDNA cloning analysis as described in Example 1 below in detail. The gene of SEQ ID NO: 2 was searched through computer bioinformatic software programs and, as a result, found that the isolated gene is an *OsCIPK9* gene encoding one of phosphoproteins (CIPK: Calcineurin B-like (CBL) protein Interacting Protein Kinases) binding to more than about 31 Calcineurin B-like (CBL) proteins present in rice. The base sequence (SEQ ID NO: 3) of the *Ds* flanking DNA and the base sequence of the gene, which was not modified with *Ds*, were analyzed and, as a result, it was found that the mutant had a 4.7 kb *Ds* element inserted into the 927th base from the ATG (start codon) of the *OsCIPK9* locus. An allele resulting from the insertion of *Ds* was named *OsCIPK9::Ds*.

Example 1 below describes the discovery of the *OsCIPK9* gene, experiments relating to base sequence analysis, and Northern analysis showing that the mutant *OsCIPK9::Ds* does not express *OsCIPK9* mRNA.

(2) Analysis of expression of *OsCIPK9* gene

Whether the analyzed *OsCIPK9* gene is a gene involved in stresses, that is, whether the application of stresses leads to an increase in the expression of the *OsCIPK9* gene, was examined through RNA Northern hybridization.

It was observed that the expression of the gene in wild-type rice plants was low in normal conditions, but when wound stress was applied, the gene was expressed at a very high level. Also, it was observed through GUS staining that *OsCIPK9::Ds* was expressed at a high level through a GUS coding region present in *Ds*, indicating the same results as in the Northern blot analysis.

Example 2 below relates to the analysis of expression pattern of the *OsCIPK9* gene as described above.

(3) Verification of function of gene through *OsCIPK9* mutant (*OsCIPK9::Ds*)

Whether the *OsCIPK9* mutant (*OsCIPK9::Ds*) produces callose upon the application of various stresses was examined. Whether callose is present was

analyzed by adding an aniline blue chemical, which emits fluorescent light through specific binding to callose, to the plant tissue, and observing the plant tissue with UV light (see Sprau. F., 1955, Ber. Dtsch. Bot. Ges. 68:239-246; Schrich, W, 1956, Protoplasma 47:487-530; Hood, M.E., Shew, H.D., 1996, Phytopathology 86:704-708).

(i) It was observed that, upon being wound, in a stamen producing pollens, and upon exposure to heavy metals such as aluminum, a wild-type plant was strongly stained with aniline blue, indicating the deposition of callose, but the mutant did not show any aniline blue fluorescence.

(ii) In order to observe whether the deposition of callose, caused by diseases and insect pests, is determined by *OsCIPK9*, the deposition of callose at a wound site caused by rice blight was examined.

In the wild-type plant, a strong aniline blue stain was observed, whereas, in the mutant plant, an aniline blue stain was not observed in the wound site caused by rice blight, indicating that callose was not deposited in the mutant.

(iii) In order to further examine the deposition of callose, observation was conducted using a callose-specific antibody. The roots of the wild-type plant and the mutant plant were exposed to heavy metal, and then were subjected to immunolocation using the callose antibody. It was found that the wild-type plant had much callose deposited therein, but the mutant plant had less callose deposited therein.

(iv) It was also observed that callose was much produced in a transgenic plant in which *OsCIPK9*, the expression level of which is very low in normal conditions, was overexpressed using an overexpression promoter. Such results are definite experimental demonstrations indicating that *OsCIPK9* plays a very important role in the formation of callose.

In Example 3 below, the mutant plant and the wild-type plant were used to demonstrate that the *OsCIPK9* gene is involved in the production and deposition of callose, and the production of callose in the *OsCIPK9*-overexpressed plant was analyzed.

(4) Resistance of mutant plant to rice blast disease

It was experimentally demonstrated that the *OsCIPK9* mutant ((*OsCIPK9::Ds*) did not deposit callose, and thus acquired resistance to the rice blast fungus that induces diseases in the wild-type plant.

Example 5 below is an experiment showing that the *OsCIPK9* mutant acquires disease resistance which is not present in the wild-type plant.

(5) Acquisition of herbicide paraquat tolerance of mutant plant

Herbicide paraquat has a mechanism by which ROS (radical oxygen species, superoxide, hydroxyl group, hydrogen peroxide, etc.) is over-produced in cells, particularly chloroplasts, to kill the cells.

The tolerance of the *OsCIPK9* mutant (*OsCIPK9::Ds*) to herbicide paraquat was tested and, as a result, it could be seen that the *OsCIPK9* mutant had a remarkably high tolerance to paraquat, compared to the wild-type plant, and thus the growth thereof was normally maintained. The level of MDA (malondialdehyde), a product resulting from radicals, was measured in order to examine the breakdown of unsaturated fatty acids in the cell membrane, caused by ROS. The measurement results showed that a high concentration of lipid oxidation product MDA was produced in the wild-type plant, but was not substantially produced in the *OsCIPK9* mutant.

Such results show that the *OsCIPK9* mutant has strong tolerance to active oxygen.

Example 6 below is an experiment showing that the *OsCIPK9* mutant acquires herbicide paraquat resistance which is not present in the wild-type plant.

【Advantageous Effects】

As described above, according to the present invention, the stress resistance of plants can be improved by inducing *OsCIPK9* mutation through various mutagens, including insertional mutagens (T-DNA, transposable elements), chemical mutagens (MMS (methyl methane sulfonate), EMS (ethyl methane sulfonate), dDES (diethylsulfate), NTG or NG (nitrosoguanidine)) and

physical mutagens (UV, X-ray or gamma-ray).

【Description of Drawings】

FIG. 1 is a conceptual diagram showing the progression of TAIL-PCR applied in the present invention.

FIG. 2 is an electrophoresis photograph of TAIL-PCR products.

FIG. 3 shows the chromosomal structure of an *OsCIPK9::Ds* allele.

FIG. 4 is a photograph showing that the expression of *OsCIPK9* mRNA in an *OsCIPK9::Ds* plant.

FIGS. 5 and 6 are photographs showing that an *OsCIPK9* gene is rapidly expressed in response to a wound.

FIG. 7 is a photograph showing the deposition of callose in the wound site and stamen pollen of each of a mutant plant (*OsCIPK9::Ds*) and a wild-type plant.

FIG. 8 is a photograph showing the deposition of callose in a wound site by rice blast disease according to the present invention.

FIG. 9 is an immunolocalization photograph showing the difference between a wild-type plant and a mutant plant with respect to roots exposed to an aluminum solution.

FIG. 10 is a photograph showing the rice blast resistance of an *OsCIPK9* mutant (*OsCIPK9::Ds*).

FIG. 11 is a photograph showing the herbicide tolerance of the *OsCIPK9* mutant (*OsCIPK9::Ds*).

FIG. 12 is a graphic diagram showing the results of measurement for lipoperoxide levels that indicate damages caused by herbicide.

FIG. 13 is a conceptual diagram of an overexpression vector according to the present invention.

FIG. 14 is a photograph showing that an excess amount of callose is deposited in an *OsCIPK9*-overexpressed transgenic plant.

FIG. 15 is a photograph showing the breakdown of leaf chlorophyll caused by herbicide.

FIG. 16 and FIG. 17 is a table showing the amino acid sequence homology between an *OsCIPK9* gene product and other similar proteins.

【Best Mode】

Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are presented for illustrative purposes only and are not to be construed to limit the scope or content of the present invention. In these examples, the expression of the inventive gene in rice was inhibited or the gene was overexpressed; however, it will be readily conceived by those skilled in the art that the stress resistance of other plants, particularly monocotyledonous plants, can be regulated by inhibiting the expression of said gene in the plants or overexpressing the gene.

Example 1: Selection of specific gene, and analysis of mutated gene and mutant expression

Using the *Ac/Ds* tagging system constructed by the present inventors, a mutant having GUS highly expressed in the vascular system was selected from a rice mutant population, and a gene related thereto was investigated and analyzed.

(1) Identification of related gene through TAIL-PCR

DNA of the selected mutant was extracted, and cloned by PCR. The base sequence of the cloned *Ds* flanking DNA was analyzed to identify the related gene. Specifically, the base sequence of the flanking DNA was determined through TAIL-PCR using transposable element *Ds* primer and five kinds of AD (arbitrary degenerate) primers. These primers were designed in order to analyze the flanking DNA sequence in the *Ac/Ds* tagging system used in the present invention (Liu et al., 1995, Plant J. 8:457-463), and were constructed directly by the present inventors or provided by other research group (Ueli Crossniklaus, University of Zurich). The primer sequences are shown in Table 1 below. TAIL-PCR was previously conducted using each of five kinds of variously degenerated AD primers (AD 1-5) and, as a result, PCR

products were produced from all these AD primers.

The leaf of rice was crushed in liquid nitrogen, and then 1 x lysis buffer [10 x lysis buffer (3.5 M NaCl, 0.1 M Tris-HCl pH 7.6, 0.01M EDTA pH 8.0), 7M urea, 2% SDS, 50 mM EDTA] was added thereto, followed by further stirring. Then, the solution was shaken at 37 °C for 10 minutes to centrifuge it.

The same amount of P/C/I (phenol/chloroform/isoamylalcohol = 25:24:1) was added thereto and vortexed, and then the solution was centrifuged for 10 minutes to separate the supernatant. 0.6-fold volume of IPA (iso-propanol) was added to the supernatant and centrifuged at room temperature for 20 minutes to obtain pellets. 400 ml of 70% ethanol was added to the pellets and centrifuged for 10 minutes, and then the ethanol was completely removed at room temperature. Then, an adequate amount of TE (10 mM Tris-HCl, 1 mM EDTA) was added to prepare a genomic DNA solution.

A first-step PCR reaction was conducted using a total of 20 ml of a PCR solution comprising 1 mg of the genomic DNA as a template and Ds 5-1 and AD primers. The first-step PCR product was 50-fold diluted, and a second-step PCR reaction was conducted using a total of 10 ml of a PCR solution comprising 1 ml of the dilution as a template and Ds 5-2 and AD primers. The second-step PCR product was 50-fold diluted and a third-step PCR reaction was conducted using 1 ml of the second dilution as a template and Ds 5-3 and AD primers (FIG. 1).

[Table 1]

Name	SEQ ID NO	Base sequence
Ds 5-1	5	CCG TTT ACC GTT TTG TAT ATC CCG
Ds 5-2	6	CGT TCC GTT TTC GTT TTT TAC C
Ds 5-3	7	GTACGGAATTCTCCCATCCT
Ds 3-4	8	GTT ACC GAC CGT TTT CAT CC
Ds 3-37	9	TAT GAA AAT GAA AAC GGT AGA GG
Ds 3-155	10	ACC CGA CCG GAT CGT ATC GGT
AD-1	11	NTC GA(G/C) T(A/T)T (G/C)G(A/T) GTT
AD-2	12	NGT CGA (G/C)(A/T)G ANA (A/T)GA A
AD-3	13	(A/T)GT GNA G((A/T)A NCA NAG A
AD-4	14	(G/C)TT NTA (G/C)T NCT NTG C
AD-5	15	(A/T)CA GNT G(A/T)T NGT NCT G

As can be seen in Table 1, although the same AD primer was used in the three Tail-PCR reaction steps, the Ds primers were closer to the terminus of Ds in the PCR reaction steps, and thus the size of the PCR products was also decreased by the difference in position between the DS primers.

The second-step to third-step PCR products were electrophoresed (FIG. 2) to clone DNA bands where the size of the second-step and third-step PCR products became 80 bp corresponding to the distance between the Ds primers used in the respective PCR reaction steps, thereby identifying *Ds* flanking DNA. The isolated *Ds* flanking DNA was sequenced according to a conventional method, thus obtaining SEQ ID NO: 3.

FIG. 2 is a photograph showing the results of electrophoresis of the second-step and third-step PCR products resulting from the first-step to third-step PCR reactions conducted using 1 mg of the genomic DNA as a template and the primer set of Ds5/AD2 or Ds3/AD2. In FIG. 2, M represents a size marker, II represents the second-step TAIL-PCR product, and III represents the third-step TAIL-PCR product. The first-step PCR reaction product showed an excessively weak band, and thus was not shown in a separate photograph. In FIG. 2, each of Ds5 + AD2 and Ds3 + AD2 represents the primer

set used in the PCR reaction steps.

As can be seen in a conceptual diagram of FIG. 1, the *Ds* flanking DNA could be obtained through the three TAIL-PCR reaction steps. As can be seen in the photograph of FIG. 2, the primers used in the second-step and third-step PCR reactions had a difference of about 80 bp in their position, and thus DNA from the *Ds* insertion site could be identified by comparing the sizes of DNA bands from the second-step and third-step PCR reactions. There was a difference of about 80 bp in size between the second-step PCR product and the third-step PCR product. DNA of the band thus confirmed was cloned.

SEQ ID NO: 3 analyzed by cloning was subjected to computer bioinformatic search in NCBI (National Center of Biotechnology Information, USA) and, as a result, it was found that *Ds* was inserted into the gene predicted as *OsCIPK9*.

Since there was only information on the genomic DNA in NCBI, cDNA was cloned through a cDNA library prepared in this laboratory, and the base sequence thereof was analyzed to confirm the exons and introns in the gene and to identify a protein coding region. It was found that the *OsCIPK9* cDNA had a 1362-bp nucleotide sequence in the protein coding region between ATG (start codon) and TAA (stop codon). The base sequence between the start codon and stop codon of *OsCIPK9* is shown in SEQ ID NO:4.

The base sequences of the genomic DNA (SEQ ID NO: 2) and cDNA (SEQ ID NO: 4) were comparatively compared and, as a result, it was found that *OsCIPK9* was a gene consisting of 16 exons and 15 introns and making a protein of a total of 454 amino acids. Also, it could be seen that *Ds* was inserted into the first intron.

The isolated gene was found to be a gene encoding one of 31 phosphoproteins (CIPK: Calcinurin B-like (CBL) protein Interacting Protein Kinases) binding to calcineurin B-like proteins present in rice. The allele resulting from the insertion of *Ds* was named *OsCIPK9::Ds*.

(2) It could be found through flanking sequence analysis and GUS expression that, in the *OsCIPK9* mutant according to the present invention, *Ds*

was located at the first intron in the way that a transcription orientation of of a reporter gene is the same as the host gene, *OsCIPK9* (FIG. 3). In FIG. 3, small squares represent exons, black solid lines between the squares represent introns, and an inverted triangle at the terminus of the first intron represents the inserted *Ds*.

Northern analysis revealed that the *OsCIPK9* mutant did not normally expressed mRNA due to the insertion of *Ds* (FIG. 4). Leaves collected from a wild-type plant and the mutant plant, which were grown in soil for 20 days without stresses, were crushed in liquid nitrogen, guanidium thiocyanate extraction buffer was added thereto, and RNA was extracted from the leaves. About 20 mg of the extracted DNA was loaded onto 1.3% formaldehyde agarose gel (1.3 g agarose, 72 ml dH₂O, 10 ml 10 X MOPs/EDTA, 18 ml formaldehyde), which has been pre-run at 50 V for 30 minutes, and the gel was electrophoresed at 50 V for 3 hours and at 100 V for 2 hours. After the electrophoresis, the gel was washed with water for 5 minutes, and then immersed in 10 x SCC for 1 hour (10 x SCC was replaced at 20-minute intervals). The RNA on the gel was transferred to a nitrocellulose membrane.

The membrane was washed with 3 x SCC for about 5 minutes, was placed in a UV cross-linker, dried in an oven at 80 °C for 2 hours, and then pre-soaked for about 6 hours. Meanwhile, the N-terminal end of the *OsCIPK9* gene was cut with *SacI*, and the 400-bp DNA fragment resulting therefrom was eluted, labeled with an isotope and incubated at room temperature for about 3 hours, thus obtaining a probe. The probe was hybridized in the pre-soaked membrane for 12 hours, and then washed with a washing solution. Then, the membrane was exposed to an X-ray film in a cassette. After 1-3 days, the film was developed (FIG. 4).

As can be seen in FIG. 4, the *OsCIPK9* mutant plant (m/m) did not express mRNA due to the insertion of *Ds*, unlike the wild-type plant (+/+).

Example 2: Analysis of expression pattern of *OsCIPK9* gene

The expression pattern of the *OsCIPK9* gene in stress conditions was examined.

It was observed that the *OsCIPK9* gene was very weakly expressed in normal growth conditions, but was very strongly expressed when wounded.

After the leaves were wounded, the expression of *OsCIPK9* mRNA was observed at varying time points by Northern hybridization, and a reporter gene, GUS, in the inserted *Ds* was expressed using X-gluc and observed, and as a result, the same results as in the Northern analysis were obtained.

(1) Analysis of expression pattern through Northern hybridization

The leaves of a wild-type rice plant were wounded with a knife or forceps and then collected at varying time points. The collected leaves were tested according to the Northern analysis method described in Example 2 above (FIG. 5). In FIG. 5, the time denotes the time elapsed after wounding, and the lower photograph (EtBr) is an EtBr stained gel taken with a UV camera in order to examine relatively the relative amount of RNA loaded on the gel.

As can be seen in FIG. 5, the expression of the *OsCIPK9* gene was rapidly increased after wounding, suggesting that the inventive gene very sensitively responded to wounds.

(2) Analysis of expression pattern caused by wound through GUS staining

Rice seeds were surface-sterilized with Sportak for 12 hours and then washed clean with flowing water. The washed seeds were planted in a Petri dish and allowed to germinate for 2 days in a dark state. Then, the plant was transferred to a growth chamber, and after 15 days, the leaf and root of the plant were examined.

The leaf and root of rice were wounded with a knife or forceps, and after 30 minutes, the wound sample was collected, immersed in a GUS solution (50 mM Na phosphate buffer pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferrocyanide, 200 mg/ml chloram-phenicol, 1 mg/ml X-gluc) and left to stand at 37 °C in the dark for two nights and three days. Then, the chlorophyll was removed with 70% ethanol, followed by microscopic observation (FIG. 6).

As can be seen in FIG. 6, blue-color GUS was expressed specifically only at the wounding site, suggesting that the inventive gene was expressed

specifically at the wounding site.

Thus, it was found through GUS staining that the *OsCIPK9* gene was expressed within 30 minutes after the leaf and root were wounded (FIG. 5). Also, based on the Northern blot analysis of RNA extracted at varying time points after the leaf was wounded, it was demonstrated that the *OsCIPK9* gene was expressed in response to the wound.

Example 3: Analysis of function of inventive *OsCIPK9* gene

Callose deposition in the wild-type plant and callose deposition in the mutant were compared to each other in various stress conditions known to cause callose deposition. As a result, it was found that *OsCIPK9* gene was involved in callose deposition. Whether callose was present was observed using UV fluorescence after adding an aniline blue chemical that emits fluorescence through specific binding to callose.

Upon being wound, in a stamen site making pollen, or exposure to heavy metals such as aluminum, which are generally known to cause callose deposition, the wild-type plant was shown to be stained with aniline blue, but the mutant did not show any aniline blue fluorescence. In FIG. 4, m/m represents the mutant (*OsCIPK9::Ds*) having the inventive gene *OsCIPK9*, and +/+ represents a plant (*OsCIPK9-Rev*) in which Ds was removed to restore the normal function of the plant.

(1) Examination of callose deposition caused by wound

Rice seeds were surface-sterilized with Sportak for 12 hours and washed clean with flowing water for 12 hours. The washed seeds were planted in a growth chamber, and after 15 days, the leaf and root of the plant were used. The rice leaf was wounded with a knife and left to stand for 24 hours. Then, the wounded rice leaf was immersed in a fixing solution (acetic acid: EtOH=1:2) for at least 12 hours while replacing the fixing solution with a fresh solution at 4-hr intervals. The leaf was taken out of the solution and placed in 1M KOH solution. Then, the leaf was treated in a microwave oven for 2 minutes and washed with sterilized water. The leaf was placed on a slide, and about 200 ml of an aniline blue dye (0.05% aniline blue dye in

0.067M K_2HPO_4 at pH 9.0) was added thereto. The resulting leaf was covered with a cover glass, and after 10 minutes, observed under a UV microscope (FIG. 7A).

As can be seen in FIG. 7A, a strong aniline blue stain that indicates callose deposition at the wounding site was observed in the wild-type plant (+/+), whereas an aniline blue stain was not observed at the wounding site of the mutant, suggesting that callose was not deposited in the mutant.

(2) Examination of callose deposition at anther before heading

The anther before heading was collected, immersed in a fixing solution (10% acetic acid in ethanol) for at least 12 hours and then immersed in 1M NaOH for 12 hours. The anther was washed three times with 50 mM KPO_4 buffer and then observed according to the method as described in the above section (1) (FIG. 7B).

In this case, in the mutant (m/m) in which the inventive gene was mutated, the production of callose was not observed at the anther, unlike the wild-type plant (+/+).

(3) Examination of callose deposition caused by rice blast disease

Rice seeds were surface-sterilized with Sportak for 12 hours and washed clean with flowing water for 12 hours. The washed seeds were planted in a growth chamber, and after 15 days, the leaf and root of the plant were used.

The proliferation of *Magnaporthe grisea* (KJ-105a) causing rice blast disease in rice was carried out as follows. Rice blast fungus was inoculated onto a rice bran agar (20 g rice bran powder, 20 g sugar, 1 liter deionized water) plate using a sterilized bar and was cultured at 25-28 °C for 7 days. Onto the plate containing the rice blast fungus cultured thereon, 20 ml of 0.05% Tween 20 solution was poured, and the fungus was separated from the medium using a sterilized rubber scraper. The separated fungus was filtered using a cotton gauze.

Rice blast fungus was sprayed onto the plant leaf at a concentration of 1×10^5 spores/ml, the plant was incubated in a growth chamber at 26-28 °C,

and after 48 hours. The rice leaf having a wounding site caused by rice blast disease was observed according to the method as described in the above section (1) (FIG. 8).

As can be seen in FIG. 8, a strong aniline blue stain that indicates callose deposition at the wounding site caused by rice blast disease was observed in the wild-type plant (+/+). However, in the mutant (m/m), an aniline blue stain was not observed at the wounding site caused by rice blast disease, suggesting that callose was not deposited in the mutant.

(4) Examination of callose deposited in root through antibody in response to heavy metal

Rice seeds were surface-sterilized with Sportak for 12 hours, washed clean with flowing water for 12 hours, and then allowed to germinate. When the root was grown to a length of 2 cm, the root was immersed in 20 μ M aluminum for 3 hours and washed with sterilized water. The full plant was fixed in a fixation solution (3.7% paraformaldehyde, 0.2% picric acid, 50 mM potassium phosphate, 5 mM EGTA pH 6.8) at room temperature for 2 hours and then washed with a washing buffer (50 mM potassium phosphate, 5 mM EGTA, pH 6.8). In order to fix the sample to an OCT (optimal cutting temperature)-embedding medium, the sample was immersed in each of 30% and 60% OCT for 30 minutes and in 100% OCT for 1 hour, such that the sample could be penetrate with the OCT medium. Finally, the sample was fixed to 100% OCT. The fixed sample was cut to a thickness of 10 μ m using a cryostat at -20 $^{\circ}$ C and then transferred onto a slide. The slide having the sample transferred thereto was left to stand in a freezer at -70 $^{\circ}$ C overnight and then completely dried in an incubator at 37 $^{\circ}$ C for 2-3 hours. The dried slide was left to stand in a solution of 3% BSA and 1% NP-40 in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 , pH 7.4) at room temperature for 30 minutes. After 30 minutes, the slide was taken out of the solution and treated with a 1:50 dilution of a callose antibody, followed by incubation at 37 $^{\circ}$ C for about 2 hours. Then, the slide was washed with PBS buffer for 10-15 minutes and treated with a 1:50 dilution of a second antibody labeled with Texas-RED,

followed by incubation at 37 °C for about 2 hours. The slide was taken of the solution and washed clean with PBS for 1 hour. The slide was covered with a coverslip and left to stand in a dark state for 1 hour. Then the slide was observed with a microscope (green excitation filter 546/10 nm, dichroic mirror 580 nm, orange-red barrier filter 590 nm) (FIG. 9). As can be seen in FIG. 9, the deposition of a large amount of callose was observed in the root of the wild-type plant (+/+), whereas the deposition of callose in the mutant (m/m) was reduced.

As shown in FIG. 9, in the root of the wild-type plant (+/+) exposed to the aluminum solution, the deposition of a large amount of callose, shown as a red color, was observed, however, in the mutant (m/m), the deposition of callose was reduced.

Example 4: Construction of *OsCIPK9*-overexpressed transgenic plant and analysis of characteristic thereof

In order to examine what is a phenomenon (phenotype) occurring when the *OsCIPK9* gene is overexpressed, an overexpression vector was constructed using a 35S promoter, a potent promoter, and was used to transform a rice plant.

(1) Construction of transformation vector

OsCIPK9 cDNA was fused to the 35S promoter, which was then inserted into vector pCAMBIA 1303 (CAMBIA, Australia) having GUS and GFP, thus constructing overexpression vector pCAMBIA-CIPK9. The vector pCAMBIA-CIPK9 was a vector made for plant transformation and consisted of a 1,236-bp base sequence. Also, it had hygromycin- and kanamycin-resistant genes as selection markers for plant transformation, and could express GUS and GFP in the form of a fusion protein.

The overexpression vector pCAMBIA-CIPK9 according to the present invention was constructed through the following procedures: (i) the 35S promoter and CIPK cDNA were cloned into a pBSK plasmid vector (STRATAGENE, USA). (ii) T-DNA vector pCAMBIA 1303 and a plasmid vector, containing CaMV35S promoter and *CIPK9* cDNA, were digested with *Bgl*III and *Spe*I enzymes, respectively. (iii) the 35S promoter and the DNA of *CIPK9* were eluted on gel

and then ligated to pCAMBIA 1303 at 16 °C overnight. (iv) the ligated DNA was transformed into *E. coli* competent cells, and DNA was extracted from the resulting clone and was digested with *Bgl*III and *Spe*I enzymes to confirm whether the DNA was ligated.

The main elements of the overexpression vector pCAMBIA-CIPK9 according to the present invention are shown in FIG. 13. As shown in FIG. 13, *OsCIPK9* cDNA according to the present invention is inserted between the 35S promoter and the GUS gene.

Since the 35S promoter and the pCAMBIA 1303 are currently easily available and the *OsCIPK9* cDNA was obtained as described in the present invention, the vector was not deposited in the International Depository Authority.

(2) Construction of overexpressed plant

The transformation of plants was conducted using a conventional *Agrobacterium* method.

First, pCAMBIA-CIPK9 containing *OsCIPK9* cDNA was transformed into the *Agrobacterium* LBA4404 strain. The bacterial strain was cultured at 30 °C for 3 days, and then DNA was extracted from the strain to confirm co-integration. The confirmed strain was cultured for 3 days and used for infection. The transformed *Agrobacterium* cells were infected to the calli of rice and subjected to dark room culture (AAM, 2N6-AS medium) for 3 days, selective culture (N6-Hyg 50 mg) in a dark room for 3-4 weeks, pre-regeneration (N6-7-CH) in a dark room for 10 days, regeneration I (N6S3-CH-I) in a light room for at least 3 weeks, and regeneration II (N6S3-CH-II) in a light room up to shooting. The cultured cells were then cultured in MS medium in a light room, thus obtaining a transformed plant. Whether the plant was transformed was confirmed through PCR and Southern blot analysis using genomic DNA extracted from the plant.

It could be seen that, in the *OsCIPK9* gene-overexpressed transgenic plant thus constructed, callose was made in an amount larger than in the wild-type plant even in normal growth conditions (FIG. 14).

Example 5: Analysis of resistance of mutant to rice blast disease

The seeds of the wild-type plant and the transgenic plant (rice) according to the present invention were surface-sterilized with Sportak for 12 hours, and then washed clean with flowing water for 12 hours. The washed seeds were germinated in a container, were transferred, and grown in a greenhouse for 20 days (about 3-4 leaf stage).

The plants infected with rice blast fungus according to the method described in Example 2 above were left to stand in a growth chamber at 26-28 °C for 24 hours, and then moved into a greenhouse. After 7 days, whether rice blast disease occurred in the plants was observed (FIG. 10).

As shown in FIG. 10, the results of observation conducted at 7 days after treatment with the rice blast fungus showed that rice blast resistance, which did not appear in the wild-type plant (+/+), appeared in the mutant (m/m). In FIG. 10, Rev represents a plant having a revertant allele normally restored by the removal of Ds, which has been inserted into the *OsCIPK9::Ds* allele. The revertant plant (Rev) and the overexpressed plant (OX) did not show disease resistance, like the wild-type plant. It was observed that, in the overexpressed plant, larger lesions occurred.

Example 6: Examination of tolerance of mutant to herbicide paraquat

(1) In order to examine tolerance to herbicide paraquat, the seeds of the *OsCIPK9* mutant and the wild-type plant (rice) were surface-sterilized with Sportak for 12 hours and then washed with flowing water for 12 hours. The washed seeds were planted in soil and grown in a greenhouse for 2 weeks (2-3-leaf stage).

200 g ai (active ingredient)/10a of paraquat was dissolved in 200 ml of 0.1% surfactant Tween 20 and 20% acetone to prepare a herbicide solution, which was then uniformly spread to the mutant plant and the wild-type plant, which have been grown in 1 m² of a space for 2 weeks. At about 2 hours after the spreading, the herbicide solution was dried, and each of the plants was left to stand in a plant incubator at 30 °C and a light intensity of 230

mmol/s.m² for 10 hours. Then, the plants was recorded as an photo image and sampled. The herbicide tolerance of the plants was measured as either the degree of wilting or the whitening phenomenon of the plant leaf (FIG. 11).

The left panel of FIG. 11 shows the wild-type plant (+/+) and the mutant (m/m), which were not treated with the solution containing no paraquat herbicide, and the right panel shows the plants at 10 hours after treatment with the herbicide. The lower photograph is an enlarged photograph of the upper photograph. As can be seen in FIG. 11, the wild-type plant withered so that the leaves drooped and the whitening phenomenon started to appear, however, the mutant plant maintained normal conditions.

(2) In order to examine the breakdown of the unsaturated fatty acid of the cell membrane, caused by ROS, the level of MDA (malondialdehyde) was measured.

The collected sample was crushed in liquid nitrogen and centrifuged in the presence of 4 ml ethanol at 12000 rpm for 15 minutes. Then, 2 ml of the supernatant was taken, and 2 ml of 20% TCA (trichloroacetic acid) solution, 1 ml of TBA (2-thiobarbituric acid) solution and 1 ml of BHT (butylated hydroxytoluene) solution were added thereto and lightly shaken. The sample was boiled for 15 minutes, left to stand on ice for 5 minutes and then centrifuged at 12000 rpm for 15 minutes. 4 ml of the supernatant was collected, and measured for absorbance at 440 nm, 532 nm and 600 nm. The measurement values were substituted into the following equation to calculate MDA values.

$$\text{nmol (MDA)/gr (sample fresh weight)} = \frac{[(A_{532}-A_{600})-(A_{440}-A_{600}) \times 0.0571] \times 6.3694 \times 6 \times (2 + \text{FW})}{\text{FW}}$$

wherein FW = fresh weight.

The MDA values obtained through the triple experiment were shown as bar graphs (FIG. 12).

As shown in FIG. 12, the wild-type plant had a MDA value of 12.55 3.28 nmol/gr when untreated with the herbicide, and a MDA value 110.66 22.43 at 10 hours after treatment with the herbicide, however, the *OsCIPK9*

mutant had a MDA value of 21.02 - 4.52 when untreated with the herbicide, and a MDA value of 12.03 - 2.55 at 10 hours after herbicide treatment, and thus showed little or no difference in MDA value.

(3) The herbicide tolerance of the overexpressed plant was compared to those of the wild-type plant and the mutant plant.

The wild-type, mutant and overexpressed plants were grown in soil for about 14 days, and the leaves of the plants cut, immersed in 1 μ M paraquat, and left to stand in a plant culture chamber for 4 days. Then, the chlorophyll breakdown degrees caused by paraquat were comparatively observed (FIG. 15). As shown in FIG. 15, it was observed that the leaves of the wild-type plant (+/+), the mutant (m/m) and the overexpressed plant (OX) maintained the chlorophyll for a long time in the solution containing no herbicide (samples in the upper portion of FIG. 15). However, it was observed that, in the solution containing paraquat, the leaves of the wild-type plant and the overexpressed plant were turned more yellow compared to those of the mutant (samples in the lower portion of FIG. 15). Particularly, it was observed that the chlorophyll of the overexpressed plant was broken faster than that of the wild-type plant, resulting in discoloration.

Through the above experiments, it could be seen that the tolerance of the *OsCIPK9* mutant was significantly increased compared to that of the wild-type plant, and the tolerance of the overexpressed plant was significantly decreased. Thus, it was demonstrated that the *OsCIPK9* gene is a gene associated with tolerance to ROS (active oxygen).

Meanwhile, as shown in FIGS. 16 and 17, in monocotyledonous plants, including rice, maize, wheat and the like, there are protein genes, the function of which is not yet found and which have an amino acid sequence homology of at least 90% to the amino acid sequence of the *OsCIPK9* gene product. Thus, any person skilled in the art to which the present invention pertains can understand that these genes would also have the property and function of inducing callose deposition and an increase in resistance, like the *OsCIPK9* gene. The name, SEQ ID NO and origin of these gene products are

shown in Table 2 below.

[Table 2]

Name	SEQ ID NO	Origin
OSCIPK08	16	Rice (<i>Oryza sativa</i>)
OSCIPK24	17	Rice (<i>Oryza sativa</i>)
TC2471256	18	Wheat (<i>Triticum aestivum</i>)
AZM-303	19	Maize (<i>Zea mays</i>)
AZM-68301	20	Maize (<i>Zea mays</i>)
AZM-12380	21	Maize (<i>Zea mays</i>)
PUEGQ89TD	22	Maize (<i>Zea mays</i>)

【Industrial Applicability】

As described above, according to the present invention, it is possible to use the *OsCIPK9* gene as a main gene, which is involved in the production and deposition of callose and determines stress resistance.

Also, according to the present invention, it is possible to use monocotyledonous plants even in a more severe environment by regulating the expression of stress-related genes.

【CLAIMS】**【Claim 1】**

A protein, which functions to produce callose in a plant and has an amino acid sequence showing a homology of at least 90% to SEQ ID NO: 1.

【Claim 2】

The protein of Claim 1, which is an OsCIPK protein having an amino acid sequence of SEQ ID NO: 1.

【Claim 3】

A gene encoding the protein of Claim 1.

【Claim 4】

The gene of Claim 3, which is any one selected from the group consisting of an *OsCIPK9* gene having a base sequence of SEQ ID NO: 2, an *OsCIPK9* gene having a base sequence of SEQ ID NO: 4, 20SCIPK08 having a base sequence of SEQ ID NO: 16, OSCIPK24 having a base sequence of SEQ ID NO: 17, TC2471256 having a base sequence of SEQ ID NO: 18, AZM-303 having a base sequence of SEQ ID NO: 19, AZM-68301 having a base sequence of SEQ ID NO: 20, AZM-12380 having a base sequence of SEQ ID NO: 21, and PUEGQ89TD having a base sequence of SEQ ID NO: 22.

【Claim 5】

An expression vector for plants, which comprises the gene of Claim 3.

【Claim 6】

An expression vector for plants, which comprises the gene of Claim 4.

【Claim 7】

The expression vector of Claim 6, which is expression vector pCAMBIA-CIPK for plants.

【Claim 8】

A method of inducing the production and deposition of callose in a plant, the method comprising transforming the plant with the expression vector of any one of Claims 5 to 7.

【Claim 9】

A method of obtaining a rice blast-resistant plant, the method

comprising inducing the mutation of the gene of Claim 3 or 4.

【Claim 10】

A rice blast-resistant plant, in which the gene of Claim 3 or 4 is mutated.

【Claim 11】

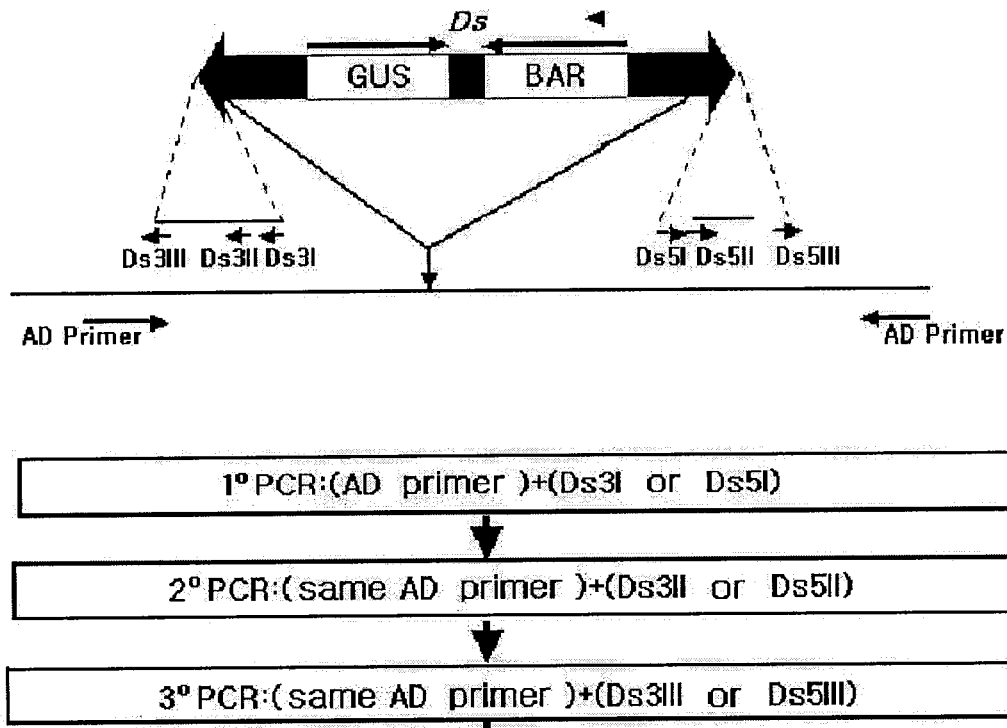
A method of obtaining an herbicide paraquat-resistant plant, the method comprising inducing the mutation of the gene of Claim 3 or 4.

【Claim 12】

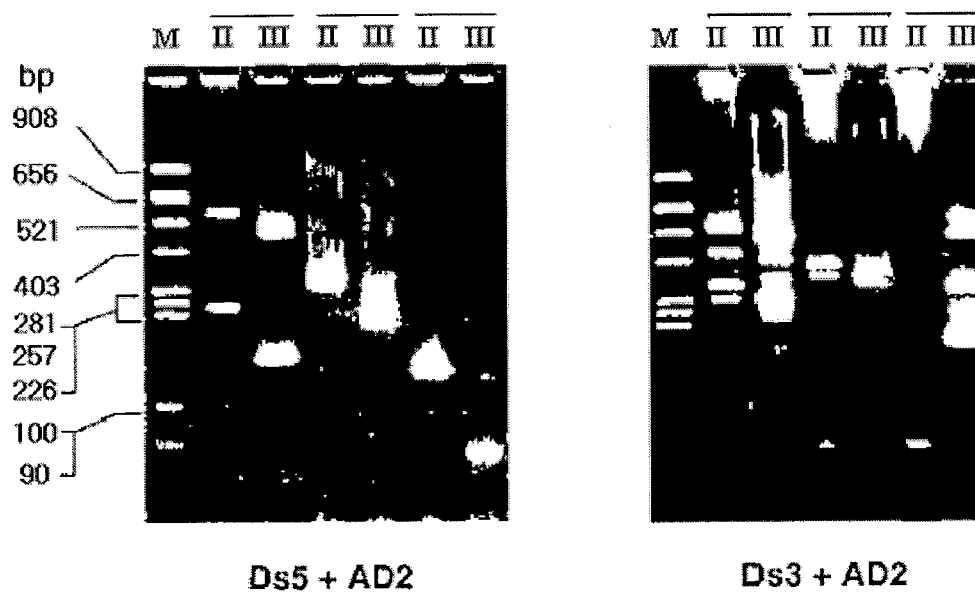
An herbicide paraquat-resistant plant, in which the gene of Claim 3 or 4 is mutated.

【DRAWINGS】

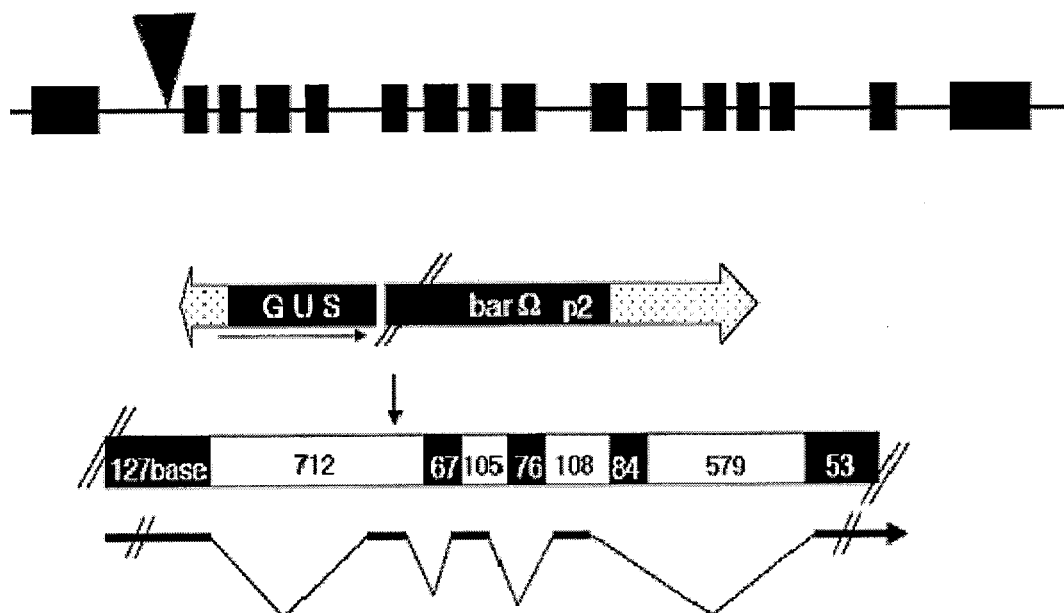
【Figure 1】



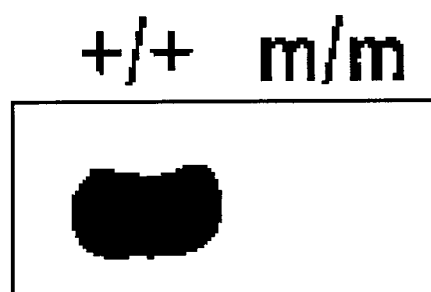
【Figure 2】



【Figure 3】

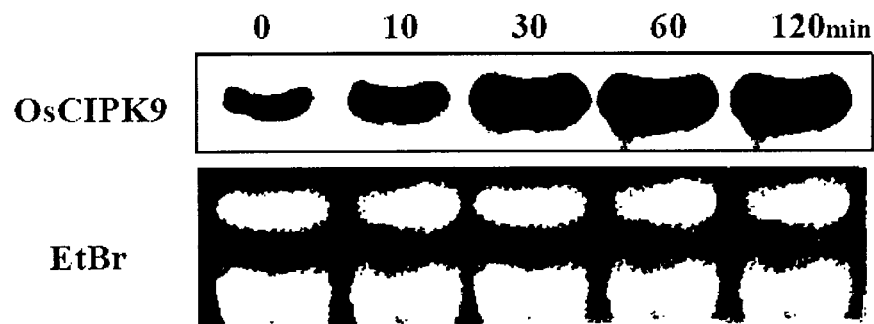


【Figure 4】

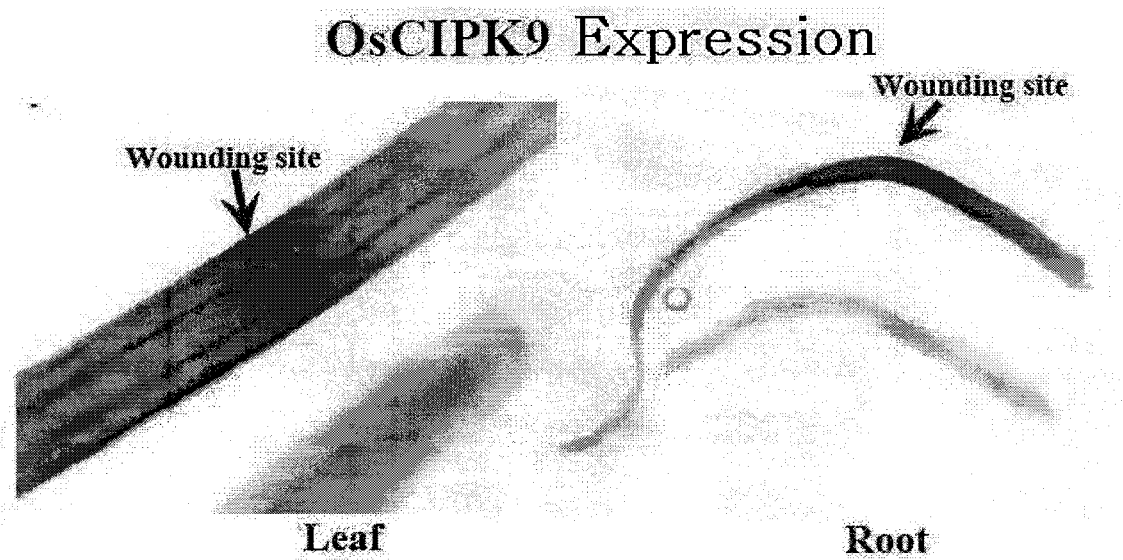


Probe : OsCIPK09

【Figure 5】



【Figure 6】

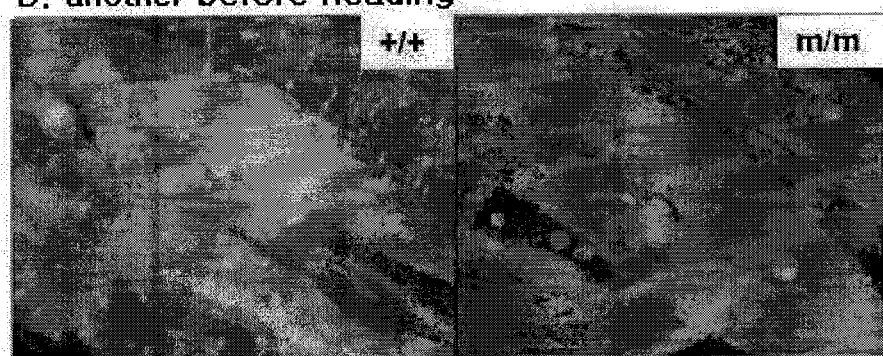


【Figure 7】

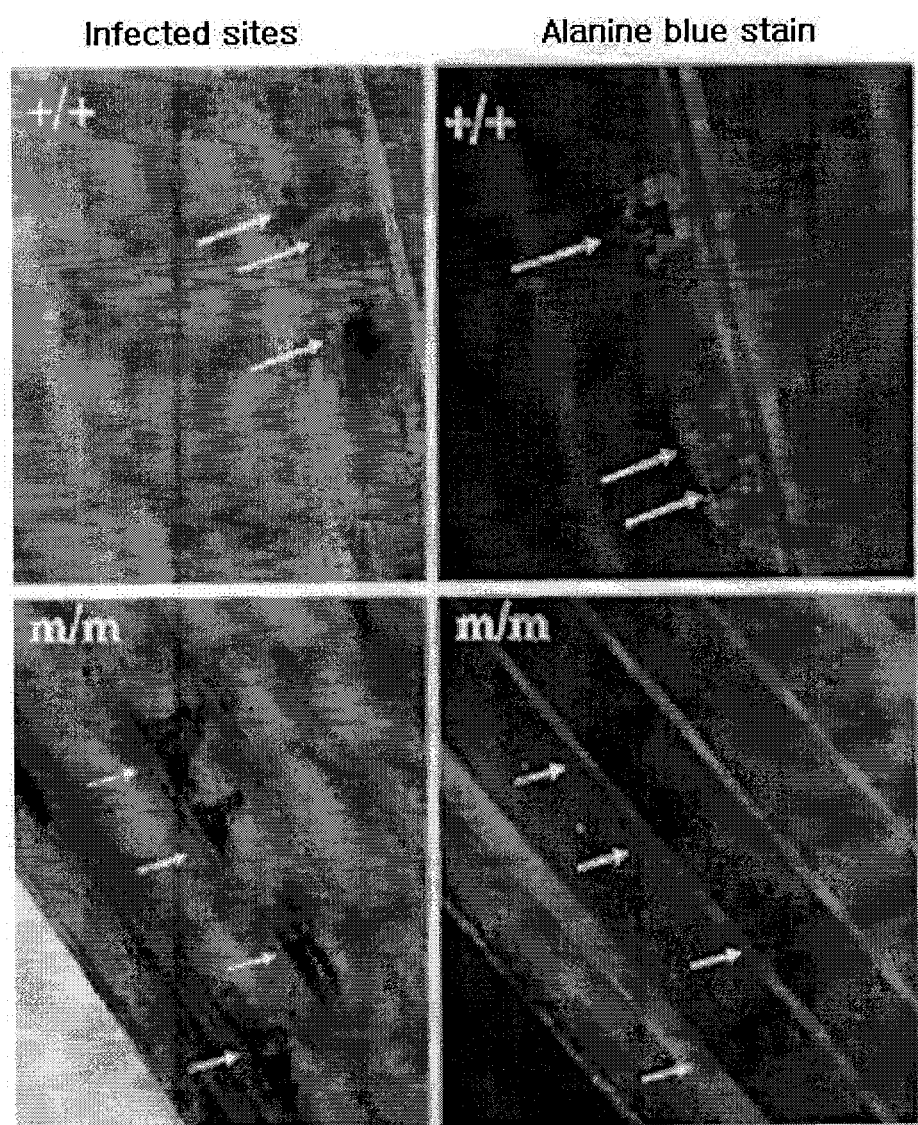
A. 1 day after Wounding



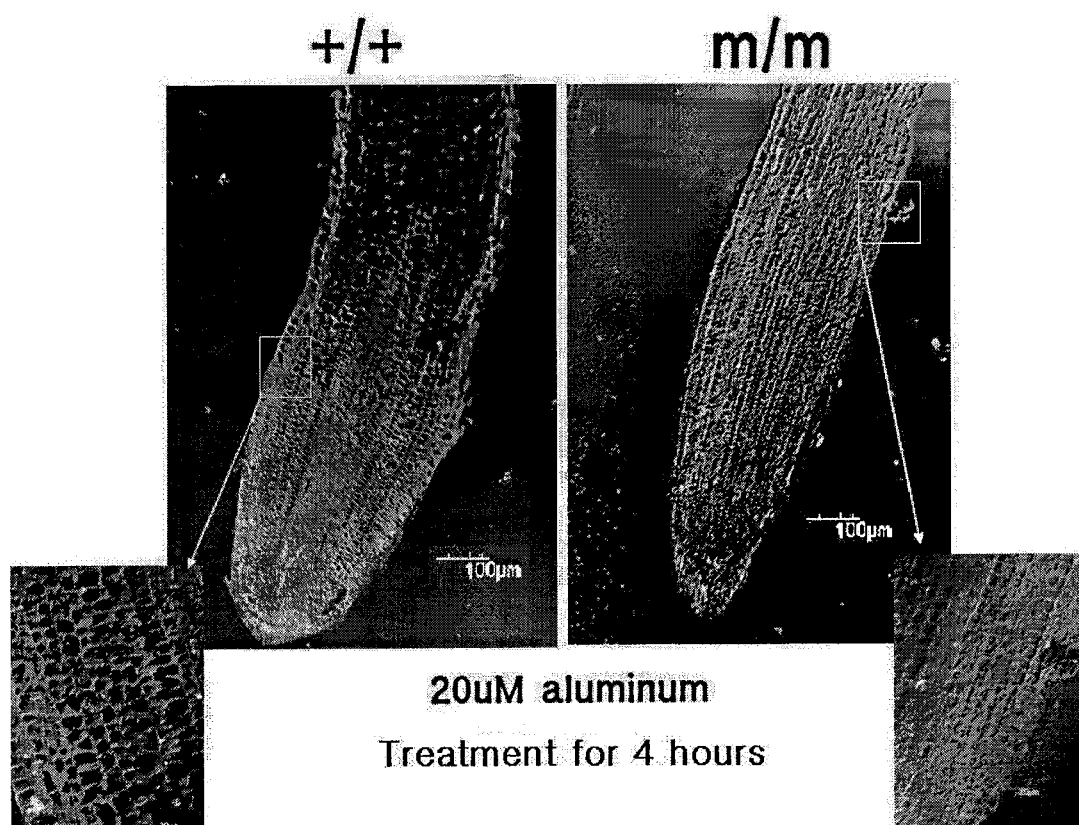
B. another before heading



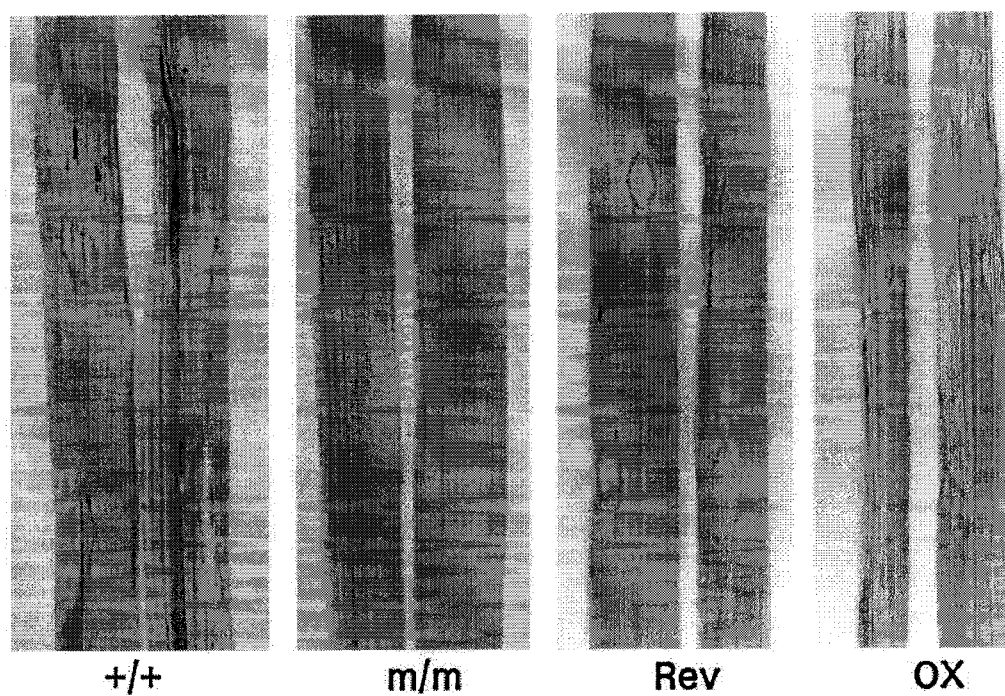
【Figure 8】



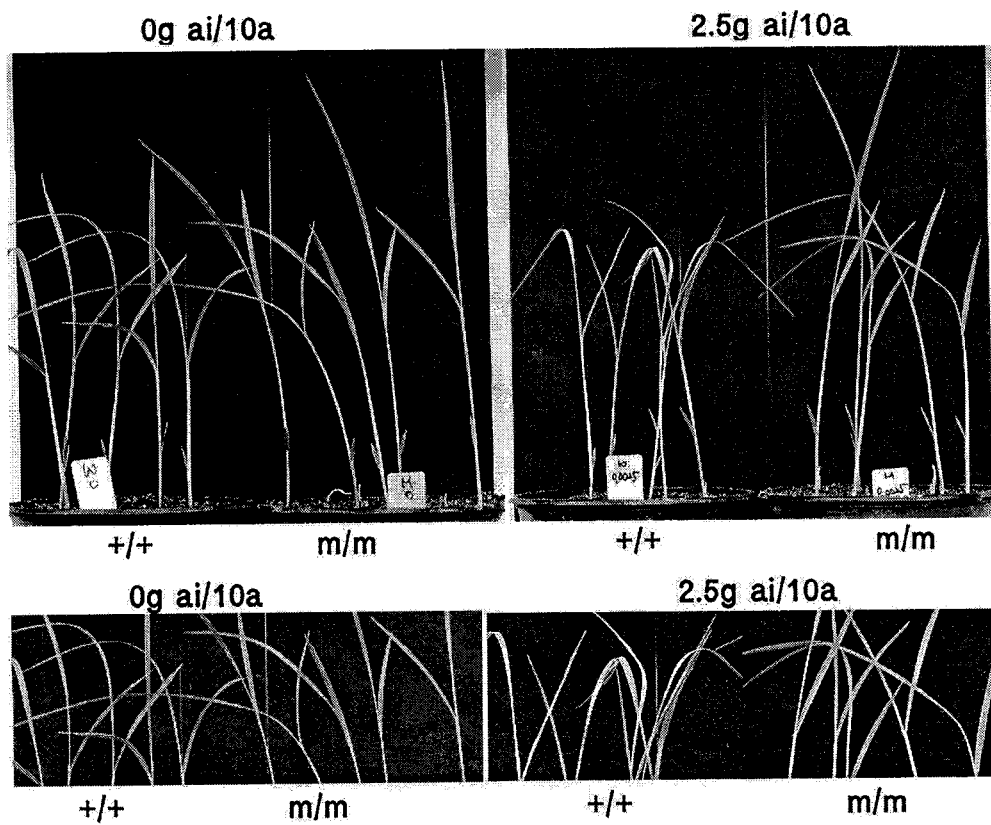
【Figure 9】



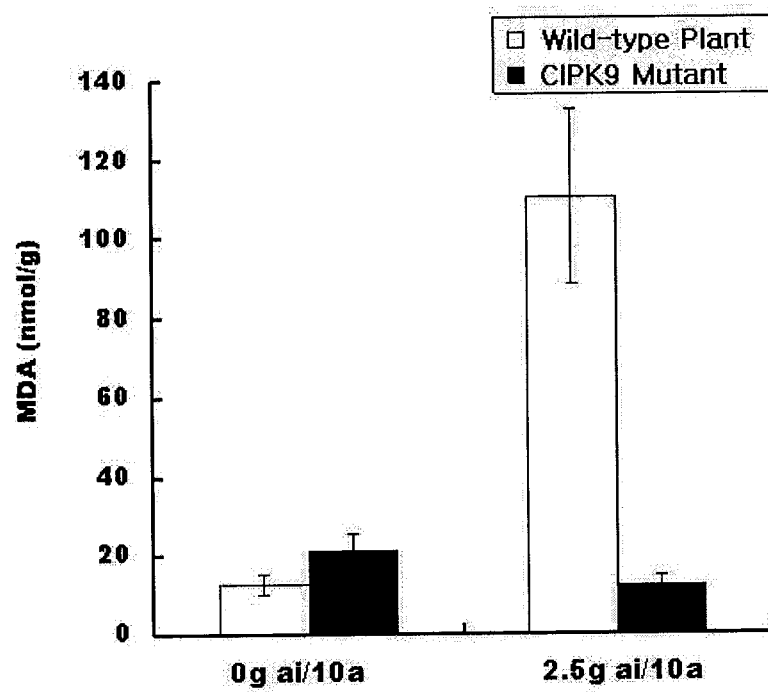
【Figure 10】



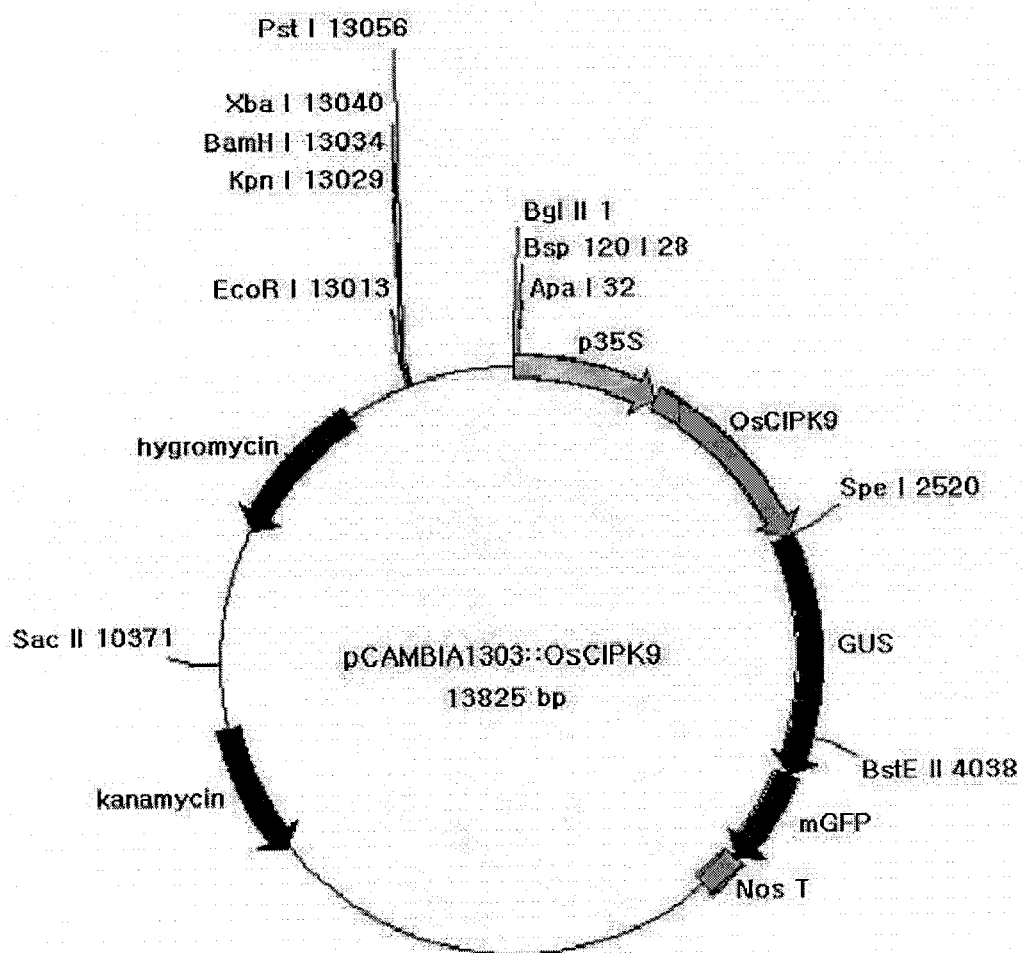
【Figure 11】



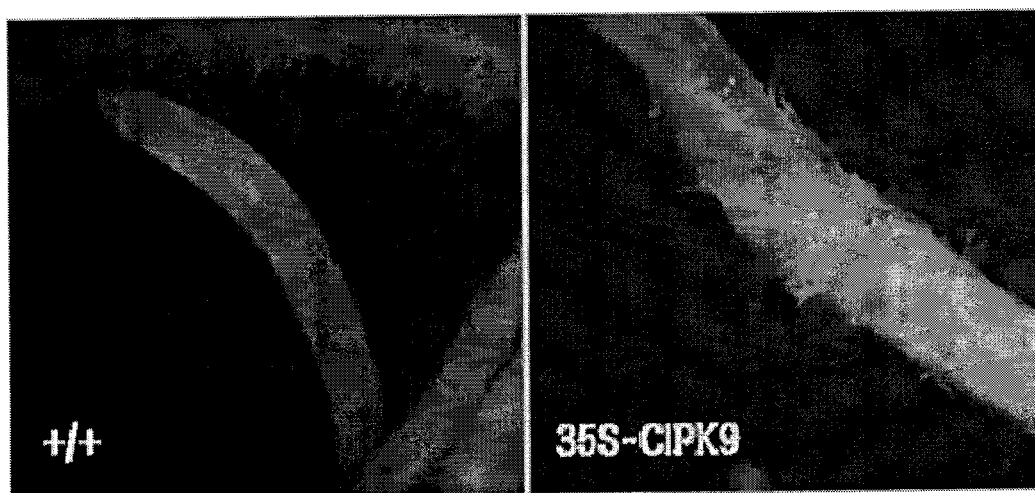
【Figure 12】



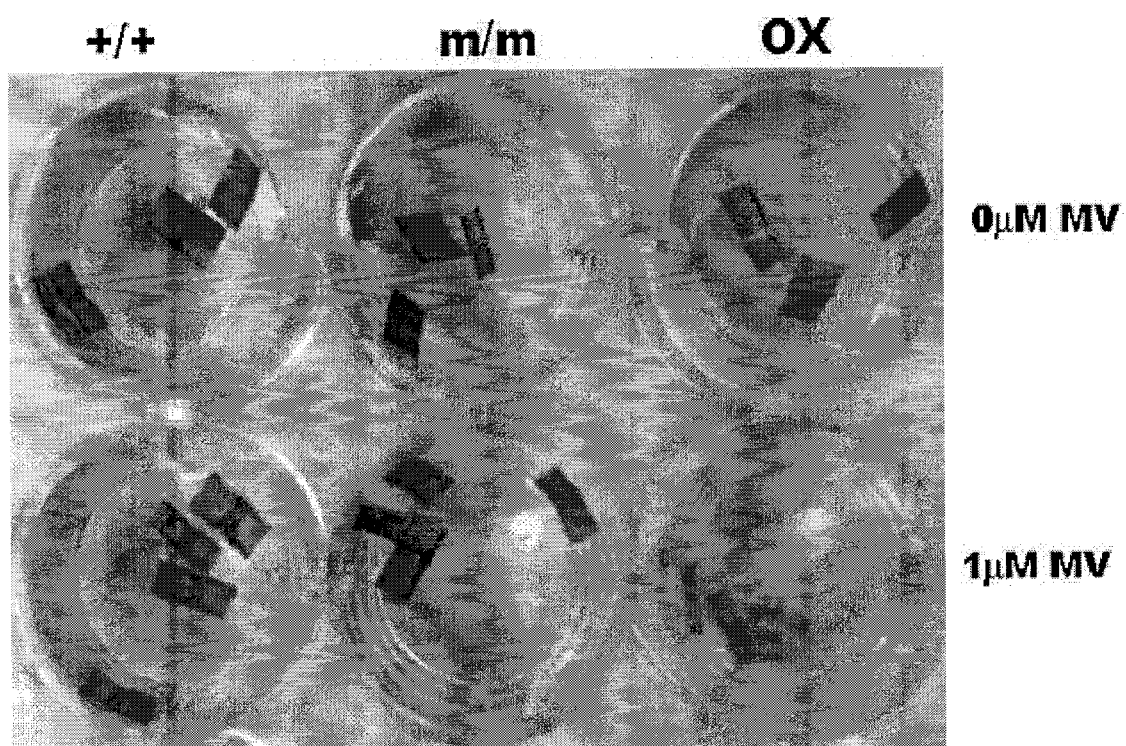
【Figure 13】



【Figure 14】



【Figure 15】



[Figure 16]

```

TC2471256 -----MVEQK-----GNIIMERYEIGKLLGQGSFAKVYHGR 31
AZM_303 -----MESR-----GKIIMERYELGRLLGEGTFCKVHYAR 30
AZM_12380 MAATPPSSQERRPLSSSSASAAASLAGKPR----GGGLILGRYELGRLLGEGTFKAVYQAR 55
AZM4_68301 -----MMIAAAEEGEGKKKG-----GGTVIQGRYEMGRVLGHGMFGRVHVAR 41
Q=CIPK09 -----MIEAEAEALGAGA--CPSRRTTBVGRVELGKTI GEGSFAKVEVAR 43
PUEG089TD -----MIEAEAEALGAGAGACPARRTTBVGRVELGKTI GEGSFAKVEVAR 45
Q=CIPK08 -----MVG-----GGALR-----RVGKYEVGRTI GEGTFKAVEFAQ 31
Q=CIPK24 -----MGGEFGMAIGRKK-----RVGRYEVGRTI GQGTFAKVEFAV 36
                                     :*:*: :*.*.*:

```



```

TC2471256 NIKNSQSVAIKVIDKEKILKCELDQIRREISVMNLVHPCIVQLYEVMA TKTKIYFILE 91
AZM_303 NLESNQSVAIKVIDKQOTILEVGLSEQIRREITTHRLVAHKNIWQLHEVMA TRNKIYFVME 90
AZM_12380 SADSGEPVAIKVLDKEKAMRHGLVPHIKREIAILRRVH PNIWRLFEVMA TKSKIYFVME 115
AZM4_68301 DLRTGRSVAVKVVAKKVVVRAGNNEQIKREIIVMKRVSH PNIWELHEVMA TRSKIYLALE 101
Q=CIPK09 DTRTGDTLAIKVLD RNEVLRHKMVEQIKREISTMKLIKH PNVVQLHEVMA SKSKIYAVLE 103
PUEG089TD DTRTGDTLAIKVLD RNEVLRHKMVEQIKREISTMKLIKH PNVVQLHEVMA SKSKIYAVLE 105
Q=CIPK08 NTESGESVANKVVD RQSI LKHKMLDQIKREISIMKLVH PNVVRLHEVLA SRKKIYFILE 91
Q=CIPK24 DADTGAASVANKVLDKDTILNHRMLHQIKREISIMKIVH PNIWRLNEVLAGTKIYFILE 96
. . . :*:*: :. . : :*:*: :. . : * :*. * *:*: :*

```



```

TC2471256 YVKGGE LFNKVR-RGRLKFEVARKYFQQLISAIDFCHSRGVYHRDLKPENLLDENRMLK 150
AZM_303 YVKGGE LFEKVAKRKITEVVAHKYFQQLISAVDYCHSRGVYHRDLKPENLLDENRMLK 150
AZM_12380 LVRGGE LFGKVA-KERLKEDTARRVFQQLVSAVGFCHARGVFHRDLKPENLLVDEHGDLE 174
AZM4_68301 LVRGGE LFGRIVRLGRVREDARRVFQQLVSAVD FCHSRGVYHRDLKPENLLDEAGNLK 161
Q=CIPK09 YVDGGE LFDKI VNSGRIGEDEARRYFHOLINAVDYCHSRGVYHRDLKPENLLDSHGALK 163
PUEG089TD YVDGGE LFDKI VNSGRIGEDEARRYFHOLINAVDYCHSRGVYHRDLKPENLLDSHGALK 165
Q=CIPK08 FITGGE LFDKIIRHGRIGEDARRVFQQLIDGVD FCHSKGVYHRDLKPENLLDSQGMK 151
Q=CIPK24 LITGGE LFDKIARQCKIRENEARKYFQQLIDAINVCHSKGVYHRDLKPENLLDSRGMK 156
: ***** :. . : * :*:*: :. . :*:*: :*:*****:*. **

```



```

TC2471256 ISDFGLSALAECKRQDGLLHTTCGTPAYVAPEVINRKGVDGARADVACGVILVLLAGY 210
AZM_303 VSDFGLSALSECKRQDGLLHTTCGTPAYVAPEVISKIGYDGA KSDIWSCGVILFVLVAGY 210
AZM_12380 VSDFGLSAVIDQFHPDGLLHTTCGTPSYVAPEVLARRGYDGA KADIWSCGIILFVLEAGY 234
AZM4_68301 VVD FGLSALADHARADGLLHTLCGTPGYAAPEVL RDKGYDGA FADLWSCGVILVLLAGS 221
Q=CIPK09 VSDFGLSAFAPUTKEDGLLHTACGTPNYVAPEVLADKGYDGM AADVWSCGIILFVLEAGY 223
PUEG089TD VSDFGLSAFAPUTKEDGLLHTACGTPNYVAPEVLADKGYDGM AADVWSCGIILFVLEAGY 225
Q=CIPK08 ISDFGLSAWPAQG--GALLRTTCGTPNYVAPEVL SHKGYDGA LADTUSCGVILVLLAGY 209
Q=CIPK24 VSDFGLSTLAQKG--VGLLHTTCGTPNYVAPEVL SNNGYDGS AADVWSCGVILVLLAGY 214
: ***** :. . :*: * :*:*: :. . :*:*: :*:*****:*. **

```



```

TC2471256 LPFDKNI VIMYKKICKAEFKWPSWFS SDIRKLLRRILD PNPATRI SVSLIMEDPWIRVG 270
AZM_303 LPFDGPNLMEMYRKIOHGEFRCFPGWFSRKL OKLLYKIND PNPSTRISI OKIKESTWFRKG 270
AZM_12380 LPFHDONLMAMYRKIYRGEFRCFPRWFSKOLSS LLNRI LDINPETRITVKEVMESRUFOKG 294
AZM4_68301 LPFPDDNI VTLYRKAGRGDYRCFANLST DARRLI PRLD PNPTRISVAGLVETPWFKK- 280
Q=CIPK09 LPFD DPNLMTLYKLI CKAKVSCP HWFSSGARKFKRI LD PNPCTRITIAQILEDDWFKKD 283
PUEG089TD LPFD DPNLMTLYKLI CKAKVSCP HWFSSGARKFKRI LD PNPCTRITIAQILEDDWFKKD 285
Q=CIPK08 LPFIDEVDLITLYGKIESAEYSFPANFPNGAKS LIHRI LD PNPDKRIRIELIRNDEWFKKN 269
Q=CIPK24 LPFIEEDDLPTLYDKITAGOFSCFYWFS PGATSLIHRIL DPNPKTRITIEQIREDTWFKKT 274
*** :. . : * :*:*: :. . :*:*: :*:*****:*. **

```

【Figure 17】

```

TC2471256      LNSDLLNKTIPDTDKVDKVVHVD-----MDST 296
AZM_303        PEE---NRILKERTLNENTTKN-----VAPV 293
AZM_12380      FRP--VRIFYVEDDQVHS LADGDMMPLEPSEPPPPPPPPPPQQDDGGEESGWESDSS 352
AZM4_68301     -----TSISRPVSIE-----LPPA 294
0sCIPK09      YKP-----PLFEQGEDVSLD-----DVDAA 303
PUEGQ89TD     YKP-----PLFEQGEDVSLD-----DVDAA 305
0sCIPK08      YEP-----TREIESEEVNLD-----DVWAA 289
0sCIPK24      YVA-----IKRGEDENVLD-----DVQAV 294
               . : .

TC2471256      FGNLSNNINECKQEAENL-----TSINAFDIISLSSGFDLSAMFEDENSKE----ESKF 346
AZM_303        LGVRRKKNAHEDVKPMNV-----TNLNAFEIISFSKGFDSLGMFIVKEWRN----EARF 343
AZM_12380      VASCPATLSSEERRQRPLGSLTRPASINAFDIISFSKGFDSLGLFEERGS-----EYRF 406
AZM4_68301     FAD-PAPAKEEAKEDEPE-----TLNAFHLISLSEGFDSLPLFEGDSAKGRDGGGLF 346
0sCIPK09      FDCSEENLVAEKREK-----PESMNAFALISRSQGFNLGNLFE-KENMGMVKRETSF 354
PUEGQ89TD     FDCSEENLVAEKREK-----PESMNAFALISRSQGFNLGNLFE-KENMGMVKRETSF 356
0sCIPK08      FDDPEED--ADETLDDEAG---PLTLNAFDLIISQGLNLAALFD-RRQD-YDKLQNR 341
0sCIPK24      FDNIEDKYVSEQVTHNDGG---PLVVNAFEMITLSQGLDLSALFD-ROOE-FVKROTRF 348
               . :*** :* *.*:*. :* *

TC2471256      TSTNTATTITKKLEDVAKNLR--LKFLKKNGLLKMEGS-KPGRKGVMSINAEIFQITPD 403
AZM_303        TSDKSASTIISKLEDVAKALN--LRVRKKDNGVVKMQR-KEGRNGVLQFDIEIFEVTT 400
AZM_12380      ISAEPMQTIITKLEETAKVKS--FFVRKKD-WRVSIET-REGLKGPLTIGAEIFELTPS 462
AZM4_68301     ATREPASGVISRLEGVAARGGGRMRVTKSGARGVRLEGAERGGAKRLAVAADIFSVAPS 406
0sCIPK09      TSQCTPQEIMSKIEEACGPLG--FNVRKQN-YKMKLKGQ-KTGRKGYLSVATEYFEVAPS 410
PUEGQ89TD     TSQCTPQEIMSKIEEACGPLG--FNVRKQN-YKMKLKGQ-KTGRKGYLSVATEYFEVAPS 412
0sCIPK08      LSRKPAKYIMSSMEVVAQSMG--YKTHIRN-YKMRVEGL-NANKTSHLAVMLEIFEVAPS 397
0sCIPK24      VSRKPAKTIVATIEVVAETMG--LKVHSQN-YKLRLEGV-SSNRMSFFAVVLQYFEVAPS 404
               : . : :* . . : :* . . : :*.:..

TC2471256      FHLVEFTKINGDTLEYOKVKOE-MRPALKDIVWANGEOPOPSLNEOS- 451
AZM_303        YHIIEMKQTSQDSLEYRQLLEEGIRPALKDIVLA----- 434
AZM_12380      LVVVEVKKKAGDKEETDDFCNRELKPGMQHLVHHNGSVFNIPSDTE---- 508
AZM4_68301     VLVVDVKKDGDTLEYRSFCSEELRPALQDIVWGAADPTPTAAV----- 451
0sCIPK09      LHMVELRKTGGDTLEYHNFFYN-FSELKDIVWK-SESDAKAAKKR---- 454
PUEGQ89TD     LHMVELRKTGGDTLEYHNFFYN-FSELKDIVWK-SESDAKAAKKR---- 456
0sCIPK08      IFMIELQRAAGDTSYNKFINN-YCSKLDDIIWNFPIEKSKSRISRLSKR 446
0sCIPK24      LFMVDVRKVAGDTLEYHRFYKN-LCNKMESIIWRPIEVSAKSALLRTATC 453
               :.. : ** : : . : : : :

```

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/003514

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/003514**A. CLASSIFICATION OF SUBJECT MATTER****C07K 14/415(2006.01)i**

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)

C07K 14/415

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 practicable, search terms used)

eKIPASS "callose, glucan, stress, aluminum", NCBI, PubMed, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Parre, E. et al. 'More than a leak sealant. The mechanical properties of callose in pollen tubes' Plant Physiology, January 2005, Vol. 137, pp 274-286 See abstract	1-12
A	US 6495740 B1(Arioli, A. et al.) 17 December 2002 See abstract and claims	1-12
A	Bhuja, P. et al. 'Accumulation of 1,3-beta-D-glucans, in response to aluminum and cytosolic calcium in Triticum aestivum' Plant Cell Physiol., May 2004, Vol. 45(5), pp 543-549 See entire document	1-12
A	Jacobs, A.K. et al. 'An arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation' The Plant Cell, November 2003, Vol. 15, pp 2503-2513 See abstract	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 DECEMBER 2006 (11.12.2006)

Date of mailing of the international search report

12 DECEMBER 2006 (12.12.2006)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

PARK, JEONG UNG

Telephone No. 82-42-481-8159



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2006/003514

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6495740 B1	17. 12. 02	US 2003126643 A1 JP 2001510326 T2 EP 956353 A1	03. 07. 03 31. 07. 01 17. 11. 99