

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
15 September 2005 (15.09.2005)

PCT

(10) International Publication Number
WO 2005/085279 A1

(51) International Patent Classification⁷: **C07K 14/415**

(21) International Application Number:
PCT/KR2005/000663

(22) International Filing Date: 9 March 2005 (09.03.2005)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
10-2004-0015804 9 March 2004 (09.03.2004) KR

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(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, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

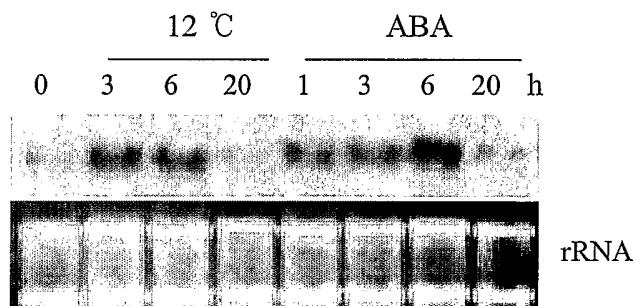
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, 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
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

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: STRESS-INDUCIBLE OSASR1 GENE AND PROTEIN ENHANCING TOLERANCE TO ABIOTIC STRESS



(57) Abstract: The present invention relates to abiotic stress-inducible OsAsr1 gene and protein enhancing plant tolerance to cold, salt or drought stress, where OsAsr1 is overexpressed in the plant. The OsAsr1 gene and protein of the present invention is very useful for enhancing plant tolerance to cold, salt or drought stress.

STRESS-INDUCIBLE OsAsr1 GENE AND PROTEIN ENHANCING TOLERANCE TO ABIOTIC STRESS

FIELD OF THE INVENTION

5 The present invention relates to abiotic stress-inducible OsAsr1 gene and protein enhancing plant tolerance to cold, salt or drought stress.

BACKGROUND OF THE INVENTION

10 As sessile organisms, plants must cope with environmental stresses, e.g., low temperatures, drought and salt stress. After such exposures, a series of changes occurs in their patterns of gene expression (Guy, 1999), a response that affects growth rates, productivity, and species distribution. Rice, a cereal crop of tropical or subtropical origin,
15 frequently suffers from chilling injury, and plants exhibit various symptoms such as chlorosis, necrosis, or growth retardation (De Datta, 1981). In temperate regions, rice frequently confronts chilling at the seedling stage as well as cool-summer damage during flowering. To understand the abiotic stress-response mechanism, researchers have
20 isolated a number of genes that encode abiotic stress-inducible proteins in several plant species (Guy, 1999; Thomashow, 1999). *Calcineurin B-like 1 (CBL1)* gene is highly inducible by drought, salt and cold stresses. In addition, *CBL1*-overexpressing plants showed enhanced tolerance to salt and drought (Cheong, Y.H., Kim, K.N., Pandey, G.K., Gupta, R.,
25 Grant, J.J., and Luan, S. 2003. *CBL1*, a calcium sensor that differentially regulates salt, drought, and cold responses in *Arabidopsis*. *Plant Cell* 15:1833-1845). The genes induced under low temperatures are thought to function in protecting cells by producing various gene products.

For example, *Arabidopsis* MAP kinases (ATMPK4 and ATMPK6) and C-repeat/DRE binding factor (CBF/DREB) transcription factors have roles in cold-stress signal transduction (Mizoguchi et al., 1993; Stockinger et al., 1997), while lipid desaturase is involved in membrane modifications during chilling (Gibson et al., 1994). Cold-induced pathogenesis-related proteins, e.g., chitinase-like and thaumatin-like proteins, function as antifreeze proteins (Hilovaara-Teijo et al., 1999). Moreover, chaperones, late embryogenesis abundant (LEA) proteins, calmodulin-related proteins, and 14-3-3 proteins might contribute to enhanced freezing tolerance (Thomashow, 1999): The enzymes required for biosynthesis of various osmoprotectants, such as sugars, prolines, and betaines are also important to a plant's osmotic adjustment (Kishor et al., 1995; Igarashi et al., 1997; Scott et al., 1999).

The *Asr* genes, which are responsive to ABA, osmotic stress, and ripening, have been identified in various species, including tomato, potato, apricot, loblolly pine, lily, maize, pummelo, grape and rice (Iusem et al., 1993; Canel et al., 1995; Silhavy et al., 1995; Wang et al., 1998; Chang et al., 1996; Mbeguie-A-Mbeguie et al., 1997; Vaidyanathan et al., 1999; Hong et al., 2002; Jeanneau et al., 2002; Cakir et al., 2003). High levels of the tomato *Asr1* mRNA can be detected in ripe fruit and in leaves subjected to water stress (Hagit et al., 1995). The level of lily ASR proteins is also increased through desiccation during pollen maturation (Wang et al., 1998). The physiological roles of ASR proteins are unclear yet. The localization of tomato ASR1 proteins in nucleus proposed the nature of tomato ASR proteins as nonhistone chromosomal proteins (Rossi and Iusem, 1994). Also, the structural and functional similarity of some ASR proteins with Late Embryo Abundant (LEA) or dehydrin proteins suggests a possible

role of ASR in the seed development (Maskin et al., 2001; Silhavy et al., 1995). Many known ASR proteins contain two conserved regions of a putative Zn-binding site at the N-terminal region and a putative nuclear localization sequence (NLS) at the C-terminal region of ~70 amino acids
5 (Cakir et al., 2003; Silhavy et al., 1995). These facts as well as the binding of grape ASR (VvMSA) to the promoter sequence of a monosaccharide transporter gene suggested that VvMSA is a component of the transcription-regulating complex involved in sugar and ABA signaling (Cakir et al., 2003).

10 Vaidyanathan et al. (1999) have identified an ASR cDNA, *OsAsr1*, from 'Pokkali' rice, where *OsAsr1* was both ABA- and osmotic (NaCl) stress-inducible. However, the functions of *OsAsr1* having hydrophilic alpha helix structure is almost unknown.

15 Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention
20 pertains.

DETAILED DESCRIPTION OF THIS INVENTION

The present inventors have made intensive study to find genes capable of enhancing tolerance to abiotic stress. As a result, the
25 inventors have found that *OsAsr1* (*Oryza sativa* responsive to ABA, osmotic stress, and ripening gene 1) gene was induced by stress in *Oryza sativa* and a plant transformed with the gene showed an enhanced tolerance to cold, salt or drought stress.

Accordingly, it is an object of this invention to provide an abiotic stress-inducible OsAsr1 protein enhancing plant tolerance to cold, salt or drought stress.

It is another object of this invention to provide a nucleic acid
5 which comprises a nucleotide sequence encoding the abiotic-stress inducible OsAsr1 protein.

It is other object of this invention to provide a vector comprising a nucleic acid comprising a nucleotide sequence encoding the abiotic-stress inducible OsAsr1 protein.

10 It is other object of this invention to provide a transformant transformed with the vector of the present invention.

It is other object of this invention to provide a method for preparing a transgenic plant showing an enhanced tolerance to cold, salt, or drought stress.

15 It is other object of this invention to provide a transgenic plant showing an enhanced tolerance to cold, salt, or drought stress.

Other objects and advantages of the present invention will become apparent from examples to follow, appended claims and drawings.

20

In one aspect of this invention, there is provided an OsAsr1 protein, comprising amino acid sequence of SEQ ID NO 2, which is inducible by abiotic stress, enhancing plant tolerance to cold, salt or drought stress, when the protein is overexpressed in the plant, or a homologue protein
25 sharing at least 80% amino acid sequence identity with the OsAsr1 protein.

The present inventors have made intensive study to find genes capable of enhancing tolerance to abiotic stress. As a result, the

inventors have found that *OsAsr1* (*Oryza sativa* responsive to ABA, osmotic stress, and ripening gene 1) gene was induced by stress in *Oryza sativa* and a plant transformed with the gene showed an enhanced tolerance to cold, salt or drought stress.

5 The term "abiotic stress" as used herein refers to stress caused by abiological factors such as cold, salt and drought.

 The term "cold stress" as used herein refers to stress caused by low temperatures, preferably below 15°C, more preferably 12°C and most preferably 10°C.

 The abiotic-stress inducible *OsAsr1* protein of the present
10 invention is considered to include acid sequences which have substantial identity to the aforementioned amino acid sequence and are capable of enhancing tolerance to cold, salt or drought stress. The phrase "substantial identity" refers to that an amino acid sequence has at least 80%, preferably at least 90%, most preferably at least 95% amino
15 acid sequence identity, when the aforementioned amino acid sequence of the present invention is compared and aligned for maximum correspondence with an amino acid sequence, as measured using conventional sequence comparison program (for example: Clustal X and PROSIS).

20 Meanwhile, the stress-inducible *OsAsr1* protein of the present invention may be defined as follows: (a) protein isolated from *Oryza sativa* L.; (b) protein having a Zn-binding motif in the region of N-end; and (c) protein having a nuclear localization domain in the region of C-end.

25

 In one aspect of this invention, there is provided a nucleic acid which comprises a nucleotide sequence encoding the abiotic-stress

inducible OsAsr1 protein.

In the present invention, the term "nucleic acid" is considered to include DNA (gDNA and cDNA) and RNA. Nucleoside, a basic unit
5 constituting nucleic acid, includes the natural nucleosides as well as the nucleosides having modified base moieties and/or modified sugar moieties (Scheit, *Nucleotide Analogs*, John Wiley, New York, 1980); Uhlman and Peyman, *Chemical Reviews*, 90:543-584 (1990))

Most preferably, the nucleic acid of the present invention
10 comprises 67-480 nucleotide sequence in SEQ ID NO:1. The nucleic acid of the present invention encoding abiotic stress-inducible OsAsr1 protein is considered to include nucleotide sequences which have substantial identity to the aforementioned nucleic acid. The phrase "substantial identity" refers to that an nucleotide sequence has at
15 least 80%, preferably at least 90%, most preferably at least 95% nucleotide sequence identity, when the aforementioned nucleotide sequence of the present invention is compared and aligned for maximum correspondence with an nucleotide sequence, as measured using conventional sequence comparison program (for example: Clustal X and
20 PROSIS).

In one aspect of this invention, there is provided a vector comprising a nucleic acid which comprises a nucleotide sequence encoding the abiotic-stress inducible OsAsr1 protein.

25 According to a preferred embodiment, the vector of the present invention comprises (a) nucleotide sequence encoding abiotic stress-inducible OsAsr1 protein and (b) promoter operably linked to the nucleotide.

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, signal sequence, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence affects transcription and/or translation of the nucleic acid corresponding to the second sequence.

The vector system of this invention may be constructed according to the known methods in the art as described in Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2001), which is incorporated herein by reference.

Typically, the vector may be constructed for cloning or expression. In addition, the vector may be constructed for use in prokaryotic or eukaryotic host cells.

For example, where the vector is constructed for expression in prokaryotic cells, it generally carries a strong promoter to initiate transcription (e.g., pL λ promoter, *trp* promoter, *lac* promoter, T7 promoter and *tac* promoter), a ribosome binding site for translation initiation and a transcription/translation termination sequence. In particular, where *E. coli* is used as a host cell, a promoter and operator in operon for tryptophan biosynthesis in *E. coli* (Yanofsky, C., *J. Bacteriol.*, 158:1018-1024 (1984)) and a leftward promoter of phage λ (pL λ promoter, Herskowitz, I. and Hagen, D., *Ann. Rev. Genet.*, 14:399-445 (1980)) may be employed as a control sequence.

The vector used in the present invention may be prepared from plasmid (e.g.: pSC101, ColE1, pBR322, pUC8/9, pHC79, pGEX series, pET series and pUC19), phage (e.g.: λ gt4, λ B, λ -Charon, λ Δ z1 and M13 etc)

or virus (e.g.: SV40) used conventionally in the art.

Where the vector of the present invention is an expression vector constructed for eukaryotic host cell, a promoter derived from the genome of mammalian cells (e.g., metallothionein promoter) or mammalian virus (e.g., adenovirus late promoter; vaccinia virus 7.5K promoter, SV40 promoter, cytomegalovirus promoter and *tk* promoter of HSV) may be used. The vector generally contains a polyadenylation site as a transcription termination sequence.

The vector of this invention may further comprise a nucleotide sequence to conveniently purify the abiotic stress-inducible OsAsr1 protein expressed, which includes but not limited to, glutathione S-transferase (Pharmacia, USA), maltose binding protein (NEB, USA), FLAG (IBI, USA) and 6X His (hexahistidine; Quiagen, USA). Due to the additional sequence, the protein expressed in host can be purified with affinity chromatography in a rapid and feasible manner.

It is preferable that the expression vector of this invention carries one or more markers which make it possible to select the transformed host, for example, genes conferring the resistance to antibiotics such as ampicillin, gentamycin, carbenicillin, chloramphenicol, streptomycin, kanamycin, geneticin, neomycin, geneticin and tetracycline.

Since the gene of the present invention has been isolated from plant and is capable of enhancing plant tolerance to abiotic stress, it is most useful for plants.

Accordingly, the vector of the present invention comprises (i) nucleotide sequence encoding abiotic stress-inducible OsAsr1 protein and (ii) a promoter that functions in plant cells to cause the production of an RNA molecule operably linked to the nucleotide sequence of (i) ;

and (iii) a 3'-nontranslated region that functions in plant cells to cause the polyadenylation of the 3'-end of said RNA molecule.

According to a preferred embodiment of the present invention, where the expression vector is constructed for a plant cell, numerous
5 plant-functional promoters known in the art may be used, including the maize ubiquitin promoter, the cauliflower mosaic virus (CaMV) 35S promoter, the nopaline synthetase (nos) promoter, the Figwort mosaic virus 35S promoter, the sugarcane bacilliform virus promoter, the commelina yellow mottle virus promoter, the light-inducible promoter
10 from the small subunit of the ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO), the rice cytosolic triosephosphate isomerase (TPI) promoter, the adenine phosphoribosyltransferase (APRT) promoter of Arabidopsis, and octopine synthase promoters.

According to a preferred embodiment of the present invention,
15 the 3'-non-translated region causing polyadenylation in this invention may include that from the nopaline synthase, gene of Agrobacterium tumefaciens (nos 3' end) (Bevan et al., Nucleic Acids Research, 11(2) :369-385(1983)), that from the octopine synthase gene of Agrobacterium tumefaciens, the 3'-end of the protease inhibitor I or
20 II genes from potato or tomato, the CaMV 35S terminator.

The vector may alternatively further carry a gene coding for reporter molecule (e.g. luciferase and P-glucuronidase).

The vector may contain antibiotic (e.g. neomycin, carbenicillin, kanamycin, spectinomycin and hygromycin) resistance genes (e.g.
25 neomycin phosphotransferase (nptII), hygromycin phosphotransferase (hpt) as selective markers.

In further aspect of this invention, there is provided a

transformant transformed with the above-described vector of the present invention.

The hosts useful in cloning and expressing the vector of the present invention are well known to those skilled in the art. For example, *E. coli* JM109, *E. coli* BL21, *E. coli* RR1, *E. coli* LE392, *E. coli* B, *E. coli* X 1776, *E. coli* W3110, *Bacillus subtilis*, *Bacillus thuringensis*, *Salmonella typhimurium*, *Serratia marcescens* and various *Pseudomonas* may be employed.

As eukaryotic cell, yeast (*Saccharomyces cerevisiae*), insect cell, human cell (e.g., CHO cell lines (Chinese hamster ovary), W138, BHK, COS-7, 293, HepG2, 3T3, RIN and MDCK cell lines) and plant cell may be used.

Since OsAsr1 gene of the present invention is useful for plant, the transformants include callus derived from plant cells or tissues.

The transformation of a host cell can be carried out by a large number of methods known to one skilled in the art. For example, in case of using prokaryotic cells as host, CaCl₂ method (Cohen, S.N. et al., Proc. Natl. Acad. Sci. USA, 9:2110-2114 (1973)), Hanahan method (Cohen, S.N. et al., Proc. Natl. Acad. Sci. USA, 9:2110-2114 (1973); and Hanahan, D., J. Mol. Biol., 166:557-580 (1983)) and electroporation (Dower, W.J. et al., Nucleic Acids Res. 16:6127-6145 (1988))' can be used for transformation. Also, in case of using eukaryotic cells as host, microinjection (Capecchi, M.R., Cell, 22:479 (1980)), calcium phosphate precipitation (Graham, F.L. et al., virology, 52:456 (1973)), electroporation (Neumann, E. et al., EMBO J., 1:841 (1982)), liposome-mediated transfection (Wong, T.K. et al., Gene, 10:87 (1980)), DEAE-dextran treatment (Gopal, Mol. Cell Biol., 5:1188-1190 (1985)), and particle bombardment (Yang et al., Proc. Natl. Acad. Sci.,

87:9568-9572 (1990)) can be use for transformation.

The vector introduced to host cell is expressed in the host cell, thereby, a mass of abiotic stress-inducible OsAsr1 is obtained.

5 In one aspect of this invention, there is provided a method for preparing transgenic plant showing an enhanced tolerance to cold, salt or drought stress, which comprises the steps of: (a) transforming a plant cell or tissue with the vector of the present invention; (b) selecting a transformed plant cell or tissue; and (c) regenerating the transformed
10 plant cell or tissue to obtain a transgenic plant.

In one aspect of this invention, there is provided a transformant which shows an enhanced tolerance to cold, salt or drought stress.

The explants used in the present invention are plant cells or
15 tissues. Employing callus is preferred in case of using plant tissues.

The transformation of plant cells may be carried out according to the conventional methods known one of skill in the art, including electroporation (Neumann, E. et al., EMBO J., 5 1:841 (1982)), particle bombardment (Yang et al., Proc. Natl. Acad. Sci., 87: 9568-9572 (1990))
20 and Agrobacterium-mediated transformation (U.S. Pat. Nos. 5,004,863, 5, 349,124 and 5,416,011). Among them, Agrobacterium-mediated transformation is the most preferable.

The selection of transformed cells may be carried out with exposing the transformed cultures to a selective agent such as a metabolic
25 inhibitor, an antibiotic and herbicide. Cells which have been transformed and have a stably integrated marker gene conferring resistance to the selective agent will grow and divide in culture. The exemplary marker includes, but not limited to, a hygromycin phosphotransferase gene,

a glyphosphate resistance gene and a neomycin phosphotransferase (nptII) system.

The development or regeneration of plants from either plant protoplasts or various explants is well known in the art. The development
5 or regeneration of plants containing the foreign gene of interest introduced by *Agrobacterium* may be achieved by methods well known in the art (U.S. Pat. Nos. 5,004,863, 5,349,124 and 5,416,011).

The method of the present method may be applicable to various plants, preferably to corps such as rice, barley, wheat and maize, more
10 preferably rice and most preferably japonica cultivars.

In the present invention, transformation is preferably performed with *Agrobacterium* system, more preferably with *Agrobacterium tumefaciens*-binary vector system.

An embodiment of a method using *Agrobacterium tumefaciens*-binary
15 vector system comprises the steps of: (a') inoculating an explant material from the plant with *Agrobacterium tumefaciens* harboring a vector, in which the vector is capable of being inserted into a genome of a cell from the plant and contains the following nucleotide sequences: (i) the nucleotide sequence encoding abiotic stress-inducible OsAsr1
20 protein; (ii) a promoter that functions in plant cells to cause the production of an RNA molecule operably linked to the nucleotide sequence of (i); and (iii) a 3'-non-translated region that functions in plant cells to cause the polyadenylation of the 3'-end of the RNA molecule;
(b') regenerating the inoculated explant material on a regeneration
25 medium to obtain regenerated transformants.

Transformation of plant cells derived is carried out with *Agrobacterium tumefaciens* harboring Ti plasmid (Depicker, A. et al., Plant cell transformation by *Agrobacterium* plasmids. In Genetic

Engineering of Plants, Plenum Press, New York (1983)).

More preferably, binary vector system such as pBin19, pRD400, pRD320, pGA1611 and pGA1991 is used for transformation (An, G. et al., Binary vectors" In Plant Gene Res. Manual, Martinus Nijhoff Publisher, New York(1986); An et al. 1988; and Lee et al., 1999). The binary vector
5 useful in this invention carries: (i) a promoter capable of operating in plant cell; (ii) a structural gene operably linked to the promoter; and (iii) a polyadenylation signal sequence. The vector may alternatively further carry a gene coding for reporter molecule (for example, luciferase and p-glucuronidase). Examples of the promoter used in the binary vector
10 include but not limited to cauliflower mosaic Virus 35S promoter, 1' promoter, 2' promoter and promoter nopaline synthetase (nos) promoter.

Inoculation of the explant with *Agrobacterium tumefaciens* involves procedures known in the art. Most preferably, the inoculation
15 involves immersing the explant in the culture of *Agrobacterium tumefaciens* to coculture. *Agrobacterium tumefaciens* is infected into plant cells.

The explant transformed with *Agrobacterium tumefaciens* is regenerated in a regeneration medium. The transgenic plant is
20 finally produced on a rooting medium.

The transformed plant produced according to the present invention may be confirmed using procedures known in the art. For example, using DNA sample from tissues of the transformed plant, PCR is carried out to elucidate exogenous gene incorporated into a genome of the transformed
25 plant. Alternatively, Northern or Southern Blotting may be performed for confirming the transformation as described in Maniatis et, al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).

The *OsAsr1* gene and protein of the present invention is very useful for enhancing plant tolerance to cold, salt or drought stress

5 BRIF DESCRIPTION OF THE DRAWINGS

Figure 1 represents comparison of amino acid sequence of the deduced *OsAsr1* protein with other known Asr proteins performed with the CLUSTALW program. Putative Zn^{2+} DNA binding site and nuclear localization signal (NLS) were underlined. White letters in a black
10 box indicate 16 out of 16 matches.

Figure 2 is a dendrogram representing a phylogenetic analysis of various Asr proteins with rice homologues. The rice homologues were retrieved from TBLASTN search against the indica rice WGS contigs. The dendrogram was constructed using the ClustalX and Mega2 program. The
15 respective accession number is described next to the plant name. The lengths of horizontal lines reflect the evolutionary distance.

Figure 3 represents southern blot analysis of *OsAsr1*. Twenty μ g of genomic DNA from three japonica cultivars, Namyang 21 (1), Odae (2), Dongjin (3), was cut with *EcoRI* (E), *HindIII* (H), and *BamHI* (B), and
20 hybridized with radiolabeled *OsAsr1* probe. Positions and sizes in kb of *HindIII* digested *OsAsr1* DNA are indicated.

Figures 4a-4e represent *OsAsr1* expression analysis under the treatments of various abiotic stress and ABA: Figures 4a-4c represent northern blot analysis of *OsAsr1*. Thirty (A) or 10 μ g (B and C) of total
25 RNA was separated, blotted, and hybridized with radiolabeled *OsAsr1* probe. Figure 4a represents *OsAsr1* expression in the various organs or tissues. Lanes: 1, callus; 2, shoot of seedling; 3, root of seedling;

4, mature leaf; 5, leaf sheath of flag leaves; 6, highest internode (between node I and II) at prehead stage; 7, 1-2 cm panicle; 8, 3-8 cm panicle; 9, mature panicle prior to anthesis, 10, developing seeds of 3 days after pollination (DAP); 11, developing seeds of 6 DAP. Figure 4b represents the cold inducible expression of *OsAsr1*. Mature plants were treated at 12 °C for 4 d. L, leaves; CL, cold-treated leaves; F, florets; CF, cold-treated florets. Figure 4c represents expression of *OsAsr1* by different temperature and ABA. Seedlings grown at 30°C were treated with 4 °C, 12 °C, or 10µM ABA for 3 hr, 6 hr and 20 hr. Figure 4d represents analysis of *OsAsr1* expression under various abiotic stresses and ABA. *Salt* was used for the drought-, cold-, salt-, and ABA-response controls. Transcript of rice *actin* gene shows an internal control for PCR analysis. Figure 4e represents analysis of *OsAsr1* expression levels using real-time PCR. Error bars represent standard deviation. 8-d seedlings were treated with 4°C (c), 250 mM NaCl (s), drought (d) or 100 µM ABA (A) and then were periodically harvested.

Figures 5a and 5b represent *in situ* localization of *OsAsr1* mRNA. Cross sections of the rice flowers of 4 days before heading and leaves exposed to 12°C for 4 days were hybridized with digoxigenin-labeled antisense (A, C, E, and G) or sense (B, D, F, and H) *OsAsr1* probes. Higher magnification of the cross section is shown (C, D, G, and H). an, anther; lm, lemma; pa, palea; l.e.p, lower epidermis of palea; LVB, large vascular bundle; mc, motor cell; xy, xylem; ph, phloem; me, mesophyll. Bar = 0.3 mm.

Figures 6a-6c represent analysis of transgenic plants expressing *OsAsr1* in sense and antisense orientation. Figure 6a represents construction of *OsAsr1* sense (pSK167) and antisense (pSK168) expression

vector for rice transformation. P_{UBI} , maize ubiquitin promoter; P_{35S} , CaMV 35S promoter; Tnos, terminator sequence of the nopaline synthase gene; T7, terminator sequence of the transcripts 7; *hph*, the hygromycin phosphotransferase gene for selection of transgenic rice callus; RB and
5 LB, the right and left border sequences, respectively, of the Ti plasmid from *Agrobacterium tumefaciens*. Figure 6b represents southern blot analysis of transgene. Southern blot was conducted using 10 μ g of genomic DNA cut with *Hind*III from transgenic plants. The ubiquitin promoter fragment in the transformation vector was used as a probe. Positions
10 and sizes in kb of *Hind*III-digested DNA are indicated. Figure 6c represents expression of *OsAsr1* in the sense transgenic rice (*Ubiquitin::OsAsr1*). Leaf total RNA from wild type segregant (WS) and sense transgenic plants under normal growth condition was separated, blotted, and hybridized with radiolabeled *OsAsr1* probe. Number
15 represents each transgenic line. EtBr-stained rRNA bands indicate an equal amount of loading.

Figure 7 represents stress tolerance of *Ubiquitin::OsAsr1* plants as judged by the measurement of chlorophyll fluorescence. Changes in the chlorophyll fluorescence of the extended leaves under cold stress
20 was measured. Functional damage to photosynthesis was estimated by measuring the mean and standard error of F_v/F_m values.

Figure 8 represents expression of *OsAsr1* in *CBF1* transgenic rice. Total RNA from mature leaves of two strong *CBF1*-expressors (18-2 and 18-3) and the wild type non-transgenic line (NT) under control (30 °C)
25 or cold (4 °C) treated condition was hybridized with radiolabeled *OsAsr1* probe. EtBr-stained rRNA bands indicate an equal amount of loading.

Figure 9 represents salt tolerance of transgenic rice plants

expressing *OsAsr1* in sense (9a) or antisense (9b) orientation. 12-day old seedlings treated with 200 mM NaCl for 1 d (for sense plants) or 12 h (for antisense plants) were recovered to normal condition for 2 d.

5 Figure 10 represents wilting ratio of rice cultivars and transgenic *OsAsr1* rice plants. 12-day old seedlings treated 200 mM NaCl for 1 d (for sense plants; A) or 12 h (for antisense plants; B) were recovered to normal condition for 2 d.

10 Figure 11 represents drought tolerance transgenic rice plants expressing *OsAsr1* in sense (11a) or antisense (11b) orientation. 14-day old seedlings treated no water for 4 d were returned to normal growth conditions for 7 d.

15 Figure 12. wilting ratio of rice cultivars and transgenic *OsAsr1* rice plants. 14-day old seedlings treated no water for 4 d were recovered to normal condition for 7 d.

The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

20

EXAMPLES

MATERIALS AND METHODS

Plant samples and bacterial strains

25 'Dongjin', a cultivar of japonica rice (*Oryza sativa*), was used for constructing our seed-coat cDNA library and for generating transgenic plants. The other cultivars for cold tolerance are listed in Table 1, 2 and 3. Seeds were surface-sterilized and then germinated either in

water or on an MS agar medium (Murashige and Skoog, 1962). Also, seeds were germinated in Yoshida solution (Yoshida et al., 1976). They were grown under hydroponically controlled conditions having 29°C day/21°C night temperatures, 16-h-light/8-h-dark cycle, and 83% relative humidity.

The seedlings were transferred to soil and kept in a growth chamber at 30 °C under continuous light (intensity of 60 to 70 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). For the cold treatment, 11-d-old seedlings exposed to 4 °C or 12 °C in the dark were harvested. For the ABA, 5-d-old seedlings transferred to an MS liquid medium containing 10 μM ABA were harvested. For the salt or ABA treatments, seedlings were transferred to an Yoshida solution containing either 250 mM NaCl or 100 μM ABA and then were periodically harvested. For drought treatment, the seedlings were dried in the air and then periodically harvested. To study the effect of cold stress at the reproductive stage, field-grown rice plants at ca. 4 d before heading were transferred to the greenhouse (30 °C, 14-h light/10-h dark) for 2 d of adapting. Afterward, they were treated for 4 d at 12 °C under a 14-h photoperiod (ca. 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). All tissue samples were immediately frozen with liquid nitrogen and stored at -70 °C. The *E. coli* strain, XL-1 Blue MRF' { Δ (mcrA)183 Δ (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB, lacI^qZ Δ M15 Tn10 (Tet^r)]} was used as a host for molecular cloning.

Construction of cDNA library and EST Analysis

The coats from 4- to 8-day-old seeds were manually dissected under a microscope. A cDNA library was then constructed from poly A RNA prepared from the seed coats. cDNA clones were randomly selected from the library,

and their 5' ends were sequenced. The DNA preparation, sequencing, and computer analysis were performed as described by Hong et al. (1998). Briefly, template DNAs were prepared by the alkaline lysis method, and the inserts were sequenced with an ABI PRISM™ BigDye™ Terminator Cycle Sequencing kit (Amersham). Computer software, such as DNAsis, Prosis (Hitachi), ClustalX, ClustalW, and GeneDoc (Thompson et al., 1994; Nicholas and Nicholas, 1997), were used for our sequence analysis. The Genbank, EMBL, and Swiss-Prot databases were then searched for amino-acid sequence homology using the BLASTX algorithm (Altschul et al., 1997).

DNA and RNA gel- blot analysis

DNA gel-blot analyses were conducted on three japonica cultivars - 'Namyang 21', 'Dongjin', and 'Odae'. Namyang 21 is cold-sensitive (the cold tolerance index, CTI = 7), Odae is cold-tolerant (CTI = 3), and Dongjin is intermediate (CTI = 5) (Lim, 1998). In the CTI value given between 0 to 9, 0 is most tolerant, 9 most sensitive. Genomic DNA was extracted from their seedlings according to the cetyltrimethylammonium bromide method (Roger and Bendich, 1988). Ten micrograms of DNA, digested with restriction enzymes for 6 h at 37 °C, was separated on a 0.8% agarose gel, then transferred to a Hybond-N membrane (Amersham) using a vacuum transfer system (Hoefer). For RNA gel-blot analyses, 10 µg of total RNA was resolved on a 1.3% formaldehyde agarose gel and blotted onto a nylon membrane. The DNA and RNA blot analyses were performed using the radiolabeled *OsAsr1* probe. To prepare this probe, *OsAsr1* cDNA fragments were radioactively labeled with [α -³²P] dCTP (3000 ci mmol⁻¹), following the random priming method. Unincorporated nucleotides were removed via G-50 Sephadex column chromatography. After

hybridization, the membrane was washed with $2 \times \text{SSC}$, 0.1% SDS at RT for 15 min; $1 \times \text{SSC}$, 0.1% SDS at RT for 15 min; and $0.1 \times \text{SSC}$, 0.1% SDS at RT for 15 min. Hybridization signals were detected with an image analyzer (BAS-1500, Fuji) and exposed on HyperfilmTM MP film (Amersham).

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RNA in situ hybridization

Rice flowers and leaves were fixed overnight at 4 °C in 2% (wt/vol) paraformaldehyde plus 2.5% (vol/vol) glutaraldehyde in a 50-mM PIPES buffer (pH 7.2). The fixed tissues were dehydrated by graded concentrations of ethanol, then embedded in a paraplast medium (Oxford labware, USA). The embedded tissues were sliced into 7- μm sections with a rotary microtome (Leica, Germany), and each section was attached to a silanized glass slide (Matsunami, Japan). Paraffin was removed through a graded series of ethanol concentrations, and the samples were dried for 1 h. Dioxygenin-labeled sense or antisense RNA probes were prepared from the linearized pBluscript carrying the *OsAsr1* cDNA, using either T3 or T7 RNA polymerase. The sections were hybridized with the probes at 48 °C for 16 h in a hybridization solution, then washed in a solution containing $2 \times \text{SSC}$, $1 \times \text{SSC}$, and $0.1 \times \text{SSC}$ for 15 min at 50°C. The hybridizing probe was detected colorimetrically using an anti-DIG conjugated alkaline phosphatase (Boehringer Mannheim). Photographs were taken under bright-field microscopy (Nikon Eclipse 600).

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Production and analysis of transgenic rice plants

Full-length *OsAsr1* cDNA was introduced into the binary vector pGA1611 (An et al., 1988; Kim et al., 2003), in the sense and antisense orientations downstream of the maize *ubiquitin* promoter (Christensen

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et al., 1992). Rice transformation was performed via the *Agrobacterium*-mediated cocultivation method (Jeon et al., 1999; Lee et al., 1999).

Rice seeds of which lemma and palea have been removed were placed on 2N6 medium containing 2 mgL^{-1} 2,4-D and cultivated for one month to induce callus. *Agrobacterium* cultured in AB medium containing 30 mgL^{-1} hygromycin B and 3 mgL^{-1} tetracycline for 3 days were cocultured with the induced calli in 2N6-ASB medium at 20°C under dark condition for 3 days. Then, they were placed in 2N6-CH40 medium containing 40 mgL^{-1} hygromycin B and 250 mgL^{-1} cefotaxime for 3 weeks. Well growing calli were selected and regenerated on MS medium containing 0.1 mgL^{-1} NAA, 2 mg L^{-1} kinetin, 2% sorbitol, 1.6% fitagar, 50 mgL^{-1} hygromycin B and 250 mgL^{-1} cefotaxime under continuous light for 3 days. The regenerated individuals were transferred to soil and grown in the greenhouse. All transgenic rice plants were generated on a 40-mg L^{-1} hygromycin B-containing medium, and were transferred to the greenhouse after regeneration. PCR analysis to verify those transgenic plants was performed using the forward primer (5'-CAC CCT GTT GTT TGG TG-3') and the reverse primer (5'-GCG GGA CTC TAA TCA TAA AAA CC-3'). PCR conditions included 1 min at 94°C , 1 min at 54°C , and 1 min at 72°C , through 30 cycles.

Determination of chlorophyll fluorescence

About 5-cm-long segments of the extended leaves from mature plants were floated on MS liquid media at 4°C under white fluorescent light ($260 \mu\text{mol m}^{-2} \text{ sec}^{-1}$) for 0, 6, 12, or 24 h. After 30 min of dark-adaptation, chlorophyll fluorescence signals were measured with a Plant Efficiency

Analyzer (Hansatech). All experiments were repeated four times.

Determination of cold stress

Rates of survival were calculated for plants exposed to cold stress
5 by assessing either their degree of wilting (Saijo et al., 2000) or
the amount of their regrowth (Lee et al., 1993). Ten day-old seedlings
(at the three-leaf stage), that had been raised in a growth chamber (16-h
light/8-h dark; light intensity of $60 \mu\text{mol m}^{-2} \text{sec}^{-1}$; 30°C), were exposed
to 4°C for 3, 4, 5, 6, 7, 10, or 12 d under continuous light ($60 \mu\text{mol}$
10 $\text{m}^{-2} \text{sec}^{-1}$). Afterward, they were returned to standard growth-chamber
conditions for 10 d to allow for recovery. The wilting ratio was based
on the level of prominent chlorosis and withering of the leaves. To
analyze regrowth, seedlings that had previously been exposed to 4°C
for 4 d were returned to the growth chamber for 13 d of recovery. Regrowth
15 was defined as the production of new, fourth leaves on the seedlings.

Determination of drought and salt stress

Plants were grown for about 2 weeks in the growth chamber (30°C ,
with light/dark cycles of 16/8 h) and then used to measure survival
20 rate after stress treatment. For the drought-survival test, they received
no water for 4 d. Afterwards they were returned to normal growth conditions
for 7 d and numbers of wilted and healthy plants were counted (Lee,
S.C., Huh, K.W., An, K., An, G., and Kim, S. R. 2004b Ectopic expression
of a cold-inducible transcription factor. CBF1/DREB1b, in transgenic
25 rice (*Oryza sativa* L.) Mol. Cells 18: 107-114). For the salt stress
treatment, 10-d-old seedlings were incubated in a nutrient solution
of 0.1 % (w/v) Hyponex (Hyponex) for 2 days and then transferred to fresh

nutrient solution containing 200 mM NaCl for 1 d (for sense plants) or 12 h (for antisense plants) at 30 °C with a photoperiod of 16 h (50-60 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ light). After salt stress, the roots of the seedlings were rinsed with water, and then grown in a fresh nutrient solution without NaCl under the normal condition. Wilting after stresses was determined as three and four leaves of the plant completely wilted showing prominent chlorosis.

Results

10 Identification of *OsAsr1* cDNA from the rice seed-coat library

The cDNA clone isolated from our developing seed coats is highly homologous with *OsAsr1*, found previously by Vaidyanathan et al. (1999). This clone is 838 bp long and comprises a 66-bp, 5' untranslated region (UTR), a 417-bp open reading frame, a 325-bp 3' UTR, and a poly (A)-tail (data not shown). It is 4 bp shorter at the 5' end than the previously reported clone, but its nucleotide sequence exactly matches *OsAsr1* except for a T-to-C change at the 3' UTR (304 bp downstream of the termination codon). Because the previously isolated cDNA was from 'Pokkali', an indica rice, and the clone identified here is from the japonica type 'Dongjin', we propose that this one-nucleotide variation at the 3' UTR is due to the difference in cultivars.

An *OsAsr1* genomic sequence was retrieved from the indica rice WGS genomic database (Yu et al., 2002). *In-silico* analysis of the clone predicted that core sequences of the ABA response element (ABRE), ACGT, are present at positions -261, -610 and -616 from the first ATG codon. An important cold-responsive *cis* element, C-repeat/drought responsive element (CRT/DRE) sequence, GCCGAC, is also found at -714. Putative

Myb (-143, -149, and -698), Myc (-71, -512, and -694) and bZIP (-202 and -711) binding sites are also found. A putative Zn-binding His-residue and the putative NLS domain are present in the N-terminal and the C-terminal region of OsAsr1 protein, respectively (Figure 1). A comparison of the cDNA and the genomic sequences revealed a 119-bp-long intron in the *OsAsr1* gene. TBLASTN searches (Altschul et al., 1997), using the OsAsr1 protein sequence (accession no. AAB96681) as the query, were able to retrieve five homologues in the scaffolds (002913, 037286, 026604, 023736, and 081294). Their phylogenetic relationship presented as a dendrogram indicates that OsAsr1 has the highest homology with ZmAsr1 of maize (Figure 2). ASR proteins had been categorized into 4 main groups (Hong et al., 2002) and OsAsr1 belongs to group I related to ZmAsr1. More than 100 rice *OsAsr1* cDNA sequences are registered in the GenBank database, indicating that the gene belongs to an abundantly expressed gene family. Moreover, the *OsAsr1* homologues have been found only in plant species.

DNA gel-blot analysis of OsAsr1

The results of our DNA gel-blot analyses on three japonica cultivars showed only one copy of the *OsAsr1* gene (Figure 3). This observation matches that previously made with 'Pokkali' (Vaidyanathan et al., 1999). No significant polymorphism was found with regard to restriction-fragment lengths for these three cultivars of different cold tolerance.

Induction of OsAsr1 by cold stress

The inventors of the present invention used RNA gel-blot analyses to examine gene expression at different tissues under various abiotic

condition. The *OsAsr1* transcript was detectable in all the tissues and organs except in calluses (Figure 4a). *OsAsr1* transcript was induced by cold, drought, salt, and ABA treatment. It reached their maximum after 3 h of drought and salt stresses (Figure 4d and 4e). The transcript size was about 0.9 kb, indicating that the *OsAsr1* cDNA clone is nearly full-length. The transcript was present at high levels in the shoots and roots of seedlings, sheath of flag leaves, and most abundantly in the internodes between node I and II, demonstrating organ-preferential expression.

OsAsr1 was expressed at the basal level in the leaves of mature plants, but more abundantly in mature flowers. Interestingly, low temperatures elevated overall transcript levels in both organ types (Figure 4b). Cold treatment increased the transcript level also at the seedling stage, but more significantly at 12 °C instead of 4 °C (Figure 4c). The cold-responsive *OsAsr1* accumulation was restricted to shoots, demonstrating the organ-specific stress response of the gene expression.

Because *OsAsr1* is ABA-inducible (Vaidyanathan et al., 1999), we compared the induction kinetics of cold stress versus that of ABA. Whereas transcript levels reached their maximum after 3 h of cold stress, ABA treatment caused levels to increase more slowly, achieving their highest point only after 6 h (Figure 4c). The expression of *OsAsr1* in the physiological range of growth temperature (12, 20, and 30 °C) was compared between the cold-tolerant cultivar Odae and the cold-sensitive cultivar Namyang 21. However, we did not observe any significant difference of the gene expression between the cultivars.

RNA *in-situ* hybridization was performed to further elucidate the cold-induced expression pattern of the *OsAsr1* gene at the tissue level.

In the leaves, induction was confined to the mesophyll tissues, and was not present in the epidermal or vascular tissues (Figure 5e and g). Because the flower is a primary target organ of cold stress, hybridization was also performed for the flowers. Immature flowers at 4 d before heading were cold-treated at 12 °C for 4 d. Afterward, *OsAsr1* transcript was detectable in the parenchyma cells of the palea and lemma from those stressed flowers (Figure 5a and c). In the control flowers that had not been cold-stressed, the *OsAsr1* expression pattern was similar to that of the cold-stressed flowers although the expression level was much lower.

Expression of *OsAsr1* cDNA in transgenic plants

Binary vectors containing *OsAsr1* cDNA were constructed in the sense (pSK167) or antisense (pSK168) orientation under the maize ubiquitin promoter (Figure 6a), to determine the function of *OsAsr1*. Twenty transgenic plants were generated via the *Agrobacterium* co-cultivation method, and integration of the transgene into the rice genome was examined by DNA gel-blot analyses. The copy number was usually one to two, although three or more copies of the introduced genes were detected (Figure 6b). Transgenic plants showed no significant morphological changes in their T1 and T2 generations.

To examine whether the changed expression of *OsAsr1* has a role in the cold tolerance of the transgenic rice, RNA gel-blot analyses were conducted (Figure 6c). Overall, the *OsAsr1* transcript level was higher in the transgenic plants compared with the wild-type segregants. Size of the hybridized transcript in the transgenic plants was slightly bigger than those of wild type. Two strong over-expressers, S2 and S15,

were selected for further analyses.

Chlorophyll fluorescence of transgenic plants under cold stress

Chlorophyll fluorescence was measured as an indicator of chilling tolerance after cold treatment (4°C). The ratio of Fv to Fm, which represents the activity of Photosystem II, is used to assess functional damage in plants (Genty et al., 1989). For our wild-type segregants, Fv/Fm progressively decreased following chilling. This decline illustrates the extent of photoinhibition caused by cold stress (Krause, 1994). Values for Fv/Fm had been 0.84 ± 0.01 before stress was induced. Following the 6-h cold treatment, Fv/Fm decreased slightly, and no significant difference in values was found between the transgenic and the wild-type segregants. After 24 h of treatment, however, the Fv/Fm values for the wild-type were reduced significantly, to 0.25 ± 0.021 (Figure 7). In contrast, the Fv/Fm values for the over-expressers S2 and S15 were 0.53 ± 0.049 and 0.60 ± 0.067 , respectively. These ratios were about two-fold higher those calculated for the wild-type (Figure 7), which indicates that the transgenic plants had a higher degree of cold tolerance.

Survival test

The rate of survival for transgenic plants at the seedling stage was measured in wilting and regrowth tests. The critical length of the cold treatment was first determined by assessing the amount of wilting (i.e., the ratio of the number of wilted seedlings to the total number of cold-treated seedlings) after stress.

The cold-tolerant cultivars, 'Odae' and 'Stejaree 45', had wilting

ratios of 10/18 (55.6%) and 8/20 (40%), respectively, after 5 d of exposure to low temperatures (Table 1). This wilting frequency increased to about 75% after 6 d of stress. In contrast, the parental line of our *OsAsr1* transformation, 'Dongjin', exhibited wilting symptoms in 12 of 17 plants (70.6%) after 5 d of stress. The wilting frequency increased to about 94.% after 6 d of stress. A cold-sensitive indica x japonica cultivar, 'Milyang 23', showed 100% wilting after only 4 d. Therefore, it is concluded that the critical length of time for tolerating low-temperature stress by 'Dongjin' was about 5 to 6 d. Based on that assumption, the inventors chose to cold-treat our T2 transgenic plants for 6 d. Of the 34 seedlings obtained from the S2 plants, 23 (68%) showed wilting after chilling while 10 (67%) of 15 seedlings from the S15 plants exhibited those symptoms. In contrast, 63 (96%) of 66 seedlings derived from the wild-type segregating plants had wilted. The antisense transgenic plants were examined as well, with the occurrence of wilting in A3, A12, and A14 being 9/9 (100%), 9/11 (82%), and 10/11 (91%), respectively.

The regrowth test, conducted in the T3 generation, involved analyzing homozygous lines for the transgene after hygromycin selection. Of the 35 seedlings from S15 plants, 14 (40%) showed active development of a fourth leaf. In contrast, the S18 plants, which are from a weaker *OsAsr1*-expressing line, presented only 7 of 35 plants (20%) with signs of regrowth. Nevertheless, the wild type-segregating control plants had even lower rates of regrowth, i.e., 9%, or 9 out of 105 plants. Similar results were seen from the A3 plants, in which only 7 of 70 (10%) showed any response. Taken together, these results indicate that the transgenic plants over-expressing the *OsAsr1* transcript gained increased tolerance to cold stress. However, the level of tolerance did not change noticeably when *OsAsr1* expression was suppressed in the

antisense plants.

Table 1

Cold stress tolerance of rice cultivars and rice transgenic *OsAsr 1*
5 rice

Cultivars or lines	Cold treatment period (days)							
	0	3	4	5	6	7	10	12
Odae	0/18*	2/20	5/20	10/18	13/17	17/18	19/19	19/19
Stejaree 45	0/18	0/20	6/20	8/20	15/20	18/19	20/20	19/19
Hapchunaengmi	0/18	0/20	4/20	8/20	10/20	18/19	20/20	19/19
Chuchung	0/18	0/19	5/20	9/20	18/19	19/19	20/20	20/20
Dongjin	0/20	6/20	6/20	12/17	17/18	17/18	20/20	19/19
Namyang	0/19	10/16	12/20	16/18	18/18	19/19	19/19	20/20
Milyang	0/19	13/18	17/17	17/17	17/17	19/19	19/19	20/20
Transgenic S2	-	-	-	-	23/34	-	-	-
Transgenic S15	-	-	-	-	10/15	-	-	-
Wild type segregant	-	-	-	-	63/66	-	-	-
* no. of wilted seedlings/no. of cold treated seedlings								

Expression of *OsAsr1* cDNA in transgenic rice plants expressing *Arabidopsis CBF1*

Since a CRT/DRE core sequence is located in the putative promoter
10 region of *OsAsr1*, the gene may be controlled by CBF1. To this end, we
have generated transgenic rice plants that ectopically expressed
Arabidopsis CBF1 cDNA. RNA gel-blot experiments showed that expression
of the *OsAsr1* gene was elevated in transgenic plants (18-2 and 18-3)
that strongly expressed the CBF1 transcription factor (Figure 8).

Expression of *OsAsr1* in the transgenic plants was further increased by cold stress.

Salt tolerance of transgenic OsAsr1 plants

5 The rate of survival for transgenic plants at the seedling stage was measured in wilting tests. For accurate experiments, I showed the salt tolerance with two wild type rice cultivars, a japonica cultivar, Dongjin and an indica cultivar, 'Tachung Native 1' (TN1). Ten-d-old seedlings were transferred to a nutrient solution, 0.1% (w/v) Hyponex
10 solution, containing 200mM NaCl. Sense transgenic plants were treated for 1 d and antisense transgenic plants were treated for 12 h at 30 °C, and then returned into a fresh nutrient solution without NaCl under normal condition for 2 d. Wilting after stresses was determined as three and four leaves of the plant completely wilted showing prominent
15 chlorosis.

 The salt-tolerant cultivar, TN1, had wilting ratios of 36.1 ± 8.4 % after 2 d of recovery (Figure 9, Figure 10, Table 2). In contrast, the parental line of our *OsAsr1* transformation, 'Dongjin', exhibited wilting symptoms in 66 of 86 plants (77 ± 22.5 %) after 2 d of recovery. 39 (67 ± 4.1 %)
20 of 58 seedlings derived from the wild-type segregating plants had wilted after 2 d of recovery. Two strong over-expressers, S2 and S15, were selected for stress tolerance tests. The plants of each T3 line were homozygous plants. Homozygous T3 transgenic plants are salt stressed for 1 d for sense and 12 h for antisense plants. These salt-treated
25 times were referred to the literature (Kim S.H., PhD thesis, Sogang University, 2004). Of the 94 seedlings obtained from the S2-1 plants, 26 (27 ± 11 %) showed wilting. Also, of the 88 seedlings obtained from

the S2-2 plants, 27 ($31 \pm 8\%$) showed wilting. However, 33 ($45 \pm 22\%$) of 73 seedlings from the S15-1 plants exhibited those symptoms and 34 ($39 \pm 5\%$) of 88 seedlings from the S15-2 plants showed wilting. In contrast, the antisense transgenic plants were examined after 12 h of stress, with the occurrence of wilting in A3-1 and A3-2 being 38/53 ($72 \pm 3\%$) and 30/67 ($45 \pm 23\%$), respectively. After 5 d of recovery, the wilting ratio was increased in 64/73 ($73 \pm 4\%$) plants of TN1, 86/86 ($100 \pm 0\%$) plants of Dongjin, 58/58 ($100 \pm 0\%$) plants of NT-S2, 87/94 ($93 \pm 3\%$) plants of S2-1, 75/88 ($85 \pm 2\%$) plants of S2-2, 71/73 ($97 \pm 4\%$) plants of S15-1, and 87/88 ($99 \pm 2\%$) plants of S15-2. All experiments were repeated three times. Based on these data, it is concluded that overexpression of *OsAsr1* could confer salt tolerance on rice plants and that the *OsAsr1* antisense lines show increased salt sensitivity than wild type.

Table 2

Salt stress tolerance of the rice cultivars and transgenic *OsAsr1* rice

Line	Wilted	Total	Wilting ^a (%)
TN1	32	88	36 ± 8
Dongjin	66	86	77 ± 23
NT-S2	39	58	67 ± 4
S2 -1	26	94	27 ± 11
S2 -2	27	88	31 ± 8
S15-1	33	73	45 ± 22
S15-2	34	88	39 ± 5
A3 -1	38	53	72 ± 3
A3 -2	30	67	45 ± 23

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NT-S2	17	50	34±4
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^aNumbers of wilting plants/ numbers of total plants (percentage of numbers of wilting plants). Numbers of wilting plants indicate three and four leaves of plants wilted 2d recovery after salt stress.

5 Drought tolerance of transgenic *OsAsr1* plants

Drought tolerance of *OsAsr1* transgenic plants were assessed. For accurate experiments, the inventors showed that the drought tolerance with two wild type rice cultivars, Sangnambathyeo and Dongjin (Table 3, Figures 1 and 12). 14-d-old seedlings were grown in the growth chamber
 10 (30 °C, with light/dark cycles of 16/8 h) and then received no water for 4 d. We chose to drought-treat our T3 transgenic plants for 4 d. Wilting after stresses was determined as three and four leaves of the plant completely wilted showing prominent chlorosis. These drought-treated times are referred to the literature (Lee, S.C., Huh,
 15 K.W., An. K., An. G., and Kim, S. R. 2004b Ectopic expression of a cold-inducible transcription factor. CBF1/DREB1b, in transgenic rice (*Oryza sativa* L.) Mol. Cells 18: 107-114). Of the 60 S2-1 seedlings, 11 (18±12%) showed wilting. Also, among the 61 S2-2 seedlings, 15 (25±10%) showed wilting. However, 14 (27±13%) of 51 seedlings from the S15-1
 20 plants exhibited those symptoms and 17 (29±4%) of 58 seedlings from the S15-2 plants showed wilting after 4 d of stress. The inventors also examined the antisense transgenic plants for the drought tolerance. The antisense plants A3-1 and A3-2 showed sensitivity of 41/45 (91±12%) and 38/51 (75±3%), respectively. All experiments were repeated three
 25 times. These data suggest that overexpression of *OsAsr1* could confer drought tolerance on rice plants and that the *OsAsr1* antisense lines

show more drought sensitivity than wild type.

Table 3

Drought stress tolerance of the rice cultivars and transgenic *OsAsr1* rice plants.

Line	Wilted ^a	Total	Wilting (%)
SNB	25	57	44±16
Dongjin	64	100	64± 6
NT-S2	35	59	59± 3
S2 -1	11	60	18±12
S2 -2	15	61	25±10
S15-1	14	50	28±13
S15-2	17	58	29±4
A3 -1	38	53	90±12
A3 -2	30	67	79±3

^a Numbers of WT and T3 plant, three and four leaves of which completely wilted 7 d recovery after drought stress.

As described above, the present invention provides abiotic stress-inducible *OsAsr1* protein enhancing tolerance to cold, salt, or drought stress. In addition, the present invention provides nucleic acid which comprises a nucleotide sequence encoding the abiotic-stress inducible *OsAsr1* protein. The present invention provides a vector and a transformant. Furthermore, the present invention provides transgenic plants showing an enhanced tolerance to cold, salt, or drought stress

and a method for preparing thereof. The *OsAsr1* gene and protein of the present invention is very useful for enhancing plant tolerance to cold, salt or drought stress.

5 Having described specific examples of the present invention, it is to be understood that such examples are only preferred embodiments and should not be construed as limiting the scope of the invention. Therefore, the substantive scope of the invention may be determined by appended claims and their equivalents

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What is claimed is:

1. An OsAsr1 protein comprising amino acid sequence of in SEQ ID NO 2, which is inducible by abiotic stress, enhancing plant tolerance to cold, salt or drought stress when the protein is overexpressed in the plant,
5 or a homologue protein sharing at least 80% amino acid sequence identity with the OsAsr1 protein.

2. A nucleic acid which comprises a nucleotide sequence encoding the abiotic-stress inducible OsAsr1 protein according to claim 1.

10

3. The nucleic acid according to claim 2, wherein the nucleotide sequence corresponds to 67-480 nucleotide sequence in SEQ ID NO 1.

4. A vector comprising the nucleic acid according to claim 2 or 3.

15

5. A transformant transformed with the vector according to claim 4.

6. A method for preparing a transgenic plant showing an enhanced tolerance to cold, salt or drought stress, which comprises the steps of:

20 (a) transforming a plant cell or tissue with the vector according to claim 4;

(b) selecting a transformed plant cell or tissue; and

(c) regenerating the transformed plant cell or tissue to obtain a transgenic plant.

25

7. The method according to claim 6, wherein the plant is rice, barley, wheat or maize.

8. A transgenic plant prepared by the method according to claim 6 or 7 which shows an enhanced tolerance to cold, salt or drought stress.

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Fig 1

OsAsr1	: MAEEK--HHHHLF	-----KDDEPATGVDS--YGEGVYT-----	: 32
Maize	: MAEEKH--HHHHLF	-----KDEQEQLAGGGYGESA EY-----	: 35
Tomato1	: MAEEKH-QHHHHLF	-----	: 16
Tomato2	: MEEKK--HHHHLF	-----	: 15
Tomato3	: MAEEK--QHHLRF	-----	: 14
Tomato4	: MAEEKH-QHHHHLF	-----	: 16
Potato1	: --KHH--FGGLFH	-----NEEDTPVEKTTYEETTYGESEKSTSYGEKTSYDHTYGEKTTTFGDDNKYGE	: 65
Potato2	: MEEKK--HHHHLF	-----DK	: 16
Grape	: MSEEK--HHHHLF	-----DKPVDDAVPYSDNAYSDDTYS-----	: 35
Apricot	: MSEEK--HHHGLF	-----HKDEDRPIETSDYPQSGGYSDEGR TGS--GYGGGGGGYGGGGGGYDGGGGGGYD	: 64
Melon	: MAEEEQGHHHHHLF	-----	: 17
Pummelo			: -
Pine1	: MSEEKQ--HHHHLF	-----EDG--GE	: 20
Pine2		-----EDENDNPPSEVVYFETTTA--YGD	: 25
Pine3		-----EDG--	: 7
Pine4	: MSEEKH--HHHLLH	-----KDESENVSPSEVVC AETTTA--YGD	: 37

Zn²⁺ DNA binding site

OsAsr1	:	SETVTTEVVAG-----	:	43
Maize	:	TEATVTEVVST-----	:	46
Tomato1	:	-----NKE-----	:	19
Tomato2	:	-----DKA-----	:	18
Tomato3	:	-----NRE-----	:	17
Tomato4	:	-----NKE-----	:	19
Potato1	:	KTSYGGDDTYGEKPTSYGGDNTYGEKTSYGKGDDNKYGEKTSYGEGDDNKYGEKTSYGDSGYGEKPSYGGG	:	135
Potato2	:	-----	:	-
Grape	:	DTSYATDGVSG-----	:	46
Apricot	:	NTAYSGEGRPGSGYGGGG-----	:	82
Melon	:	-----KEG-----	:	20
Pummelo	:	-----MAG-----	:	3
Pine1	:	QVIESTDVIYAA-----	:	31
Pine2	:	EVIQSADVYAT-----	:	36
Pine3	:	---ESADVDE-----	:	15
Pine4	:	EVIOASADVYAA-----	:	48

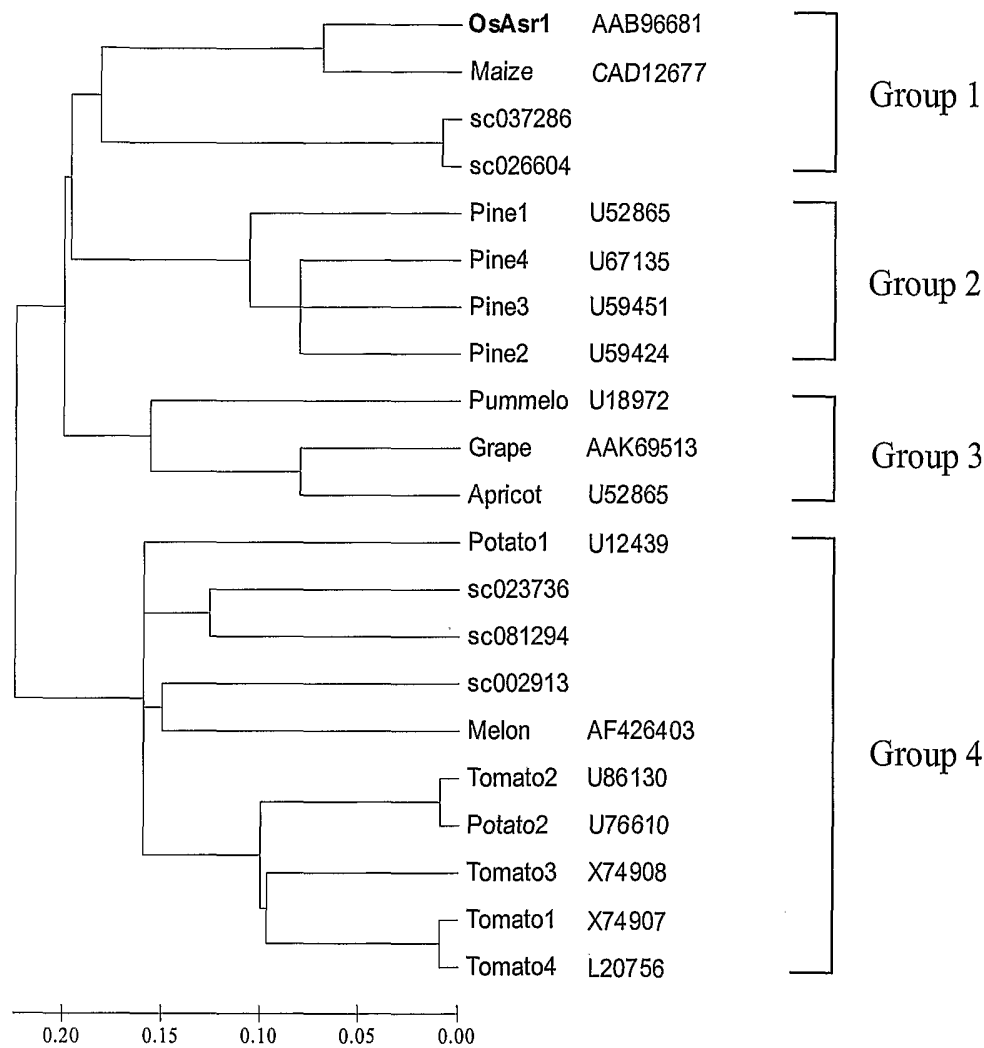
OsAsr1	:	-----GQDE-----	YERYK	E	E	H	K	K	H	K	H	E	A	A	L	A	F	A	F	Y	:	80					
Maize	:	-----GNE-----	YDEMK	E	E	H	K	K	H	K	H	E	A	A	I	A	F	A	Y	:	82						
Tomato1	:	-----DEGG-----	PVDYE	V	V	H	H	S	L	E	K	I	E	L	A	V	A	L	A	H	:	56					
Tomato2	:	-----EEG-----	PVDYE	I	I	H	K	K	L	E	Q	I	K	L	T	V	A	A	Y	H	:	54					
Tomato3	:	-----EGGG-----	PVDHK	K	V	H	H	S	L	Q	K	I	E	L	A	V	A	R	Y	H	:	54					
Tomato4	:	-----DEGG-----	PVDYE	V	V	H	H	S	L	E	K	I	E	L	A	V	A	L	H	:	56						
Potato1	:	DNKYGEKTSYGNEEGGYGGVGGETTNYEENESETK	SDPMKE	K	K	H	K	L	E	E	T	G	L	A	V	A	F	A	H	:	205						
Potato2	:	-----EEEG-----	PVDYE	K	T	K	E	H	H	L	E	Q	I	G	K	L	G	T	V	A	G	A	A	L	H	:	92
Grape	:	-----YAAETTEVLADD-----	PAPDWR	E	E	H	K	K	L	E	H	L	E	L	A	V	A	A	Y	A	:	52					
Apricot	:	-----GYGESADYSDGGRYKETAAAYGTTGTNE-----	SEIDMK	E	E	H	K	K	L	E	H	L	S	E	P	A	A	V	F	H	:	143					
Melon	:	-----EESS-----	DVVDYK	K	K	H	K	K	L	E	H	L	E	L	A	V	A	A	Y	H	:	58					
Pummelo	:	-----KEEE-----	FDMK	E	E	H	K	K	L	E	H	L	E	L	G	T	A	G	A	P	P	R	L	S	:	39	
Pine1	:	-----GNVD-----	EYEKAK	D	E	H	K	K	M	E	H	L	E	M	T	V	A	F	A	H	:	69					
Pine2	:	-----GNVNSD-----	EYEKAR	E	E	H	K	K	M	E	E	V	E	G	L	X	M	T	A	F	H	:	76				
Pine3	:	-----GNVKSE-----	EKAK	E	E	H	K	K	L	E	E	T	G	L	A	V	T	G	P	E	:	53					
Pine4	:	-----GEVNDK-----	FAEYEKAR	E	E	H	K	K	L	E	E	T	G	L	T	V	A	F	A	H	:	91					

OsAsr1	:	EAKKKE	N	R	H	T	E	I	A	T	V	G	A	F	A	F	H	B	KD	H	S	A	B	E	S	T	G	E	K	K	H	H	L	F	G	-	138			
Maize	:	EAKKKE	H	R	H	E	E	V	A	A	V	S	E	P	A	F	H	B	KD	H	K	D	A	B	A	G	E	K	K	H	H	F	F	G	-	140				
Tomato1	:	EAKKKE	H	R	K	E	E	T	M	A	V	V	A	E	P	A	F	H	B	DA	K	E	K	K	V	E	G	G	H	H	H	H	H	-	144					
Tomato2	:	EAKKKE	H	R	K	E	E	T	M	A	V	V	A	E	P	A	F	H	B	DA	K	E	E	K	K	L	R	G	D	T	T	I	S	S	K	L	F	-	145	
Tomato3	:	EAKKKK	N	K	K	K	K	I	A	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	178				
Tomato4	:	EAKKKE	H	R	K	E	E	T	M	A	V	V	A	E	P	A	F	H	B	DA	K	E	K	K	V	E	G	G	H	H	H	H	-	-	-	144				
Potato1	:	EAKKEI	N	K	K	E	B	G	I	A	A	I	A	E	P	A	F	H	B	E	A	K	E	E	E	A	E	G	K	K	H	H	H	H	F	N	-	263		
Potato2	:	KHEAK	K	D	P	H	A	K	H	K	I	E	I	A	A	-	A	V	G	A	-	F	A	L	H	E	-	H	H	L	F	-	-	-	-	-	109			
Grape	:	EAKKEK	H	R	K	E	E	I	P	A	A	V	G	A	F	A	F	H	B	E	A	K	E	D	E	A	H	G	K	K	H	H	L	F	-	149				
Apricot	:	ESKKK	H	R	K	E	E	I	A	A	V	S	S	E	P	A	F	H	B	E	A	K	E	D	E	S	H	G	K	K	H	H	L	F	-	200				
Melon	:	EAKKKS	S	H	S	E	K	K	V	G	A	V	V	A	E	P	A	F	H	B	E	A	K	R	E	K	-	-	-	-	-	-	-	-	-	-	112			
Pummelo	:	EAKKE	D	H	R	E	E	I	A	A	G	L	S	E	P	A	F	H	B	E	A	K	E	D	O	A	H	G	K	S	T	T	I	S	F	N	-	98		
Pine1	:	ADKKK	H	R	H	E	E	V	A	A	V	E	A	G	V	F	H	B	E	S	K	E	E	K	A	E	G	K	K	H	H	L	F	-	126					
Pine2	:	EAEKK	H	R	H	E	E	I	A	A	V	E	A	G	V	F	H	B	E	S	K	R	A	E	A	A	Q	Q	K	K	H	-	-	-	-	-	131			
Pine3	:	EEKKK	H	R	E	E	E	V	A	A	A	T	A	G	E	V	L	H	R	D	V	E	D	K	R	E	K	A	E	G	K	K	H	-	-	-	108			
Pine4	:	ASKKK	N	A	R	H	E	E	I	A	A	V	E	A	G	V	F	H	B	E	S	K	E	E	K	A	E	G	K	K	H	H	L	F	V	R	C	H	-	153

NLS

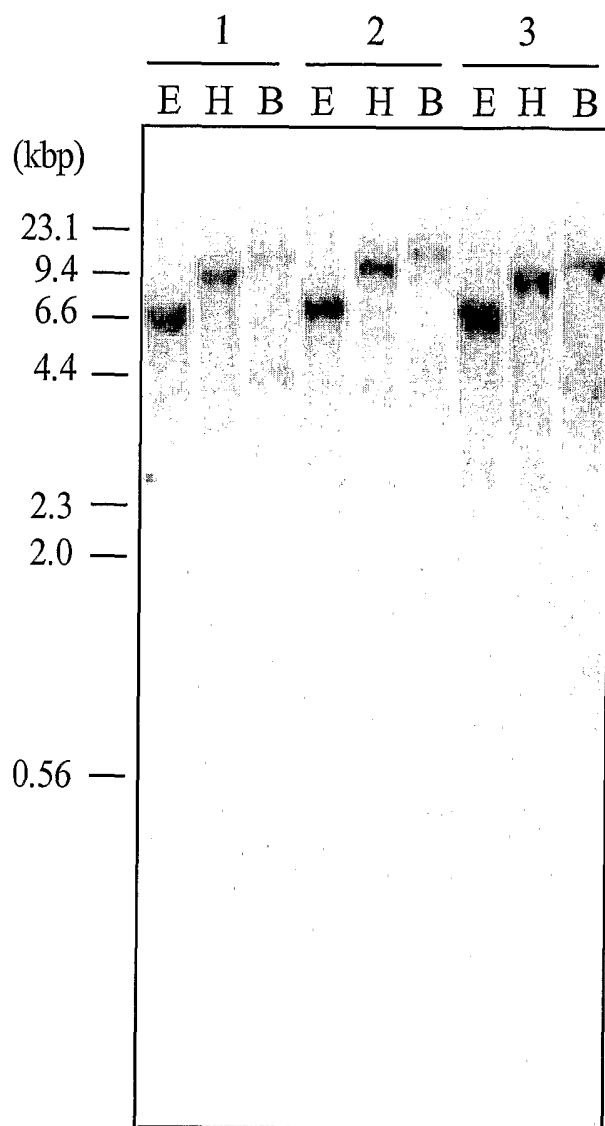
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Fig 2



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Fig 3



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Fig 4a

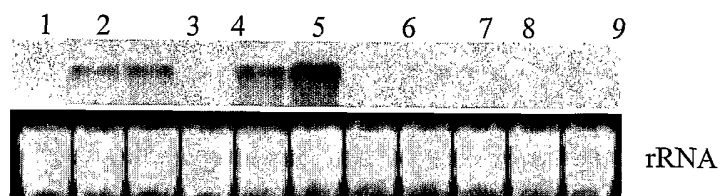


Fig 4b

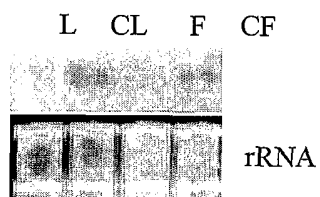


Fig 4c

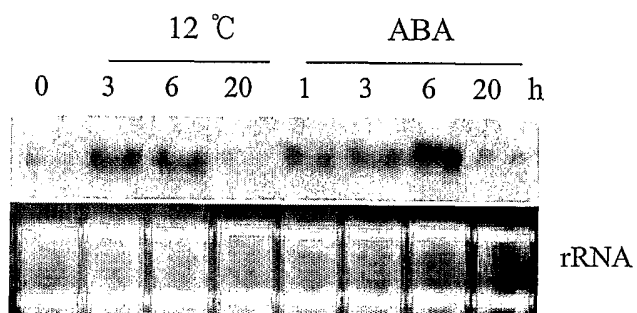


Fig 4d

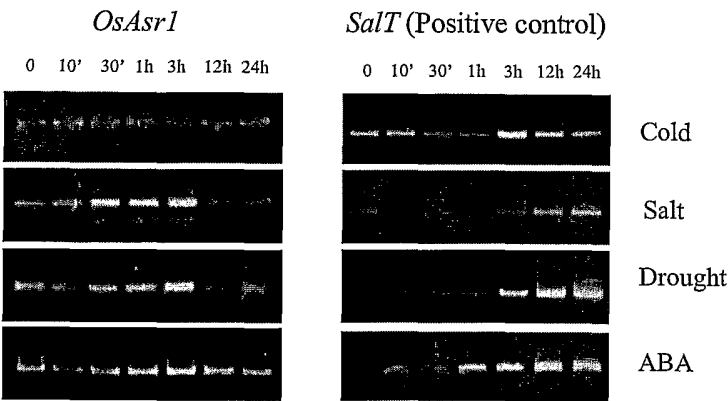
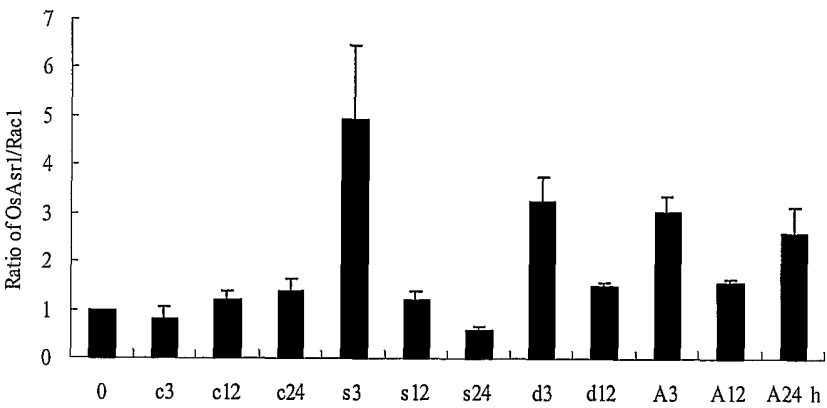


Fig 4e



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Fig 5a

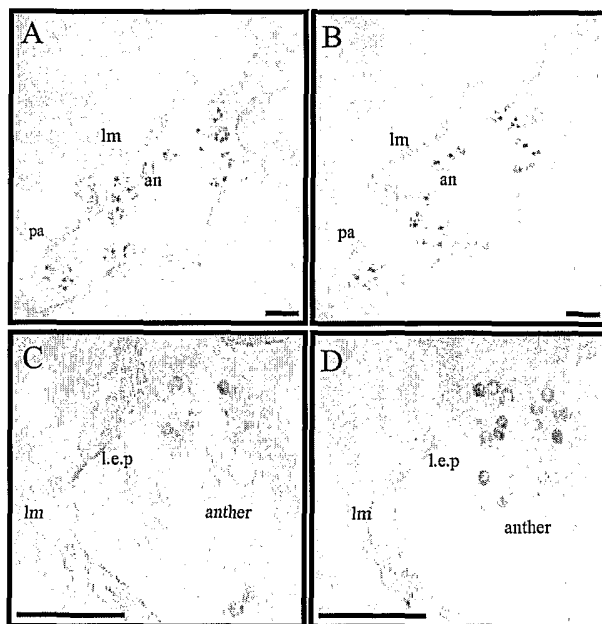


Fig 5b

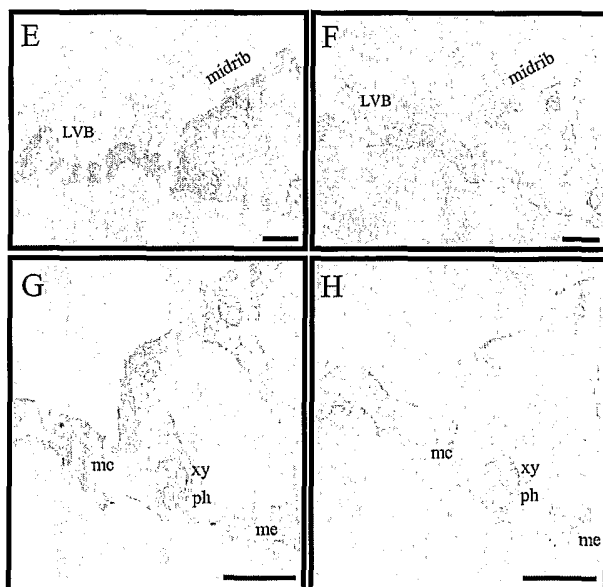


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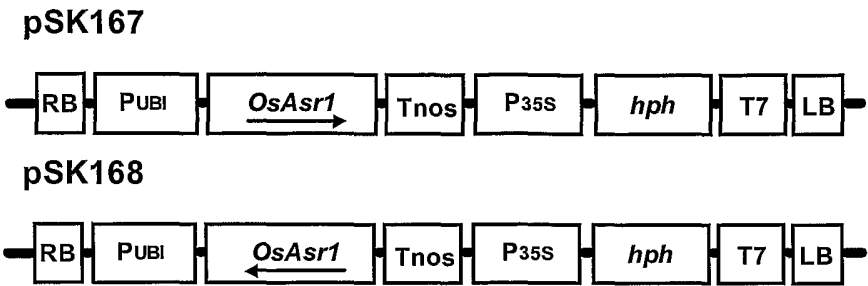


Fig 6b

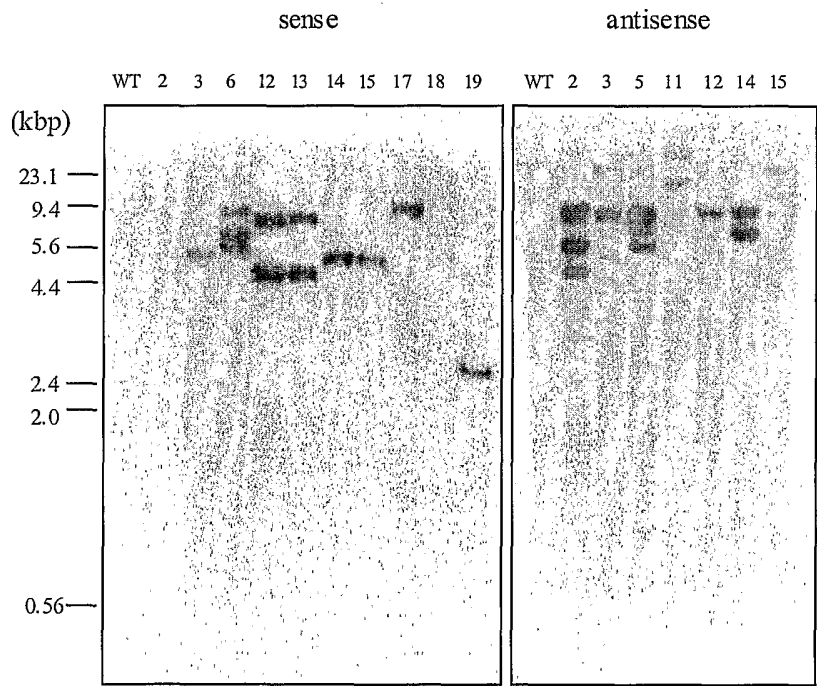
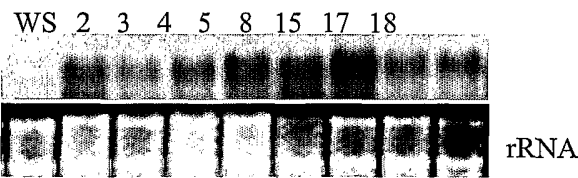
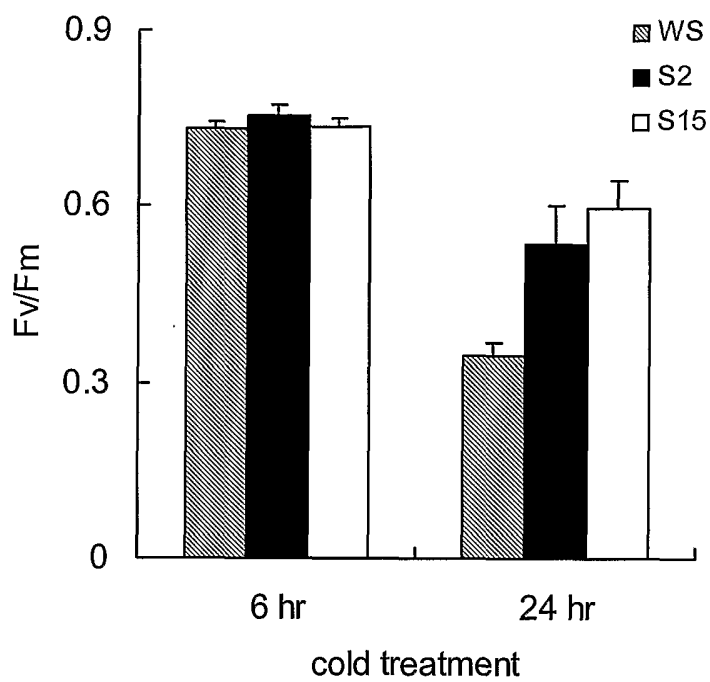


Fig 6c



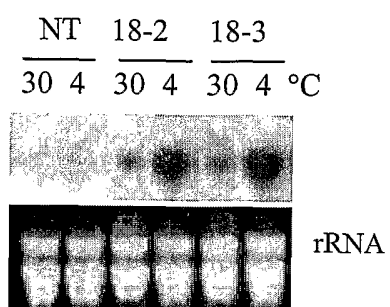
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Fig 7



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Fig 8



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Fig 9a

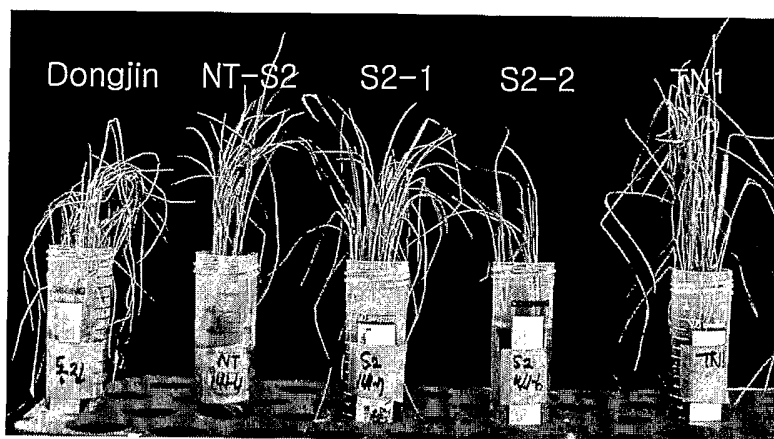


Fig 9b



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Fig 10a

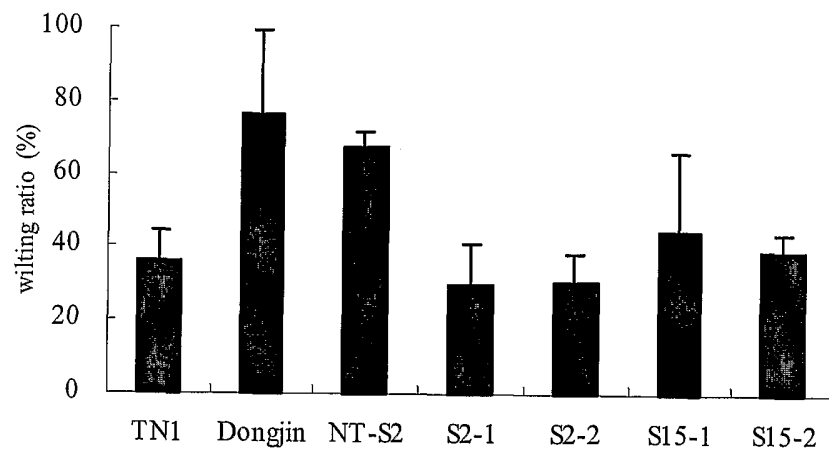
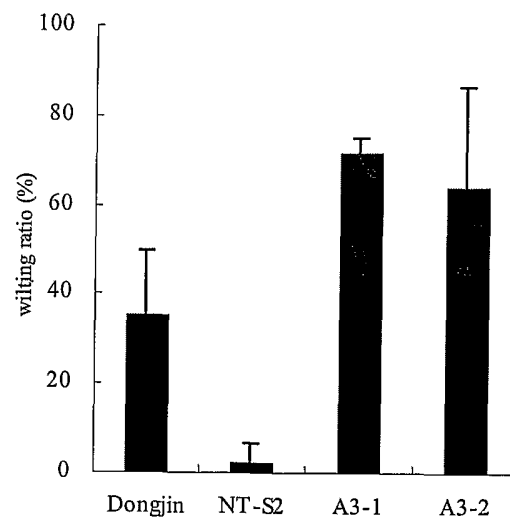


Fig 10b



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Fig 11a

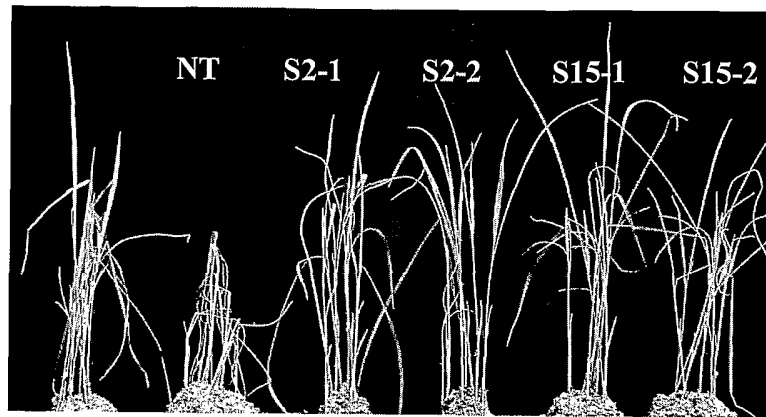
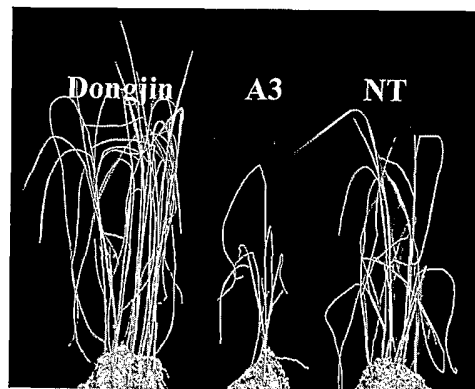
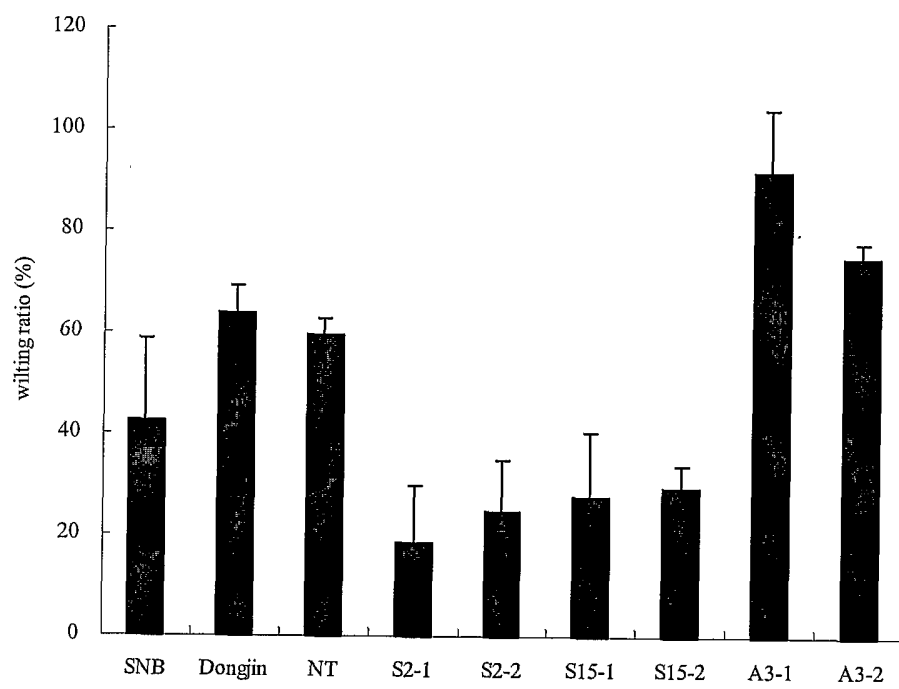


Fig 11b



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Fig 12



Sequence Listing

<110> Sogang University

<120> Stress-Inducible OsAsr1 Gene and Protein Enhancing Tolerance to Abiotic stress

<160> 2

<170> KopatentIn 1.71

<210> 1

<211> 838

<212> DNA

<213> Oryza sativa

<220>

<221> CDS

<222> (67)..(480)

<400> 1

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Met Ala Glu Glu Lys His His His His Leu Phe His His Lys
1 5 10

aag gac gac gag ccg gcc acc gga gta gac tcc tac ggc gag ggc gtc 156
Lys Asp Asp Glu Pro Ala Thr Gly Val Asp Ser Tyr Gly Glu Gly Val
15 20 25 30

tac acg tcg gag acg gtg acc acc gag gtg gtc gcc ggc ggc cag gac 204
Tyr Thr Ser Glu Thr Val Thr Thr Glu Val Val Ala Gly Gly Gln Asp
35 40 45

gag tac gag agg tac aag aag gag gag aag cag cac aag cac aag cag 252
Glu Tyr Glu Arg Tyr Lys Lys Glu Glu Lys Gln His Lys His Lys Gln
50 55 60

cac ctc ggc gag gcc ggc gcc ctc gcc gcc ggc gcc ttc gcc ctg tat 300
His Leu Gly Glu Ala Gly Ala Leu Ala Ala Gly Ala Phe Ala Leu Tyr
65 70 75

Sequence Listing

gag aag cac gag gcg aag aag gac ccg gag aac gcg cac agg cac aag 348
 Glu Lys His Glu Ala Lys Lys Asp Pro Glu Asn Ala His Arg His Lys
 80 85 90

atc acg gag gag atc gcg gcc acg gcg gcg gtc ggc gcc ggc ggc tac 396
 Ile Thr Glu Glu Ile Ala Ala Thr Ala Ala Val Gly Ala Gly Gly Tyr
 95 100 105 110

gcc ttc cac gag cac cac gag aag aag aag gac cac aag agc gcc gag 444
 Ala Phe His Glu His His Glu Lys Lys Lys Asp His Lys Ser Ala Glu
 115 120 125

gag tcc acc ggc gag aag aag cac cac ctc ttc ggc tgatcgacct 490
 Glu Ser Thr Gly Glu Lys Lys His His Leu Phe Gly
 130 135

catcacaacg tcgccggcgg cggcgacgac ctgcccgtag gtcgccggcc gccgtgtcgt 550

cgatttgtgt gtgtaataat ttgtcttctt ctgcatgcgt ggtgttgctg tttttcacia 610

gagtctccgg cctcgaccag tgagaggctg acaggtgggg ccgtgagttc agcttgtgtt 670

gcttgatttt ctctgcagcc ttgccctctg tgtgtccaaa taagtgggtg gcatggctct 730

ctccgtgtca tgtatcaatg tatttttatc tgtactttgt acaagtgaag caatatttat 790

cgaaccttga cttttctcaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 838

<210> 2

<211> 138

<212> PRT

<213> Oryza sativa

<400> 2

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Asp Glu Pro Ala Thr Gly Val Asp Ser Tyr Gly Glu Gly Val Tyr Thr

Sequence Listing

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35	40	45
Glu Arg Tyr Lys Lys Glu Glu Lys Gln His Lys His Lys Gln His Leu		
50	55	60
Gly Glu Ala Gly Ala Leu Ala Ala Gly Ala Phe Ala Leu Tyr Glu Lys		
65	70	75
80		
His Glu Ala Lys Lys Asp Pro Glu Asn Ala His Arg His Lys Ile Thr		
85	90	95
Glu Glu Ile Ala Ala Thr Ala Ala Val Gly Ala Gly Gly Tyr Ala Phe		
100	105	110
His Glu His His Glu Lys Lys Lys Asp His Lys Ser Ala Glu Glu Ser		
115	120	125
Thr Gly Glu Lys Lys His His Leu Phe Gly		
130	135	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2005/000663

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C07K 14/415**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7: C07K, C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubMed, NCBI, Delphion, eKIPASS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	NCBI Accession No. AAB96681 (Mar. 17, 2000)	1-3 4-8
Y	Ajay, K.G., et al. "Trehalose accumulation in rice plants confers high tolerance levels tolerance levels to different abiotic stresses" PNAS, Nov., 2002, Vol. 99: pages 15898-15903, See entire document.	4-8
A	Hong, J.K., et al. "Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its in situ localization in pepper (Capsicum annuum)" Physiol Plant, Apr., 2002, Vol. 114(4): pages 549-558, See entire document.	1-8
A	Kim, T.E., et al. "ABA and polyamines act independently in primary leaves of cold-stressed tomato (Lycopersicon esculentum)" Physiol Plant, Jul., 2002, Vol. 115(3): pages 370-376, See entire document.	1-8



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

12 JULY 2005 (12.07.2005)

Date of mailing of the international search report

12 JULY 2005 (12.07.2005)

Name and mailing address of the ISA/KR

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Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

PARK, JEONG UNG

Telephone No. 82-42-481-8159



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2005/000663

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



in written format



in computer readable form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in computer readable 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: