NOVEL TRANSCRIPTIONAL FACTOR ENHANCING THE RESISTANCE OF PLANTS TO OSMOTIC STRESS

AtSIZ

EtBr

Cold Drought

0 0.5 1 6 0.5 1 6 (h)

AtSIZ

EtBr

NaCl ABA

0 0.5 1 6 0.5 1 6 (h)

AtSIZ

EtBr


[Continued on next page]
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NOVEL TRANSCRIPTIONAL FACTOR ENHANCING THE RESISTANCE OF PLANTS TO OSMOTIC STRESS

Technical Field

The present invention relates to a transcription factor of a plant, AtSIZ, induced by osmotic stress; a gene encoding the transcription factor; and a method for enhancing stress resistance in plants by using the gene.

Background Art

Environmental stresses such as high concentration of salt, drought and cold inhibit plant growth to limit harvest yields in many important agricultural fields. In terms of these stresses, osmotic stress caused by diverse external conditions, for example, high salt, dehydration and cold, is a crucial problem for farmers.

Therefore, with even a slight increase of tolerance to such osmotic stress, it is expected that there would be a considerable improvement in agricultural productivity and yield of crops. For this reason, much research into regulatory mechanisms of plants, with respect to osmotic stress, and regulatory factors involved in the mechanism, have been ongoing. Recent studies have revealed that plants employ elaborate mechanism to partially adapt to osmotic stress, and one of important requirements for such stress-adaptation is the transcriptional activation of a gene encoding a protein necessary to such adaptation (Jang et al., Plant Mol. Biol., 37: 839-847, 1998; Liu et al.,
Many genes induced by certain stresses were isolated, and their characteristics have been widely studied, being helpful to understand mechanisms involved in adaptation to osmotic stress. From these studies, it has become clear that there are multiple signaling pathways that lead to induction of osmotic stress responsive genes (Jonak et al., Proc. Natl. Acad. Sci. USA, 93: 11274-11279, 1996; Ishitani et al., Plant Cell 9: 1935-1949, 1997), and these pathways include ABA (abscisic acid)-dependent or ABA-independent pathway (La Rosa et al., Plant Physiol., 85: 174-181, 1987; Savoure et al., Mol. Gen. Genet., 254: 104-109, 1997). In addition, it was discovered that some signaling pathways are common to all osmotic stress conditions, such as high salt, dehydration and cold (Jang et al., Plant Mol. Biol., 37: 839-847, 1998; Liu et al., Science, 280: 1943-1945, 1998; Pardo et al., Proc. Natl. Acad. Sci. USA, 95: 9681-9686, 1998; Lie et al., Proc. Natl. Acad. Sci. USA, 97: 3730-3734, 2000).

As described above, transcriptional control plays a pivotal role in the adaptation responses and is likely to be regulated by specific transcription factors, and several stress-inducible genes encoding such transcription factors or their homologues have been isolated and characterized (Tague et al., Plant Mol. Biol. 28: 267-279, 1995; Bastola et al., Plant Mol. Biol., 24: 701-713, 1998; Kasuga et al., Nat. Biotechnol., 17: 287-291, 1999; van Der Kroel et al., Plant Physiol., 121: 1153-1162, 1999; Nakashima et al., Plant Mol. Biol., 42: 657-665, 2000). Further research provided information that transcription factors encoded by several genes of the discovered genes have a zinc finger motif. Examples of such transcription factors include Atmyb2, ATHB-7,

Specifically, Atmyb2 in the Arabidopsis plant is induced by dehydration stress, and then bound to a conserved MYB recognition sequence, and induction of an Arabidopsis homeobox gene ATHB-2 is caused by dehydration or abscisic acid treatment. Mlip15 in corn is a transcription factor isolog having a bZIP motif, and is induced at low temperature. Alfin1, a zinc finger protein, is induced by salt stress and has an MsPRP2 promoter-binding site. The Alfin1 protein plays an important role in regulating MsPRP2 expression in the root of alfalfa and consequently contributes to the salt-tolerance in this plant. There is a recent report in which a gene family coding a DRE/CRT binding protein was isolated and characterized (Liu et al., Plant Cell, 10: 1391-1406, 1998). According to the report, such a gene family includes DREB1 (dehydration-inducible element binding protein) and DREB2, which binds to 9 consensus sequences found in promoter regions of a variety of dehydration- or low temperature-inducible genes such as RD29A, Cor6.6 and RD17. However, the transcription factors described above are just a part of the factors constituting a large group of transcription factors involved in a response to osmotic stress.

Since, with respect to such stress-inducible genes, transcription factors can regulate their transcriptions, they may be useful to construct a plant resistant to stress by causing the transcription factors to be overexpressed or inhibited in the plant.
Disclosure of the Invention

Accordingly, the present inventors have conducted studies to find a novel gene involved in resistance of plants to environmental stress, particularly, osmotic stress. As a result, they found that a novel protein, AtSIZ, which is isolated from *Arabidopsis thaliana* and has zinc finger motifs, exhibits a regulatory activity on transcription of stress-inducible genes, and is further capable of enhancing resistance of the plant to osmotic stress upon overexpression of a gene coding AtSIZ.

Thus, it is an object of the invention to provide a novel plant transcription factor induced by osmotic stress, which induces expressions of diverse stress-inducible genes.

It is another object of the invention to provide a gene encoding the transcription factor and an eukaryotic expression vector comprising the gene.

It is yet another object of the invention to provide a method for enhancing resistance of a plant to osmotic stress by transformation of the plant with the gene.

In accordance with one aspect of the present invention, the above and other objects can be accomplished by the provision of a plant transcription factor, AtSIZ comprising the amino acid sequence of SEQ ID NO: 2 having 596 amino acid residues.

The transcription factor, AtSIZ is isolated from *Arabidopsis thaliana*, and has three C_{3}H-type zinc finger motifs at amino acid residues 211 through 325. It has a transcription activation domain at the C-terminal region. Further, AtSIZ activates transcriptions of a variety of genes induced by osmotic stress, for example, *COR15a*
(cold-regulated protein) which confers resistance to cold, and RD29 which confers resistance to dehydration, cold and high concentrations of salt.

AtSIZ shows the same ability to promote transcription of stress-induced genes when approximately 206 amino acid residues are deleted from its C-terminal region. With an additional deletion of 45 amino acid residues, however, its transcription-promoting activity is significantly reduced. For this reason, it is expected that the region containing amino acid residues 345 through 390 plays a critical role in AtSIZ transcription regulatory activity. Moreover, a part of the AtSIZ protein consisting of amino acid residues 1 through 390 with a deletion of amino acid residues 391 through 596 at its C-terminus can be used in constructing a plant resistant to osmotic stress. In accordance with the invention, there is thus provided a part of AtSIZ protein consisting of amino acid residues 1 through 390 of the amino acid sequence of SEQ ID NO: 2.

In accordance with another aspect of the present invention, there is provided an AtSIZ gene encoding the transcription factor, AtSIZ. The gene has a nucleotide sequence represented by SEQ ID NO: 1 and is induced by osmotic stress, especially, a high concentration of NaCl.

When a mutation occurs in the AtSIZ gene, a plant becomes sensitive to osmotic stress. As a result, the mutant plant accumulates anthocyanin in its leaves and has broken leaf edges, and small and etiolated leaves are seen. On the other hand, plants which overexpress AtSIZ show a high survival rate even under conditions where a wild type plant cannot survive, due to osmotic stress upon being treated with a high concentration of NaCl.

Finally, in accordance with yet another aspect of the present invention, there
is provided a method for enhancing resistance of a plant to osmotic stress by introducing an \textit{AtSIZ} gene to the plant, constructing a transformed plant which overexpresses the gene. Using the method, productivity of the plant can be considerably increased. For transformation, the \textit{AtSIZ} gene may be a full-length cDNA of \textit{AtSIZ} or a partial gene encoding a part (amino acid residues 1 through 390) of the \textit{AtSIZ} protein comprising only a region exhibiting transcription-promoting ability of stress-inducible genes.

The term "osmotic stress-inducible gene" or "osmotic stress-responsive gene" as used herein refers to a gene encoding a protein induced by osmotic stress caused by exposure of the plant to a high concentration of salt, low temperature, dehydration, or exogenous ABA treatment; or a gene involved in exhibiting tolerance or resistance to osmotic stress. The term "\textit{AtSIZ-complement}" refers to a recombinant vector comprising an active \textit{AtSIZ} gene, which can express the transcription factor \textit{AtSIZ} in an \textit{AtSIZ}-inactivated mutant. The term "\textit{AtSIZ-complemented mutant}" refers to a mutant plant transformed with the \textit{AtSIZ-complement}.

Additionally, a description herein referring to a gene encoding \textit{AtSIZ}, the transcription factor, is in italic, that is, "\textit{AtSIZ} gene" or "\textit{AtSIZ}", while the protein encoded by the gene is represented as "\textit{AtSIZ} protein" or "\textit{AtSIZ}".

\textit{Brief Description of the Drawings}

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description
taken in conjunction with the accompanying drawings.

Fig. 1 shows a comparison of amino acid sequences of other zinc finger containing proteins, with respect to an amino acid sequence of AtSIZ deduced from a nucleotide sequence of an AtSIZ gene.

Fig. 2 shows Northern blot analysis of an expression pattern of an AtSIZ gene in diverse plant tissues such as flower (F), leaf (L), root (R) and silique (S).

Fig. 3 shows Northern blot analysis of an expression pattern of an AtSIZ gene induced by diverse osmotic stresses (the numerals represent treatment time of osmotic stress).

Figs. 4 shows results of PCR screening and Southern blot analysis for selecting and confirming a mutant harboring a T-DNA insert within an AtSIZ gene and a location of the T-DNA insert in AtSIZ, based on the result of nucleotide sequencing: A) is a agarose gel of primary PCR products, wherein LB represents a primer specific for the left border of T-DNA; B) is a agarose gel of tertiary PCR products; and C) shows a location of T-DNA within AtSIZ in a selected mutant harboring T-DNA.

Fig. 5 is a result of examining phenotype of T-DNA insertion mutant: A) shows Northern blot analysis of expression patterns of AtSIZ in a T-DNA insertion mutant (mt) and a wild type (WT), and B) shows comparisons of phenotypes in leaves of plants cultivated after treatment with varying concentrations of NaCl (b, c and d represent 25, 50 and 100 mM, respectively), with respect to the plant cultivated under general growth condition (a), wherein the numerals 2, 4, 6 and 8 represent a 2nd, 4th, 6th, and 8th leaf from the base of the plants, respectively.

Fig. 6 is a result of examining the sensitivity to NaCl stress by
complementing *AtSIZ* cDNA into T-DNA insertion mutant: A) shows Northern blot analysis of *AtSIZ* expressed in a T-DNA-complemented mutant plant (mt/*AtSIZ*), a T-DNA insertion mutant (mt) and a wild type (WT) and B) shows comparisons of phenotypes in leaves of the plants cultivated after treatment with varying concentrations of NaCl (a, b, c and d represent 0, 25, 50 and 100 mM, respectively), wherein the numerals 2, 4, 6 and 8 represent a 2nd, 4th, 6th, and 8th leaf from the base of the plants, respectively.

Fig. 7 shows Northern blot analysis of expression patterns of *AtSIZ*, *COR5a* and *RD29A* in a wild type (WT), a T-DNA insertion mutant (mt) and T-DNA-complemented mutant plants (2-2 and 5-4 represent independent mutant lines thereof), wherein NaCl refers to 6 hrs treatment with 150 mM NaCl, and cold refers to 6 hrs treatment of a low temperature of 4 °C.

Fig. 8 shows Northern blot analysis of expression patterns of *AtSIZ* in a wild type (WT) and T-DNA-complemented mutant plants (1-6, 2-4 and 19-4 represent independent mutant lines thereof) (A); and a photograph showing plants exhibiting different sensitivities to NaCl (B).

Fig. 9a is a schematic diagram showing GAL4-*AtSIZ* fused constructs having deletions at their C-terminal regions, which have diverse lengths of *AtSIZ* fragments linked to a gene encoding a GAL4 DNA-binding domain (GAL4 BD), wherein Rich-E represents a region rich in glutamate residues.

Fig. 9b shows results of β-galactosidase activity assays in yeast transformed with the respective diverse lengths of *AtSIZ*, as represented in Fig. 9a.
Best Mode for Carrying Out the Invention

The inventors intended to isolate a novel gene induced by osmotic stress. Control cDNAs derived from a plant not exposed to stress were subjected to hybridization with individual single cDNAs from a cDNA library of a plant exposed to high salt stress. Thus, constitutively expressed cDNAs in the plant were subtracted, constructing a subtraction library.

More specifically, the control cDNAs were labeled with biotin. Then, they were subjected to hybridization with individual single cDNAs obtained from the cDNA library which comprises cDNAs induced by salt stress. Based on a binding property of biotin to streptavidin, the hybridized cDNAs were removed by applying a membrane coated with streptavidin. Through the course of removal, the residual single cDNAs are genes specifically induced by stress from a high concentration of salt, and were subsequently subjected to sequencing analysis. As a result, 15 clones of genes expressed by osmotic stress were found. Among the clones found, a novel clone whose nucleotide sequence was not known, OS183, was employed as a probe for finding a full-length cDNA. The full-length cDNA was isolated. The cDNA has a size of 2267 bp with a putative molecular weight of approximately 66 kDa and an open reading frame encoding 596 amino acids, which was designated as AtSIZ (Arabidopsis thaliana Stress-Induced Zinc finger).

A nucleotide sequence of the AtSIZ gene is represented by SEQ ID NO: 1, and an amino acid sequence of an AtSIZ protein deduced therefrom is represented by SEQ ID NO: 2. The AtSIZ protein encoded by the gene shares high homology with other
proteins having zinc finger motifs (see Fig. 1). It has CxH type zinc finger motifs comprising a consensus sequence of CX₇CX₇CX₇H (C is Cysteine, H is Histidine and X is any amino acid) in the middle of the amino acid sequence. In addition, AtSIZ has an acidic region with 6 consecutive glutamate residues located upstream of the zinc finger region.

In an embodiment of the invention, with the aim of examining expression patterns of the AtSIZ, total RNAs were isolated from tissues of flowers, leaves, roots of Arabidopsis thaliana and siliques, respectively, and subjected to Northern blot analysis. It was seen that AtSIZ is expressed at different levels according to the tissues, and the root tissues show the highest level of expression, followed by the flower and leaf tissues in order, but there is little expression in sillage tissues (see Fig. 2). Meanwhile, since AtSIZ was first isolated where the Arabidopsis plant was treated with a relatively high concentration of NaCl, it might be necessary to also examine its expression patterns according to intensity and variety of osmotic stress. Seedlings of Arabidopsis were exposed to a higher concentration of NaCl, dehydration, cold and exogenous abscisic acid (ABA), respectively, and subjected to Northern blot analysis. The results showed that though there were differences in AtSIZ expression levels, its expression was commonly induced by all the above treatments, demonstrating that AtSIZ is involved in a response to general osmotic stress. Further, considering that their expression patterns varied according to the stress conditions, it was found that the expression of AtSIZ is regulated according to certain stress conditions (see Fig.3).

In another embodiment of the invention, it was found that when a mutation occurs in the AtSIZ gene, the mutant plant is sensitive to NaCl stress, while when the
AtSIZ is overexpressed, the plant shows an increased resistance to NaCl stress.

Specifically, a mutant plant which harbors a T-DNA within AtSIZ was selected from transformed plants (ABRC, USA) harboring a T-DNA insert. With respect to individual genomic DNAs obtained from the group of the transformed plants, PCR was performed using a combination of a primer specific for the 3'-end or 5'-end of the AtSIZ gene and a primer specific for the T-DNA right border (RB) or T-DNA left border (LB). Then, a Southern blot was prepared using a labeled AtSIZ cDNA as a probe for hybridization with the PCR products. As shown in Fig. 4, where a combination of primers specific for LB of the T-DNA and 3'-end of AtSIZ were used, an amplified PCR product was hybridized with an AtSIZ cDNA, indicating that a T-DNA was inserted within AtSIZ. With sequencing, the identity of the PCR product thus obtained was confirmed and the location of the inserted T-DNA was determined. It was found that T-DNA was inserted at nucleotide 615 of the AtSIZ gene.

Using the selected mutant plant whose AtSIZ was mutated via T-DNA insertion, physiological roles of the AtSIZ gene were examined. Such mutant plants were exposed to a variety of stress conditions, and their phenotypes were examined. The mutant plants accumulated anthocyanin in their leaves. While wild type plants showed no response to the concentration of 25 mM NaCl, the mutant plants had broken leaves at their edges. In addition, with a higher concentration of NaCl, the leaves of the mutants were smaller than those of wild type plants and had the yellow phenotype. These results demonstrate that the mutant plants, which had a mutation in AtSIZ, are much more sensitive to NaCl stress than the wide-type plants. Accordingly, this indicates that AtSIZ is involved in resistance to NaCl stress.
In another embodiment of the invention, a complementation test was carried to clearly confirm the physiological activities of \textit{AtSIZ}. That is, complementation ability of the mutants was tested to prove that such phenotype changes of the mutant plants were attributed to a T-DNA insertion in the \textit{AtSIZ} gene.

An \textit{AtSIZ} cDNA was inserted in pBIB-HYG, a binary vector carrying a hygromycin resistance marker so as to construct an \textit{AtSIZ} complement. The complement was introduced to the mutant plant which has a mutation in \textit{AtSIZ} caused by a T-DNA insertion, using an \textit{Agrobacterium}-mediated vacuum infiltration method. Northern blot was performed to confirm the \textit{AtSIZ} complementation. It was seen that \textit{AtSIZ} of the \textit{AtSIZ}-complemented mutant plant was expressed at a high level. With respect to a wild type plant, the sensitivities of \textit{AtSIZ}-inactivated mutant and \textit{AtSIZ}-complemented mutant plants, to NaCl stress were compared. The \textit{AtSIZ}-complemented mutant plant exhibited NaCl resistance at a level similar to the wild type plant (see Fig. 6). These results demonstrate that the NaCl sensitivity phenotype of the \textit{AtSIZ}-inactivated mutant is due to a T-DNA insertion in the \textit{AtSIZ} gene.

In another embodiment of the invention, it was examined whether \textit{AtSIZ}, a transcription factor, is involved in regulating transcription of other genes induced by NaCl stress. As stress-inducible genes, there are \textit{COR15a} and \textit{RD29A}, whose expression can be induced by exogenous ABA treatment or a variety of osmotic stresses.

\textit{COR15a} is one of \textit{COR} genes (cold-regulated) found in plants which undergo cold adaptation, and it is possible that their gene products may be involved in freezing resistance (Artus \textit{et al.}, \textit{Proc. Natl. Acad. Sci. USA}, 93(23): 13404-13409, 1996).
According to a recent report, constitutive expression of *COR15a* raises a survival rate of *Arabidopsis* under cold conditions. A low temperature induces disruption of both the physical continuity and permeability of the plasma membrane, which is responsible for osmotic control. Such damage allows substances in the cytoplasm and organelles to leak, and causes fusion of the cell membrane with an endomembranes, for example, outer membrane of chloroplasts, thereby lowering freezing resistance. *COR15a* is a stress-inducible gene raising a survival rate of plants by attenuating responses of the cell membrane and chloroplast to freezing.

*RD29* is a gene of the *Arabidopsis* plant whose expression is induced by dehydration, cold, or a high concentration of salt. There are two kinds of *RD29A* and *RD29B*. A promoter of *RD29A* exists in most organelles and tissues of plants growing where moisture is deficient, such that the plants have increased resistance to dehydration stress (Yamaguchi et al., *Mol. Gen. Genet.*, 236: 331-340, 1993).

Expression levels of *COR15a* and *RD29A* in wild type, *AtSIZ*-inactivated mutant and *AtSIZ*-complemented mutant plants, in response to a high concentration of NaCl and cold stress, were examined. A mutation of *AtSIZ* specifically inhibited induction of *COR15a* expression in response to NaCl stress, while failing to inhibit its expression in response to cold stress (see Fig. 7). On the other hand, *RD29A* expression was highly induced in the *AtSIZ*-inactivated mutant in response to NaCl or cold stress, indicating that a mutation in *AtSIZ* does not affect *RD29A* expression induction by NaCl or cold stress. These results strongly imply that *AtSIZ* is involved in induction of expression of stress-inducible genes, specifically in response to NaCl stress.
Involvement of AtSIZ in the response to NaCl stress is further supported by an experiment using a plant overexpressing AtSIZ. A transformed plant which expresses AtSIZ at a level of 10 to 20 times higher than a wild type plant showed increased resistance to stress from a high concentration of NaCl (see Fig. 8).

In yet another embodiment of the invention, the activity of the AtSIZ protein as a transcription factor and its active site were determined. Using a yeast 1-hybrid technique, it was proved that AtSIZ has transcription promoting activity. The hybrid technique is based on the fact that, if AtSIZ has transcription activating ability, when AtSIZ is expressed so as to bind to a GAL4 DNA-binding protein, a reporter gene, gal1-LacZ transcription would be activated. In yeast, AtSIZ activated transcription of gal1-LacZ, demonstrating that AtSIZ has transcription promoting activity. The deletion test showed that where 206 amino acid residues are deleted from the C-terminal of the AtSIZ amino acid sequence of SEQ ID NO: 2, the transcription-promoting activity of AtSIZ is not changed. An additional deletion of 45 amino acid residues, however, significantly reduced its transcription-promoting activity. For this reason, it is expected that the region containing amino acid residues 345 through 390 plays a critical role in the transcription activation activity of AtSIZ (see Fig. 9a and Fig. 9b). Thus, a part of the AtSIZ protein consisting of amino acid residues 1 through 390 can be used for increasing resistance to osmotic stress in plants.

Hereinafter, the present invention will be described in detail, in conjunction with various examples. These examples are provided only for illustrative purposes, and the present invention is not to be construed as being limited to those examples.
Example 1: Cultivation of Arabidopsis thaliana

All Arabidopsis plants used in experiments of the invention were cultivated in a culture room at 22°C or a green house controlled to maintain a cycle of 16 hrs light/8 hrs darkness and 70% relative humidity. The seedlings were cultured on Murashige-Skoog (MS) agar plates, or for a chemical treatment, in 250 ml culture flasks containing MS liquid media while stirring at 100 rpm. The plant tissues of choice were collected and immediately frozen under liquid nitrogen.

Example 2: Isolation of osmotic stress-inducible genes

To isolate genes whose expressions are induced by osmotic stress, especially, salt stress, a subtraction library was constructed, and then cDNAs were randomly selected from the library and screened by sequencing them. For the subtraction library, cDNAs derived from plants exposed to salt stress were subjected to hybridization with cDNAs derived from the control plant which was not exposed to salt stress, thereby subtracting the constitutively expressed cDNAs.

2-1: Culture of the Arabidopsis plant

The Arabidopsis plants were cultured under the same conditions as in Example 1. After 1 week, with the purpose of obtaining stress-induced cDNAs, the seedlings were exposed to salt stress by changing the media with MS media containing 0.15 M NaCl. The seedlings were cultured for an additional 1 to 6 hrs while stirring. All
seedlings were immediately frozen and stored at - 80°C.

2-2: Extraction of RNA and construction of cDNA library

To prepare an osmotic stress-derived cDNA library, total RNA was first extracted from the frozen seedling which was treated with 0.15 M NaCl for 6 hrs, using a lithium chloride/phenol extraction method. The total RNA was treated according to the protocol of an mRNA isolation kit (Pharmacia, USA) to isolate poly(A)$^+$ RNA. As a control, salt-untreated seedlings were subjected to the above procedure to isolate poly(A)$^+$ RNA. Then, with respect to osmotic stress-derived cDNA, the cDNA library was constructed using a cDNA synthesis kit (Stratagene, USA). The individual cDNAs were ligated to λ ZAP II (Stratagene, USA), finally constructing a λ ZAP II cDNA library.

2-3: Construction of subtraction library

With regard to the λ ZAP II cDNA library, a quantity of filamentous phages were obtained, and individual single stranded DNAs were purified therefrom. To remove clones bearing constitutively expressed cDNAs from the λ ZAP II cDNA library derived from the stress-applied plant, subtraction was performed by hybridization with the control cDNA.

To label the control cDNA with biotin, an excess amount of double stranded cDNA prepared above was subjected to PCR incorporating biotinylated dUTP.

In detail, 100 ng of the control double cDNA was employed as a template. As for primers, oligonucleotides having the nucleotide sequences of SEQ ID NO: 3 and
SEQ ID NO: 4, respectively, were synthesized and 50 ng each was employed. 50 μM of biotin-16-dUTP was supplied in a PCR reaction mixture, thereby producing biotin-labeled cDNA. The PCR condition was 30 sec at 94°C, 30 sec at 38°C, 30 sec at 72°C, and 50 cycles thereof.

To subtract constitutively expressed cDNAs, hybridization was carried out. 0.1 μg of the single stranded cDNA from the λ ZAP II cDNA library and 1 μg of the biotinylated control cDNA probe were added to a hybridization cocktail (containing 50 mM Tris-HCl, pH 7.5, 0.25 M NaCl and 1.0 mM EDTA) at 65°C overnight. The hybridization solution was incubated with membranes coated with streptavidin on ice for 2 hrs with occasional stirring, followed by removing the membranes. Since streptavidin has a high affinity for biotin, cDNA samples hybridized with the biotinylated control cDNA can be removed by eliminating the membranes coated with streptavidin. That is, after hybridization, only cDNAs which are specifically expressed by salt stress remained in the hybridization solution. These cDNAs were extracted by employing phenol/chloroform and chloroform. Then, the cDNAs were added with 2 μg carrier tRNA and cold ethanol at -20°C, thereby being precipitated. The cDNAs thus obtained were transfected to an E. coli host cell by electroporation method. In this way, the subtraction library was prepared. In such a library, clones were randomly selected, and their DNA sequences were analyzed using an automatic sequencer.

From the sequencing analysis, 15 osmotic stress-inducible ESTs (Expressed Sequence Tags) were found. Among these, 4 cDNAs were found to be novel genes. Specifically, the clone carrying a 0.8 kb DNA was employed in experiments thereafter.
Example 3: Isolation of AtSIZ cDNA

An amino acid sequence deduced from the nucleotide sequence of said clone was subjected to homology search using NCBI’s Blast program. The amino acid sequence showed a high degree of homology to other proteins having zinc finger motifs. Database search revealed that the sequence shares 55.7 % homology with \( \text{C}_3\text{H}\)-isolog, 23.7 % with \( \text{C}_3\text{H}\)-Znfp2, and 19.1 % with PEI1, as represented in Fig. 1. Therefore, the inventors designated the protein expressed by the gene isolated above as AtSIZ (\textit{Arabidopsis thaliana} Stress-Induced Zinc Finger).

Since the cDNA clone has a part of the gene AtSIZ, it is required to find a clone carrying full-length AtSIZ. To obtain the full-length gene, an AtSIZ insert was employed as a probe to screen the \( \lambda \) ZAP II cDNA library constructed in Example 2. One clone carrying the full-length cDNA was isolated. The cDNA has a size of 2267 bp (SEQ ID NO: 1) with a putative molecular weight of approximately 66 kDa and an open reading frame encoding 596 amino acids. The full-length cDNA was subcloned to pBluescript, constructing a recombinant plasmid. The recombinant plasmid was transformed to \textit{E. coli}, and the transformed cell was deposited in the Korean Collection for Type Cultures (KCTC) affiliated to Korea Research Institute of Bioscience and Biotechnology (KRIBB), under deposit No. KCTC 0886BP on Nov. 3, 2000.

An amino acid sequence of AtSIZ protein which is deduced from the full-length cDNA, was subjected to homology comparison using a BlastX program of NCBI. The comparison was performed between amino acid sequences of \( \text{C}_3\text{H}\)-isolog (protein accession No. g1871192), \( \text{C}_3\text{H}\)-Znfp2 (g3643609), and PEI1 (g2961542). It
was seen that homology with respect to the above proteins having zinc finger motifs is
highest in the middle region of the amino acid sequence involving zinc finger motifs
(see Fig. 1). Referring to Fig. 1, the amino acids identical to amino acids for AtSIZ
were marked with black color. Based on the analysis result, the zinc finger region of
the deduced protein AtSIZ has a consensus sequence of CX₂CX₃CX₃H, demonstrating
C₃H-type zinc finger. In addition, AtSIZ has an acidic region with 6 consecutive
 glutamate residues upstream of the zinc finger region.

Example 4: Induction of AtSIZ expression by diverse osmotic stresses

To determine functions of AtSIZ, expression patterns of AtSIZ by diverse
osmotic stresses in diverse tissues were examined.

4-1: Expression patterns according to diverse tissues

Total RNAs were isolated from tissues of flowers, leaves, roots and siliques,
respectively, using a LiCl/phenol method. Northern blot was prepared to assess the
transcription levels in these tissues, employing AtSIZ cDNA as a probe. 15 µg total
RNA from each tissue was heat-treated at 65°C for 15 min to loosen its secondary
structure, mixed with formaldehyde gel loading buffer (50 % glycerol, 1 mM EDTA,
pH 8.0, 0.25 % bromophenol blue, 0.25 % xylene cyanol FF in distilled water). Each
sample was loaded on 1 % agarose gel containing 2.2 M formaldehyde, followed by
slow electrophoresis at a voltage of 4 V/cm. The gel with developed RNAs was
immersed in DEPC-H₂O to remove formaldehyde. Then, the gel was transferred to a
nylon membrane by capillary transfer for about 16 hrs, followed by heat treatment at 80°C for 1 hr, thereby immobilizing RNAs. As a probe for hybridization, *AtSIZ* cDNA was labeled with [α-^32^P] dCTP with the aid of a random primer labeling kit (Boeringer Mannheim, Germany). That the same amount of respective total RNAs was loaded into each well in the gel was confirmed by staining with ethidium bromide. Finally, for the detection of hybridization, the membrane was exposed to X-ray film at -70°C. The results are shown in Fig. 2. It was seen that *AtSIZ* is expressed at different levels according to the tissues. The root tissues show the highest level of expression, followed by the flower and leaf tissues in order, but there is little expression in silique tissues.

4-2: Expression patterns according to osmotic stress variety

In addition to the effect of 0.15 M NaCl on the expression of *AtSIZ* in *Arabidopsis* plant, the expression patterns were further examined according to osmotic stress intensity and variety. The seedlings of *Arabidopsis* were exposed to a high salt concentration of 150 mM NaCl, dehydration, a low temperature of 4°C and exogenous abscisic acid (ABA), respectively. Total RNAs were isolated and subjected to Northern blot analysis using an analogous method as in Example 4-1. The results are shown in Fig. 3. The expression of *AtSIZ* was induced by all the above treatments, indicating that *AtSIZ* is involved in responses to general osmotic stress. Despite such a common expression of *AtSIZ*, its expression pattern was different according to the stress conditions. As for the exogenous ABA and 150 mM NaCl treatment, the expression showed a peak 1 hr after each treatment, followed by a decrease thereafter. As for the
low temperature treatment, the expression was induced at a low level at first, followed by a large increase 6 hours after treatment, implying that there are at least two mechanisms for the expression induction of AtSIZ by a low temperature stress. On the other hand, as for the dehydration treatment, the expression showed a peak within 30 min after treatment and such an expression level was maintained for more than 6 hrs after treatment. Therefore, these results demonstrate that the expression of AtSIZ is regulated at a variable level according to stress conditions.

Example 5: Isolation of an Arabidopsis mutant plant with T-DNA inserted in AtSIZ gene

It is necessary to compare a wild type plant with an AtSIZ gene-inactivated plant to understand physiological roles of AtSIZ.

To select a mutant having T-DNA inserted within an AtSIZ gene, a PCR screening method (McKinney et al., Plant J., 4: 613-622, 1995) was used to probe genomic DNAs (ABRC, USA) of a group of transformed plants harboring a T-DNA tag. The three round PCR screening was performed using a combination of primers specific for the 3'-end and 5'-end (SEQ ID NO: 5 and SEQ ID NO: 6, respectively) of the AtSIZ gene of SEQ ID NO: 1 and primers specific for the T-DNA right border (RB) and T-DNA left border (LB)(SEQ ID NO: 7 and SEQ ID NO: 8, respectively). As for respective templates, individual genomic DNAs (ABRC, USA) obtained from a group of transformed plants harboring T-DNA inserts were employed.

The PCR product thus obtained was subjected to Southern blot analysis
employing a $^{32}$P labeled *AtSIZ* cDNA as a probe for detecting hybridization. The result is shown in Fig. 4; A. Using a combination of primers specific for LB of T-DNA and 3’-end of *AtSIZ*, an amplified PCR product was found, indicating that the PCR product has a T-DNA insert within an *AtSIZ* gene. In this way, the transformed plant having a T-DNA inserted within an *AtSIZ* gene was selected.

Confirmation of disruption of the *AtSIZ* gene in such a transformed plant was carried out by Southern blot analysis of genomic DNA obtained therefrom (Data not shown). Moreover, a transformation marker gene, *npt II*, was employed as a probe in performing a Southern blot to confirm that there is only one copy of T-DNA in the genome of the plant (Data not shown). Meanwhile, an inactivated homozygous mutant plant was selected relying on kanamycin resistance, and its DNA was extracted, followed by PCR amplification to confirm a T-DNA insertion.

To prove that said PCR product is identical to the *AtSIZ* gene, and a location of the T-DNA insertion, the PCR product was subcloned to a pBluescript vector, followed by sequencing. It was found that T-DNA was inserted at nucleotide 615 of the *AtSIZ* cDNA sequence (See Fig. 4; C).

Example 6: Determination of phenotype of T-DNA insertion mutant

Phenotype of mutant plants was examined for determining physiological role of *AtSIZ*.

First, total RNA was extracted from the homozygous mutant and wild type plants subjected to Northern blot analysis to confirm no expression of *AtSIZ* in mutant
plants.

Wild type (WT) and mutant (mt) plants were cultivated in soil for 3 weeks, and thereafter, the plants were cultured in liquid media with a treatment of variable concentrations of NaCl for 10 days. As for the mutants, although no expression of \textit{AtSIZ} was found by Northern blot (Fig. 5; A), little difference of phenotype could be seen as compared to wild type (WT) plants, when grown in soil under a general growth conditions. However, the mutants were highly sensitive to NaCl stress. The mutant plants accumulated anthocyanin in their leaves. Where the plants were treated with 25 mM NaCl, WT plants did not show any response, while levels of mutant plants developed broken edges (Fig. 5; B, lower part of panel b). Also, where the plants were treated with 50 mM NaCl, WT plants did not show any response to the stress (Fig. 5; B, upper part of panel c), while mutant plants had smaller leaves than those of WT, and the leaves were yellow (Fig. 5; B, lower part of panel c). Further, where the plants were treated with 100 mM NaCl, mutant plants had smaller leaves with strong etiolation, while the leaves of WT plants were a little etiolated in their edges. These results demonstrate that the mutant plants are much more sensitive to NaCl stress than the wild type plants.

Example 7: Complementation test

The mutant plants were isolated from transgenic lines inserted with T-DNA. Therefore, complementation ability of the mutants was tested to prove that such osmotic stress-sensitive phenotypes of the mutant plants were attributable to a T-DNA insertion
in the \textit{AtSIZ} gene.

7-1: Construction of an \textit{AtSIZ} complement and production of an \textit{AtSIZ}-complemented mutant using the complement

An \textit{AtSIZ} cDNA was inserted in pBIB-HYG, a binary vector carrying a hygromycin resistance marker gene (Becker D., Institut fur Genetic der Universitat zu K6ln, FRG, \textit{Nucleic Acid Res.}, 180(1): 203, 1990) so as to construct an \textit{AtSIZ} complement.

First, the \textit{AtSIZ} subcloned in pBluescript was digested with \textit{Xho I}, and the 5-overhang of the \textit{Xho I} site was filled in with a mixture of Klenow and dNTPs to make a blunt ended DNA. Then, the DNA was restricted with \textit{Xba I} to yield an \textit{AtSIZ} insert. The insert was ligated into an \textit{Xba I/Eco} 136 II site of pBIB-HYG vector, ensuring that the insert is located between the CaMV 35S promoter and the Nos terminator, thereby constructing a recombinant vector capable of expressing a transcription factor encoded by \textit{AtSIZ}.

The recombinant vector thus constructed was used to transform a mutant plant harboring an inactivated \textit{AtSIZ} gene. Using an \textit{Agrobacterium}-mediated vacuum infiltration method (Clough \textit{et al.}, \textit{Plant J.}, 16: 735-743, 1998), the mutant plant was transformed, whereby the transcription factor \textit{AtSIZ} can be complemented. The transformed plants were selected on a MS plate containing 50 \textmu g/ml hygromycin. Such selected plants were transferred to soil, and their seeds were obtained. These seeds were again selected on a hygromycin containing MS plate. In this way, homozygous T2 lines of \textit{AtSIZ}-complemented mutants were obtained. Total RNA was
extracted from wild type, mutant and the AtSIZ-complemented homozygous T2 mutant plants, followed by Northern blot analysis employing $^{32}$P-labeled AtSIZ cDNA as a probe. Fig. 6; A shows that AtSIZ-complemented homozygous T2 mutant lines 2-2 and 5-4 contain large amounts of transcripts of the AtSIZ gene, while the AtSIZ-inactivated mutant contains few of the transcripts.

7-2: NaCl stress sensitivity test of AtSIZ-complemented mutant plants

Ability of the AtSIZ-complemented homozygous mutant plants to resist NaCl stress was examined by measuring the sensitivity to NaCl stress at various NaCl concentrations. Wild type, mutant and AtSIZ-complemented homozygous T2 mutant (line 2-2) plants were cultivated in soil for 3 weeks. Then, the plants were immersed in 0, 25, 50 and 100 mM NaCl solutions, respectively, every 3 days, followed by drying and culturing them. These steps were repeated through 10 days.

As shown in Fig. 6; B, the AtSIZ-complemented homozygous mutant plants exhibited NaCl tolerance at a level similar to the wild type plants. This demonstrates that phenotype of AtSIZ-inactivated mutants against NaCl stress can be changed by an AtSIZ complement. That is, it was proved that NaCl sensitivity of the AtSIZ-inactivated mutants is due to T-DNA insertion in AtSIZ.

Example 8: Effect of AtSIZ on activation of transcription of stress inducible-genes

On the basis of the experimental results showing that sensitivity to NaCl stress
increases in AtSIZ-inactivated mutants, it was examined whether AtSIZ is involved in regulating transcription of other genes whose expressions are induced by NaCl stress. Total RNAs were extracted from wild type, mutant and AtSIZ-complemented mutant plants, respectively, followed by Northern blot analysis.

Each of the plants above was cultured in a liquid MS medium for 1 week, and exposed to 150 mM NaCl and a low temperature of 4°C for 6 hrs. As stress-inducible genes, COR15a and RD29A (Urao et al., Plant Cell, 5: 1529-1539, 1993; Baker et al., Plant Mol. Biol., 24: 701-713, 1994), served as reporter genes. Their expressions can be induced by exogenous ABA treatment or a variety of osmotic stresses. Northern blot analysis was performed employing 32P-labeled cDNAs of AtSIZ, COR15a (Thomashow M.F., GenBank accession No. U01377), and RD29A (Yamaguchi-Shinozaki, GenBank accession No. D13044) as probes.

Expression patterns of COR15a and RD29A in AtSIZ-inactivated mutant and AtSIZ-complemented mutant plants, in response to NaCl and cold stress, were examined. The results are shown in Fig. 7. As for COR15a, a strong expression induction was seen in AtSIZ-complemented mutants, while AtSIZ-inactivated mutants failed to induce the expression in response to NaCl stress. On the other hand, in response to cold stress, COR15a expression was strongly induced in the AtSIZ-inactivated mutants as well as AtSIZ-complemented mutants, showing that a mutation in AtSIZ does not affect COR15a expression induction by cold stress. As for RD29A, the expression was highly induced in the AtSIZ-inactivated mutants as well as AtSIZ-complemented mutants in response to NaCl or cold stress, indicating that a mutation in AtSIZ does not affect RD29A expression induction by NaCl or cold stress.
Consequently, it is demonstrated that *AtSIZ* has an effect of transcription activation of certain osmotic stress-inducible genes, specifically in response to NaCl stress.

Example 9: Increase of stress resistance of a plant due to *AtSIZ* overexpression

It was confirmed that the *AtSIZ* gene is involved in response to NaCl stress by the results mentioned above. Therefore, a transformed plant which overexpresses *AtSIZ* was constructed, and its resistance to NaCl stress was examined.

First, to construct a recombinant vector carrying an *AtSIZ* gene, *AtSIZ* cDNA was inserted in a pBI121 binary vector (Dr. Goodman, Harvard Medical School, Genetics Department) having a CaMV (*Cauliflower mosaic virus*) 35S promoter, replacing a GUS (β-glucuronidase) coding region. Vacuum infiltration was performed to transfer the recombinant vector to a wild type *Arabidopsis* plant. A detailed protocol is described as follows. The *AtSIZ* subcloned in pBluescript was digested with *Spe* I, and the 5-overhang was filled in with a mix of Klenow and dNTPs to make a blunt ended DNA. Then, *Sma* I was used to restrict the DNA to yield an *AtSIZ* insert. The insert was ligated into pBI121 which was restricted with *Sma* I and *Eco* 136 II, ensuring that the insert is located between the CaMV 35S promoter and the Nos transcription terminator, thereby constructing a recombinant binary plasmid, pBI121-*AtSIZ* capable of overexpressing *AtSIZ*.

The recombinant plasmid thus constructed was used to transform *Arabidopsis* plants via vacuum infiltration. For transformation, the wild type plants were grown in
pots for 4 weeks at 22°C. First bolts were clipped off. Infiltration was performed when secondary bolts had a length of 2 to 10 cm. Meanwhile, 3 days after clipping the first bolts, a colony of Agrobacterium transformed with the pBI121-AtSIZ was pre-cultured overnight in a 5 ml LB broth, followed by culturing in a 500 ml LB medium until its turbidity reached to 2.0 at OD_{600}. The cells were harvested by centrifugation at 5,500 x g and resuspended to 0.8 at OD_{600} in a 5% sucrose solution. The suspension was added with a surfactant, Silwet L-77 to a final concentration of 0.05%, and the solution was transferred to a beaker. The pots were inverted so only the above-ground parts of the plants were immersed in the Agrobacterium solution for 30 seconds. The pots were removed, laid on their side and let stand in the dark for 24 hrs. The next day, the pots were placed upright and cultivated in a green house at 22°C until seeds were mature. Dry seeds were harvested. The transformed plants were selected on a MS plate containing 50 mg/L kanamycin. In this way, homozygous T2 lines of AtSIZ-overexpressing mutants were obtained. Total RNA was extracted from the plants, followed by Northern blot analysis employing ^{32}P-labeled AtSIZ cDNA as a probe. Fig. 8; A shows that the transgenic lines 1-6, 2-4 and 19-4 exhibit strong AtSIZ expression, indicating that the AtSIZ insert was highly expressed.

Responding to a high concentration of NaCl stress, such AtSIZ-overexpressing mutant plants were examined for their phenotypes. The plants were cultivated for 2 weeks and treated with 200 mM NaCl every 3 days. Phenotypes were observed 10 days after treatment. Fig. 8; B shows appearances of a wild type (WT) and a transgenic line 19-4 plants. For reference, other transgenic lines had the same appearance as the lineage 19-4, so they are not presented in Fig. 8. With respect to the
plants, NaCl resistance was quantified. Their seeds were treated with 300 mM NaCl for 10 days and then sown. 20 plants each of wild type and AtSIZ-overexpressing mutant were tested in three independent experiments. The respective mean data are represented in Fig. 8; B. The AtSIZ-overexpressing plants showed resistance to the high concentration of NaCl, and specifically, 3 week-cultivated plants survived at a rate of about 70 % (resistance 70 ± 5), while wild type plants failed to survive. These results demonstrate that AtSIZ is involved in the plant’s adaptability to intense NaCl stress.

Example 10: Analysis of functional domains of the transcription factor, AtSIZ

Sequencing of AtSIZ gave information that AtSIZ encodes C$_2$H$_2$-type zinc finger polypeptides. Here, it was determined whether an AtSIZ protein acts as a transcription factor, like other zinc finger-containing proteins, and identified which regions activate transcription. A deletion analysis of the AtSIZ gene was carried out. Sequential deletions from the C-terminal of the AtSIZ protein were made, individually inserted in a vector, pAS2-1 downstream of a region encoding a GAL4 DNA binding domain and then, effect of such deletions was assessed using Yeast 1-hybrid system (Clonetech, USA). In detail, each of the full-length sequence (amino acid residues 1 - 596) and diverse lengths of C-terminal-deleted sequences (amino acid residues 1 - 470, 1 - 390, 1 - 345, 1 - 201 and 1 - 133) was inserted into the BamHI site of pAS2-1 (Clonetech, USA), ensuring that it could be expressed as a fusion protein linked to a 3'-end of the GAL4 binding domain. The recombinant vectors thus constructed and a control pAS2-
1 were respectively introduced to a yeast strain, Y190 (Clontech, USA), and then
tryptophan positive clones were selected as transformants. Using a color development
assay method (Li et al., Science, 262: 1870-1874, 1993), the transcription activation of
the gene coding β-galactosidase was examined. The selected yeast cells were placed
on Whatman 3MM filter paper and soaked with X-gal solution, then let stand at room
temperature for 3 hrs, thereby color being developed.

As shown in Fig. 9b, it can be seen that the full-length AtSIZ protein was fused
with the GAL4 DNA-binding domain so it strongly induced expression of β-
galactosidase. This result demonstrates that AtSIZ is a strong transcription factor.
Where 206 amino acids were deleted from the C-terminus (that is, Del 1 and Del 2), no
changes in transcription-activation activity were seen. On the other hand, where an
additional deletion of 45 amino acid residues was made (that is, Del 3; corresponding to
amino acid residues 1 - 345 of a sequence of SEQ ID NO: 2), such a deletion construct
showed a very low induction level of β-galactosidase. Moreover, where the zinc finger
region was deleted (that is, Del 4; amino acid residues 1 - 201), no β-galactosidase
activity was exhibited. Therefore, these results demonstrate that AtSIZ plays a role as
a transcription factor in plant cells. Further, it is clear that 206 amino acids from the
C-terminus of the AtSIZ amino acid sequence of SEQ ID NO: 2 are not necessary for
the transcription regulating activity.

Industrial Applicability

As apparent from the above description, the present invention provides a
novel transcription factor induced by osmotic stress, which is a protein designated as AtSIZ and having zinc finger motifs, its gene being AtSIZ. Inactivation of AtSIZ makes a plant sensitive to osmotic stress, while overexpression thereof confers the plant with resistance to osmotic stress. Further, AtSIZ exhibits activity as a transcription factor with respect to certain osmotic stress-inducible genes, its active site being revealed herein. Accordingly, it is possible to transform a plant with an expression vector carrying the AtSIZ, making the plant overexpress AtSIZ, thus constructing a plant resistant to osmotic stress. Consequently, it is possible to accomplish a considerable increase in plant productivity.

Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.
INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Genomine Inc.
    Environmental Eng. Bldg. 226, Pohang University of Science & Technology,
    #San31, Hyoja-dong, Nam-ku, Pohang 790-784,
    Republic of Korea

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What is claimed is:

1. A transcription factor comprising amino acid residues 1-390 of the amino acid sequence of SEQ ID NO: 2.

2. The transcription factor according to claim 1, wherein the factor is isolated from *Arabidopsis thaliana*.

3. The transcription factor according to claim 1, wherein the factor has C_{2}H-type zinc finger motif, and a transcription activation domain at the C-terminal region.

4. The transcription factor according to any one of claims 1 to 3, wherein the factor comprises the amino acid sequence of SEQ ID NO: 2 and is designated as protein AtSIZ.

5. A gene encoding the transcription factor according to claim 1.

6. The gene according to claim 5, wherein the gene comprises the nucleotide sequence of SEQ ID NO: 1 and is designated as gene AtSIZ.

7. An eukaryotic expression vector comprising the gene according to claim 5.

8. The eukaryotic expression vector according to claim 7, wherein the vector
comprises the *AtSIZ* gene having nucleotide sequence of SEQ ID NO: 1 (deposit number: KCTC 0886BP).

9. A method for enhancing resistance to osmotic stress in a plant, comprising the step of transforming the plant with the expression vector according to claim 7 so as to overexpress the transcription factor according to claim 1.

10. The method according to claim 9, wherein the osmotic stress includes stress induced by high concentration of salt, low temperature, dehydration or abscisic acid treatment.

11. A transformed plant whose resistance to osmotic stress is enhanced by the method according to claim 9 or claim 10.
FIG. 2

AtSIZ

EtBr
FIG. 3

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AtSIZ

EtBr

Cold

Drought

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AtSIZ

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FIG. 4

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C

615

T-DNA
FIG. 5

A

WT  mt

AtSIZ

B

WT

8  6  4

mt

a

8  6  4

b

WT

8  6  4

mt

c

8  6  4

d

6  4  2
FIG. 6

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AtSIZ

B

**WT**

**mt**

**mt/AtSIZ (2-2)**

(a) (b)

**WT**

**mt**

**mt/AtSIZ (2-2)**

(c) (d)
FIG. 7

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AtSIZ

COR15a

RD29A

EtBr
FIG. 9b
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120
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174
Met Cys Gly

1
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Ala Lys Ser Asn Leu Cys Ser Ser Lys Thr Leu Thr Glu Val Glu Phe
5 10 15

atg agg cag aaa tca gaa gac gga gct tcc gcc acg tgt ctc ctc gaa
Met Arg Gln Lys Ser Glu Asp Gly Ala Ser Ala Thr Cys Leu Leu Glu
20 25 30 35

ttc gcc gcc tgt gat ctt tca tcc ttt aag aga gag atc gaa gag
Phe Ala Ala Cys Asp Leu Ser Ser Phe Lys Arg Glu Ile Glu Glu
40 45 50

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Asn Pro Ser Val Glu Ile Asp Glu Ser Gly Phe Trp Tyr Cys Arg Arg
55 60 65

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Val Gly Ser Lys Met Gly Phe Glu Glu Arg Thr Pro Leu Met Val
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Leu His Cys Ala Val Ser Gly Ser Val Ser Ile Val Glu Ile Ile
120 125 130

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Lys Ile Leu Leu Asp Ala Ala Ala Ser Pro Asn Cys Val Asp Ala Asn
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Gly Asn Lys Pro Val Asp Leu Leu Ala Lys Asp Ser Arg Phe Val Pro
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165 170 175

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Met Glu Leu Arg Phe Arg Gly Leu Asp Asn Arg Arg Leu Gly Asp Leu
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Lys Pro Ser Asn Leu Glu Glu Thr Phe Gly Ser Tyr Asp Ser Ala Ser
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Val Met Gln Leu Gln Ser Pro Ser Arg His Ser Gln Met Asn His Tyr
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Pro Ser Ser Pro Val Arg Gln Pro Pro Pro His Gly Phe Glu Ser Ser
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Ala Ala Met Ala Ala Ala Val Met Asn Ala Arg Ser Ser Ala Phe Ala
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Lys Arg Ser Leu Ser Phe Lys Pro Ala Pro Val Ala Ser Asn Val Ser
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Asp Trp Gly Ser Pro Asn Gly Lys Leu Glu Trp Gly Met Gln Ile Tyr
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Glu Leu Asn Lys Leu Arg Arg Ser Ala Ser Phe Gly Ile His Gly Asn
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Asn Asn Asn Ser Val Ser Arg Pro Ala Arg Asp Tyr Ser Asp Glu Pro
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Asp Val Ser Trp Val Asn Ser Leu Val Lys Glu Asn Ala Pro Glu Arg
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Val Asn Glu Arg Val Gly Asn Thr Val Asn Gly Ala Ala Ser Arg Asp
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5' primer for PCR of AtSIZ gene

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3' primer of PCR of AtSIZ gene

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right border primer for PCR of T-DNA

togggcctaa cttttggtg
left border primer for PCR of T-DNA

gaacatcggctctcaatgca
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC7 C12N 15/82
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)
IPC7 C12N 15/82

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)
NCBI pubmed, blast database, blastn database, DDBJ, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>A</td>
<td>Plant Cell. 1999 Jun;11(6):1019-32.</td>
<td>7-10, 11</td>
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<td>A</td>
<td>Plant J 1994 Nov;6(5):749-758</td>
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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
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  "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
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"&" document member of the same patent family

Date of the actual completion of the international search
07 SEPTEMBER 2001 (07.09.2001)

Date of mailing of the international search report
11 SEPTEMBER 2001 (11.09.2001)

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Form PCT/ISA/210 (second sheet) (July 1998)