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A Transmembrane Hybrid-Type Histidine Kinase in Arabidopsis Functions as an Osmosensor

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Water deficit and the resulting osmotic stress affect plant growth. To understand how plant cells monitor and respond to osmotic change from water stress, we isolated a cDNA from dehydrated Arabidopsis plants. This cDNA encodes a novel hybrid-type histidine kinase, ATHK1. Restriction fragment length polymorphism mapping showed that the *ATHK1* gene is on chromosome 2. The predicted ATHK1 protein has two putative transmembrane regions in the N-terminal half and has structural similarity to the yeast osmosensor synthetic lethal of N-end rule 1 (SLN1). The ATHK1 transcript was more abundant in roots than other tissues under normal growth conditions and accumulated under conditions of high or low osmolarity. Histochemical analysis of β-glucuronidase activities driven by the *ATHK1* promoter further indicates that the *ATHK1* gene is transcriptionally upregulated in response to changes in external osmolarity. Overexpression of the ATHK1 cDNA suppressed the lethality of the temperature-sensitive osmosensing-defective yeast mutant *sln1-ts*. By contrast, ATHK1 cDNAs in which conserved His or Asp residues had been substituted failed to complement the *sln1-ts* mutant, indicating that ATHK1 functions as a histidine kinase. Introduction of the ATHK1 cDNA into the yeast double mutant *sln1*Δ *sho1*Δ, which lacks two osmosensors, suppressed lethality in high-salinity media and activated the high-osmolarity glycerol response 1 (HOG1) mitogen-activated protein kinase (MAPK). These results imply that ATHK1 functions as an osmosensor and transmits the stress signal to a downstream MAPK cascade.

INTRODUCTION

Plants have multiple physiological and biochemical systems that enable them to tolerate environmental stresses. Water deficit is the most serious factor limiting plant growth and productivity, and it occurs not only during drought but also with high salinity and low temperature. A change in osmotic potential in cells caused by water loss triggers various molecular responses in plants (Bray, 1997). To date, many genes induced by drought, salinity, or cold stress have been identified and studied (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996, 1997). However, little is known about how plant cells detect water deficits.

In bacteria, histidine kinases function as sensor molecules that transduce extracellular signals (including chemotactic factors, changes in osmolarity, and nutrient deficiency) to the cytoplasm. This transduction is mediated by phosphotransfer to the cognate response regulator (Parkinson and Kofoid, 1992; Parkinson, 1993; Alex and Simon, 1994; Swanson et al., 1994; Chang and Meyerowitz, 1995; Mizuno et al., 1996; Mizuno, 1997, 1998; Wurgler-Murphy et al., 1997; Chang and Stewart, 1998). This simple signaling unit is called a two-component system. Typically, the two-component system is composed of two types of proteins, a sensory histidine kinase and a response regulator. A typical histidine kinase contains an N-terminal input domain and a C-terminal transmitter domain with an invariant histidine residue. A typical response regulator contains an N-terminal receiver domain with an invariant aspartate residue and a C-terminal output domain. The input domain of the sensory histidine kinase detects environmental variables and selectively promotes autophosphorylation of a histidine residue within its transmitter domain. Following autophosphorylation, the phosphoryl group is transferred to the invariant aspartate residue in the receiver domain of the cognate response regulator. A change in the phosphorylation state of the response regulator alters the effector activity of the output domain to control the transcription of signal-responsive genes.

Recently, several histidine kinase–encoding genes have been cloned in eukaryotes. Some of these have been shown to be involved in osmosensing (Ota and Varshavsky, 1993; Maeda et al., 1994, 1995; Posas et al., 1996; Wang et al., 1996), development (Alex et al., 1996; Schuster et al., 1996),

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and plant hormone responses (Chang et al., 1993; Hua et al., 1995, 1998; Wilkinson et al., 1995; Kakimoto, 1996; Payton et al., 1996; Vriezen et al., 1997; Lashbrook et al., 1998; Sakai et al., 1998). It is thus apparent that the two-component system is not confined to prokaryotes. A two-component histidine kinase functions as an osmosensor in yeast (Maeda et al., 1994, 1995); however, all of the plant histidine kinases described to date are involved in perception of the plant hormones ethylene and cytokinin (Chang et al., 1993; Hua et al., 1995, 1998; Wilkinson et al., 1995; Kakimoto, 1996; Payton et al., 1996; Vriezen et al., 1997; Lashbrook et al., 1998; Sakai et al., 1998). These observations prompted us to isolate histidine kinases and to investigate their roles in osmosensing by plants. In this report, we provide evidence, using yeast mutants, that a novel Arabidopsis histidine kinase has a potential function as an osmosensor. We also present an analysis of the upregulation of the histidine kinase gene by changes in external osmolarity. Possible functions of the histidine kinase in osmosensing in plants are discussed.

RESULTS

ATHK1 Is an Aradidopsis Hybrid-Type Histidine Kinase

To isolate histidine kinases, we used a polymerase chain reaction (PCR) strategy with degenerate oligonucleotide primers and cDNAs prepared from dehydrated Arabidopsis rosette plants. One PCR fragment that encodes a novel histidine kinase was identified and then used to screen a cDNA library. The cDNA clone with the longest insert (~4 kb), designated ATHK1 (for *Arabidopsis thaliana* histidine kinase 1), encoded a polypeptide of 1207 amino acid residues with a predicted molecular weight of 135 kD (Figure 1A). We also screened a genomic library using the cDNA clone as a probe and isolated a genomic clone that contains the 1.6-kb upstream region of the *ATHK1* gene. The predicted protein, ATHK1, contains both a transmitter and a receiver domain with a conserved histidine residue in the transmitter domain and a conserved aspartate residue in the receiver domain (Figure

A

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MRGDSFSMSIENLPDSPMGSRKKKMQIRKVFDKMTEWVTPWRSNLESPREMMILRGDVEQDEFQYASSHCLSSYYSVFVVRLAIMVMLAILIGLLTVLTW     100
HFTRIYTKQSLQTLAYGLRYELLQRPVLRMWSVLNTTSELTTAQVKLSEYVIKKYDKPTTQEELVEMYQAMKDVTWALFASAKALNAITINYRNGFVQAF     200
HRDPASSSTFYIFSDLKNYSISGTGPEDVSGWNNKSIHGNMSAIWYQQQLDPVTGENLGKPLKIPPDDLINIAGISQVPDGEASWHVTVSKYMDSPLLSA     300
ALPVFDASNKSIVAVVGVTTALYSVGQLMRDLVEVHGGHIYLTSQEGYLLATSTDGPLLKNTSNGPQLMKATDSEEWVIKSGAQWLEKTYGSKRPHVVHA     400
ENVKLGDQRYYIDSFYLNLKRLPIVGVVIIPRKFIMGKVDERAFKTLIILISASVCIFFIGCVCILILTNGVSKEMKLRAELIRQLDARRRAEASSNYKS     500
QFLANMSHELRTPMAAVIGLLDILISDDCLSNEQYATVTQIRKCSTALLRLLNNILDLSKVESGKLVLEEAEFDLGRELEGLVDMFSVQCINHNVETVLD     600
LSDDMPALVRGDSARLVQIFANLISNSIKFTTTGHIILRGWCENINSLHDEMSVSVDRRKPWAPMKTKQVQHRNHLQKSCKNANKMVLWFEVDDTGCGID     700
PSKWDSVFESFEQADPSTTRTHGGTGLGLCIVRNLVNKMGGEIKVVQKNGLGTLMRLYLILSTPDTVDQNIQPDFSKYGLVVMLSMYGSTARMITSKWLR     800
KHGIATVEASDWNELTQIIRDLLETGSRDNSFDSQHNISDPLRAELSNIVEIKNPVFVIVVDIGVLDLTTNIWKEQLNYLDRFSNKAKFAWLLKHDTSNT     900
VKTELRRKGHVMMVNKPLYKAKMIQILEAVIKNRKRGLCNDLRNRGNGSDESHDCLEIDPTQFDTCSSDDSSETSGEKQVDKSVKPSTLHSPVLKNYLID    1000
ATTSNDDSTSASMTQKNPEEEDWKDRLYSGIALDGKNQKSLEGIRFLLAEDTPVLQRVATIMLEKMGATVTAVWDGQQAVDSLNYKSINAQAPTEEHKSF    1100
EEETANKVTTRETSLRNSSPYDLILMDCQMPKMDGYEATKAIRRAEIGTELHIPIVALTAHAMSSDEAKCLEVGMDAYLTKPIDRKLMVSTILSLTKPSA    1200
FQTSLSA                                                                                                    1207
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B

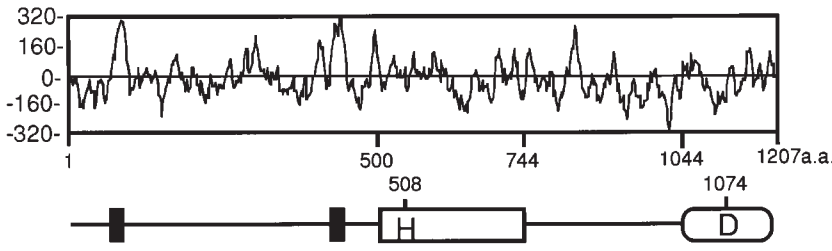


Figure 1. ATHK1 Is a Hybrid-Type Histidine Kinase.

(A) Amino acid sequence of ATHK1 deduced from the nucleotide sequence. Two putative transmembrane regions are indicated by boldface underlines. The transmitter and receiver domains are indicated by light underlines. The potential phosphorylation sites, His-508 and Asp-1074, are shown by asterisks. The nucleotide sequence appears in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases as accession number AB010914.

(B) Structure of ATHK1. The hydrophobicity plot was calculated by the hydropathy algorithm (Kyte and Doolittle, 1982). An open box and oval indicate transmitter and receiver domains, respectively. Two filled boxes represent putative transmembrane regions. a.a., amino acids; D, aspartate residue; H, histidine residue.

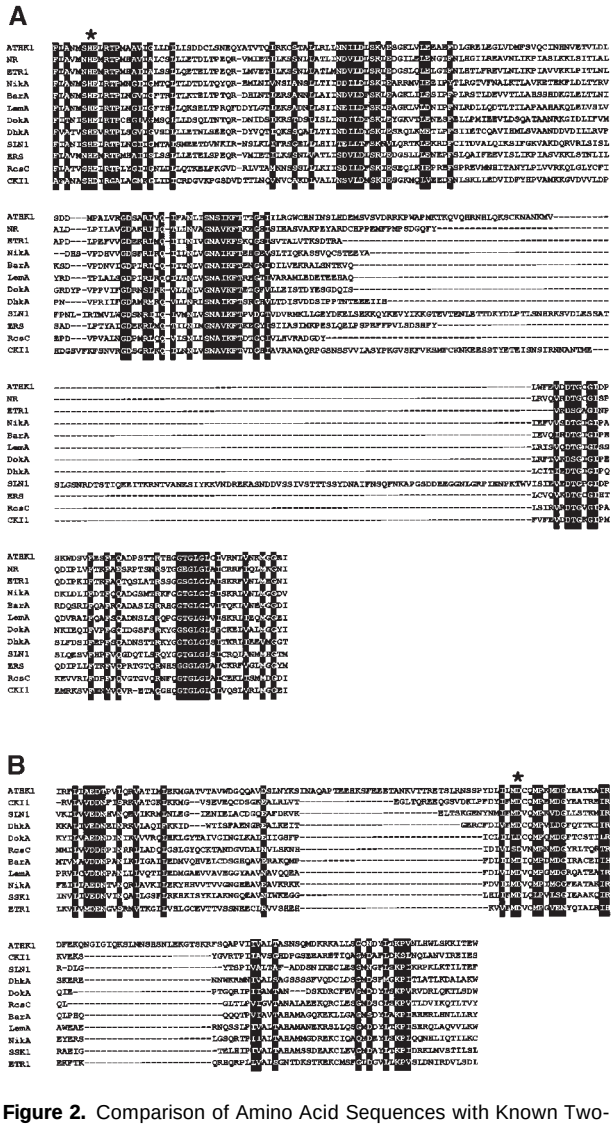


Figure 2. Comparison of Amino Acid Sequences with Known Two-Component System Molecules.

(A) Alignment of amino acid sequences of the transmitter domain.
(B) Alignment of amino acid sequences of the receiver domain.
Predicted amino acid sequence of ATHK1 aligned with those of *S. cerevisiae* SLN1 (Ota and Varshavsky, 1993), Arabidopsis ETR1 (Chang et al., 1993), Arabidopsis ERS (Hua et al., 1995), tomato NR (Wilkinson et al., 1995), Arabidopsis CKI1 (Kakimoto, 1996), *S. cerevisiae* SSK1 (Maeda et al., 1994), *E. coli* BarA (Nagasawa et al., 1992), *E. coli* RcsC (Stout and Gottesman, 1990), *Pseudomonas syringae* LemA (Hrabak and Willis, 1992), *Neurospora crassa* NikA (Alex et al., 1996), *Dictyostelium discoideum* DokA (Schuster et al., 1996), and *D. discoideum* DhkA (Wang et al., 1996). Conserved amino acid residues are shown with a black background; dashes indicate gaps introduced to maximize alignment. Asterisks indicate potential phosphorylation sites.

1A). Hydrophobicity analysis revealed that ATHK1 has two hydrophobic regions in the N-terminal half (Figure 1B). This suggests that ATHK1 is a transmembrane protein.

Comparisons of amino acid sequences of the transmitter and receiver domains of ATHK1 with those of known histidine kinases are shown in Figures 2A and 2B. The transmitter domain of ATHK1 shows the highest degree of similarity to that of the Arabidopsis cytokinin independent 1 (CKI1) protein (30%), and the receiver domain shows the highest degree of similarity with that of synthetic lethal of N-end rule 1 (SLN1) protein from *Saccharomyces cerevisiae* (29%). Thus, although three putative ethylene receptors, ethylene resistant 1 (ETR1), ethylene response sensor (ERS), and never-ripe (NR), exhibit a high degree of similarity (70 to 80%) with each other (Hua et al., 1995; Wilkinson et al., 1995), ATHK1 exhibits no marked similarity to particular histidine kinases found in plants. However, the significant structural similarity to the yeast osmosensor SLN1 suggests that ATHK1 may be an osmosensor in plants.

Genomic DNA gel blot analysis revealed no extra bands that hybridized with the ATHK1 cDNA probe under high-stringency conditions, suggesting that the *ATHK1* gene is present as a single copy (Figure 3). However, under low-stringency conditions, a few extra bands, shown by arrowheads, were detected, suggesting that there may be a few related genes in the Arabidopsis genome (Figure 3).

We determined the chromosomal position of the *ATHK1* gene by restriction fragment length polymorphism (RFLP) mapping using P1 clones. When restriction fragments of genomic DNA were probed with a P1 plasmid DNA (MZL17) carrying the *ATHK1* gene, HindIII-digested bands revealed a polymorphism between the ecotypes Landsberg *erecta* and Columbia. Therefore, we analyzed the RFLP segregation patterns of genomic DNA digested with HindIII from 99 recombinant inbred lines. Figure 4 shows the approximate chromosomal position of the *ATHK1* locus on chromosome 2, flanked by the markers mi139 and m216. This map position is consistent with the report that the bacterial artificial chromosome clone T17A5, which also carries the *ATHK1* gene, maps to yeast artificial chromosome clone CIC8B10 on chromosome 2 (http://nucleus. cshl.org/protarab/).

ATHK1 Acts as a Histidine Kinase in Yeast

Because ATHK1 is structurally related to the yeast osmosensor SLN1, we analyzed its biological function by using yeast *sln1* mutants. First, we introduced the ATHK1 cDNA into the temperature-sensitive *sln1* mutant *sln1-ts* under the control of the galactose-inducible *GAL1* promoter to examine the catalytic activity of ATHK1. The *sln1-ts* mutation is lethal because of the constitutive activation of the high-osmolarity glycerol response 1 (HOG1) mitogen-activated kinase (MAPK) cascade at a restrictive temperature, 37°C (Maeda et al., 1994). Overexpression of the ATHK1 cDNA (ATHK1FL) suppressed the phenotype of *sln1-ts* mutant in a galactose-dependent

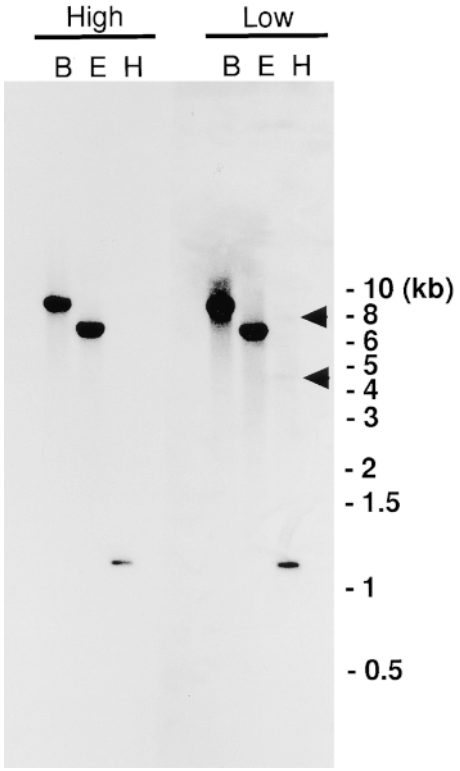


Figure 3. Genomic DNA Gel Blot Analysis of the *ATHK1* Gene.

Genomic DNA (5 μg) was digested with BamHI (B), EcoRI (E), and HindIII (H), fractionated in a 0.7% agarose gel, and transferred to a nitrocellulose membrane. The membrane was hybridized with ^{32}P-labeled PCR fragment and washed with 0.1 × SSC, 0.1% SDS, at 50°C (high-stringency conditions) or 2 × SSC, 0.5% SDS, at 37°C (low-stringency conditions). DNA markers are indicated at right in kilobases.

manner (Figure 5). By contrast, cDNAs in which either putative phosphorylation site had been substituted—His-508 (ATHK1H508V; His-508 to Val) or Asp-1074 (ATHK1D1074E; Asp-1074 to Glu)—failed to complement the *sln1-ts* mutation (Figure 5). These results suggest that ATHK1 acts as a histidine kinase in yeast.

Similarly, deletions of both the N-terminal half (ATHK1SOL) and the predicted extracellular domain between the two hydrophobic regions (ATHK1dExD) resulted in failure to complement the *sln1-ts* mutation. This indicates that ATHK1 contains an extracellular domain that plays an important role in the activation of ATHK1 (Figure 5). The deletion of the extracellular domain may affect the activity of ATHK1 due to a structural change. Interestingly, the lethality of the *sln1-ts* mutants was not complemented by the expression of ETR1, an ethylene receptor in Arabidopsis (Chang et al., 1993). To determine whether the suppression of the *sln1-ts* mutation by ATHK1 requires SLN1 to be active in the *sln1-ts* mutants, we introduced the ATHK1 cDNA into an *sln1* deletion mutant (*sln1*Δ). ATHK1 was able to complement *sln1*Δ, indicating that ATHK1 can functionally substitute for SLN1 (data not shown). Thus, ATHK1 seems to be active, at least in yeast, without any protein modifications or a requirement for external signals. Apparently, ATHK1 is capable of inactivating the HOG1 MAPK cascade by performing phosphotransfer to the downstream signal transduction components that ordinarily are modified by SLN1.

ATHK1 Confers High-Osmolarity Tolerance to the *sln1*Δ *sho1*Δ Yeast Double Mutant

S. cerevisiae contains two osmosensors: synthetic high-osmolarity-sensitive 1 (SHO1), a protein that contains transmembrane domains, and SLN1. Mutants lacking both SLN1 and SHO1 are lethal under high-osmolarity conditions. If ATHK1 expressed in yeast becomes inactive in response to increases in osmolarity, mutants lacking both SLN1 and SHO1 should be able to grow upon activation of the HOG1 MAPK cascade. To analyze the function of ATHK1 as an osmosensor in yeast, we introduced the ATHK1 cDNA into the *sln1* and *sho1* deletion mutants (*sln1*Δ *sho1*Δ) and then examined their ability to grow on high-osmolarity media. As shown in Figure 6, the *sln1*Δ *sho1*Δ double mutant expressing either ATHK1 (*sln1*Δ *sho1*Δ ATHK1) or SLN1 (*sln1*Δ

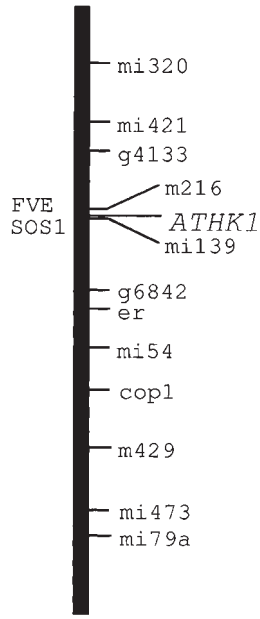


Figure 4. Location of the *ATHK1* Gene on Chromosome 2.

Mapping of the *ATHK1* gene was achieved by RFLP linkage analysis with the F_8 progeny of 99 recombinant inbred lines generated between Landsberg *erecta* and Columbia ecotypes.

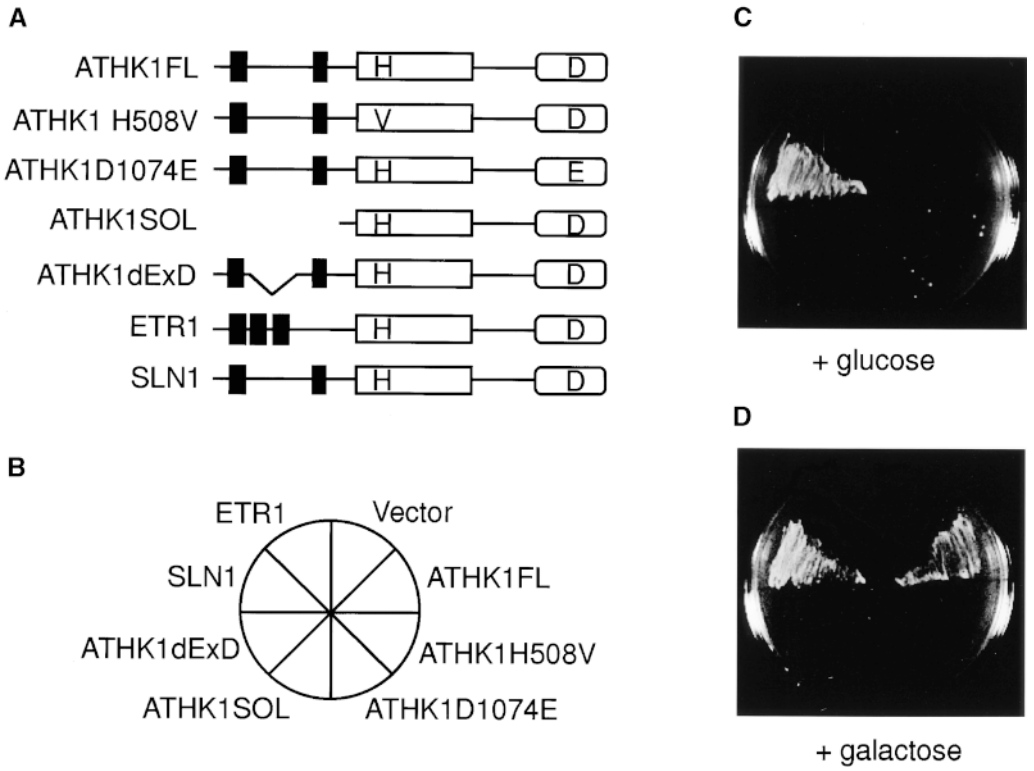


Figure 5. Complementation of the *sln1-ts* Mutation by Expression of the ATHK1 cDNA.

(A) Constructs used in this experiment. D, aspartate residue; E, glutamate residue; H, histidine residue; V, valine residue.
(B) Strains used in this experiment.
(C) and **(D)** The *sln1-ts* mutants carrying either vector alone, the indicated expression plasmids of ATHK1 and its mutants, ETR1, or SLN1, were cultured on plates supplemented with 2% glucose **(C)** or 2% galactose **(D)** for 3 days at 37°C.

*sho1*Δ SLN1) grew as well as wild-type cells on YPD medium containing 0.9 M NaCl, but the *hog1* deletion mutants (*hog1*Δ) showed reduced growth under these conditions. However, introduction of ATHK1D1074E did not confer high-osmolarity tolerance to the *sln1*Δ *sho1*Δ double mutant (*sln1*Δ *sho1*Δ ATHK1D1074E). This suggests that the catalytic activity of ATHK1 is required for growth under high-salinity conditions. The growth of the *sln1*Δ mutants expressing either ATHK1 (*sln1*Δ ATHK1) or SLN1 (*sln1*Δ SLN1) was not affected by increases in osmolarity in the medium (Figure 6). These results imply that the activity of ATHK1 was stopped in response to increases in external osmolarity, and consequently the HOG1 MAPK cascade was activated.

ATHK1 Senses and Transduces Changes in External Osmolarity to the HOG1 MAPK Cascade in Yeast

To confirm that the tolerance of the *sln1*Δ *sho1*Δ ATHK1 cells to high osmolarity was due to activation of the HOG1 MAPK cascade, we analyzed tyrosine phosphorylation of the HOG1 MAPK by immunoblot analysis with an antiphosphotyrosine antibody. Treatment of the *sln1*Δ *sho1*Δ ATHK1 cells, the wild-type cells, and the *sln1*Δ *sho1*Δ SLN1 cells with 0.4 M NaCl induced tyrosine phosphorylation of HOG1 (Figure 7A). No phosphorylated bands were detected at the same position in the *hog1*Δ cells, indicating that the corresponding band was tyrosine-phosphorylated HOG1 (Figure 7A). Tyrosine phosphorylation of HOG1 also was not detected in a double mutant lacking both the suppressor of sensor kinase 1 (SSK1) and SHO1 (*ssk1*Δ *sho1*Δ). This indicates that the NaCl-dependent tyrosine phosphorylation of HOG1 in the *sln1*Δ *sho1*Δ ATHK1 cells was mediated by ATHK1. Furthermore, the amount of phosphorylation of HOG1 in the *sln1*Δ *sho1*Δ ATHK1 cells depended on the concentration of NaCl; phosphorylation rapidly increased within 5 min after exposure to high osmolarity and returned to a basal level after 60 min (Figure 6B). This response was similar to that observed in the *sln1*Δ *sho1*Δ SLN1 cells (Figure 6B). Thus, ATHK1 seems to sense changes in external

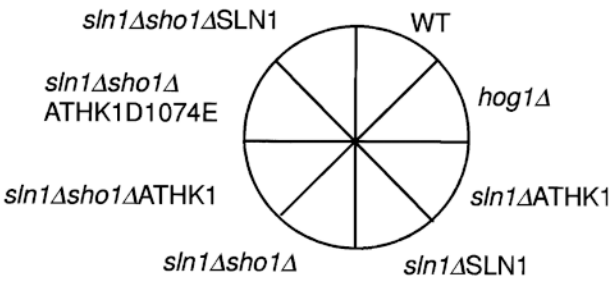
osmolarity, as does SLN1, and then transduces the information to the downstream cytoplasmic targets in yeast.

The ATHK1 mRNA Is More Abundant in Roots and Accumulates under High- and Low-Osmolarity Conditions

We examined the expression of the *ATHK1* gene in normally grown Arabidopsis organs using RNA gel blot analysis. The *ATHK1* mRNA was more abundant in roots than in other organs, suggesting a functional importance of ATHK1 in root tissue (Figure 8A). We then examined the induction of the *ATHK1* gene under a variety of stress conditions. *ATHK1* mRNA significantly accumulated under conditions of high salinity and low temperature (Figure 8B). Moreover, treatment with distilled water and dehydration also slightly stimulated the induction of the *ATHK1* gene (Figure 8B). These results suggest that a change in osmolarity may trigger the induction of the *ATHK1* gene. Extra transcripts of ~2 kb in length were also detected in each lane. These were probably degraded or alternatively spliced products. We also examined the protein level of ATHK1 after stress treatments by protein gel blotting with an antiserum raised against recombinant ATHK1 proteins. However, we could not detect a specific band due to very low abundance of the endogenous ATHK1 in wild-type Arabidopsis (data not shown).

We then constructed a chimeric gene consisting of the *ATHK1* promoter fused to a β-glucuronidase (*GUS*) reporter gene (*ATHK1p–GUS*) to analyze the distribution of expression of the GUS activity driven by the ATHK1 promoter in transgenic Arabidopsis plants (Figure 9A). Strong GUS activity was detected in leaf bases and roots of rosette plants when the transgenic plants were exposed to either high- or low-osmolarity solutions (Figures 9C and 9D). By contrast, weak GUS activity was observed in transgenic plants that had been exposed to isotonic solution (GM solution) and in untreated plants (Figures 9B and 9G). These results further suggest that the expression of the *ATHK1* gene is transcriptionally regulated in response to changes in external osmolarity.

A



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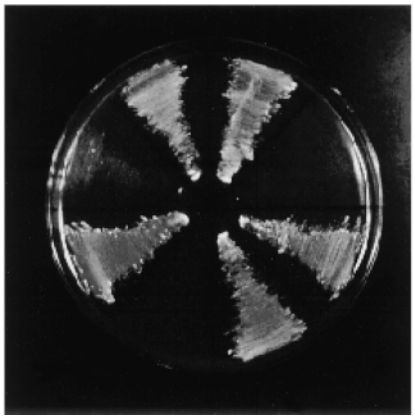

Figure 6. Sensitivity to High Osmolarity of the *sln1*Δ *sho1*Δ Double Mutant Transformed with the ATHK1 cDNA.

(A) Strains used in this experiment. WT, wild-type; *hog1*Δ, *hog1* deletion mutant; *sln1*Δ ATHK1, *sln1*Δ deletion mutant expressing ATHK1; *sln1*Δ SLN1, *sln1*Δ deletion mutant expressing SLN1; *sln1*Δ *sho1*Δ ATHK1, *sln1*Δ *sho1*Δ double deletion mutant expressing ATHK1; *sln1*Δ *sho1*Δ ATHK1D1074E, *sln1*Δ *sho1*Δ double mutant expressing ATHK1D1074E; and *sln1*Δ *sho1*Δ SLN1, *sln1*Δ *sho1*Δ double mutant expressing SLN1.
(B) Yeast strains cultured on YPD media containing 0.9 M NaCl for 3 days at 30°C.

DISCUSSION

In this study, we isolated a cDNA for a hybrid-type histidine kinase that has structural similarity to the yeast osmosensor SLN1. The ATHK1 protein contains both a transmitter and a receiver domain with conserved histidine and aspartate residues, respectively, and a transmembrane domain with two hydrophobic regions toward the N terminus, as does SLN1 (Figure 1B). ATHK1 complemented both *sln1-ts* and *sln1*Δ mutants, whereas ATHK1 that was mutated at the invariant His or Asp positions did not (Figure 5). These results indicate that ATHK1 has a histidine kinase activity. ATHK1 also complemented *sln1*Δ *sho1*Δ double deletion mutants that lacked two osmosensors, SLN1 and SHO1, whereas ATHK1 mutated at an Asp did not (Figure 6). Moreover, high-salinity-dependent tyrosine phosphorylation of the HOG1 MAP kinase was detected in *sln1*Δ *sho1*Δ double deletion mutants (Figure 7). These results indicate that ATHK1 became inactive in response to high salinity and activated the HOG1 MAPK cascade, as does SLN1 in yeast. Thus, we showed, by analyzing both sensing (input) and catalytic (output) activities with yeast osmosensing-defective mutants, that Arabidopsis ATHK1 can act as an osmosensor in yeast in vivo.

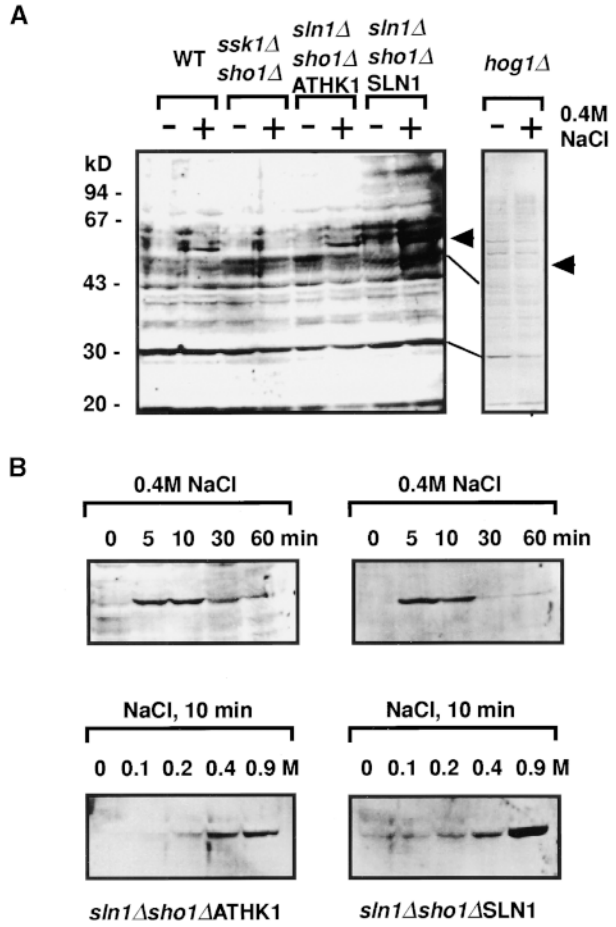

Figure 7. ATHK1 Senses and Transduces Changes in External Osmolarity in Yeast.

(A) NaCl-dependent tyrosine phosphorylation of the HOG1 MAPK by ATHK1. Wild-type (WT) cells, the *ssk1 sho1* deletion mutant (*ssk1*Δ *sho1*Δ), the *sln1*Δ *sho1*Δ double mutant expressing either ATHK1 (*sln1*Δ *sho1*Δ ATHK1) or SLN1 (*sln1*Δ *sho1*Δ SLN1), and *hog1* deletion mutants (*hog1*Δ) were cultured in YPD medium. The cells were treated without (−) or with (+) 0.4 M NaCl for 10 min and subjected to SDS-PAGE. The tyrosine phosphorylation of HOG1 was analyzed by immunoblotting with the anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY). Arrowheads indicate the tyrosine-phosphorylated HOG1. The sizes of protein markers are indicated at left in kilodaltons.

(B) Transient tyrosine phosphorylation of HOG1 MAP kinase in the *sln1*Δ *sho1*Δ double mutant expressing ATHK1 in response to high osmolarity. *sln1*Δ *sho1*Δ mutants expressing either ATHK1 (*sln1*Δ*sho1*ΔATHK1) or SLN1 (*sln1*Δ*sho1*ΔSLN1) were cultured in YPD medium. The cells were treated with 0.4 M NaCl for the indicated periods (top) or for 10 min with the indicated concentrations of NaCl (bottom). The tyrosine phosphorylation of HOG1 was analyzed by immunoblotting with an anti-phosphotyrosine antibody.

To date, histidine kinases that play roles in the perception of plant hormone signals have been found in plants (Chang et al., 1993; Hua et al., 1995, 1998; Wilkinson et al., 1995; Kakimoto, 1996; Payton et al., 1996; Vriezen et al., 1997; Lashbrook et al., 1998; Sakai et al., 1998). However, two-component systems are thought to be involved in sensing osmotic stress, which is a common environmental signal not only for unicellular organisms, such as bacteria and yeast, but also for higher plants. In *S. cerevisiae*, SLN1 acts as an osmosensory protein, sequentially phosphorylating a phosphorelay intermediater, tyrosine phosphatase dependent 1 (YPD1), and the response regulator SSK1, under conditions of normal osmolarity (Posas et al., 1996). These three protein factors perform a four-step phosphorelay (His-Asp-His-Asp). Recently, several cDNAs encoding response regulators (Brandstatter and Kieber, 1998; Imamura et al., 1998; Sakakibara et al., 1998; Urao et al., 1998) and three cDNAs

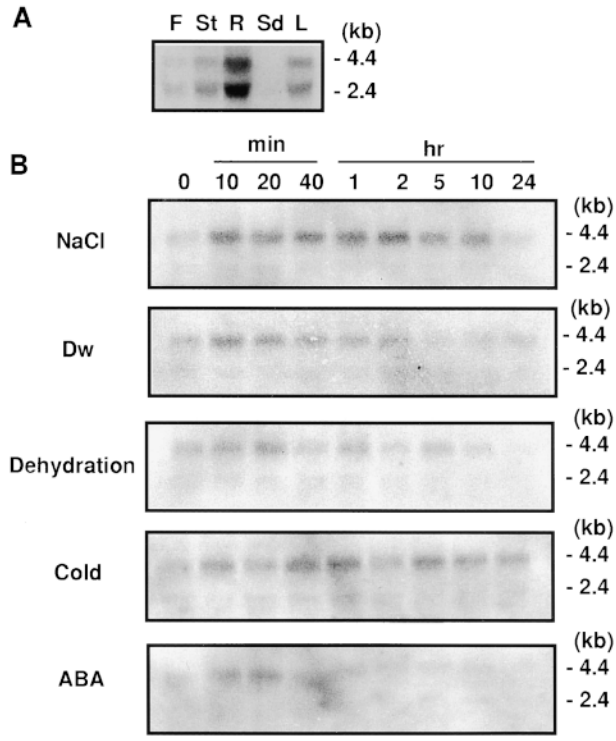

Figure 8. Expression of the *ATHK1* Gene in Organs and under Stress Conditions.

(A) Each lane was loaded with 30 μg of total RNA prepared from flowers (F), stems (St), roots (R), developing seeds (Sd), and leaves (L) from whole rosette plants.

(B) Plants were treated with 250 mM NaCl (NaCl), distilled water (Dw), dehydration, low temperature at 4°C (Cold), or 100 μM abscisic acid (ABA). The blots were probed with the ^{32}P-labeled ATHK1 cDNA.

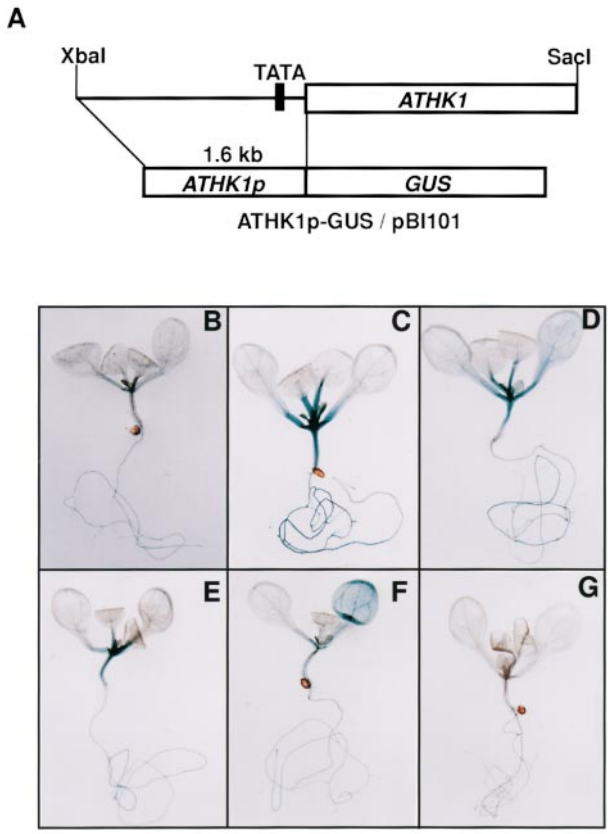

Figure 9. Histochemical Localization of GUS Activity in Transgenic Arabidopsis Plants.

(A) Shown is the construct used in this experiment. A PCR fragment containing 1.6 kb of the upstream region from the initiation site of translation (ATG) of the *ATHK1* gene was fused to the coding region of a *GUS* reporter gene, as described in Methods.
(B) to **(G)** Histochemical localization of GUS activity in transgenic Arabidopsis plants.
(B) Plant grown under normal conditions.
(C) Plant exposed to 250 mM NaCl.
(D) Plant exposed to distilled water.
(E) Dehydrated plant.
(F) Plant exposed to 4°C.
(G) Plant exposed to isotonic solution.

encoding phosphorelay intermediaters that possess a histidine-containing phosphotransfer domain (Miyata et al., 1998; Suzuki et al., 1998) have been found in Arabidopsis and maize. These observations further suggest that plants have an osmosensing and signaling system similar to that of yeast. In addition, a MAPK cascade that consists of SSK2/SSK22 (MAPK kinase kinase [MAPKKK]), PBS2 (MAPKK), and HOG1 (MAPK) operates downstream of the SLN1–YPD1–SSK1 two-component osmosensing system in *S. cerevisiae*. At high osmolarity, unphosphorylated SSK1 activates SSK2/SSK22, resulting in the activation of PBS2 by Ser-Thr phosphorylation. Then, phosphorylated PBS2 activates HOG1 by Thr-Tyr phosphorylation. A MAPK cascade is also thought to be involved in osmotic stress response in higher plants (Hirt, 1997; Mizoguchi et al., 1997). Expression of genes for MAPKs of various plants, such as *ATMPK3* in Arabidopsis and *MMK4* in alfalfa, is transcriptionally induced by drought, low temperature, and mechanical stress. Moreover, two genes for protein kinases, MAPKKK (*ATMEKK1*) and ribosomal S6 kinase (*ATPK19*), are induced by similar stresses (Mizoguchi et al., 1996). Recently, activation of MMK4 at the post-translational level by these same stresses was shown (Jonak et al., 1996). These observations indicate that a MAPK cascade analogous to the yeast HOG1 pathway might function in plants in a signal transduction cascade in response to osmotic stress.

RNA gel blot analysis revealed that the *ATHK1* transcript was more abundant in roots than in other tissues under normal growth conditions and that it accumulated under conditions of high or low osmolarity (Figure 8). Roots represent the interface between a plant and its soil environment and are involved in nutrient acquisition and responses to environmental conditions, such as gravity, light, water, and temperature (Jackson, 1997). Several ion transporter genes, which are involved in nutrient uptake, are expressed predominantly in roots. Interestingly, the levels of the transcripts are regulated by each of their substrates (Lauter et al., 1996; Muchhal et al., 1996). The expression of the GUS activity driven by the *ATHK1* promoter was strongly induced in roots in response to changes in external osmolarity (Figure 9). These results further indicate that the expression of the *ATHK1* gene is transcriptionally upregulated by changes in external osmolarity.

A possible advantage of the upregulation of sensor molecules is speculated as follows. Increased osmotic pressure may cause a conformational change in ATHK1; consequently, its kinase activity may be inactivated. The newly produced ATHK1 under high-osmolarity conditions would also be inactive in the membrane. The accumulation of the inactive ATHK1 may be required to enhance the response or to reduce the response quickly through the reactivation of the kinase activity of ATHK1 when osmotic stress is relieved. Recently, the Arabidopsis ethylene receptors ERS, ERS2, and ETR2 have been shown to be upregulated by ethylene (Hua et al., 1998). The upregulation of the ethylene receptors is suggested to be a mechanism for adaptation in ethylene responses by production of additional proteins that can repress the ethylene response (Hua and Meyerowitz, 1998).

The mRNAs of several genes involved in signal transduction pathways, such as protein kinases, protein phosphatases, a phospholipase C, calmodulins, monomeric small GTP binding proteins, and transcription factors, also accumulate in response to environmental stimuli or stresses (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997). Under stress conditions, it appears that the transcriptional regulation of genes for such signal transducers confers higher

sensitivity and signaling efficiency to the cellular transducing processes. Thus, a high level of expression of ATHK1 in roots and stressed plants may be necessary for efficient sensing of environmental changes. Although analysis of transgenic plants is now in progress to elucidate the physiological function of ATHK1 in plant cells, we suggest that ATHK1 senses changes in osmolarity in cells caused by dehydration, salinity, and cold stresses, and subsequently transduces the stress signals to the nucleus through a protein phosphorylation cascade, probably a MAPK cascade.

METHODS

Plant Materials

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on GM agar plates (Valvekens et al., 1988) under continuous illumination of ~2000 lux at 22°C for 3 to 4 weeks and were used for the construction of a cDNA library and in stress treatments. Plants used for the analysis of tissue-specific expression were grown in 15-cm pots filled with a 1:1 mixture of perlite and vermiculite and were watered with 0.1% Hyponex (Hyponex, Inc., Tokyo, Japan).

For the growth regulator and salt treatments, plants were transferred to and grown hydroponically in water of either 100 μM abscisic acid or 250 mM NaCl under dim light. For temperature treatments, plants were grown under continuous light at temperatures of 40 or 4°C. For dehydration treatments, plants were removed from the agar and desiccated in plastic dishes at 22°C and 60% humidity under dim light.

Molecular Cloning

Two oligonucleotide primers, 5′-CAC/TGAG/AATGA/CGIACICC-3′ and 5′-ATIGCIAG/AICCIAG/AICCIG/CT/AICCICC-3′, corresponding to the amino acid sequences HEMRTP (H box) and GGSGLGLAI (G2 box), respectively, were synthesized and phosphorylated with T4 polynucleotide kinase, as described by Sambrook et al. (1989). Polymerase chain reaction (PCR) was performed in a 100-μL reaction mixture containing 10 mM Tris-HCl, pH 8.9, 80 mM KCl, 1.5 mM $MgCl_2$, 0.5 mg mL^{-1} BSA, 0.1% deoxycholate, 0.1% Triton X-100, 0.2 mM deoxynucleotide triphosphates, 1 nmol of primer, 10 ng of cDNA, and 2.0 units of Tth DNA polymerase (Toyobo, Ltd., Tokyo, Japan). The reaction mixture was overlaid with 100 μL of liquid paraffin and subjected to 30 cycles of amplification at 94°C for 1 min (denaturation), 40°C for 2 min (annealing), and 72°C for 2 min (polymerization) followed by final incubation at 72°C for 7 min. cDNA templates for amplification by PCR were prepared from poly(A)$^+$ RNA by using the cDNA synthesis tool System Plus (Amersham International, Little Chalfont, UK).

cDNA Library Screening

The PCR fragments were purified on a 5% polyacrylamide gel and cloned into the SmaI site of pBluescript II SK− (Stratagene, La Jolla, CA). DNA sequences were determined by the dye–primer cycle sequencing method using a DNA sequencer (model 373A; Applied Biosystems, San Jose, CA). Gene Works software (InteriGenetics, Inc., Mountain View, CA) was used for the analysis of DNA and amino acid sequences. To isolate full-length cDNA, we screened by plaque hybridization an Arabidopsis cDNA library (constructed with λZAPII), prepared from rosette plants that had been dehydrated for 1 hr (Kiyosue et al., 1994), as described by Sambrook et al. (1989). Positive plaques were purified, and the cDNA inserts were subcloned into a pBluescript phagemid by an in vivo excision process, according to the manufacturer's instructions (Stratagene).

Restriction Fragment Length Polymorphism Mapping

The Arabidopsis P1 library (Liu et al., 1995) was screened using a DNA fragment of *ATHK1* as a probe. A P1 plasmid DNA carrying the *ATHK1* gene was sheared by vigorous vortexing, labeled by random priming using a ^{32}P-dCTP labeling kit (BCA Best; Takara, Kyoto, Japan) according to the manufacturer's protocol, and used for restriction fragment length polymorphism (RFLP) mapping with the recombinant inbred lines that had been generated between two ecotypes, Landsberg *erecta* and Columbia (Lister and Dean, 1993), as described by Liu et al. (1995, 1996). Segregation data for RFLP markers on 99 recombinant inbred lines (Lister and Dean, 1993) were analyzed using the Mapmaker mapping program (Lander et al., 1987), as described by Liu et al. (1996). The scoring data for 67 markers (provided by C. Dean, John Innes Research Institute, Colney, UK) and those of 129 mi markers (Liu et al., 1996) were used for the calculation.

Complementation Analysis of the Temperature-Sensitive *SLN1* Mutants

Yeast (*Saccharomyces cerevisiae*) strain HS13 with a temperature-sensitive *SLN1* allele (Maeda et al., 1994) was used for complementation experiments. Expression plasmids used in this experiment were constructed with the multicopy expression vector pNV7, in which expression is under the control of the galactose-inducible *GAL1* gene promoter (Jonston, 1987). The ATHK1 expression plasmid pNV-ATHK1FL was constructed by subcloning a BamHI fragment containing the complete open reading frame from the ATHK1 cDNA into the BamHI site of pNV7.

To construct the catalytically inactive mutants pNV-ATHK1H508V and pNV-ATHK1D1074E, we subcloned BamHI-XbaI and SpeI fragments from the ATHK1 cDNA containing His-508 and Asp-1074 residues, respectively, into pBluescript II SK−. Nucleotide substitutions were generated by a site-directed mutagenesis kit (Chameleon; Stratagene) with oligonucleotides 5′-pTTGGCAAATATGAGTGTTGAGTTGAGGACACCA-3′ (H508V; His [CAT] replaced by Val [GTT]) and 5′-pTTTTTGGCATCTGGCACTCCATGAGGATCAAG-3′ (D1074E; Asp [GAC] replaced by Glu [CTC]). Each mutation was confirmed by DNA sequencing. Subsequently, the mutated fragments were cloned into their parental plasmids; then each BamHI fragment containing the complete open reading frame was subcloned into the BamHI site of pNV7.

To construct the deletion mutant of the extracellular domain, we subcloned pNV-ATHK1dExD, a BamHI-XbaI fragment from the ATHK1 cDNA, into pBluescript II SK− and then mutated it with the oligonucleotide 5′-pGCCTTAGATACGAAGATCTTCAGCGTCCAGTC-3′

(GCT replaced by AGA, resulting in a BglII site [AGATCT]) to introduce an additional BglII site at amino acid 122. The plasmid was digested with BglII to remove an internal BglII fragment (amino acids 122 to 231) and religated, resulting in an in-frame deletion. The fragment encoding the protein in which the extracellular domain had been deleted was excised with XhoI and XbaI and used to replace the XhoI-XbaI fragment of pNV-ATHK1FL. To construct the N-terminal-half deletion mutant pNV-ATHK1SOL, a cDNA fragment (amino acids 477 to 1207) was amplified by PCR by using oligonucleotides incorporating BamHI or KpnI sites at the 5′ ends (5′-CAGTGGATCCAT-GAAGCTAAGAGCAGAACTGATAAGG-3′ [for amino acids 477 to 485; the BamHI site is underlined] and 5′-ATAGGGTACCGTTAG-ATTTTTACCCGATTCTTCA-3′ [for amino acids 1200 to 1207; the KpnI site is underlined]). The PCR fragments were digested with BamHI and KpnI and then cloned into the BamHI-KpnI site of pNV7.

The ETR1 expression plasmid pNV-ETR1 was constructed by insertion of a BamHI fragment from the ETR1 cDNA into the BamHI site of pNV7. The SLN1 expression plasmid cloned in pRS414, pPD2146 (Maeda et al., 1994), was used as a positive control. These plasmids were introduced into yeast cells by using a lithium-acetate method (Ito et al., 1983).

Complementation Analysis of the *sln1* and *sho1* Deletion Mutant (*sln1*Δ *sho1*Δ)

To construct the *sln1* deletion mutant (*sln1*Δ), strain TM141 (*MAT* α, *ura3*, *leu2*, *trp1*, *his3*) (Maeda et al., 1994) was transformed with the *PTP2* expression vector under the control of the *GAL1* promoter (pGP22; *GAL1p*::*PTP2* in pRS413) (Maeda et al., 1994). The resulting transformants were mated with the strain TM205 (*MAT* α, *ura3*, *leu2*, *his3*, *sln1*::*LEU2*, *ssk1*::*URA3*) (Maeda et al., 1994). The heterozygous diploid cells were induced to sporulate, and *sln1* deletion mutants (*sln1*::*LEU2*, *GAL1p*::*PTP2*) were obtained by random spore analysis, as described by Ausubel et al. (1987). The *sln1* deletion mutants were transformed with either YEp-ATHK1FL or pRS-SLN1, and their ability to grow on YPD medium (1% yeast extract, 2% polypeptone, 2% glucose [rather than galactose]) was examined.

Expression plasmids used in this experiment were constructed with a multicopy expression vector, YEpGAP, in which expression is under the control of the constitutive *GAP* promoter (Rosenberg et al., 1990). The plasmids YEp-ATHK1FL and YEp-ATHK1D1074E were constructed by insertion of BamHI fragments containing the complete open reading frame from pNV-ATHK1FL and pNV-ATHK1D1074E, respectively, into the BamHI site of YEpGAP. The *hog1* deletion mutant (*hog1*Δ) was constructed by introduction of the *hog1*::*LEU2* disruption vector pHG11 (Maeda et al., 1994) into TM141. The disruption of *hog1*::*LEU2* was confirmed by the failure of growth on YPD medium containing 0.9 M NaCl. The *sln1 sho1* deletion double mutant (*sln1*Δ *sho1*Δ) was constructed by transformation of the *sln1* deletion mutant with the *sho1*::*URA3* disruption vector pDOS82 (Maeda et al., 1995). The disruption of *sho1* by *URA3* was confirmed by PCR amplification using the oligonucleotides 5′-TCT-GGATTCTGAACTACGGCG-3′ and 5′-ATCATCGTCATCAGCGTCG-3′ corresponding to both flanking sequences at the *URA3* insertion point and the genomic DNA isolated from Ura^+ transformants. The *sln1* and *sho1* deletion mutants (*sln1*Δ *sho1*Δ) were then transformed with YEp-ATHK1FL, YEp-ATHK1D1074E, or pRS-SLN1. The *ssk1* and *sho1* deletion strain TM301 (*MAT*α, *ura3 leu2 his3 trp1 ssk1*::*HIS3 sho1*::*TRP1*) was kindly provided by T. Maeda (Cancer Institute, Tokyo, Japan) and used as a negative control.

Analysis of the Tyrosine Phosphorylation of HOG1 MAP Kinase

The yeast strains were cultured on YPD medium. When the cell density of the culture reached 10^7 cells per mL, NaCl was added and incubation was continued for various periods. The cells (in 1.5 mL of culture medium) were harvested by centrifugation, resuspended in 40 μL of SDS sample buffer, immediately boiled, and then resolved by 10% SDS-PAGE. Proteins were transferred to a polyvinylidine difluoride membrane (Immobilon-P; Millipore Ltd., Tokyo, Japan), and the blot was blocked in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Triton X-100) containing 3% BSA and then incubated with antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) in TBST buffer containing 1% BSA. The blot was washed three times with TBST buffer and then incubated with anti–mouse IgG antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in TBST buffer containing 1% BSA. After three washes with TBST buffer, the immune complexes were visualized by using the enhanced chemiluminescence detection system (Amersham International).

RNA Gel Blot Analysis

Total RNA was isolated by the phenol-SDS method described by Ausubel et al. (1987). Thirty micrograms of total RNA was fractionated on a 1% agarose gel containing formaldehyde and blotted onto a nylon membrane (Hybond-N; Amersham International). The membrane was hybridized with the ^{32}P-labeled PCR fragment in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 25 mM sodium phosphate buffer, pH 6.5, 10 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 250 μg mL^{-1} of denatured salmon sperm DNA at 42°C. The membrane was washed twice with 0.1 × SSC and 0.1% SDS at 60°C for 15 min and subjected to autoradiography.

Histochemical Analysis of GUS Activity

A genomic DNA library (Clontech, Palo Alto, CA) was screened using the full-length cDNA clone as a probe. Based on DNA gel blot analysis of the purified λ DNA digested with several enzymes, a 3.2-kb XbaI-SacI DNA fragment was subcloned, and part of it was sequenced. A DNA fragment containing 1.6 kb of the upstream region of the *ATHK1* gene was amplified by PCR by using oligonucleotides incorporating SalI or BamHI sites at the 5′ ends (5′-GAGGTCGAC-GGTATCGATAAGCT-3′ [the SalI site is underlined] and 5′-TCG-GATCCGTTTCAGATGATCCCAAATCAT-3′ [the BamHI site is underlined]). The PCR fragments were digested with SalI and BamHI and then cloned into the SalI-BamHI site of a promoterless β-glucuronidase (GUS) expression vector, pBI101.1 (Clontech). The resulting construct, *ATHK1p–GUS*, was transferred from *Escherichia coli* DH5α into *Agrobacterium tumefaciens* C58C1Rif by triparental mating with *E. coli* that contained plasmid pRK2013. Arabidopsis was transformed by vacuum infiltration with Agrobacterium that contained *ATHK1p–GUS*, as described by Bechtold et al. (1993). Histochemical localization of GUS activities in the transgenic plants was performed by incubating the transgenic unbolted plants in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2% DMSO, 0.5 mM potassium ferrocyanide, 2 mg mL^{-1} X-gluc) at 37°C for 6 to 12 hr. The stained plants were fixed in 5% formaldehyde, 5% acetic

acid, and 20% ethanol and then washed for 30 min with 50 to 100% ethanol to remove the chlorophyll.

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REFERENCES

Alex, L.A., and Simon, M.I. (1994). Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. Trends Genet. **10,** 133–138.

Alex, L.A., Borkovich, K.A., and Simon, M.I. (1996). Hyphal development in *Neurospora crassa*: Involvement of a two-component histidine kinase. Proc. Natl. Acad. Sci. USA **93,** 3416–3421.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K., eds (1987). Current Protocols in Molecular Biology. (New York: Greene Publishing Associates and Wiley Inter-Science).

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.R. Acad. Sci. Paris Ser. III Sci. Vie **316,** 1194–1199.

Brandstatter, I., and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10,** 1009–1019.

Bray, E.A. (1997). Plant responses to water deficit. Trends Plant Sci. **2,** 48–54.

Chang, C., and Meyerowitz, E.M. (1995). The ethylene hormone response in *Arabidopsis*: A eukaryotic two-component signaling system. Proc. Natl. Acad. Sci. USA **92,** 4129–4133.

Chang, C., and Stewart, R.C. (1998). The two-component system. Plant Physiol. **117,** 723–731.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). *Arabidopsis* ethylene-response gene *ETR1*: Similarity of product to two-component regulators. Science **262,** 539–544.

Hirt, H. (1997). Multiple roles of MAP kinases in plant signal transduction. Trends Plant Sci. **2,** 11–15.

Hrabak, E.M., and Willis, D.K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. J. Bacteriol. **174,** 3011–3020.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94,** 261–271.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by *Arabidopsis ERS* gene. Science **269,** 1712–1714.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). *EIN4* and *ERS2* are members of the putative ethylene receptor gene family in *Arabidopsis*. Plant Cell **10,** 1321–1332.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C., and Mizuno, T. (1998). Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95,** 2691–2696.

Ingram, J., and Bartels, D. (1996). The molecular basis of dehydration tolerance in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 377–403.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153,** 163–168.

Jackson, M. (1997). Hormones from roots as signals for the shoots of stressed plants. Trends Plant Sci. **2,** 22–28.

Jonak, C., Kiegerl, S., Lighterink, W., Barker, P.J., Huskisson, N.S., and Hirt, H. (1996). Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. Proc. Natl. Acad. Sci. USA **93,** 11274–11279.

Jonston, M. (1987). A model fungal gene regulatory mechanism, the *GAL4* genes of *Saccharomyces cerevisiae*. Microbiol. Rev. **51,** 458–476.

Kakimoto, T. (1996). CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274,** 982–985.

Kiyosue, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in *Arabidopsis thaliana* L.; identification of three ERDs as HSP cognate genes. Plant Mol. Biol. **25,** 791–798.

Kyte, L., and Doolittle, R.F. (1982). A simple model for displaying the hydropathic character of a protein. J. Mol. Biol. **157,** 105–132.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Day, M.J., Lincoln, S.E., and Newberg, L. (1987). Mapmaker: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1,** 174–181.

Lashbrook, C.C., Tieman, D.M., and Klee, H.J. (1998). Differential regulation of the tomato *ETR* gene family throughout plant development. Plant J. **15,** 243–252.

Lauter, F.-R., Ninnemann, F.-R., Bucher, M., Riesmeier, J.W., and Frommer, W.B. (1996). Preferential expression of an ammonium transporter and of two putative nitrate transporters in root hairs of tomato. Proc. Natl. Acad. Sci. USA **93,** 8139–8144.

Lister, C., and Dean, C. (1993). Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. Plant J. **4,** 745–750.

Liu, Y.G., Mitsukawa, N., Vazquez, T.A., and Whittier, R.F. (1995). Generation of a high-quality P1 library of *Arabidopsis* suitable for chromosome walking. Plant J. **7,** 351–358.

Liu, Y.G., Mitsukawa, N., Vazquez, T.A., and Whittier, R.F. (1996). Isolation and mapping of a new set of 129 RFLP markers in *Arabidopsis thaliana* using recombinant inbred lines. Plant J. **10,** 733–736.

Maeda, T., Wurgler-Murphy, S.M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369,** 242–245.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **269,** 554–558.

Miyata, S., Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Characterization of genes for two-component phosphorelay mediators with a single HPtdomain in *Arabidopsis thaliana*. FEBS Lett. **437,** 11–14.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **93,** 765–769.

Mizoguchi, T., Ichimura, K., and Shinozaki, K. (1997). Environmental stress response in plants: The role of mitogen-activated protein kinases. Trends Biotechnol. **15,** 15–19.

Mizuno, T. (1997). Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. DNA Res. **4,** 161–168.

Mizuno, T. (1998). His-Asp phosphotransfer signal transduction. J. Biochem. **123,** 555–563.

Mizuno, T., Kaneko, T., and Tabata, S. (1996). Compilation of all genes encoding bacterial two-component signal transducers in the genome of the Cyanobacterium, *Synechocystis* sp. strain PCC 6803. DNA Res. **3,** 401–414.

Muchhal, U.S., Pardo, J.M., and Raghothama, K.G. (1996). Phosphate transporters from the higher plant *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **93,** 10519–10523.

Nagasawa, S., Tokishita, S., Aiba, H., and Mizuno, T. (1992). A novel sensor-regulator protein that belongs to the homologous family of signal transduction proteins involved in adaptive responses in *Escherichia coli*. Mol. Microbiol. **6,** 799–807.

Ota, I.M., and Varshavsky, A. (1993). A yeast protein similar to bacterial two-component regulators. Science **262,** 566–569.

Parkinson, J.S. (1993). Signal transduction schemes of bacteria. Cell **73,** 857–871.

Parkinson, J.S., and Kofoid, E.C. (1992). Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26,** 71–112.

Payton, S., Fray, R.G., Brown, S., and Grierson, D. (1996). Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. Plant Mol. Biol. **31,** 1227–1231.

Posas, F., Wurgler-Murphy, S.M., Maeda, T., Witten, E.A., Thai, T.C., and Saito, H. (1996). Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1–YPD1–SSK1 "two-component" osmosensor. Cell **86,** 865–875.

Rosenberg, S., Coit, D., and Tekamp-Olson, P. (1990). Glyceraldehyde-3-phosphate dehydrogenase derived expression cassettes for constitutive synthesis of heterologous proteins. Methods Enzymol. **185,** 341–351.

Sakai, H., Hua, J., Chen, Q.G., Chang, C., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). *ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95,** 5812–5817.

Sakakibara, H., Suzuki, M., Takei, K., Deji, A., Taniguchi, M., and Sugiyama, T. (1998). A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. Plant J. **14,** 337–344.

Sambrook, K., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schuster, S.C., Noegel, A.A., Oehme, F., Gerisch, G., and Simon, M.I. (1996). The hybrid histidine kinase DokA is part of the osmotic response system of *Dictyostelium*. EMBO J. **15,** 3880–3889.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (1996). Molecular responses to drought and cold stress. Curr. Opin. Biotechnol. **7,** 161–167.

Shinozaki, K., and Yamaguchi-Shinozaki, K. (1997). Gene expression and signal transduction in water-stress response. Plant Physiol. **115,** 327–334.

Stout, V., and Gottesman, S. (1990). RcsB and RcsC: A two-component regulator of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **172,** 659–669.

Suzuki, T., Imamura, A., Ueguchi, C., and Mizuno, T. (1998). Histidine-containing phosphotransfer (HPt) signal transduction implicated in His-to-Asp phosphorelay in *Arabidopsis*. Plant Cell Physiol. **39,** 1258–1268.

Swanson, R.V., Alex, L.A., and Simon, M.I. (1994). Histidine and aspartate phosphorylation: Two-component systems and limits of homology. Trends Biochem. Sci. **19,** 485–490.

Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1998). Stress-responsive expression of genes for two-component response regulator-like proteins in *Arabidopsis thaliana*. FEBS Lett. **427,** 175–178.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*–mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

Vriezen, W.H., van Rijn, C.P.E., Voesenek, L.A.C., and Mariani, C. (1997). A homolog of the *Arabidopsis thaliana ERS* gene is actively regulated in *Rumex palustris* upon flooding. Plant J. **11,** 1265–1271.

Wang, N., Shaulsky, G., Escalante, R., and Loomis, W.F. (1996). A two-component histidine kinase gene that functions in *Dictyostelium* development. EMBO J. **15,** 3890–3898.

Wilkinson, J.Q., Lanahan, M.B., Yen, H.-C., Giovannoni, H.-C., and Klee, H.J. (1995). An ethylene-inducible component of signal transduction encoded by *Never-Ripe*. Science **270,** 1807–1809.

Wurgler-Murphy, S.M., and Saito, H. (1997). Two-component signal transduction and MAPK cascades. Trends Biochem. Sci. **22,** 172–176.