Plant Physiology, April 2000, Vol. 122, pp. 1355–1363, www.plantphysiol.org © 2000 American Society of Plant Physiologists

Calcium-Independent Activation of Salicylic Acid-Induced Protein Kinase and a 40-Kilodalton Protein Kinase by Hyperosmotic Stress[1]

Mary Elizabeth Hoyos and Shuqun Zhang*

Department of Biochemistry, University of Missouri-Columbia, 117 Schweitzer Hall, Columbia, Missouri 65211

Reversible protein phosphorylation/dephosphorylation plays important roles in signaling the plant adaptive responses to salinity/drought stresses. Two protein kinases with molecular masses of 48 and 40 kD are activated in tobacco cells exposed to NaCl. The 48-kD protein kinase was identified as SIPK (salicylic acid-induced protein kinase), a member of the tobacco MAPK (mitogen-activated protein kinase) family that is activated by various other stress stimuli. The activation of the 40-kD protein kinase is rapid and dose-dependent. Other osmolytes such as Pro and sorbitol activate these two kinases with similar kinetics. The activation of 40-kD protein kinase is specific for hyperosmotic stress, as hypotonic stress does not activate it. Therefore, this 40-kD kinase was named HOSAK (high osmotic stress-activated kinase). HOSAK is a Ca^{2+}-independent kinase and uses myelin basic protein (MBP) and histone equally well as substrates. The kinase inhibitor K252a rapidly activates HOSAK in tobacco cells, implicating a dephosphorylation mechanism for HOSAK activation. Activation of both SIPK and HOSAK by high osmotic stress is Ca^{2+} and abscisic acid (ABA) independent. Furthermore, mutation in *SOS3* locus does not affect the activation of either kinase in Arabidopsis seedlings. These results suggest that SIPK and 40-kD HOSAK are two new components in a Ca^{2+}- and ABA-independent pathway that may lead to plant adaptation to hyperosmotic stress.

Hyperosmotic stress, such as that caused by exposure of cells to high concentrations of NaCl, sorbitol, or Pro causes imbalance of the cellular ions, change of cell volume or turgor pressure, and alterations of the activity and stability of macromolecules. Different plant species employ a variety of mechanisms to cope with osmotic stress. However, the basic cellular responses appear to be conserved among all plants (Zhu et al., 1997). Some of these cellular responses, such as osmotic adjustment by synthesizing compatible osmolytes, are even shared by all organisms (Burg et al., 1996). Numerous studies have delineated cellular changes that occur upon exposure to osmotic stress in plants. A number of osmotic-responsive genes, as well as the cis-elements and transcription factors regulating their expression, have recently been described (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 1997; Bressan et al., 1998).

Expression of osmotic responsive genes is complex. Some genes respond to osmotic stress rapidly, whereas others are activated later after ABA accumulates. Therefore, both ABA-dependent and ABA-independent pathways are involved in plant adaptation to osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 1997). Cytosolic Ca^{2+} plays an important role in both pathways. For example, in a transient maize protoplast system, the constitutively active catalytic domain of Arabidopsis Ca^{2+}-dependent protein kinases induced the expression of the ABA-responsive *HVA1* promoter (Sheen, 1996). Another potential downstream target of Ca^{2+} is calcineurin (CaN), a Ca^{2+}/calmodulin-dependent PP-2B protein phosphatase composed of a catalytic and a regulatory subunit. Several calcineurin B-like genes, which may encode the regulatory subunits of CaN, have been identified in Arabidopsis recently (Kudla et al., 1999). Also, co-expression of a truncated form of the catalytic subunit and the regulatory subunit of yeast CaN in transgenic tobacco plants enhances their NaCl tolerance (Pardo et al., 1998). However, the most convincing evidence that CaN is a downstream target of Ca^{2+} in salt stress signal transduction pathways comes from the cloning of the *SOS3* gene (Liu and Zhu, 1998). *SOS3* encodes a protein that has high homology to the yeast CaN regulatory subunit and is activated by Ca^{2+}. A mutation in this locus, *sos3*, results in the hypersensitivity of Arabidopsis to NaCl and LiCl. Increased Ca^{2+} abrogated this hypersensitivity, and millimolar concentrations of Ca^{2+} suppressed the mutant phenotype (Liu and Zhu, 1997).

Genetic, molecular, and biochemical evidence demonstrated the involvement of both protein kinase (a mitogen-activated protein kinase [MAPK]-like kinase) and phosphatases (ABI1 and ABI2) in the ABA signaling pathway (Leung et al., 1994, 1997; Knetsch et al., 1996). Activation of protein kinases by hypotonic stress has also been reported in the halotolerant green alga *Dunaliella tertiolecta* (Yuasa and Muto, 1996) and in tobacco cells (Cazalé et al., 1999). In addition, transcripts of several protein kinases are induced under high-NaCl conditions and/or after application of exogenous ABA (Anderberg and Walker-Simmons, 1992; Urao et al., 1994; Hwang and Goodman, 1995; Mizoguchi et al., 1996; Lee et al., 1999; Piao et al., 1999).

In yeast, high osmolarity is sensed by two partially redundant membrane osmosensors, SHO1 and a three-component signaling protein complex SLN1/YPD1/SSK1. Three MAPK kinase kinases (MAPKKKs), SSK2, SSK22, and STE11 are downstream of the osmosensors and acti-

[1] This work was supported by a grant from the University of Missouri Research Board (to S.Z.).

* Corresponding author; e-mail zhangsh@missouri.edu; fax 573–884–4812.

vate a single MAPKK, PBS2. PBS2 in turn activates a single MAPK, HOG1, which leads to the expression of several genes involved in the biosynthesis of glycerol and tolerance of high osmolarity (Maeda et al., 1994, 1995; Posas et al., 1996; Wurgler-Murphy and Saito, 1997; Gustin et al., 1998). It is believed that a similar signaling pathway operates in plant cells (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 1997). Very recently, *ATHK1*, a transmembrane hybrid-type His kinase was identified in Arabidopsis that may function as an osmosensor (Urao et al., 1999). ATHK1 is structurally similar to the yeast osmosensor SLN1. Overexpression of *ATHK1* rescues the lethality of the temperature-sensitive, osmosensing-defective yeast mutant *sln1-ts*, suggesting AtHK1 is able to transfer the stress signal to the downstream HOG1 in yeast. However, the MAPK cascade equivalent to HOG1 in plants is yet to be identified. We demonstrate in this paper that SIPK (salicylic acid-induced protein kinase), a tobacco MAPK, is activated by osmotic stresses and could be the MAPK downstream of osmosensor in plant. In addition, we discovered a 40-kD kinase that is rapidly and strongly activated by hyperosmotic but not hypotonic stress. This kinase is named HOSAK for high-osmotic-stress-activated kinase. HOSAK activation is Ca^{2+}- and ABA-independent, and may occur through a dephosphorylation process.

MATERIALS AND METHODS

Treatment of Tobacco Cells and Arabidopsis Seedlings

Tobacco (*Nicotiana tabacum*) cell suspension cultures were maintained and treated as previously described (Zhang and Klessig, 1997). Log-phase cells were used at 3 to 4 d after a 1:10 dilution. Treatment was done in the original flasks to avoid any stresses associated with transfer. For high-osmotic treatment, the appropriate amount of solid NaCl or another osmolyte was added directly to the culture. For hypotonic stress treatment, an equal volume of Murashige and Skoog (MS) medium without Suc was added to the culture. At various times, 10-mL cells (0.2–0.3 g fresh weight) were harvested by filtration. The cells were quickly frozen in liquid nitrogen and stored at −80°C until analysis.

Arabidopsis seeds were surface-sterilized in 30% (v/v) bleach for 12 min and rinsed thoroughly with sterile distilled water. After cold treatment at 4°C overnight, the seeds were germinated and grown for 15 d under continuous light at 22°C without agitation in Petri dishes containing 30 mL of one-half-strength MS salts supplemented with 0.25% (w/v) Suc and 0.025% (w/v) MES buffer, pH 5.7. Seedlings were treated with NaCl by the addition of 5 M stock solution. Samples were taken at various times, quick-frozen in liquid nitrogen, and stored at −80°C until use.

Preparation of Protein Extracts

To prepare extracts from treated cells, cells were mixed with two volumes (w/v) of extraction buffer (100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol [DTT], 10 mM Na_3VO4, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 5 μg/mL antipain, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 10% [v/v] glycerol) and sonicated (model 550 Sonic Dismembrator, Fisher Scientific, Loughborough, Leicestershire, UK) until all of the cells were disrupted. After centrifugation at 20,000*g* for 30 min, supernatants were transferred into clean tubes, quickly frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA).

In-Gel Kinase Activity Assay

In-gel kinase activity assays were performed as described previously (Zhang and Klessig, 1997). Extracts containing 10 μg of protein were electrophoresed on 10% (w/v) SDS-polyacrylamide gels with a kinase substrate, i.e. MBP (0.25 mg/mL), histone III-SS (0.25 mg/mL), casein (0.25 mg/mL), or myosin light chain (0.25 mg/mL), imbedded in the separating gel. After electrophoresis, SDS was removed by washing the gel with washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg/mL bovine serum albumin (BSA), and 0.1% Triton X-100 [v/v]) three times, each for 30 min at room temperature. The kinases were then allowed to renature in 25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4°C overnight with three changes of buffer. The gel was then incubated at room temperature in 30 mL of reaction buffer (25 mM HEPES, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, and 0.1 mM Na_3VO4) with 200 nM ATP plus 50 μCi γ-^{32}P-ATP (3,000 Ci/mmol) for 60 min. The reaction was stopped by transferring the gel into 5% trichloroacetic acid (TCA; w/v)/1% NaPPi (w/v). The unincorporated γ-^{32}P-ATP was removed by washing in the same solution for at least 5 h with five changes. The gel was dried onto 3MM paper (Whatman, Clifton, NJ) and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Prestained size markers (Bio-Rad) were used to calculate the size of kinases.

Immuno-Complex Kinase Activity Assay

Protein extract (50 μg) was incubated with phospho-Tyr-specific monoclonal antibody 4G10 (2 μg; Upstate Biotechnology, Lake Placid, NY), SIPK-specific antibody Ab-p48N (2.5 μg; Zhang et al., 1998), or WIPK-specific antibody Ab-p44N (2.5 μg; Zhang and Klessig, 1998a, 1998b) in immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 2 μg/mL antipain, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 0.5% [v/v] Triton X-100, and 0.5% [v/v] Nonidet P-40) at 4°C for 4 h on a rocker. About 20 μL packed volume of protein A agarose was added, and the incubation was continued for another 2 h. Agarose bead-protein complexes were pelleted by brief centrifugation and washed three times with immunoprecipitation buffer and then three times with reaction buffer.

Kinase activity in the complex was determined by an in-gel kinase assay as described above.

RESULTS

Treatment of Tobacco Cells with NaCl, Sorbitol, or Pro Activates Two Protein Kinases with Molecular Masses of 48 and 40 kD

Protein kinases and phosphatases play important signaling roles in the plant's response to salinity/drought stresses. In yeast, two MAPK cascades, (SSK2,SSK22,STE11)/PBS2/HOG1 and BCK1/(MKK1,MKK2)/SLT2 are involved in transducing the signal from osmosensors to cellular responses under high- and low-osmotic stress, respectively (Wurgler-Murphy and Saito, 1997; Gustin et al., 1998). It is believed that a similar signaling pathway operates in plant cells (Shinozaki and Yamaguchi-shinozaki, 1997; Zhu et al., 1997). To investigate if any MAPK could be activated by osmotic stress in plants, we treated tobacco cell cultures with 250 mM of NaCl for various times. Protein extracts were prepared and kinase activity was determined by an in-gel kinase activity assay with myelin basic protein (MBP) as a substrate. As shown in Figure 1A, two protein kinases with molecular masses of 48 and 40 kD were activated in tobacco cells. The activation of both kinases was very rapid and transient. The activity of the p48 kinase peaked at 5 min and returned to basal level within 15 min. The activity of the p40 kinase peaked at 5 min and returned to a basal level within 2 h after treatment. The activation of the p40 kinase by NaCl is dose dependent and reaches a maximum at 250 mM NaCl (Fig. 1B). Cells treated with this concentration of NaCl plasmolyze within 10 min. However, they recover quickly upon being transferred to regular medium (data not shown). Both kinases are also activated in cells exposed to other osmolytes, including sorbitol and Pro (Fig. 1C), suggesting that the activation of these two kinases is caused by high osmotic potentials, rather than by ionic disturbances after exposure to NaCl. Exposure of cells to hypotonic stress by the addition of equal volume of MS medium without Suc only activates the p48 kinase transiently (Fig. 1D). Therefore, the p40 kinase is specifically responsive to hyperosmotic treatment, whereas the p48 kinase is responsive to both hyper- and hypoosmotic treatment. Similar activation and dose dependence of a 48- and a 40-kD protein kinase (HOSAK) were observed in Arabidopsis seedlings treated with NaCl (data not shown).

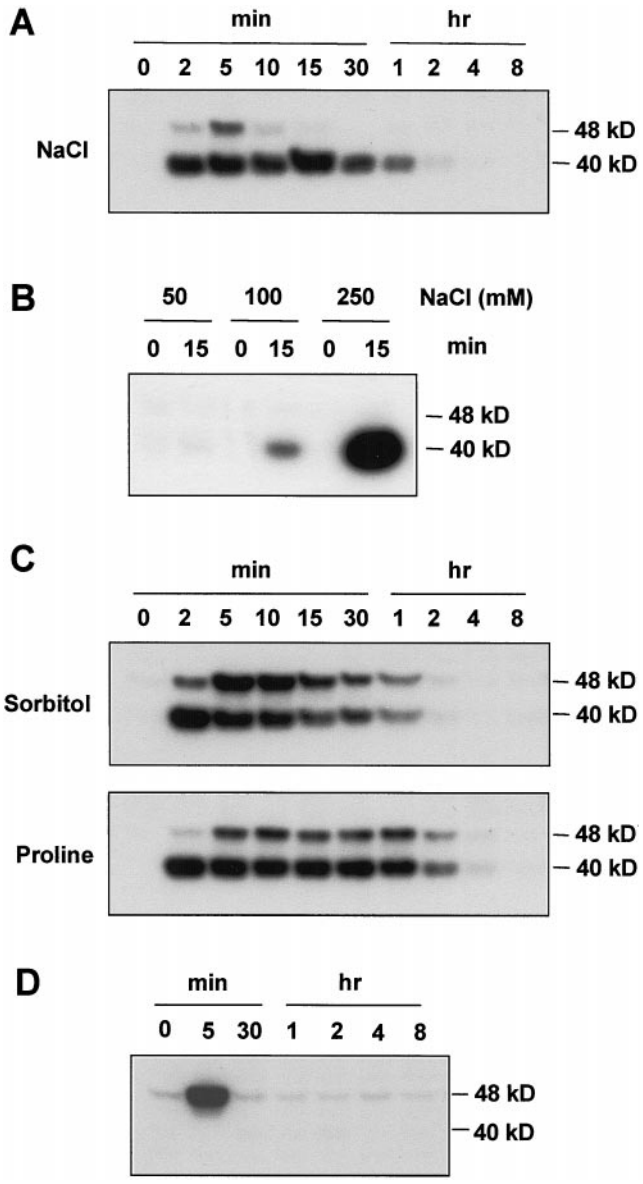

Figure 1. Salt and hyperosmotic stresses activate two kinases with molecular masses of 48 and 40 kD. A, Tobacco cells were treated with 250 mM NaCl for various times. Aliquots of cells were harvested and the kinase activities were determined by an in-gel kinase assay with MBP as a substrate. B, Cells were treated with various concentrations of NaCl. Samples were taken before (0) or 15 min after the addition of NaCl. Kinase activities were determined as in A. C, Tobacco cells were subjected to hyperosmotic stress by the addition of sorbitol (upper) or Pro (lower) to final concentration of 500 mM. Samples were taken at the indicated times and kinase activities were determined as in A. D, Tobacco cells were subjected to hypotonic stress by the addition of equal volume of MS medium without Suc. Samples were taken at indicated times, and kinase activities were analyzed for kinase activity with an in-gel kinase assay using MBP as a substrate.

The Hyperosmotic-Stress-Activated p48 Protein Kinase Is SIPK

The use of MBP as a preferred substrate suggested that the p48 kinase might be a MAPK. Another hallmark of MAPKs is their activation via dual phosphorylation of Tyr and Thr residues by MAPK kinases (Seger and Krebs, 1995). To determine if the p48 kinase is Tyr phosphorylated, extracts from treated cells were subjected to an immune complex kinase assay using the phosphotyrosine-specific monoclonal antibody 4G10. In this experiment, phosphotyrosine-containing proteins were first immunoprecipitated from protein extracts of NaCl-, sorbitol-, or Pro-treated cells, and then subjected to the in-gel kinase assay. As shown in Figure 2A, the immunoprecipitated p48

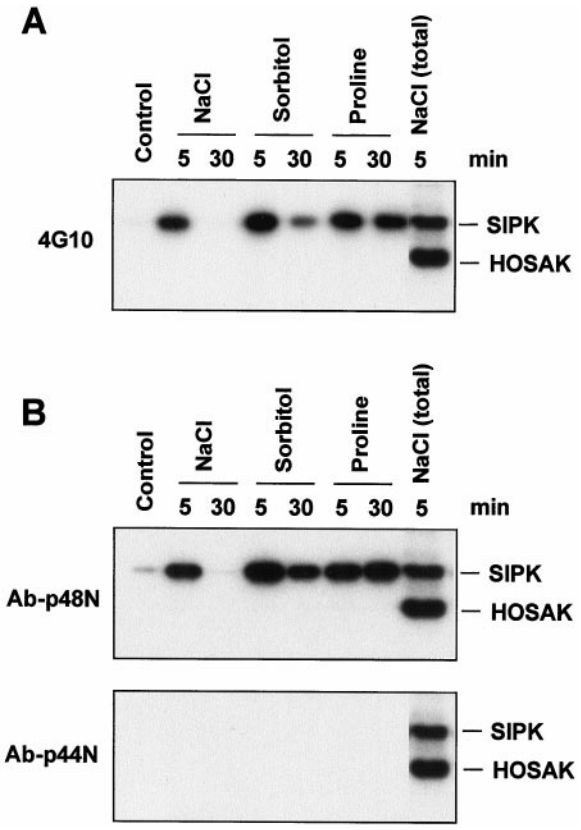

Figure 2. Hyperosmotic stress-activated p48 kinase is SIPK. A, Protein extracts (50 μg) from untreated control (0 min) or cells treated with NaCl (250 mM), sorbitol (500 mM), or Pro (500 mM) for 5 or 30 min were immunoprecipitated with 2 μg of the phospho-Tyr-specific monoclonal antibody 4G10. Kinase activity of the immune complex was subsequently determined by an in-gel kinase assay with MBP as a substrate. B, Protein extracts (50 μg) from the same set of samples as in A were immunoprecipitated with 2.5 μg of SIPK-specific antibody Ab-p48N or WIPK-specific antibody Ab-p44N. Kinase activity of the immune complex was subsequently determined by an in-gel kinase assay with MBP as a substrate.

kinase activity correlated with the p48 kinase activity in the cell extracts both qualitatively and quantitatively (Fig. 1, A and C). These results demonstrated that the p48 kinase is Tyr phosphorylated upon activation, suggesting that it is a MAPK. In contrast, the p40 HOSAK cannot be immunoprecipitated by 4G10, even though an excess amount of the antibody was used. This result suggests that the activated form of HOSAK does not contain phospho-Tyr.

SIPK, a 48-kD MAPK that is activated in tobacco under a variety of biotic and abiotic stresses, may play a role in multiple signal transduction pathways (Hoyos-Rendón, 1998; Romeis et al., 1999; Zhang and Klessig, 2000). To examine if the hyperosmotic-stress-activated p48 kinase is encoded by *SIPK*, we employed the SIPK-specific peptide antibody Ab-p48N in an immune complex kinase assay (Zhang et al., 1998). Protein extracts from tobacco cells treated with NaCl, Pro, or sorbitol were immunoprecipitated with Ab-p48N. The precipitated protein was subjected to an in-gel kinase assay with MBP as a substrate. As shown in Figure 2B, the p48 kinase activated by all three treatments can be recognized by the SIPK-specific antibody, demonstrating that the p48 hyperosmotic-stress-activated protein kinase is indeed SIPK. Using the same assay, we found that the 48-kD hypoosmotic-stress-activated kinase contains SIPK as well (data not shown). The p40 HOSAK cannot be precipitated by Ab-p48N. In addition, Ab-p44N, a WIPK-specific antibody, failed to immunoprecipitate either kinase (Fig. 2B).

HOSAK Is a Ca^{2+}-Independent Kinase and Prefers Basic Proteins as Substrates

The failure of an excess amount of 4G10 to immunoprecipitate the p40 HOSAK suggests that it is not a MAPK. Histone III-SS is a poor substrate for the p48 SIPK (Fig. 3A; Zhang and Klessig, 1997). In contrast, the p40 HOSAK phosphorylates histone III-SS and MBP equally well (Fig. 3A). Since histone III-SS is a preferred substrate for Ca^{2+}-dependent protein kinases, we examined the effect of Ca^{2+} on HOSAK activity. As shown in Figure 3B, the addition of Ca^{2+} plus omitting EGTA from the standard kinase reaction buffer did not enhance the p40 HOSAK activity, demonstrating that HOSAK is not a Ca^{2+}-dependent protein kinase. In contrast, several additional kinases with molecular mass in the range of 50 to 75 kD show up in the presence of Ca^{2+} (compare Fig. 3B versus Fig. 3A, top). They are likely to be different Ca^{2+}-dependent protein kinases that can phosphorylate both histone and MBP. Neither kinase phosphorylates the other commonly used artificial substrates, casein and myosin light chain (Fig. 3A).

The Protein Kinase Inhibitor K252a Also Activates p40 HOSAK

Detection of high-osmotic-stress-induced HOSAK activation by the in-gel kinase assay indicates that either the protein level is induced or the protein is covalently modified post-translationally upon stimulation. The increase of HOSAK activity from undetectable basal level to maximal level within 2 min after treatment favors the second possibility, suggesting that the activation is through a covalent modification, possibly phosphorylation or dephosphorylation. Treatment of tobacco cells with the protein kinase inhibitor K252a strongly activates the p40 kinase with a similar kinetics, whereas staurosporine only causes a very weak activation of the p40 kinase (Fig. 4A). The p40 kinase activated by K252a has the same substrate preference (Fig. 4B), suggesting that it is HOSAK. These results suggest that HOSAK may be maintained in an inactive state by a K252a-sensitive kinase in unstimulated cells. Upon exposure to high osmotic stress, either the HOSAK-inactivating kinase is inhibited or a protein phosphatase is activated, which will lead to the accumulation of the active dephosphorylated form of HOSAK in the cells. Pretreatment of tobacco cells with the Ser/Thr protein phosphatase inhibitors calyculin A and okadaic acid or the protein Tyr phosphatase inhibitor sodium orthovanadate did not abolish the salt-induced activation of HOSAK (Fig. 5). These results suggest the presence of a HOSAK-inactivating kinase. The other possible explanation is that the dephosphorylation

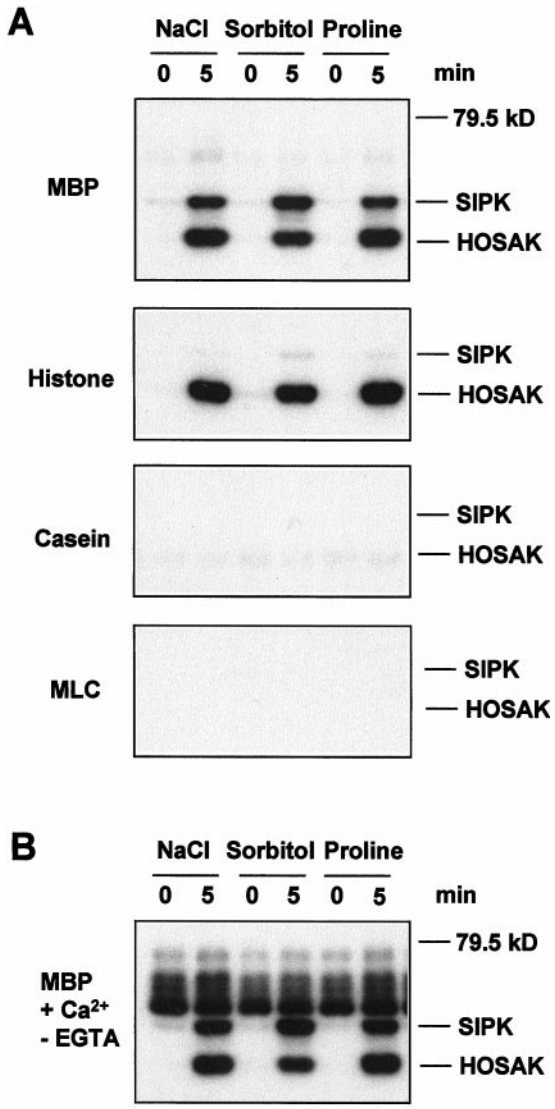

Figure 3. HOSAK preferentially phosphorylates MBP and histone and is Ca^{2+} independent. A, Kinase activities in protein extracts from control cells (0 min) or cells treated with NaCl (250 mM), sorbitol (500 mM), or Pro (500 mM) for 5 min were analyzed by an in-gel kinase assay with MBP, histone III-SS, casein, or myosin light chain as a substrate. Two millimolar EGTA and no Ca^{2+} were added in the reaction buffer. B, Kinase activities in the same protein extracts were determined in the presence of 100 μM Ca^{2+} and no EGTA with MBP as a substrate.

activation of HOSAK is mediated by a specific phosphatase that is not sensitive to these inhibitors.

Activation of HOSAK Is *SOS3*-, Ca^{2+}-, and ABA-Independent

Arabidopsis SOS3 encodes a protein with regions homologous to the EF hand Ca^{2+}-binding domain and has the highest homology with the regulatory subunit of CaN (Liu and Zhu, 1998). Mutation in this locus results in the hypersensitivity of Arabidopsis to NaCl and LiCl (Liu and Zhu, 1997). To investigate if SOS3 plays a role in the activation of the Arabidopsis p48 and p40 kinases, we examined the kinase activation in *sos3* mutant under salt stress. As shown in Figure 6, exposure of Arabidopsis wild-type plants to NaCl activates two protein kinases with the same molecular masses as those activated in tobacco. These two kinases should correspond to the p48 SIPK and p40 HOSAK in tobacco. The Arabidopsis p48 kinase may be encoded by *AtMPK6*, an Arabidopsis MAPK that shares 89% identity in its amino acid sequence with SIPK. In a phylogenetic tree constructed using all cloned plant MAPKs, AtMPK6 from Arabidopsis falls into the same group as SIPK (Zhang and Klessig, 1997). The activation of neither the p48 kinase nor the p40 kinase is affected in the *sos3* mutant seedlings (Fig.

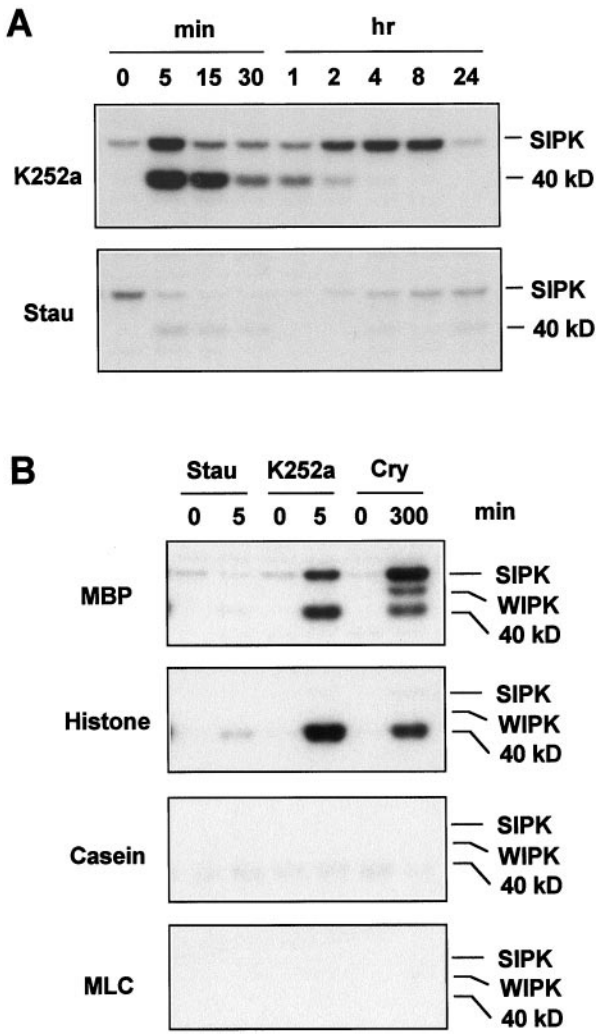

Figure 4. Treatment of tobacco cells with the kinase inhibitor K252a activates p40 HOSAK. A, Tobacco cells were treated with 0.5 μM of K252a or staurosporine (Stau) for various times. Kinase activity was determined by an in-gel kinase assay with MBP as a substrate. B, Substrate preference of the p40 kinase activated by K252a and cryptogein (Cry). Protein extracts from tobacco cells treated with 0.5 μM staurosporine, 0.5 μM K252a, or 25 nM cryptogein for either 5 min or 5 h were analyzed for kinase activities with an in-gel kinase assay using different substrates: MBP, histone III-SS, casein, or myosin light chain (MLC).

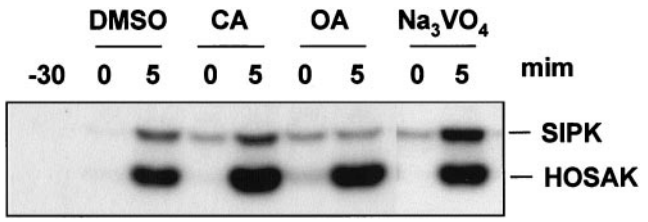

Figure 5. Inhibitors of protein phosphatase cannot block HOSAK activation by NaCl. Tobacco cells were pretreated with either solvent control (DMSO) or different phosphatase inhibitors: calyculin A (CA, 150 nM), okadaic acid (OA, 1 μM), or sodium orthovanadate (Na_3VO_4, 1 mM) for 30 min, and then treated with 250 mM NaCl. Samples were taken before (0 min) or 5 min after the addition of salt. Kinase activities in protein extracts were analyzed by an in-gel kinase assay with MBP as a substrate.

6). These results demonstrated that the activation of SIPK and HOSAK is independent of the *SOS3* pathway.

Cytosolic Ca^{2+} plays important roles in both ABA-dependent and ABA-independent pathways in response to drought and salt stresses (Knight et al., 1997, 1998; Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 1997; Sanders et al., 1999). Besides exerting its function through SOS3, Ca^{2+} is also involved in other signaling pathways in plant responses to salinity/drought (Zhu et al., 1997; Bressan et al., 1998; Sanders et al., 1999). To investigate if Ca^{2+} is required for SIPK and/or HOSAK activation by osmotic stress, a Ca^{2+} chelator and a Ca^{2+} channel blocker were employed. Tobacco cells were pretreated with various concentrations of EGTA or $LaCl_3$ for different times, and subsequently treated with NaCl. None of these pretreatments affected the activation of HOSAK (Fig. 7, only results from one concentration with a pretreatment of 2 h is shown). Cyclosporin A is an inhibitor of CaN phosphatase in vivo after binding with cyclophilin (Luan et al., 1993). Pretreatment of cells with cyclosporin A alone or together with EGTA or $LaCl_3$ did not suppress the activation of either SIPK or HOSAK, suggesting that CaN is not involved in the activation of either kinase (Fig. 7). As a result, we conclude that both SIPK and p40 HOSAK may function in a Ca^{2+}-independent pathway during plant response to salt and high osmotic stresses.

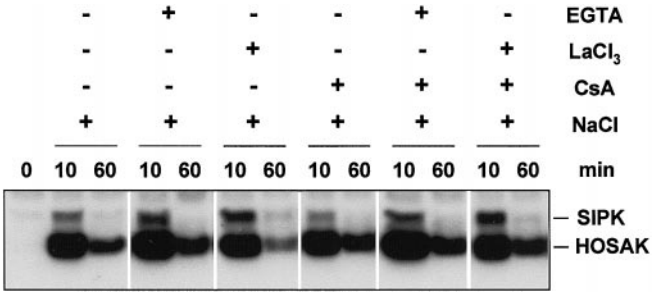

Figure 7. Activation of both SIPK and the p40 HOSAK by high salinity is Ca^{2+} independent. Tobacco cells were pretreated for 2 h with 20 mM EGTA, 5 mM $LaCl_3$, or 20 μM cyclosporin A (CsA) alone or in combination with EGTA or $LaCl_3$ as shown in the figure. −, Absent; +, present. Samples were taken at 10 min and 1 h after the addition of NaCl. Protein extracts were analyzed for kinase activity by an in-gel kinase assay using MBP as a substrate.

Plant hormone ABA is one of the most important signaling molecules in plant responses to water deficit, including stresses caused by drought, high osmolarity, salinity, freezing, and chilling (Bray, 1997). ABA levels increase in plants under these stresses, which then leads to the expression of ABA-responsive genes. There are also responses that are induced by osmotic stress through an ABA-independent pathway (Skriver and Mundy, 1990; Bray 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). The very rapid activation of SIPK and HOSAK by high osmolarity suggests that ABA is not involved. In agreement with this assumption, no elevation of kinase activity was detected after tobacco cells were treated with either 20 or 100 μM ABA, supporting the hypothesis that HOSAK activation is ABA independent (Fig. 8). ABA-induced MAPK activation reported by Knetsch et al. (1996) could be a cell-type-specific response that occurs in barley aleurone protoplasts (Heimovaara-Dijkstra et al., 2000). Currently, whether these two kinases

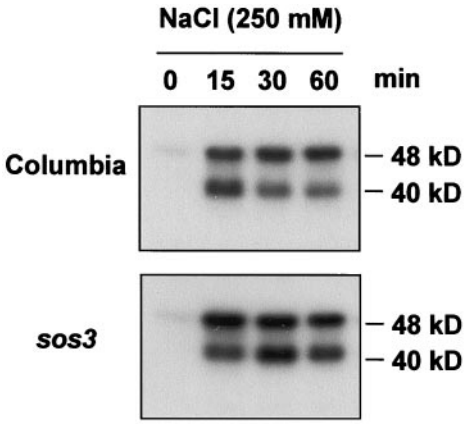

Figure 6. Activation of the p48 and p40 kinases in Arabidopsis seedlings from both wild-type and *sos3* mutant. Arabidopsis seedlings (15 d old) grown in one-half-strength MS medium supplemented with MES and Suc were treated with 250 mM NaCl for various times. Protein extracts (15 μg) were analyzed for kinase activity with an in-gel kinase assay using MBP as a substrate.

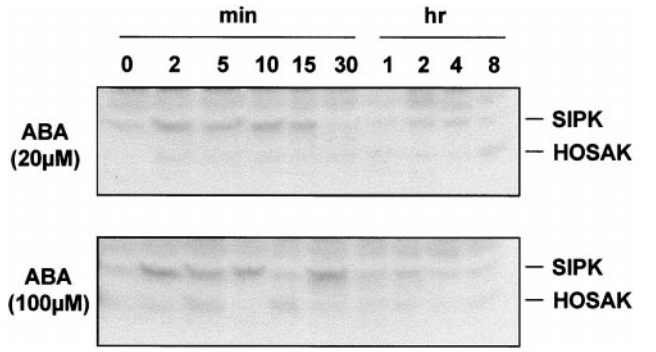

Figure 8. Treatment of tobacco cells with exogenous ABA does not activate SIPK or p40 HOSAK. Tobacco cells were treated with either 20 or 100 μM ABA for various times. Kinase activity in total protein extracts was analyzed by an in-gel kinase assay with MBP as a substrate.

are upstream of ABA and involved in regulating ABA biosynthesis in response to osmotic stress is unknown.

DISCUSSION

Phosphorylation and dephosphorylation play important signaling roles in the adaptation to osmotic stress in animals, yeast, and plants (Anderberg and Walker-Simmons, 1992; Giraudat et al., 1994; Maeda et al., 1995; Burg et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Wurgler-Murphy and Saito, 1997; Zhu et al., 1997). In the present study, we demonstrated that SIPK and a 40-kD protein kinase are rapidly activated by hyperosmotic stress in tobacco suspension cells in a dose-dependent manner (Fig. 1). The p40 kinase is activated only in cells under high osmotic stress, whereas SIPK is activated in cells under both high and low osmotic/hypotonic stresses. Two kinases with the same molecular masses as p48 SIPK and p40 HOSAK are also activated in Arabidopsis seedlings with similar kinetics (Fig. 6). The magnitude and kinetics of HOSAK activation in cells exposed to high salt (e.g. NaCl) and a high concentration of osmolytes (e.g. Pro or sorbitol) are very similar, suggesting that HOSAK is indeed activated in response to an osmotic stress rather than to the ionic disturbance caused by NaCl.

Transcripts of genes in MAPK cascade, *AtMPK3* (a MAPK) and *AtMEKK1* (a MAPKKK) are induced by salt stress in Arabidopsis (Mizoguchi et al., 1996). However, the changes of kinase activities corresponding to these genes have yet to be demonstrated. *MMK4*, the *AtMPK3* ortholog in alfalfa does not respond to salt treatment (Jonak et al., 1996). We demonstrated here that SIPK, a MAPK that belongs to a different subgroup than AtMPK3/MMK4, is activated by salt and osmotic stresses in tobacco cells. In yeast, two partially redundant membrane osmosensors, SHO1 and SLN1, sense the high osmolarity and transduce the signal into cellular responses through a MAPK cascade (Maeda et al., 1994; Posas et al., 1996; Gustin et al., 1998). It is believed that a similar signaling pathway operates in plant cells (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu et al., 1997). Very recently, *ATHK1*, a transmembrane hybrid-type His kinase that may function as an osmosensor, was identified in Arabidopsis (Urao et al., 1999). Is SIPK the equivalent of HOG1 in tobacco that functions downstream of the putative osmosensor? In yeast, the HOG1 MAPK cascade specifically responds to high osmotic stress, whereas in mammalian cells, SAPK/JNK and p38 are activated by various stress stimuli including osmotic stress (Kyriakis and Avruch, 1996; Bode et al., 1999). In plants, the activation of SIPK in response to osmotic stress is more similar to the activation of MAPKs in mammalian cells. It is unlikely that the SIPK activation can define the cellular response to hyperosmotic stress, as hypotonic stress also activates SIPK with a similar kinetics. On the other hand, SIPK activation could be an integral part of the response to hyperosmotic stress, which, in combination with the p40 HOSAK, defines a Ca^{2+}- and ABA-independent signaling pathway.

The p40 HOSAK is activated in cells exposed to high osmolarity, but not hypotonic stress, suggesting that HOSAK may be more important in defining the hyperosmotic-stress-specific responses. Is p40 HOSAK a member of the MAPK family? Although its size and the use of MBP as a substrate suggest that it might be a MAPK. There are also several pieces of evidence that argue against it. Although HOSAK phosphorylates MBP, it uses histone as a substrate equally well if not better. However, histone is not a good substrate for known MAPKs, including SIPK (Fig. 3A). More importantly, no Tyr phosphorylation is associated with HOSAK activation as determined by immune complex kinase assay using phospho-Tyr-specific antibody 4G10 (Fig. 2A). In addition, the activation of HOSAK appears to be associated with dephosphorylation rather than phosphorylation.

Rapid activation of HOSAK by the kinase inhibitor K252a suggests that the p40 HOSAK may be activated through a dephosphorylation event by a protein phosphatase. However, treatment of cell extract with several protein phosphatases including protein phosphatase-1, protein Tyr phosphatase, alkaline phosphatase, or acid phosphatase failed to activate p40 HOSAK in vitro (data not shown). In addition, pretreatment of tobacco cells with different phosphatase inhibitors did not affect HOSAK activation by NaCl (Fig. 5). One possible interpretation for these data is that HOSAK activation is mediated by a specific phosphatase. An alternative explanation is that HOSAK has multiple phosphorylation sites and its activation requires the dephosphorylation of a particular residue, while other sites remain phosphorylated, which is similar to the regulation of cyclin-dependent kinases (CDKs) and glycogen synthase kinase-3 (GSK3)-type kinases (Cook et al., 1996; Hardie, 1999; Mironov et al., 1999). Besides, the interaction with cyclin, activity of CDKs requires the phosphorylation at a conserved Thr residue in the "T loop" catalyzed by CDK-activating kinases, and in the meantime the dephosphorylation of a Tyr residue in the conserved kinase domain by a specific Tyr phosphatase, CDC25 (Hardie, 1999; Mironov et al., 1999). The activity of GSK3 is differentially controlled by phosphorylation/dephosphorylation at different residues. Phosphorylation of Ser at the N terminus causes inactivation of GSK3, which can be reversed by protein phosphatase 2A, whereas phosphorylation of Tyr residue results in an increase in GSK3 activity, which is necessary for its biological function (Wang et al., 1994; Plyte et al., 1996; Welsh et al., 1996; Hardie, 1999).

Kinase activities of the same M_r as p40 HOSAK have been demonstrated in halophytic alga *Dunaliella tertiolecta* exposed to both hypo- and hyperosmotic stresses (Yuasa and Muto, 1996). In *D. tertiolecta*, the 40-kD kinase responsive to hyperosmotic stress phosphorylates casein and histone, but not MBP. In contrast, HOSAK in tobacco phosphorylates histone and MBP equally well, but not casein. The difference in substrate preference suggests that different kinases are activated by hyperosmotic stress in algae and higher plants. It has been previously reported that a 40-kD kinase is activated in tobacco cells treated with paracitisein or cryptogein, two different fungal elicitins (Zhang et al., 1998). This 40-kD kinase has the same substrate preference as HOSAK (Fig. 4B), suggesting that they may be the same kinase. During the hypersensitive re-

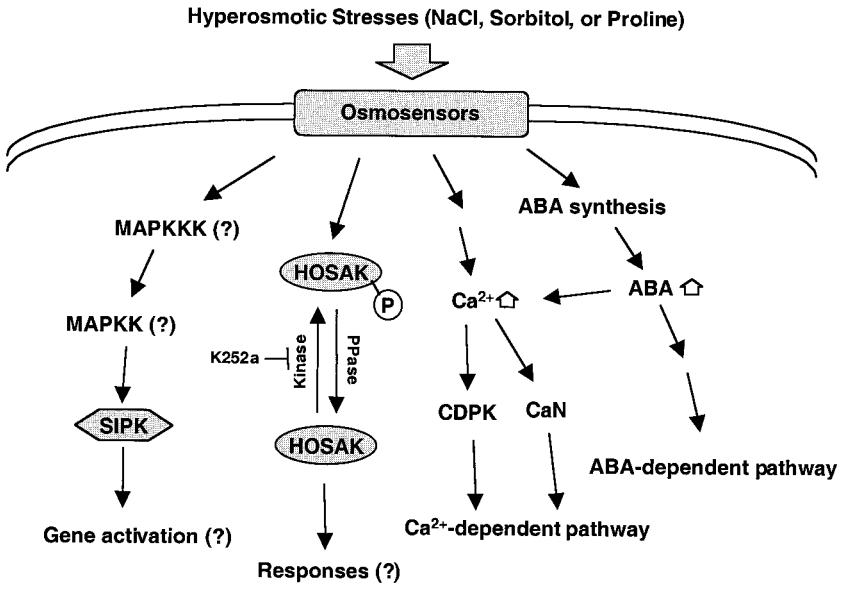

Figure 9. SIPK and p40 HOSAK are two new components in the Ca^{2+}- and ABA-independent signaling pathway that may function in a plant's response to high osmotic stresses.

sponse to pathogens or pathogen elicitors, plant cells undergo cytoplasmic condensation and loss of turgor pressure that are similar to plasmolysis of cells under high osmotic stress. Such a process may be sensed by cells as osmotic stress, and leads to the activation of the p40 HOSAK.

The exact roles of SIPK and HOSAK in the plant's response to high osmolarity remain to be defined. Based on our results, a working model was proposed (Fig. 9). In this model, SIPK and p40 HOSAK represent two new components in the Ca^{2+}- and ABA-independent signaling pathway in plants under hyperosmotic stresses. There are at least four different pathways for the signaling of drought and salt stresses, two of which are ABA dependent and two ABA independent (Shinozaki and Yamaguchi-Shinozaki, 1997). Both SIPK and HOSAK are rapidly activated before or concurrently with the increase of Ca^{2+} and ABA induced by salt or hyperosmotic stress. It is possible that one or both of these two kinases are upstream in the Ca^{2+}- and/or ABA-dependent pathways, although the activation of these two kinases is Ca^{2+} and ABA independent. Ca^{2+}-independent phosphorylation has been implicated in the transient elevation of Ca^{2+} during osmotic stress (Takahashi et al., 1997; Cessna et al., 1998). This scenario will put one or both kinases back into the ABA- and/or Ca^{2+}-dependent pathways. Currently, the purification of HOSAK is in progress. Cloning of the *HOSAK* gene based on the partial amino acid sequences will enable the study of its in vivo function in plants under osmotic stress.

ACKNOWLEDGMENTS

We thank Dr. Jian-Kang Zhu (University of Arizona, Tucson) for Arabidopsis sos mutant seeds and Drs. Douglas Randall and John Walker for critical reading of the manuscript.

Received October 29, 1999; accepted January 4, 2000.

LITERATURE CITED

Anderberg RJ, Walker-Simmons MK (1992) Isolation of a wheat cDNA clone for an abscisic acid-inducible transcript with homology to protein kinases. Proc Natl Acad Sci USA **8:** 10183–10187

Bode JG, Gatsios P, Ludwig S, Rapp UR, Häussinger D (1999) The mitogen-activated protein (MAP) kinase p38 and its upstream activator MAP kinase 6 are involved in the activation of signal transducer and activator of transcription by hyperosmolarity. J Biol Chem **274:** 30222–30227

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci **2:** 48–54

Bressan RA, Hasegawa PM, Pardo JM (1998) Plant use calcium to resolve salt stress. Trends Plant Sci **3:** 411–412

Burg MB, Known ED, Kültz D (1996) Osmotic regulation of gene expression. FASEB J **10:** 1598–1606

Cazalé A-C, Droillard M-J, Wilson C, Heberle-Bors E, Barbier-Brygoo H, Laurière C (1999) MAP kinase activation by hypoosmotic stress of tobacco cell suspensions: towards the oxidative burst response? Plant J **19:** 297–307

Cessna SG, Chandra S, Low PS (1998) Hypo-osmotic shock of tobacco cells stimulates Ca^{2+} fluxes deriving first from external and then internal Ca^{2+} stores. J Biol Chem **273:** 27286–27291

Cook D, Fry MJ, Hughes K, Sumathipala R, Woodgett JR, Dale TC (1996) Wingless inactivates glycogen synthase kinase 3 via and intracellular signaling pathway which involves a protein kinase C. EMBO J **15:** 4526–4536

Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J, Morris P-C, Bouvier-Durand M, Vartanian N (1994) Current advances in abscisic acid action and signaling. Plant Mol Biol **26:** 1557–1577

Gustin MC, Albertyn J, Alexander M, Davenport K (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev **62:** 1264–1300

Hardie DG (1999) Plant protein serine/threonine kinases: classification and functions. Annu Rev Plant Physiol Plant Mol Biol **50:** 97–131

Heimovaara-Dijkstra S, Testerink C, Wang M (2000) Mitogen-activated protein kinase and abscisic acid signal transduction. *In* H Hirt ed, MAP Kinases in Plant Signal Transduction. Springer-Verlag, Heidelberg, pp 131–144

Hoyos-Rendón ME (1998) Molecular biology of bacterial HR in tobacco plants. PhD thesis. University of Missouri, Columbia

Hwang IW, Goodman HM (1995) An *Arabidopsis thaliana* root-specific kinase homolog is induced by dehydration, ABA, and NaCl. Plant J **8:** 37–43

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, 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

Knetsch MLW, Wang M, Snaar-Jagalska BE, Heimovaara-Dijkstra S (1996) Abscisic acid induces mitogen-activated protein kinase activation in barley aleurone protoplasts. Plant Cell **8:** 1061–1067

Knight H, Brand S, Knight MR (1998) A history of stress alters drought calcium signaling pathways in *Arabidopsis*. Plant J **16:** 681–687

Knight H, Trewavas AJ, Knight MR (1997) Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. Plant J **12:** 1067–1078

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA **96:** 4718–4723

Kyriakis JM, Avruch J (1996) Protein kinase cascades activated by stress and inflammatory cytokines. BioEssays **18:** 567–577

Lee JH, Van Montagu M, Verbruggen N (1999) A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells. Proc Natl Acad Sci USA **96:** 5873–5877

Leung J, Bouvier-Durand M, Moris PC, Guerrier D, Chefdor F, Giraudat J (1994) *Arabidopsis* ABA response gene *ABI1*: features of a calcium-modulated protein phosphatase. Science **264:** 1448–1452

Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2)* and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9:** 759–771

Liu J, Zhu JK (1997) An *Arabidopsis* mutant that requires increased calcium for potassium nutrition and salt tolerance. Proc Natl Acad Sci USA **94:** 14960–14964

Liu J, Zhu JK (1998) A calcineurin sensor homolog required for plant salt tolerance. Science **280:** 1943–1945

Luan S, Li W, Rusnak F, Assmann SM, Schreiber SL (1993) Immunosuppressants implicate protein phosphatase regulation of K^+ channels in guard cells. Proc Natl Acad Sci USA **90:** 2202–2206

Maeda T, Takekawa M, Saito H (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **276:** 2054–2057

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

Mironov V, De Veylder L, Van Montagu M, Inzé D (1999) Cyclin-dependent kinases and cell division in plants: the nexus. Plant Cell **11:** 509–521

Mizoguchi T, Irie K, Hirayama T, Hayashida N, Yamaguchi-Shinozaki K, Matsumoto K, Shinozaki K (1996) A gene encoding a mitogen-activated protein 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

Pardo JM, Reddy MP, Yang S, Maggio A, Huh G-H, Matsumoto T, Coca MA, Paino-D'Urzo M, Koiwa H, Yun D-J, Watad A.A, Bressan RA, Hasegawa PM (1998) Stress signaling through Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. Proc Natl Acad Sci USA **95:** 9861–9686

Piao HL, Pih KT, Lim JH, Kang SG, Jin JB, Kim SH, Huang I (1999) An Arabidopsis *GSK3/shaggy*-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. Plant Physiol **119:** 1527–1534

Plyte SE, Feoktistova A, Burke JD, Woodgett JR, Gould KL (1996) *Schiizosaccharomyces pombe* SKP1(+) encodes a protein kinase related to mammalian glycogen synthase kinase 3 and complements a cdc 14 cytokinesis mutant. Mol and Cell Biol **16:** 179–191

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD-SSK1 "two-component" osmosensor. Cell **86:** 865–875

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones J (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence in resistance gene, elicitor, wound and salicylate responses. Plant Cell **11:** 273–297

Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell **11:** 691–706

Seger R, Krebs EG (1995) The MAPK signaling cascade. FASEB J **9:** 726–735

Sheen J (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. Science **274:** 1900–1902

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

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503–512

Takahashi K, Isobe M, Knight MR, Trewavas AJ, Muto S (1997) Hypo-osmotic shock induces increases in cytosolic Ca^{2+} in tobacco suspension culture cells. Plant Physiol **113:** 587–594

Urao T, Takeshi K, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K (1994) Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high-salt stresses in *Arabidopsis thaliana*. Mol Gen Genetics **244:** 331–340

Urao T, Yakubo B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K (1999) A transmembrane hybrid-type histidine kinase in Arabidopsis function as an osmosensor. Plant Cell **11:** 1743–1754

Wang QM, Fiol CJ, DePaoli-Roach AA, Roach PJ (1994) Glycogen synthase kinase-3B is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J Biol Chem **269:** 14566–14574

Welsh CI, Wilson C, Proud CG (1996) GSK3: a SHAGGY frog story. Trends Cell Biol **6:** 274–279

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

Yuasa T, Muto S (1996) Activation of 40-kDa protein kinases in response to hypo- and hyperosmotic shock in the halotolerant green alga *Dunaliella tertiolecta.* Plant Cell Physiol **37:** 36–42

Zhang S, Du H, Klessig DF (1998) Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophtora* spp. Plant Cell **10:** 435–449

Zhang S, Klessig DF (1997) Salicylic acid activates a 48 kD MAP kinase in tobacco. Plant Cell **9:** 809–824

Zhang S, Klessig DF (1998a) The tobacco wounding-activated MAP kinase is encoded by SIPK. Proc Natl Acad Sci USA **95:** 7225–7230

Zhang S, Klessig DF (1998b) N resistance gene-mediated de novo synthesis and activation of a tobacco MAP kinase by TMV infection. Proc Natl Acad Sci USA **95:** 7433–7438

Zhang S, Klessig DF (2000) Pathogen-induced MAP kinases in tobacco. *In* H Hirt ed, MAP Kinases in Plant Signal Transduction. Springer-Verlag, Heidelberg, pp 65–84

Zhu J-K, Hasegawa PM, Bressan RA (1997) Molecular aspects of osmotic stress in plants. Crit Rev Plant Sci **16:** 253–277