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Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress

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Summary

Plant growth is severely affected by hyper-osmotic salt conditions. Although a number of salt-induced genes have been isolated, the sensing and signal transduction of salt stress is little understood. We provide evidence that alfalfa cells have two osmo-sensing protein kinase pathways that are able to distinguish between moderate and extreme hyper-osmotic conditions. A 46 kDa protein kinase was found to be activated by elevated salt concentrations (above 125 mM NaCl). In contrast, at high salt concentrations (above 750 mM NaCl), a 38 kDa protein kinase, but not the 46 kDa kinase, became activated. By biochemical and immunological analysis, the 46 kDa kinase was identified as SIMK, a member of the family of MAPKs (mitogen-activated protein kinases). SIMK is not only activated by NaCl, but also by KCl and sorbitol, indicating that the SIMK pathway is involved in mediating general hyper-osmotic conditions. Salt stress induces rapid but transient activation of SIMK, showing maximal activity between 8 and 16 min before slow inactivation. When inactive, most mammalian and yeast MAPKs are cytoplasmic but undergo nuclear translocation upon activation. By contrast, SIMK was found to be a constitutively nuclear protein and the activity of the kinase was not correlated with changes in its intracellular compartmentation, suggesting an intra-nuclear mechanism for the regulation of SIMK activity.

Introduction

Soil salinity has become a major problem in many areas of agriculture, leading to substantial losses in crop yield. The growing need to understand the basis of salt tolerance of certain plant species and varieties has resulted in the cloning of a number of salt-induced genes and their expression in plants (for reviews, see Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). Despite considerable progress in this field, little is known about the basic mechanism of the perception and signal transduction of hyper-osmotic stress in higher plants.

Hyper-osmotic stress signalling in yeast and in mammalian cells is mediated through highly conserved MAP (mitogen-activated protein) kinase cascades (Brewster *et al.*, 1993; Galcheva-Gargova *et al.*, 1994; Han *et al.*, 1994). MAP kinase pathways are found in all eukaryotes and are involved in transducing a variety of extracellular signals including growth factors, UV radiation and osmotic stress (for review, see Waskiewicz and Cooper, 1995). MAPK cascades are usually composed of three protein kinases that upon activation undergo sequential phosphorylation (Robinson and Cobb, 1997). By phosphorylation of conserved threonine and tyrosine residues, a MAPK becomes activated by a specific MAPK kinase (MAPKK). A MAPKK kinase (MAPKKK) activates MAPKK through phosphorylation of conserved threonine and/or serine residues. MAPK pathways may integrate a variety of upstream signals through interaction with other kinases or G proteins (Robinson and Cobb, 1997). The latter factors often directly serve as coupling agent between a plasma membrane-located receptor protein that senses an extracellular stimulus and a cytoplasmic MAPK module. At the downstream end of the module, activation of the cytoplasmic MAPK module often induces translocation of the MAPK into the nucleus where the kinase activates certain sets of genes through phosphorylation of specific transcription factors (Treisman, 1996). In other cases, a given MAPK may translocate to other sites in the cytoplasm to phosphorylate specific enzymes (protein kinases, phosphatases, lipases, etc.) or cytoskeletal components (Cohen, 1997; Robinson and Cobb, 1997). By tight regulation of MAPK localization and through expression of certain signalling components and substrates in particular cells, tissues or organs, particular MAPK pathways can mediate signalling of a multitude of extracellular stimuli and bring about a large variety of specific responses.

We have investigated whether hyper-osmotic stress in plants is also mediated by MAP kinase pathways. We have found that a 46 kDa MAP kinase is activated by hyper-osmotic stress in alfalfa cells. Interestingly, extreme hyper-

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osmotic stress activates an as yet unidentified 38 kDa kinase, but not the 46 kDa MAP kinase. The 46 kDa MAP kinase is denoted as SIMK (salt stress-inducible MAP kinase) (originally named MsK7, Jonak *et al.*, 1993). SIMK was found to be a constitutively nuclear protein and did not undergo changes in its intracellular location upon salt stress activation. Our results indicate that yeast, animals and plants use a highly conserved mechanism to convey hyper-osmotic stress signals, providing a basis for investigation of hyper-osmotic stress signalling in plants at the molecular level.

Results

Distinct protein kinase pathways are activated upon moderate and extreme salt stress

To investigate whether plants use MAP kinase cascades for mediating stress signalling, suspension-cultured alfalfa cells were exposed to different concentrations of NaCl. After 15 min, protein extracts of these cells were prepared and analysed by in-gel kinase assays with myelin basic protein (MBP) as substrate (Figure 1). Exposure to more than 125 mM NaCl activated a 46 kDa protein kinase. When cells were exposed to 750 mM, the 46 kDa kinase was activated to a lesser extent than at lower salt concentrations (Figure 1). At 1 M NaCl, the 46 kDa protein kinase was not activated at all, but instead a 38 kDa protein kinase became strongly induced (Figure 1). These results show that cells activate different kinases when exposed to different hyper-osmotic salt concentrations.

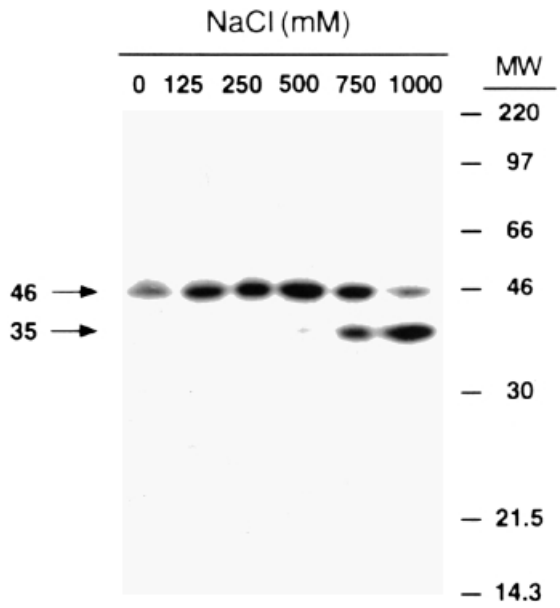

Figure 1. Different protein kinases are activated by different salt concentrations.
Suspension-cultured alfalfa cells were exposed to medium containing 0, 125, 250, 500, 750 and 1000 mM NaCl. Cell extracts were prepared at 15 min after incubation. For in-gel protein kinase assays, each lane contained 20 μg of total protein from cell extracts, which was separated by SDS–PAGE. MBP was used as a protein kinase substrate and was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel with [γ-^{32}P]ATP.

Specificity of MAP kinase antibodies

To test whether the 38 and 46 kDa kinases are members of the class of MAP kinases, mono-specific antibodies M23, M11, M14, and M24 were generated against synthetic peptides encoding the C-terminal amino acids of the SIMK, MMK2, MMK3 and SAMK alfalfa MAP kinases (Jonak *et al.*, 1993; Jonak *et al.*, 1995; Jonak *et al.*, 1996). The specificity of the antibodies was tested by immunoblotting glutathione-*S*-transferase (GST)–MAPK fusion proteins (Figure 2a). All antibodies showed mono-specific inter-

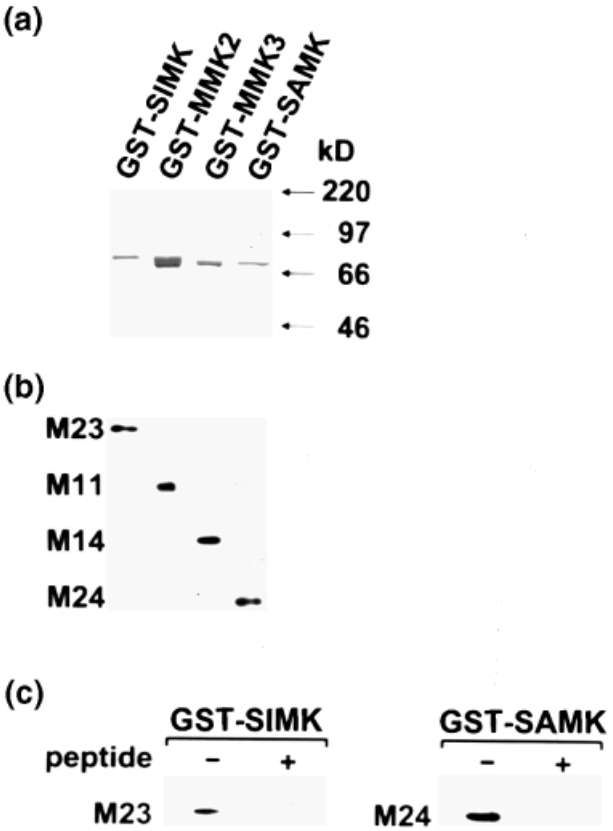

Figure 2. Specificity of MAPK antibodies.
(a) Bacterially produced and affinity-purified GST–SIMK, GST–MMK2, GST–MMK3 and GST–SAMK proteins were separated by SDS–PAGE and stained with Coomassie blue.
(b) Immunoblotting of GST–SIMK, GST–MMK2, GST–MMK3 and GST–SAMK with protein A-purified antibodies M23, M11, M14 and M24, that were prepared against synthetic peptides corresponding to the C-termini of SIMK, MMK2, MMK3 and SAMK, respectively.
(c) Immunoblotting of GST–SIMK and GST–SAMK with M23 and M24, before (–) and after pre-incubation (+) with excess M23 and M24 peptide, respectively.

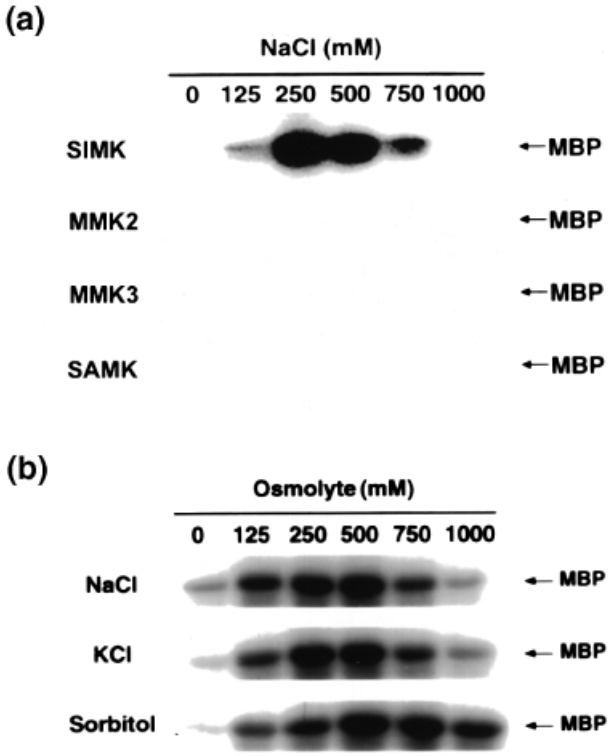

Figure 3. The SIMK pathway is activated by NaCl, KCl and sorbitol.
(a) Activation of SIMK by NaCl salt stress. For immunoprecipitation of the salt stress-induced MAP kinase, cell extracts were prepared, and 100 μg of total protein was immunoprecipitated with protein A-purified SIMK-, MMK2-, MMK3- and SAMK-specific antibodies. Kinase reactions of the immunoprecipitated proteins were performed with MBP as artificial substrate. The phosphorylation of MBP was analysed by autoradiography after SDS–PAGE.
(b) Suspension-cultured alfalfa cells were exposed to medium containing 125, 250, 500, 750 and 1000 mM NaCl, KCl or sorbitol. After incubation for 15 min, cell extracts were prepared and analysed for SIMK activity.

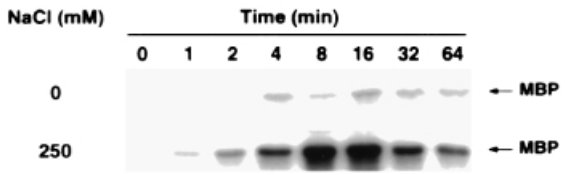

Figure 4. Transient activation of SIMK by salt stress.
Suspension-cultured alfalfa cells were exposed to medium containing 250 mM NaCl or medium alone. After incubation for 0, 1, 2, 4, 8, 16, 32 and 64 min, cell extracts were prepared from the cells and analysed for SIMK activity.

actions with the respective GST–MAPKs (Figure 2b). As shown for M23 and M24 (Figure 2c), pre-incubation of the antibodies with the respective peptides that were used for immunization blocked the specific reactions. Immunoblotting of crude cell extracts with M23, M11, M14 and M24 antibodies yielded single bands of 46 kDa for M23, and 44 kDa for M11, M14 and M24, respectively (data not shown). By in-gel kinase assays and *in vitro* kinase assays, the antibodies were shown to exclusively immunoprecipitate the respective active MAPK kinases, and immunoprecipitations could be totally competed with the peptides used to generate the antibodies (data not shown).

The salt stress-activated 46 kDa protein kinase is the SIMK MAP kinase

Different salt conditions induce the activation of protein kinases of relative molecular masses of 38 and 46 kDa that are able to use myelin basic protein as substrate. Such properties are typical of enzymes of the MAP kinase family. To test whether the 38 and 46 kDa kinases belong to the class of MAP kinases, we immunoprecipitated aliquots of cell extracts that were used for the in-gel kinase assays (Figure 1) with antibodies M23, M11, M14 and M24. The kinase activity of the immunopurified MAP kinases was then assessed by *in vitro* kinase assays. As shown by in-gel and immunokinase assays, only the SIMK-specific M23 antibody immunoprecipitated the salt stress-activated 46 kDa kinase (Figure 3a). Immunokinase assays indicated that SIMK is induced by salt conditions above 125 mM, but not at salt concentrations higher than 750 mM NaCl (Figure 3a). With the available antibodies, we were unable to immunoprecipitate an active kinase from extracts derived from high salt-treated cells. These results might suggest that the 38 kDa protein kinase is not a MAP kinase, but the high specificity of our antibodies excludes the detection of a number of other plant MAPKs. However, two tobacco protein kinases of 48 and 42 kDa have recently been identified that are also activated in response to hyper-osmotic salt stress. Whereas the 48 kDa kinase is highly related to SIMK, sequence analysis of the purified 42 kDa protein kinase showed no similarity to MAP kinases (G. Dobrowolska and G. Muzynska, personal communication).

SIMK is activated by NaCl, KCl and sorbitol

The inability to activate SIMK at high concentrations of NaCl might have been due to Na^+ ion toxicitiy rather than to hyper-osmotic stress. To test this possibility, aliquots of the same cell culture were treated with different concentrations of NaCl, KCl and sorbitol. After 15 min exposure, the activity of SIMK was determined by immunokinase assays (Figure 3b). KCl concentrations between 125 and 750 mM induced activation of SIMK, whereas a concentration of 1 M KCl did not result in activation of the kinase pathway. SIMK was also activated when sorbitol was added to the medium (Figure 3b), showing maximal activation between 500 and 750 mM sorbitol. It should be noted that sorbitol

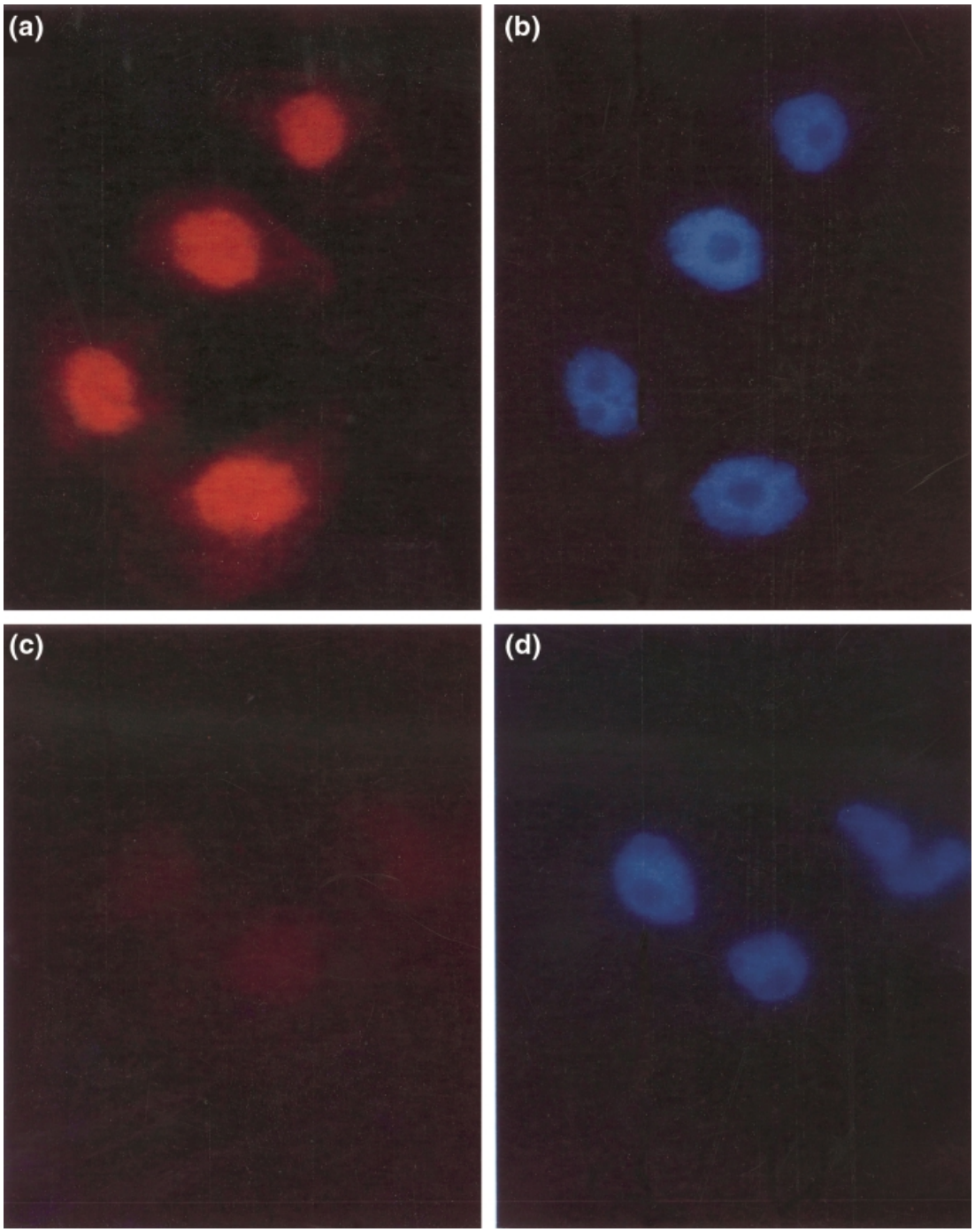

Figure 5. Constitutive nuclear localization of SIMK.
Indirect immunofluorescence of untreated alfalfa cells with SIMK-specific M23 antibody (a), stained with 4′,6′-diamino-2-phenylindole (DAPI) to visualize nuclei (b). Indirect immunofluorescence of untreated alfalfa cells with the SIMK-specific M23 antibody that was pre-incubated with excess M23 peptide (c), stained with DAPI (d). Indirect immunofluorescence was performed using a 1:500 dilution of the SIMK-specific M23 antibody (a) or M23 antibody which was pre-incubated with an excess of M23 peptide (c).

increases the osmolality to a much lower degree than NaCl or KCl, and a 1 M sorbitol solution produces the same osmotic pressure as a 0.6 M NaCl or KCl solution. Because the SIMK cascade is activated by NaCl, KCl or sorbitol, the SIMK pathway is involved in mediating a general hyper-osmotic response. Moreover, severe hyper-osmotic stress produced by either sorbitol, KCl or NaCl could not activate SIMK, indicating that Na^+ ion toxicity can

be excluded as the reason why SIMK is not stimulated at high NaCl concentrations.

SIMK is transiently activated by hyper-osmotic stress

Cells can respond to different extracellular stimuli by transient or constitutive activation of the same MAP kinase cascade activating different cellular programmes (Marshall, 1995). To examine whether hyper-osmotic stress leads to transient activation of SIMK, the time course of SIMK activation was investigated using cells that were exposed to medium containing 250 mM NaCl (Figure 4). SIMK activation was observed within 1 min after exposing cells to hyper-osmotic conditions. After maximal activation at 8–16 min, SIMK activity gradually declined but had not reached basal levels after 64 min. Because some MAP kinases can become activated by mechanical stress (Bögre *et al.*, 1996), such as stirring or shaking, cells were treated in the same way but with an equal volume of medium without NaCl. Under these conditions, SIMK was only slightly activated (Figure 4), indicating that activation of SIMK by salt treatment cannot be explained by mechanical stress alone.

Constitutive nuclear localization of SIMK

The transmission of extracellular signals from the cell surface into the nucleus by MAP kinases can involve the translocation of cytoplasmic MAPKs to the nucleus upon activation. Although this was shown for the mammalian ERK1 and ERK2 kinases (Chen *et al.*, 1992; Lenormand *et al.*, 1993), and the yeast HOG1 and Spc1/Sty1 MAPKs (Ferrigno *et al.*, 1998; Gaits *et al.*, 1998), other mechanisms must also exist, because ERK3 kinase was shown to be constitutively nuclear (Cheng *et al.*, 1996). To investigate whether SIMK is a cytoplasmic protein in unstressed cells, we performed indirect immunofluorescence microscopy with the SIMK-specific M23 antibody (Figure 5a). Comparison of the SIMK localization with the fluorescent signal emitted by 4′,6′-diamino-2-phenylindole (DAPI) to visualize nuclei (Figure 5b) revealed that SIMK was predominantly nuclear. When M23 antibody was incubated with excess M23 peptide before decorating the cells, the immunofluorescent signal was almost completely abolished (Figure 5c). This shows that the signal is generated by the specific immunolabelling of SIMK. To investigate the intracellular location of SIMK after salt stress, indirect immunofluorescence was performed after treatment with 250 mM NaCl. Under these conditions, SIMK was rapidly activated (Figure 4) but retained its nuclear localization up to 60 min after salt treatment (data not shown). These results demonstrate that SIMK has a constitutively nuclear localization and its activation is not correlated with nucleocytoplasmic translocation.

Discussion

In animals and yeast, specific MAP kinase pathways are involved in mediating responses to stress. The family of mammalian MAP kinases including the SAPK (stress-activated protein kinase)/JNK (Jun N-terminal kinase)/p38 is activated by hyper-osmotic as well as various other types of stress (Waskiewicz and Cooper, 1995). In yeast, the HOG1 MAP kinase pathway is exclusively used for mediating hyper-osmotic stress (Brewster *et al.*, 1993). We report here that a specific MAP kinase pathway is also involved in signalling hyper-osmotic stress in plants.

In alfalfa cells, salt stress activates a 46 kDa MAP kinase, identified to be SIMK. Interestingly, SIMK is only activated by moderate hyper-osmotic stress, whereas a 38 kDa protein kinase becomes activated under extreme hyper-osmotic conditions. This situtation resembles the operation of the osmo-sensing HOG1 and EHA1 pathways in yeast. Whereas the EHA1 pathway is only induced by high osmotic stress (Serrano *et al.*, 1997), the HOG1 MAP kinase pathway becomes activated by moderate hyper-osmotic conditons (Brewster *et al.*, 1993). These results suggest the existence of distinct sensors for moderate and extreme hyper-osmotic stress. Although no osmo-sensors have yet been identified in plants, two osmo-sensors, SLN1 and SHO1, are known in yeast that are both responsible for activation of the HOG1 pathway (Maeda *et al.*, 1995; Posas *et al.*, 1996). Whereas SHO1 encodes a four transmembrane protein with an SH3 domain, SLN1 has similarity to two-component histidine kinases. Two-component systems represent one of the best studied signal transduction systems in bacteria and are involved in sensing changes in environmental conditions including osmotic stress (Wurgler-Murphy and Saito, 1997). A two-component system might also be involved in osmotic stress sensing in plants, because an *Arabidopsis* two-component histidine kinase can complement yeast mutants defective in the SLN1 gene, and the plant gene is transcriptionally up-regulated by various stresses (Shinozaki and Yamaguchi-Shinozaki, 1997). Interestingly, plants appear to contain a variety of two-component histidine kinases that appear to be responsible for sensing a number of different stimuli, including the plant hormone ethylene (Chang, 1996).

MAP kinase-mediated information transfer often involves nuclear import of the MAP kinase from the cytoplasm after activation. Studies on the mammalian ERK1/ERK2 kinases indicated that the phosphorylation of the MAP kinase by the upstream MAP kinase kinase was an essential step to induce nuclear translocation (Chen *et al.*, 1992; Lenormand *et al.*, 1993). Recent data show that MAP kinases can shuttle between cytoplasmic and nuclear compartments, and that the time spent in any one compartment can be influenced by a number of parameters that may differ in a system-specific way. In

mammalian cells, MAPKKs may act as cytoplasmic anchors for inactive MAPKs (Fukuda *et al.*, 1997). Phosphorylation of MAPK is thought to induce dissociation from the MAPKK, thereby allowing nuclear import of the MAPK. Other evidence suggests that phosphorylation-induced dimerization may also contribute to nuclear import of MAPKs (Khokhlatchev *et al.*, 1998). Investigations of the fission yeast Spc1/Sty1 stress-signalling MAP kinase pathway revealed that the nuclear target of the Spc1/Sty1 kinase, the transcription factor Atf1, plays an active role in retaining the MAPK in the nucleus (Gaits *et al.*, 1998). Finally, studies on the HOG1 kinase in budding yeast added more complexity by showing that, besides regulation of entry and anchoring of the MAPK in the nucleus, nuclear export of the MAPK also contributes to the overall time of MAPK nuclear residence (Ferrigno *et al.*, 1998). Indirect immunofluorescence microscopy revealed a constitutively nuclear localization of SIMK under non-stressed conditions. No change in intracellular localization was observed after exposing cells to hyper-osmotic stress. By immunoblotting cytoplasmic and nuclear fractions, it was confirmed that a large fraction of SIMK constitutively resides in the nucleus (data not shown), suggesting that SIMK activation and inactivation are most likely intra-nuclear events. Considering our previous findings, where a MAPK showed nuclear translocation upon elicitor activation of parsley cells (Ligterink *et al.*, 1997), these findings may suggest that SIMK behaves unusually compared to other MAPKs. However, it should be noted that mammalian ERK3 was also found to have a constitutively nuclear localization (Cheng *et al.*, 1997), indicating that not all MAPKs undergo nucleo-cytoplasmic shuttling.

To investigate whether hyper-osmotic stress may be signalled by other mechanisms in intact plants, seedlings were exposed to hyper-osmotic salt conditions. SIMK was not activated in isotonic medium, but was activated by hyper-osmotic NaCl concentrations, exhibiting transient activation kinetics resembling those which were observed in suspension cultured cells (data not shown). These results show that salt stress activation of the SIMK pathway is not an artefactual response of suspension-cultured cells.

Salt stress induces a set of genes that is also induced by exogenous application of ABA. The importance of ABA as a mediator of hyper-osmotic stress is seen by its ability to induce a subset of the salt-induced genes in the absence of hyper-osmotic stress (Shinozaki and Yamaguchi-Shinozaki, 1996). Because salt stress results in rapid accumulation of ABA in plants, it was possible that SIMK activation is part of the ABA signal transduction pathway. Although ABA treatment resulted in the rapid accumulation of transcripts of the ABA-inducible *ABAMs1* gene (Jonak *et al.*, 1996), no activation of SIMK was observed (data not shown). These experiments indicate that SIMK acts independently or upstream of ABA.

In summary, our data reveal that different hyper-osmotic conditions induce the activation of distinct protein kinase pathways in alfalfa cells. Whereas the 46 kDa SIMK is activated by moderate salt stress, severe salt stress activates a 38 kDa kinase pathway. Cells can obviously distinguish between and use different signalling pathways for moderate and high hyper-osmotic conditions. Although little is so far known on how the differential activation of the pathways is brought about, and whether different sets of genes are targeted by these pathways, the identification and study of the 46 kDa SIMK and 38 kDa kinases will undoubtedly be of central importance for a molecular understanding of hyper-osmotic stress adaptation in plants.

Experimental procedures

Plant material and stress treatment

Suspension-cultured alfalfa cells (*Medicago sativa* L. cv Du Puits) were cultivated in MS medium (Murashige and Skoog, 1962) containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.1 mg l^{-1} kinetin. Cells were exposed to medium containing 125, 250, 500, 750, 1000 mM NaCl, KCl, sorbitol or medium alone. Material was collected at the indicated time points and immediately shock-frozen in liquid nitrogen before further analysis.

In-gel protein kinase assays

Cell extracts were prepared in extraction buffer (25 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM dithiothreitol, 0.1% NP40, 15 mM *p*-nitrophenylphosphate, 60 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 1 mM NaF, 1 mM phenylmethylsulphonylfluoride, 10 μg ml^{-1} each of leupeptin, aprotinin and soybean trypsin inhibitor and 5 μg ml^{-1} each of antipain, chymostatin and pepstatin). For in-gel protein kinase assays, each lane contained 20 μg of total protein which was separated by SDS–PAGE. MBP (0.5 mg ml^{-1}) was used as a substrate and was polymerized in the polyacrylamide gel. After protein renaturation, the kinase reactions were carried out in the gel with [γ-^{32}P]ATP as described previously (Bögre *et al.*, 1997).

Antibody production

M23, M11, M14 and M24 antibodies were produced against synthetic peptides, encoding the C-terminal amino acids FNPEYQQ of SIMK (Jonak *et al.*, 1993), VRFNPDPPNIN of MMK2 (Jonak *et al.*, 1995), LNFCKEQILE of MMK3, and LNPEYA of SAMK (Jonak *et al.*, 1996), respectively. The specificity of the antibodies was tested by immunoblotting glutathione-*S*-transferase (GST)–MAPK fusion proteins that were prepared as described previously (Jonak *et al.*, 1995).

Immunokinase assays

Cell extracts containing 100 μg of total protein were immunoprecipitated with 5 μg protein A-purified antibodies. The immunoprecipitated proteins were washed three times with buffer I (20 mM Tris–HCl, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100), once with

the same buffer but containing 1 M NaCl, and once with kinase buffer (20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). Kinase reactions of the immunoprecipitated protein were performed in 15 µl of kinase buffer containing 0.5 mg ml^{-1} MBP, 0.1 mM ATP and 2 µCi ^{32}P-γ-ATP. The protein kinase reaction was carried out at room temperature for 30 min. The reaction was stopped by addition of SDS sample buffer. The phosphorylation of MBP was analysed by autoradiography after SDS–PAGE.

Indirect immunofluorescence microscopy

Alfalfa cells were fixed in 3.7% formaldehyde in PBS (phosphate-buffered saline) with 0.1% Triton X-100 for 1 h. After washing in PBS, cells were treated with cell wall-degrading enzymes (1% cellulase R10 (Onozuka), 0.5% macerozyme (Calbiochem) in PBS) for 20 min. After washing in PBS, cells were attached to slides coated with poly-L-lysine (Sigma), and extracted with 1% Triton X-100 for 30 min and with 100% methanol for 10 min at –20°C. After washing with PBS and BPBS (PBS containing 1% w/v BSA), cells were incubated with 1:500 diluted protein A-purified M23 antibody (0.8 mg ml^{-1}) for 1 h at 37°C. For peptide competition, 1 µl M23 antibody was pre-incubated with 30 µg of M23 peptide for 1 h at 4°C before dilution. After washing with PBS and BPBS, 1:200 diluted secondary antibody (anti-rabbit fluorescein isothicyanate-conjugated antibody (Sigma)) was added for 45 min at room temperature. After washing in PBS, slides were mounted in an anti-fade medium (Dako) and analysed by UV microscopy (Olympus).

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