Different protein kinase families are activated by osmotic stresses in *Arabidopsis thaliana* cell suspensions

Involvement of the MAP kinases AtMPK3 and AtMPK6

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Abstract Five Ca^{2+}-independent protein kinases were rapidly activated by hypoosmotic stress, moderate or high hyperosmolarity induced by several osmolytes, sucrose, mannitol or NaCl. Three of these kinases, transiently activated by hypoosmolarity, recognised by anti-phosphorylated mitogen-activated protein (MAP) kinase antibodies, sensitive to a MAP kinase inhibitor and inactivated by the action of a tyrosine phosphatase, corresponded to MAP kinases. Using specific antibodies, two of the MAP kinases were identified as AtMPK6 and AtMPK3. The two other protein kinases, durably activated by high hyperosmolarity, did not belong to the MAP kinase family. Activation of AtMPK6 and AtMPK3 by hypoosmolarity depended on upstream protein kinases sensitive to staurosporine and on calcium influx. In contrast, these two transduction steps were not involved in the activation of the two protein kinases activated by high hyperosmolarity.

Key words: Hypoosmotic stress; Hyperosmotic stress; Mitogen-activated protein kinase; *Arabidopsis thaliana*

1. Introduction

Plants have developed complex signalling networks to sense environmental signals and adapt to unfavourable conditions. Although phosphorylation cascades induced by hyper- and hypoosmotic stresses have been fully identified in yeast, little is known about the phosphorylation events mediated by plant osmoregulation. However, the involvement of phosphorylation signalling intermediates in drought or salinity signalling pathways has been suggested, based on transcript accumulation, post-translational activation or functional complementation of yeast mutants.

Up-regulation of mRNA levels upon drought or salt stress was demonstrated, among others, for some mitogen-activated protein (MAP) kinases, *Arabidopsis* AtMPK3 [1], alfalfa MMK4 [2] and the *Arabidopsis* MAP kinase kinase kinase, AtMEKK1 [1,3]. Similar increases in mRNA levels were also reported for the two kinds of proteins, AtHK1 [4] and AtRR1–2 [5], which represent homologues of the yeast sensor histidine kinase and the two-component response regulator respectively, functioning upstream of one MAP kinase cascade allowing hyperosmolarity perception. Transcript accumulation induced by salt stress was also documented for other Ca^{2+}-independent protein kinases, like SOS 2 (salt overly sensitive), an *Arabidopsis* protein kinase homologous to the yeast SNF1 kinase [6], or AtGSK1 which corresponds to a GSK3/shaggy-like protein kinase [7].

Functional complementation of several *Saccharomyces cerevisiae* mutants affected in one component of the hyperosmolarity MAP kinase cascades has also suggested the importance in osmoregulation of several plant transduction intermediates. AtHK1 and the phosphorelay mediators AtHPs were able to reverse the phenotypes of the corresponding yeast mutants [4,8]. At the MAP kinase and MAP kinase kinase kinase levels, similar successful complementations of *Saccharomyces* mutants were obtained for PsMAPK [9] and AtMEKK1 [3], respectively. In addition, the NaCl stress-sensitive phenotype of the *mck1* mutant was rescued by AtGSK1 [7].

The most direct indications of the involvement of some plant transduction components in osmoregulation came from studies using diverse plant models and showing the activation of several protein kinases. In *Arabidopsis*, the activation of AtMPK6 by hyperosmolarity was observed in detached leaves [10] but not in cell suspensions [11]. Hyperosmotic activation of AtMPK4 was also reported in detached leaves [10]. The tobacco SIPK [12–14] and alfalfa MMK4 and MKK1, also called SAMK and SIMK respectively [2,15], were shown to be activated by different osmolytes, salt stress or drought. Interestingly, these MAP kinases were observed in all cases involved in the signalling of moderate hyperosmolarity. In contrast, the high hyperosmolarity mainly activated another protein kinase in alfalfa [15], which may be a homologue of a tobacco kinase belonging to the SNF1 family [14]. Accordingly, two soybean members of the same SNF1 family were activated in response to high hyperosmolarity [16].

Besides these elements concerning drought and salinity which correspond to hyperosmolarity situations, very little information is available on the perception of hypoosmolarity. The activation of the tobacco MAP kinases, SIPK and WIPK, was evidenced, indicating that one kinase, SIPK, may play a role in both hypo- and hyperosmotic pathways [12]. Very recently, the phosphorylation-induced activation of a tobacco Ca^{2+}-dependent protein kinase, followed by an increase in

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Abbreviations: MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein

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transcript level, was demonstrated in tobacco [17]. The specific replacement of the yeast MPK1 kinase, a MAP kinase belonging to the hypoosmolarity MAP kinase cascade, by an alfalfa MAP kinase, MMK2, was also demonstrated [18]. In *Arabidopsis*, AtMPK4 and AtMPK6 were activated by touch in detached leaves [10], although AtMPK6 activity was not modified by hypoosmolarity in cell suspensions [11].

Some upstream transduction elements of MAP kinases were also identified in an osmoregulation context. Depletion of external calcium in tobacco suspension cells suppressed the activation of protein kinases by hypoosmolarity [19] and particularly of SIPK and WIPK [12,20]. Contrary to the hypoosmotic induction, activation of SIPK by hyperosmotic stress was Ca^{2+}-independent [13]. Activation of the two tobacco MAP kinases by hypoosmolarity also depended on protein kinases sensitive to staurosporine [20]. Concerning the SNF1-like kinases, quite divergent results were observed with studies on tobacco suggesting a mechanism of activation via phosphorylation [14] or inversely by dephosphorylation [13].

In the present work, the activation of protein kinases by hypoosmolarity in *Arabidopsis* cell suspensions as well as moderate or high hyperosmolarity induced by several osmolytes was investigated. Several activated protein kinases were demonstrated to be MAP kinases and among them AtMPK3 and AtMPK6 were identified. Two other protein kinases, strongly activated by high hyperosmolarity, did not appear to belong to the MAP kinase family. Finally, the possible upstream action of other protein kinases as well as calcium influx in the different osmotic transduction pathways was also tested.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana cells (Columbia ecotype) were cultured in liquid medium containing 0.03 mM NaH_2PO_4, 0.37 mM KH_2PO_4, 0.06 mM Na_2HPO_4, 19.4 mM KNO_3, 0.45 mM $MgSO_4$, 0.9 mM $CaCl_2$, 30 μM H_3BO_3, 1.5 μM KI, 39.6 μM $MnSO_4$, 11 μM $ZnSO_4$, 0.3 μM Na_2MoO_4, 0.03 μM $CuSO_4$, 0.03 μM $CoCl_2$, 5 μM $FeSO_4$, 5 μM Na_2-EDTA, 4.1 μM nicotinic acid, 2.4 μM pyridoxine HCl, 1.2 μM thiamine HCl, 555 μM *myo*-inositol, 26.6 μM glycine, 0.01% (w/v) casein hydrolysate, 1.5% (w/v) sucrose and 1 μM 2-naphthalene acetic acid. Cells were cultured at 23°C in constant light and used after 5 days subculturing with 100 mg fresh weight/ml cell density.

2.2. Suspension cell treatments

Osmotic stresses were applied to cells equilibrated for 4 h in their culture medium containing 10 mM MES–Tris pH 6.2 and adjusted to 200 mOsm with sucrose. After equilibration, extracellular medium was replaced by either the same volume of hypoosmotic medium, 15 mOsm (10 mM MES–Tris pH 6.2, 1 mM $CaSO_4$, sucrose-free, medium A) or isoosmotic medium, 200 mOsm (medium A plus 190 mM sucrose) or hyperosmotic medium, 500 or 1000 mOsm (medium A plus 500 mM or 1000 mM sucrose). For EGTA treatments, these media are deprived of calcium. In indicated cases, sucrose was replaced by another osmolyte to get similar moderate (500 mM mannitol or 250 mM NaCl) or high (1000 mM mannitol or 650 mM NaCl) hyperosmolarities. Osmolarity was monitored using a freezing point osmometer (Roebling, Berlin, Germany). To stop treatment at indicated times, cell suspensions were filtered, frozen in liquid nitrogen and stored at −80°C until use. For treatments with gadolinium (0.5 or 1 mM), EGTA (10 mM) or staurosporine (0.5 μM), these inhibitors were added during the last 10 min of the equilibration time and during 2 or 10 min after transfer, at the same concentrations.

2.3. Preparation of protein extracts

Cells were ground in liquid nitrogen and homogenised at 4°C in extraction buffer (100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM orthovanadate, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml antipain) and centrifuged at 17 600×*g* at 4°C. The supernatant was precipitated in 10% (w/v) trichloroacetic acid (TCA) solution containing 10 mM NaF, washed twice with 80% (v/v) cold acetone and resuspended in SDS–PAGE sample buffer. Protein concentration was determined by the Bradford method with bovine serum albumin as standard [21].

2.4. In-gel kinase assay

Protein extracts (20 μg) were electrophoresed on 10% SDS–polyacrylamide gels embedded with 0.2 mg/ml myelin basic protein (MBP) or 0.5 mg/ml histone as substrates for the kinases. The gels were then treated for washing and renaturing steps as described by Zhang et al. [22]. For the activity, the gels were pre-incubated for 30 min at room temperature in kinase activity buffer (40 mM HEPES pH 7.5, 2 mM DTT, 20 mM $MgCl_2$, 1 mM EGTA, 0.1 mM orthovanadate) then 1 h in 8 ml of the same buffer supplemented with 25 μM cold ATP and 2.9 MBq [^{33}P]ATP per gel. Then the gels were washed extensively in 5% TCA (w/v) and 1% disodium pyrophosphate (w/v) solution. The protein kinase activity was revealed on the dried gels by Storm imaging system (Molecular Dynamics). For the gel treatment with kinase inhibitors, the activity buffer was completed with 500 μM apigenin or 1.5 μM staurosporine during pre-incubation and incubation steps.

2.5. In vitro treatment of protein extracts with protein tyrosine phosphatase

For protein tyrosine phosphatase treatment, 40 μg of proteins from 17 600×*g* supernatant obtained from treated cells was incubated with 5 U of YOP protein tyrosine phosphatase (New England Biolabs) in the reaction buffer supplied with the enzyme. The samples were incubated at 30°C for 30 min in the presence or absence of phosphatase inhibitor, 6 mM sodium orthovanadate. The reaction was stopped by addition of SDS–PAGE sample buffer and in-gel kinase assay was performed as described above.

2.6. Immunoprecipitation

Immunoprecipitation assays were performed either with the polyclonal human anti-phospho-p44/42 MAP kinase antibody (Cell Signaling, Biolabs) or with two polyclonal plant MAP kinase antibodies. They were raised against the 15 N-terminal amino acids of the *Arabidopsis* AtMPK6 (MDGGSGQPAADTEMT) or AtMPK3 (MNTGGGQYTDFPAVD). The 17 600×*g* supernatant was obtained as previously described except that, in extraction buffer, 10 mM was used for DTT, orthovanadate and NaF and 60 mM for β-glycerophosphate. Protein extract (100 μg for the first antibody and 200 μg for the two others) was incubated with either 12 μl anti-phospho-MAP kinase, 50 μg anti-AtMPK6 or 70 μg anti-AtMPK3 in immunoprecipitation buffer (20 mM HEPES pH 7.5, 5 mM EGTA, 5 mM EDTA, 5 mM DTT, 60 mM β-glycerophosphate, 0.1 mM orthovanadate, 10 mM NaF, 1 mM PMSF, 5 μg/ml leupeptin and antipain, 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (v/v) Nonidet P-40) for 3 h. Then 30 μl of 50% protein A-Sepharose CL4B (Sigma) was added and incubation continued for one additional hour. The immunoprecipitate was washed four times in immunoprecipitation buffer and twice in kinase buffer (20 mM Tris–HCl pH 7.5, 12 mM $MgCl_2$, 2 mM EGTA, 2 mM DTT, 0.1 mM orthovanadate), resuspended in SDS–PAGE sample buffer and then boiled for 3 min at 95°C. Supernatant fractions were electrophoresed on SDS–polyacrylamide gels embedded with MBP for in-gel kinase assay as previously described.

3. Results

3.1. Several protein kinases are activated by hypoosmotic and hyperosmotic stresses

In-gel kinase assays using MBP as a substrate were performed, in the absence of calcium, to investigate the protein kinases activated by hypo- and hyperosmotic signals (Fig. 1). When cells were transferred to hypoosmotic medium (A, Hypo), three Ca^{2+}-independent protein kinases with apparent molecular masses of 44, 39 and 37 kDa were early (2 min) and transiently activated. Cell transfer to isoosmotic medium (A,

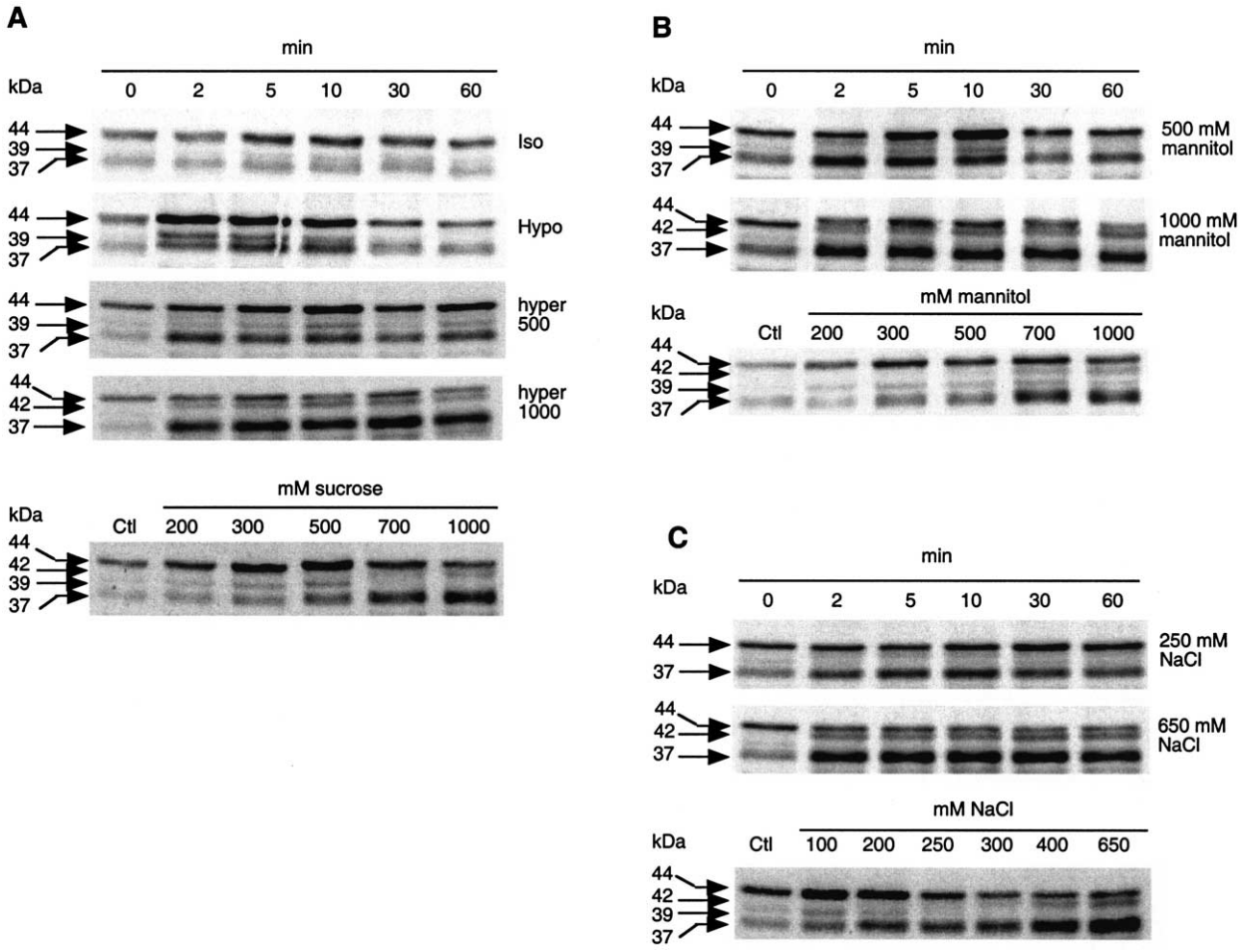

Fig. 1. Protein kinase activation in response to hypo- and hyperosmotic stresses: time kinetics and dose responses. Osmolytes used were sucrose (A), mannitol (B) and NaCl (C). Aliquots of *Arabidopsis* cell suspensions were equilibrated for 4 h in isoosmotic medium and then, at zero time, transferred to either isoosmotic (Iso), hypoosmotic (Hypo), moderate (500 mM sucrose: hyper 500, 500 mM mannitol or 250 mM NaCl) or high (1000 mM sucrose: hyper 1000, 1000 mM mannitol or 650 mM NaCl) hyperosmotic medium. Treatments were stopped at indicated times. For dose–response experiments, cells were transferred to indicated solutions for 10 min. 200 mM sucrose or mannitol and 100 mM NaCl correspond to cell transfer in isoosmotic medium. The control, Ctl or 0 min corresponds to non-transferred cells. Kinase activity was determined with an in-gel kinase assay using MBP as a substrate.

Iso) resulted in a slight and delayed (10 min) activation of 44 and 39 kDa kinases, whereas the 37 kDa protein did not show clear activation during this time course. Thus, activation of the 44 and 39 kDa kinases can be considered to be induced slightly by mechanical stress and strongly by hypoosmotic stress. Transfer of suspension cells to 500 mM sucrose (A, hyper 500) resulted in the transient activation of the 44 and 39 kDa proteins which peaked at 10 min while a constant activation of the 37 kDa lasted during 60 min. Because activation of the 39 kDa kinase by this stress was slight and not regular from one experiment to another, it was probably due to the mechanical stress as it was seen earlier, after cell transfer to isoosmotic medium. With a strong hyperosmotic stress (A, hyper 1000) two kinases of 42 and 37 kDa were early (2 min) and constantly activated during this time course. Thus, the three types of osmotic stresses resulted in distinct protein activation profiles, a transient activation of three kinases (44, 39 and 37 kDa) by hypoosmolarity, a transient (44 kDa) and sustained (37 kDa) activation of two kinases by moderate hyperosmolarity or a sustained activation of two kinases (42 and 37 kDa) by high hyperosmolarity. Dose response from 200 mM to 1000 mM sucrose, during 10 min treatment, showed kinase activations according to the concentration (bottom, Fig. 1A). The 44 kDa activation increased with the sucrose concentration until 500 mM then decreased at high osmolarities when the 37 kDa kinase activation occurred. The 39 kDa protein showed a similar dose response to the 44 kDa but with a slighter activation, probably due to mechanical stress. The 42 kDa kinase was activated only at high (1000 mM) concentration.

3.2. *Effects of mannitol and NaCl are similar to the sucrose effect*

To determine if the activation of these protein kinases by hyperosmolarity is associated specifically with sucrose stresses or corresponds to a general response to osmotic stress, two other osmolytes were tested, mannitol and NaCl. The used concentrations correspond to the different osmolarities brought by sucrose in Fig. 1A (see Section 2). When mannitol was used as osmolyte (Fig. 1B), kinetics with 500 or 1000 mM and dose response showed similar results to sucrose. When a moderate salt stress (Fig. 1C) was applied, the 44 kDa was slightly and lately (30 min) activated while the 37 kDa shows, as with sucrose or mannitol, an early (2 min) and constant

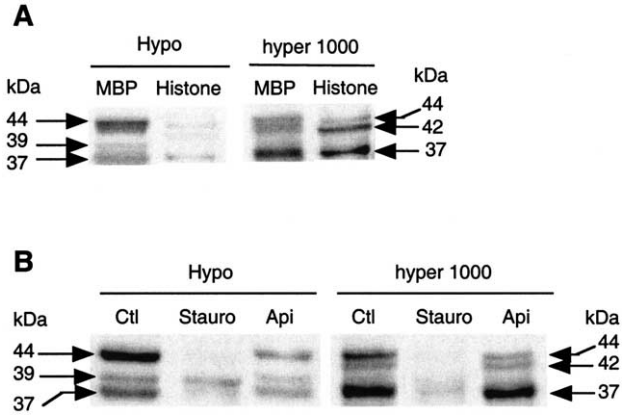

Fig. 2. Osmotically activated protein kinases: substrate dependence (A) and sensitivity to protein kinase inhibitors (B). Equilibrated cells were transferred either to hypoosmotic medium (Hypo) for 2 min or to high hyperosmotic sucrose medium (hyper 1000) for 10 min. In A, kinase activity was determined with an in-gel kinase assay using MBP or histone as a substrate. In B, protein kinase inhibitors, 1.5 μM staurosporine (Stauro) or 500 μM apigenin (Api) were added during the in vitro radioactive labelling step, when indicated. Ctl is the corresponding control without kinase inhibitor. Kinase activity was determined with an in-gel kinase assay using MBP as a substrate.

activation during this time course. No activity of the 39 kDa protein was observed during this time course. For the high salt stress (650 mM NaCl) kinetics and NaCl dose response, similar results were observed in comparison to sucrose and mannitol conditions (Fig. 1C). Thus, the comparison of the three osmolytes used indicates that the kinase activations in hyperosmotic stresses correspond to a general response to osmotic stress. In the conditions used, three kinases are activated by hyperosmotic stresses. Among these kinases, the 44 kDa is the only one to be transiently activated (in moderate hyperosmotic stress), the two others are only activated at high hyperosmotic stresses. It may be suggested that two types of kinases have a molecular weight of 37 kDa, one transiently activated by hypoosmotic stress and one constantly activated by hyperosmotic stresses. Based on these results, *Arabidopsis* cells treated for 2 min hypoosmotic, 10 min moderate (500 mM sucrose) or high hyperosmotic (1 M sucrose) stress were chosen for further characterisation of osmotic-activated protein kinases.

3.3. *Hypoosmotically induced kinases use preferentially MBP and are sensitive to apigenin, contrary to the protein kinases induced by high hyperosmolarity*

Because MAP kinases are Ca^{2+}-independent kinases transiently activated, the presence of these proteins among the kinases activated by hypoosmotic or hyperosmotic stresses was evaluated. MAP kinases are known to use preferentially MBP as a substrate. In our experiment, when histone was used as a substrate (Fig. 2A), the three hypoosmotically activated kinases 44, 39 and 37 kDa showed a very light activity whereas the two hyperosmotically activated kinases, 42 and 37 kDa, had, respectively, a higher or similar activity when compared with the MBP substrate. In tobacco cells [20], osmotically activated MAP kinases, SIPK and WIPK, have been shown to be sensitive to apigenin, an inhibitor of MAP kinases, while only SIPK was sensitive to staurosporine, a general kinase inhibitor. When protein kinase inhibitors were added during the in-gel kinase activity assay (Fig. 2B), 44 kDa kinase activity was totally prevented by staurosporine and partially inhibited by apigenin. The 39 kDa kinase activity was not affected by staurosporine but partially by apigenin. Sensitivity of the 37 kDa activity to these inhibitors depended on the type of stress, it was totally inhibited by staurosporine but partially by apigenin when cells were hypoosmotically stressed, while it was partially inhibited by staurosporine and not sensitive to apigenin when cells were hyperosmotically treated. Thus, this result would confirm the presence of two types of 37 kDa kinases, one hypoosmotically activated, apigenin-sensitive and one hyperosmotically activated, apigenin-insensitive.

3.4. *Three activated protein kinases belong to the MAP kinase family*

Anti-phospho-p44/42 MAP kinase antibody (Fig. 3A) immunoprecipitated the three kinases 44, 39 and 37 kDa activated in hypoosmotically treated cells indicating that they belong to the MAP kinase family. However, when cells were stressed by moderate or high hyperosmotic treatment this antibody immunoprecipitated only the 44 kDa kinase, indicating that the hyperosmotically activated 37 kDa kinase would not be a MAP kinase. Tyrosine phosphorylation being crucial for the activation of MAP kinases, cells extracts were treated with protein tyrosine phosphatase (PTP) in the presence or not of vanadate, a phosphatase inhibitor (Fig. 3B), in order to confirm these results. When cells were hypoosmotically treated, the PTP treatment resulted in the complete inhibition

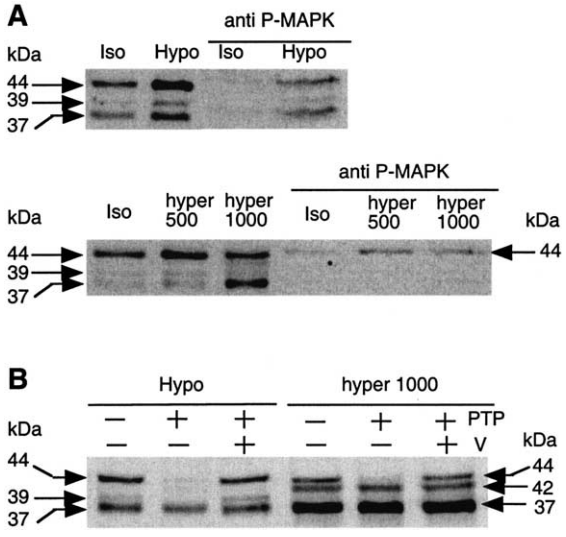

Fig. 3. Identification of several osmotically activated kinases as MAP kinases, using human anti-phospho-p44/42 MAP kinase antibody (A) or protein tyrosine phosphatase (B). Equilibrated cells were transferred for 2 min to hypoosmotic medium (Hypo) or for 10 min to moderate (hyper 500) or high (hyper 1000) hyperosmotic sucrose medium or to isoosmotic medium (Iso) for 2 or 10 min, corresponding to the controls of hypo- or hyperosmotic treatments, respectively. In A, when indicated, 100 μg of protein extract was immunoprecipitated by 12 μl anti-phospho-MAP kinase (anti P-MAPK) then kinase activity of the immunocomplex was subsequently assayed with an MBP in-gel kinase assay. In B, 40 μg of protein extract was treated with 5 U of tyrosine-specific protein phosphatase (PTP) in the presence or not of 6 mM orthovanadate (V), a PTP inhibitor, for 30 min at 30°C. Kinase activity was then tested by MBP in-gel kinase activity assay.

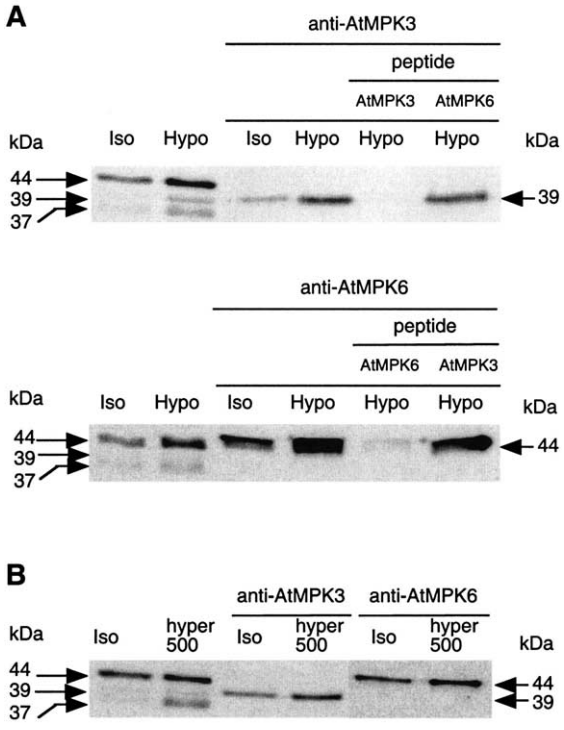

Fig. 4. Identification of AtMPK3 and AtMPK6 among the different kinases activated by osmotic stresses. In A, equilibrated cells were transferred either to isoosmotic (Iso) or to hypoosmotic (Hypo) medium for 2 min. In B, equilibrated cells were transferred to isoosmotic (Iso) or moderate (hyper 500) hyperosmotic sucrose medium for 10 min. When indicated, anti-AtMPK3 or anti-AtMPK6 antibodies were used to immunoprecipitate 200 μg of protein extract. The immunoprecipitations were also performed in the presence, as competitors, of the N-terminal AtMPK3 or AtMPK6 peptides used for immunisations, when indicated. Kinase activity of the immunocomplex was subsequently assayed with an MBP in-gel kinase assay. Kinase activity of the corresponding initial extracts is shown in the left part of each gel.

of 44 and 39 kDa kinase activities, while inhibition of the 37 kDa kinase was only partial. Inclusion of vanadate during the PTP treatment restored these activities. The two hyperosmotically activated kinases, 42 and 37 kDa, were not affected by a PTP treatment. Thus, all these results strongly suggest that the three hypoosmotically activated kinases, 44, 39 and 37 kDa, are MAP kinases while the two hyperosmotically activated kinases, 42 and 37 kDa, are not. The 44 kDa kinase activated by a moderate hyperosmotic stress also appears to be a MAP kinase and may correspond to the 44 kDa kinase activated by hypoosmolarity.

3.5. *Two MAP kinases are identified as AtMPK3 and AtMPK6*

As the hypoosmotically activated 44, 39 and 37 kDa kinases were suggested to be MAP kinases, specific antibodies against two *Arabidopsis* MAP kinases were used to test if they correspond to one of these kinases. The anti-AtMPK3 antibody immunoprecipitated the 39 kDa kinase activated by hypoosmolarity (Fig. 4A, upper part). The specificity of the precipitation was shown using successfully the corresponding peptide (AtMPK3) as a competitor, while another unrelated peptide (AtMPK6) has no effect on the immunoprecipitation of AtMPK3. Comparable results were obtained for the specificity of the anti-AtMPK6 antibody (Fig. 4A, lower part), which immunoprecipitated the 44 kDa kinase activated by hypoosmolarity. After moderate hyperosmotic stress (Fig. 4B), the two antibodies again immunoprecipitated the 39 and 44 kDa kinases. Thus, the 44 kDa kinases activated by hypoosmotic and moderate hyperosmotic stresses correspond to the same MAP kinase, AtMPK6. It can also be noticed that the slight activation of the 39 kDa kinase sometimes detectable after moderate hyperosmotic stress (see Section 3.1) was clearly evidenced here and due to AtMPK3.

3.6. *Different transduction pathways are induced by hypoosmotic or hyperosmotic stresses*

The transduction pathways leading to the activation of osmotically activated kinases were investigated (Fig. 5). The kinase inhibitor staurosporine, when added in vivo, inhibited the activation of the 44 kDa (AtMPK6) and 39 kDa (AtMPK3) while it showed no effect on the 37 kDa or the 42 kDa kinases (Fig. 5A). Therefore, AtMPK6 and AtMPK3 activation in hypo- and moderate hyperosmotically treated cells should be dependent on upstream phosphorylations by kinases sensitive to staurosporine. The 37 kDa activation in any of the three stresses and the 42 kDa, activated by high hyperosmotic stress, would not depend on upstream phosphorylations. These results suggest that hypoosmotic and moderate hyperosmotic stresses would implicate at least two different pathways, one with AtMPK6 (and AtMPK3 for hypoosmotic stress) and one with the 37 kDa kinase. When extracellular calcium was chelated by EGTA (Fig. 5B), the activation of the 44 kDa kinase (AtMPK6) and 39 kDa kinase (AtMPK3) by hypoosmotic or moderate hyperosmotic stresses was prevented. The hypoosmotic activation of the 37 kDa kinase also appeared to be prevented by EGTA. In contrast, EGTA has no effect on the 42 and 37 kDa kinase activations by hyperosmolarity. When gadolinium, an inhibitor of calcium and stretch-activated channels, was used (Fig. 5C), similar results were obtained. However, the prevention of the 44 kDa kinase activation by hypoosmotic stress was clearly visible only when the gadolinium concentration was increased to 1 mM (Fig. 5C). These results suggest that calcium influx represents a signalling event involved in the activation of the three MAP kinases by hypoosmolarity as well as the activation of AtMPK6 by moderate hyperosmolarity. In contrast, the activations of the 42 and 37 kDa kinases by hyperosmolarity appear independent of the entry of extracellular calcium.

4. Discussion

In *A. thaliana* cell suspensions, several Ca^{2+}-independent protein kinases were activated by osmotic stresses. The activation profile of these kinases by hypoosmolarity and by moderate or high hyperosmolarity induced by several osmolytes (sucrose, mannitol and NaCl) was determined. In hypoosmotically treated cells, three protein kinases with apparent molecular masses of 44, 39 and 37 kDa were strongly, rapidly and transiently activated. The two kinases of 44 and 39 kDa are also, but lightly and lately, activated by mechanical stress and this is consistent with the similarity between mechanical and hypoosmotic stresses previously shown. This relationship has also been observed in tobacco cell suspension: in contrast to hyperosmotic stress, mechanical and hypoosmotic stresses

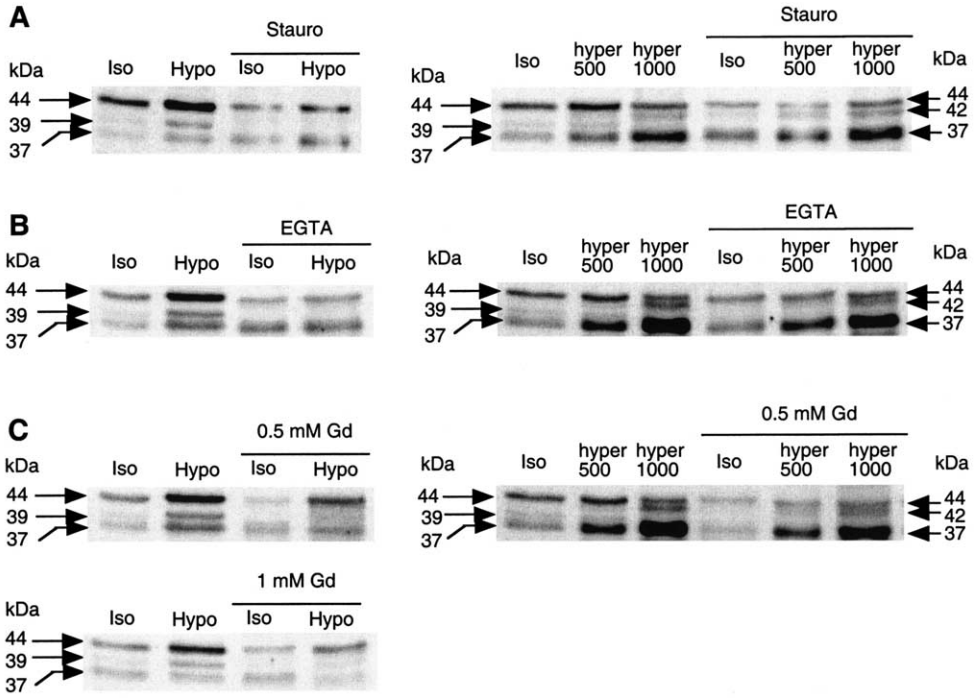

Fig. 5. Effect of staurosporine, a protein kinase inhibitor (A), EGTA, a calcium chelator (B), and gadolinium, a calcium and stretch-activated channel inhibitor (C) on the kinase activation. Equilibrated cells were transferred for 2 min to hypoosmotic medium (Hypo) or for 10 min to moderate (hyper 500) or high (hyper 1000) hyperosmotic sucrose medium or to isoosmotic medium (Iso) for 2 or 10 min, corresponding to the controls of hypo- or hyperosmotic treatments, respectively. When indicated, 0.5 μM staurosporine (Stauro), 10 mM EGTA, 0.5 mM or 1 mM gadolinium (Gd) was added during the last 10 min of the equilibration time and during 2 or 10 min after transfer, at the same concentration. Kinase activity was determined with an in-gel kinase assay using MBP as a substrate.

activated the same two kinases, SIPK and WIPK [12], and induced an oxidative burst [20].

When high hyperosmotic stress was applied, two protein kinases of 42 and 37 kDa were rapidly and durably activated, while in cells subjected to moderate hyperosmotic stress, two protein kinases of 44 and 37 kDa were activated in a transient and durable manner, respectively. When cell suspensions were exposed to different concentrations of sucrose, mannitol or NaCl, it appeared that the 44 kDa kinase was activated by moderate hyperosmolarity whereas the 42 and 37 kDa kinases were more specific to high hyperosmolarity. Similar results have already been observed in alfalfa cell suspension subjected to different concentrations of NaCl [15]. The SIMK was activated by moderate concentrations between 125 and 500 mM NaCl whereas high concentrations (750 and 1000 mM NaCl) activated an unknown protein kinase which was suggested to be homologous to the *Arabidopsis* serine/threonine kinase 1 (ASK1), a member of the SNF1-like family. Activation profiles by hyperosmolarity induced by mannitol and NaCl were similar to the profile observed when sucrose was used. This result indicates that the kinase activations observed with sucrose are due to the osmotic effect and not to sucrose metabolisation.

The protein kinases activated by osmotic stresses in *Arabidopsis* cell suspensions can be classified into two groups: three protein kinases with apparent molecular masses of 44, 39 and 37 kDa display transient activation and are more specific to hypoosmolarity, whereas two others with apparent molecular masses of 42 and 37 kDa display sustained activation and are more specific to high hyperosmolarity. The two groups of kinases differ in their enzymatic and immunochemical properties. We showed that the three transient activated kinases mainly phosphorylate MBP whereas the two durable activated kinases phosphorylate both MBP and histone. Moreover, apigenin, a MAP kinase inhibitor, partially reduced the 44, 39 and 37 kDa kinase activities induced by hypoosmolarity. These activities were also fully (for the 44 and 39 kDa kinases) or partially (for the 37 kDa kinase) inhibited by the action of a tyrosine phosphatase, with restoration of activity by addition of orthovanadate, a tyrosine phosphatase inhibitor. In contrast, the hyperosmotically activated 42 and 37 kDa kinases were insensitive to the tyrosine phosphatase. Finally, the three kinases activated by hypoosmolarity and the 44 kDa kinase activated by moderate hyperosmolarity are immunoprecipitated by antibodies raised against activated MAP kinase. Taken together, these results strongly suggest that the three kinases activated by hypoosmolarity and the 44 kDa kinase activated by moderate hyperosmolarity belong to the MAP kinase family.

Like the tobacco SIPK [12,20], the 44 kDa kinase is activated by hypo- and hyperosmotic stresses and is sensitive to staurosporine and apigenin. Like the tobacco WIPK, the 39 kDa kinase is activated by hypoosmotic stress and is sensitive to apigenin but insensitive to staurosporine. These results suggest that the 44 and 39 kDa kinases are likely to be the *Arabidopsis* homologues of SIPK and WIPK, respectively (AtMPK6 and AtMPK3). Using specific antibodies of these *Arabidopsis* kinases, the 44 and 39 kDa kinases were identified as AtMPK6 and AtMPK3, respectively. It has been shown that the three homologues, *Arabidopsis* AtMPK6 [10], tobacco SIPK [12–14] and alfalfa SIMK [15], are activated by hyperosmotic stress induced by sugar or salt. These results demon-

strate that sequence homologies can be correlated with functional homologies.

In this study, AtMPK6 activation by hyper- and hypoosmolarity was shown. This is consistent with the activation profiles observed by Ichimura et al. [10] in response to high osmolarity and to touch which is related to hypoosmotic stress [20]. However, the activation by 250 mM NaCl observed here is slight and late in comparison to the results observed in detached leaves [10]. It should be noted that Yuasa et al. [11] did not detect AtMPK6 activation by hypo- and hyperosmotic shocks in cell suspensions. These authors suggested that this may be due to the loss of upstream components of the transduction pathways leading to AtMPK6 when their cell line was established. Alternatively, this divergence between the study of Yuasa et al. [11] and the present results may be due to differences in experimental factors, like the precise nature of osmotic stress used or the immunoprecipitation conditions. The AtMPK3 activation by mechanical stress and hypoosmolarity is reported here for the first time. AtMPK6 was reported to be activated by other abiotic stresses such as low temperature, low humidity, wounding [10] or oxidative stress [11,23,24] as well as biotic stresses [23,25]. Likewise, AtMPK3 was also reported to be activated by oxidative stress [24]. These results indicate that AtMPK6 and AtMPK3 are located at the crossroads of several transduction pathways. The third MAP kinase activated in *Arabidopsis* cell suspension subjected to hypoosmolarity could be AtMPK4 which has been reported to be activated by several abiotic stresses [10].

The two other protein kinases, which do not belong to the MAP kinase family, could be members of the shaggy-like or SNF1-like families. The shaggy-like proteins are mainly involved in plant development, but recently, it has been shown that they could be implicated in stress responses such as wounding [26] and osmotic stress [7]. The SNF1-like proteins are mainly involved in carbon metabolism regulation [27], but they have also been reported to be implicated in osmotic responses [6,14,16].

Concurrently with kinase identification, upstream elements in the signal transduction pathway were investigated. Activation of AtMPK6 and AtMPK3 depended on upstream kinases sensitive to staurosporine. Similar results were observed with the tobacco homologues SIPK and WIPK [20]. In contrast, the 37 kDa MAP kinase activation by hypoosmotic shock was not affected by staurosporine, suggesting that this kinase may be involved in a different pathway from AtMPK6 and AtMPK3. In the present study, the use of EGTA, a calcium chelator, and gadolinium, an inhibitor of stretch-activated and calcium channels, led to the same effect. The activation of the three hypoosmotically activated MAP kinases was strongly reduced, as well as the activation of AtMPK6 by moderate hyperosmolarity. Coherently, SIPK and WIPK activation by hypoosmotic stress has been shown to be sensitive to gadolinium and EGTA [20] and the efficiency of gadolinium to prevent calcium influx has been demonstrated in tobacco cells [28]. Activation by high hyperosmolarity of the 37 and 42 kDa kinases was not affected by gadolinium, EGTA or staurosporine. This result suggests that the transduction pathways leading to these kinase activations do not imply calcium influx or kinase sensitive to staurosporine. These two kinases could belong to the SNF1-like or shaggy-like families and very few data are available on the possible involvement of such kinases in response to osmotic stress. In tobacco cells, Hoyos and Zhang [13] showed that the activation by hyperosmotic shock of HOSAK, a member of the SNF1-like family, did not depend on calcium influx, as we observed here for the 37 and 42 kDa hyperosmotically activated kinases. Concerning the tobacco homologues of SNF1 involved in osmoregulation, HOSAK activation seems to be associated with dephosphorylation [13] whereas Mikolajczyk et al. [14] demonstrated in vitro that an SNF1 homologue was inactivated by a phosphatase 2A.

As MAP kinases are activated by dual phosphorylation by MAP kinase kinases, we tested the involvement of MAP kinase kinases in osmotic response by using UO126, a MEK1/MEK2 inhibitor, which showed no effect on kinase activations (data not shown). Because of the wide phylogenetic distance between MEK1/MEK2 and plant MAP kinase kinases, osmotic transduction pathways may imply MAP kinase kinases insensitive to UO126. Yet two authors have reported signal transduction perturbation when UO126 was added: in one case, an increase of auxin-induced MAP kinase activation was observed in *Arabidopsis* seedlings [29] and in the other case, a decrease of harpin-induced MAP kinase activation in tobacco cells [30].

Finally, we observed different activation profiles depending on the strength of the osmotic shock, suggesting the existence of at least three osmosensing protein kinase pathways involved in signalling hypo- as well as moderate and high hyperosmotic stresses in *Arabidopsis* cell suspensions. Identification of kinase partners and substrates in an osmotic context, by transient expression of MAP kinase cascade components or coimmunoprecipitation, will provide important tools for elucidating molecular mechanisms of osmotic stress responses.

References

[1] Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. (1996) Proc. Natl. Acad. Sci. USA 93, 765–769.
[2] Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N. and Hirt, H. (1996) Proc. Natl. Acad. Sci. USA 93, 11274–11279.
[3] Covic, L., Silva, N.F. and Lew, R.R. (1999) Biochim. Biophys. Acta 21, 2–3.
[4] Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) Plant Cell 11, 1743–1754.
[5] Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) FEBS Lett. 427, 175–178.
[6] Liu, J.P., Ishitani, M., Halfter, U., Kim, C.S. and Zhu, J.K. (2000) Proc. Natl. Acad. Sci. USA 97, 3730–3734.
[7] Piao, H.L., Pih, K.T., Lim, J.H., Kang, S.G., Jin, J.B., Kim, S.H. and Hwang, I. (1999) Plant Physiol. 119, 1527–1534.
[8] Miyata, S., Urao, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) FEBS Lett. 437, 11–14.
[9] Popping, B., Gibbons, T. and Watson, M.D. (1996) Plant Mol. Biol. 31, 355–363.
[10] Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Plant J. 24, 655–665.
[11] Yuasa, Y., Ichimura, K., Mizoguchi, T. and Shinozaki, K. (2001) Plant Cell Physiol. 42, 1012–1016.
[12] Droillard, M.J., Thibivilliers, S., Cazalé, A.C., Barbier-Brygoo, H. and Laurière, C. (2000) FEBS Lett. 474, 217–222.
[13] Hoyos, M.E. and Zhang, S.Q. (2000) Plant Physiol. 122, 1355–1363.
[14] Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F. and Dobrowolska, G. (2000) Plant Cell 12, 165–178.

[15] Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Plant J. 20, 381–388.
[16] Monks, D.E., Aghoram, K., Courtney, P.D., DeWald, D.B. and Dewey, R.E. (2001) Plant Cell 13, 1205–1219.
[17] Romeis, T., Ludwig, A.A., Martin, R. and Jones, J.D.G. (2001) EMBO J. 20, 5556–5567.
[18] Jonak, C., Kiegerl, S., Lloyd, C., Chan, J. and Hirt, H. (1995) Mol. Gen. Genet. 248, 686–694.
[19] Takahashi, K., Isobe, M. and Muto, S. (1997) FEBS Lett. 401, 202–206.
[20] Cazalé, A.-C., Droillard, M.J., Wilson, C., Barbier-Brygoo, H. and Laurière, C. (1999) Plant J. 19, 297–307.
[21] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[22] Zhang, S., Du, H. and Klessig, D.F. (1998) Plant Cell 10, 435–449.
[23] Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K. and Neill, S.J. (2001) Plant Physiol. 126, 1579–1587.
[24] Kovtun, Y., Chiu, W.L., Tena, G. and Sheen, J. (2000) Proc. Natl. Acad. Sci. USA 97, 2940–2945.
[25] Nuhse, T.S., Peck, S.C., Hirt, H. and Boller, T. (2000) J. Biol. Chem. 275, 7521–7526.
[26] Jonak, C., Beisteiner, D., Beyerly, J. and Hirt, H. (2000) Plant Cell 12, 1467–1475.
[27] Halford, N.G. and Hardie, D.G. (1998) Plant Mol. Biol. 37, 735–748.
[28] Pauly, N., Knight, M., Thuleau, P., Graziana, A., Muto, S., Ranjeva, R. and Mazars, C. (2001) Cell Calcium 30, 413–421.
[29] Mockaitis, K. and Howell, S.H. (2000) Plant J. 24, 785–796.
[30] Lee, J., Klessig, D.F. and Nurnberger, T. (2001) Plant Cell 13, 1079–1093.