Protein kinases induced by osmotic stresses and elicitor molecules in tobacco cell suspensions: two crossroad MAP kinases and one osmoregulation-specific protein kinase

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Abstract Two protein kinases displaying mitogen-activated protein kinase (MAPK) properties are activated both by an hypoosmotic stress and by oligogalacturonides in tobacco cell suspensions [Cazalé et al. (1999) Plant J. 19, 297–307]. Using specific antibodies, they were identified as the salicylic acid-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK). The SIPK was also activated by an hyperosmotic stress, indicating that the same kinase may play a role both in hypo- and hyperosmotic signalling pathways, in addition to its involvement in the transduction of elicitor signals. Using immunoprecipitation followed by two-dimensional in-gel kinase assay, three molecular forms of the SIPK were observed, suggesting that additional modifications of the activated kinase may occur. In contrast to WIPK and SIPK, which are located at the crossroad of several transduction pathways initiated by elicitor or osmotic stimuli, a 44 kDa kinase, that would not belong to the MAPK family, appeared more specific to osmotic stress.

Key words: Mitogen-activated protein kinase; Osmotic stress; Tobacco

1. Introduction

Mitogen-activated protein kinases (MAPKs), which are found in all eukaryotes, constitute a large family of serine/threonine protein kinases activated via dual phosphorylation on threonine and tyrosine residues. In mammalian and yeast cells, MAPK modules, constituted of three functionally interlinked protein kinases, mediate responses to a variety of extracellular stimuli, including variations of environment osmolarity. In plants [1], a number of biotic and abiotic stimuli such as defence elicitors, wounding and osmotic stresses were reported to activate kinases displaying MAPK properties: calcium independence, ability to phosphorylate myelin basic protein (MBP), recognition by anti-mammalian MAPK antibodies and presence of phosphotyrosine residues in the active kinase. It was the case in tobacco leaves stressed by cutting [2] and in wounded [3] or UV-treated [4] tomato leaves. Similar MAPK activation was reported in studies concerning responses to pathogen attack. The bacterial protein harpin induces the activation of a 49 kDa kinase in tobacco [5] and of two 39 and 44 kDa kinases in *Arabidopsis thaliana* [6]. In tobacco, the fungal elicitor cryptogein induces the activation of two 46 and 50 kDa MAPKs [7]. Molecular identifications of these stimuli-induced MAPKs are still rare. The alfalfa MMK4 is activated in leaves submitted to wounding [8]. In parsley cells, a MMK4-related kinase is translocated to the nucleus upon activation by a peptide elicitor deriving from a *Phytophthora sojae* secreted protein, suggesting that it might interact with transcription factors that induce expression of defense genes [9]. On the other hand, two tobacco MAPKs, salicylic-induced protein kinase (SIPK, [10]) and wound-induced protein kinase (WIPK, [11]), have been shown to be activated in response to different signals. In addition to the salicylic acid induction [10], SIPK is activated by non-specific fungal elicitors [12], and both SIPK and WIPK are involved in the tobacco mosaic virus infection [13], in the elicitation by the avirulence factor Avr9 and the response to wounding [14,15].

Concerning osmotic signalling in plants, homologues of the yeast osmosensor two component system were recently cloned in *A. thaliana* [16]. Moreover, Mizoguchi et al. [17] observed interactions between one *Arabidopsis* MAPK, AtMPK4, and possible upstream kinases, using the two-hybrid analysis. Besides this first clue to the involvement of a MAPK cascade, very few enzymes of osmotic transduction cascades are identified in plant cells. Abscisic acid, which plays an important role in plant adaptation to drought and salinity, is able to activate a MAPK-related enzyme in barley aleurone protoplasts [18]. The tobacco Ntf4 is induced by pollen hydration [19] and the *Medicago* MAPK MMK4, which does not respond to high salt, is activated by drought [20]. In this last case, the transcript level of the *MMK4* gene increased after drought, without any detected variation of the corresponding protein amount. A 46 kDa MAPK of alfalfa cells called salt stress-inducible MAPK (SIMK) and corresponding to the *MMK1* gene was found to be activated by moderate hyperosmotic stress, while at severe osmotic stress, only an unidentified 38 kDa kinase is activated [21]. Interestingly, these authors observed that SIMK has a constitutively nuclear localization, indicating that its activation is not correlated with nucleo-cytoplasmic shuttling, as it is commonly admitted for other MAPKs. Among three protein kinases activated by hypoosmotic stress on BY2 tobacco cells, only the 50 kDa was phosphorylated on tyrosine upon activation [22]. Very

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Abbreviations: MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; SIPK, salicylic-induced protein kinase; WIPK, wound-induced protein kinase

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recently, a 48 kDa protein kinase induced by hyperosmotic stress on tobacco cells was identified as the SIPK [23].

In a recent work on tobacco cell suspensions, we have reported that two MAPKs with apparent molecular masses of 46 and 50 kDa are activated both by hypoosmotic stress and cell wall-derived oligogalacturonides (OGs). It was suggested that they may correspond to SIPK and WIPK. This hypothesis was tested in the present study, using highly specific anti-SIPK and anti-WIPK antibodies. On the other hand, two-dimensional in-gel kinase assay revealed the presence of three protein kinases with an apparent molecular mass of 50 kDa and differing by their isoelectrical point values [24]. It was thus investigated if these three kinases correspond to several forms of the same kinase or to three different enzymes. In addition, besides the early activated 46 and 50 kDa MAPKs, another protein kinase with an apparent molecular mass of 44 kDa was activated later after hypoosmotic stress, while a kinase of the same molecular weight was activated very early by hyperosmotic treatment [24]. The possible identity of the 44 kDa kinases induced respectively by hypo- and hyperosmotic stresses was investigated as well as the specificity of their involvement in osmoregulation.

2. Materials and methods

2.1. Plant material and treatments

Tobacco cells (*Nicotiana tabacum* cv. Xanthi) cultured as previously described [24] were used after 4 days subculturing. For osmotic treatments, cells were washed and equilibrated for 2 h in medium A which is isoosmotic to the culture medium (160 mOsm) and contained 10 mM Mes–Tris pH 5.2, 1 mM $CaSO_4$ and 150 mM sucrose. Afterwards, extracellular medium was replaced either by the same volume of hypoosmotic medium (sucrose-free medium A, 15 mOsm), or hyperosmotic medium (medium A brought to 500 mM sucrose, 640 mOsm), or fresh isoosmotic medium. The final osmotic strengths of extracellular media after transfer of the cells were about 40 and 600 mOsm for hypo- and hyperosmotic conditions, respectively. Osmolarity was monitored using a freezing point osmometer (Roebling, Berlin, Germany). For elicitation treatment, 15 μg ml^{-1} OGs was added to the 2 h isoosmotic-equilibrated cells. To stop treatments at indicated times, cell suspensions were filtered, frozen in liquid nitrogen and stored at −80°C until use.

2.2. Preparation of protein extracts

Cells were ground in liquid nitrogen and homogenized at 4°C in extraction buffer (100 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM *ortho*-vanadate, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} antipain) and centrifuged at 100 000 × *g*. The supernatant was precipitated in 10% (w/v) TCA solution containing 20 mM NaF, washed twice with 80% (v/v) cold acetone and resuspended either in sodium dodecyl sulfate (SDS)–PAGE sample buffer or in isoelectric focusing (IEF) sample buffer containing 9 M urea, 2% (w/v) CHAPS, 0.4% (v/v) Triton X-100, 15 mM DTT, 1% (v/v) Pharmalytes pH 3–10 (Pharmacia), 10 mM NaF and 8 mM PMSF. The protein concentration was determined by the Bradford method with bovine serum albumin as standard [25], modified according to Ramagli and Rodriguez [26] for protein quantification in IEF extracts.

2.3. In-gel kinase assay

Protein extracts (20 μg) were electrophoresed on 10% SDS–polyacrylamide gels embedded with 0.2 mg ml^{-1} MBP or 0.5 mg ml^{-1} casein as substrates for the kinases. The gels were then treated for washing and renaturing steps as described by Zhang et al. [12]. For the activity, the gels were preincubated 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 *ortho*-vanadate), then 1 h in 5 ml of the same buffer added with 25 μM cold ATP and 1.8 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 PhosphorImager (Molecular Dynamics, Evry, France).

For two-dimensional electrophoresis, 50 μg of proteins was loaded on a 7 cm ready-made Immobiline Drystrip pH 4–7 (Pharmacia) for IEF, followed by a 10% SDS–polyacrylamide gel embedded with MBP [24] for the in-gel kinase assay.

2.4. Antibody production

The peptides: MDGSGQQTDTMMSDAGAEQPPTAP and MADANMGAGGGQFPDFPS, corresponding to the N-terminus of SIPK and WIPK, respectively [12,27], were synthesized and conjugated to keyhole limpet hemacyanin carrier (Neosystem, Strasbourg, France). Polyclonal antibodies were raised in rabbits and purified on an activated thiol Sepharose 4B chromatography column (Pharmacia) covalently bound with the corresponding peptide where additional cysteine residue was reduced.

2.5. Immunoblotting

SDS–PAGE or two-dimensional electrophoresis separated proteins were electroblotted onto Immobilon membrane. The blot was blocked with 10% (w/v) defatted milk in Tris buffer salt (TBS) (10 mM Tris–HCl pH 7.5, 154 mM NaCl, 0.1% (v/v) Tween 20), probed either with human 1:2000 polyclonal MAPK (ERK1/ERK2) antibody (Sigma BioSciences) or with anti-SIPK or anti-WIPK antibodies (1 μg ml^{-1} for SIPK, 2 μg ml^{-1} for WIPK, final concentration in TBS buffer). Corresponding SIPK and WIPK peptides (around 40 μM) were used for competition. After incubation in alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad) in TBS, antigenic polypeptides were visualized using the NBT/BCIP kit (Bio-Rad) in Tris–acetate buffer.

2.6. Immunoprecipitation

Immunoprecipitation assays were performed either with anti-SIPK or anti-WIPK antibodies. The 100 000 × *g* protein extract (100 μg) was incubated with 5 μg anti-SIPK or 10 μg anti-WIPK in immunoprecipitation buffer (25 mM HEPES pH 7.5, 5 mM EGTA, 5 mM EDTA, 5 mM DTT, 60 mM β-glycerophosphate, 0.1 mM *ortho*-vanadate, 10 mM NaF, 1 mM PMSF, 5 μg ml^{-1} leupeptin and antipain, 150 mM NaCl, 0.5% (v/v) Triton X-100, 0.5% (v/v) NP40) for 2 h. Then 30 μl of 50% Protein A-Sepharose CL4B (Sigma) was added and incubation continued for another hour. The immunoprecipitate was washed three 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 *ortho*-vanadate), and resuspended either in SDS–PAGE sample buffer then boiled 3 min at 95°C, or in IEF buffer added with 10 mM NaF and 0.1% SDS. Supernatant fractions were electrophoresed on two-dimensional electrophoresis or SDS–polyacrylamide gels embedded with MBP for in-gel kinase assay as previously described. For two-dimensional electrophoresis, immunoprecipitates were prepared using 2.5-fold more of protein extract and antibodies.

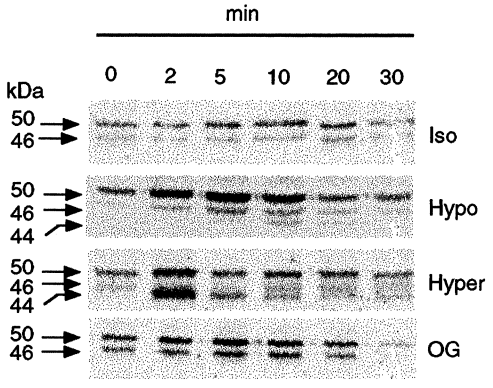

Fig. 1. Time course activation of MBP kinases in response to osmotic stresses or OG treatment. Aliquots of cell suspension were equilibrated for 2 h in isoosmotic medium and then either transferred to isoosmotic (Iso), hypoosmotic (Hypo) or hyperosmotic (Hyper) medium, or added with 15 μg ml^{-1} OGs at zero time. Treatments were stopped at indicated times. Kinase activity was determined with an in-gel kinase assay using MBP as a substrate.

3. Results

3.1. 44 kDa protein kinase(s) are activated by osmotic stresses whereas 50 and 46 kDa kinases are both induced by osmotic and elicitor signals

In-gel kinase assays using MBP as a substrate were performed to investigate the protein kinases activated by osmotic or elicitor signals (Fig. 1). When cell suspensions were transferred to hypoosmotic medium (Hypo), three protein kinases with apparent molecular masses of 50, 46 and 44 kDa were transiently activated and displayed different kinetics. The activation of 50 kDa and 46 kDa protein kinases occurred 2 min after the onset of hypoosmotic stress, peaked at 5 min and then decreased to reach the basal level at 30 min. The transient activation of 44 kDa protein kinase occurred later, 10 min after hypoosmotic stress and rapidly decreased to be very slight after 20 min. In previous sets of experiments, this 44 kDa activation was already observed, but only 30 min after transfer in hypoosmotic conditions [24]. Transfer to isoosmotic medium (Iso) resulted in a slight, delayed activation of 50 and 46 kDa kinases, whereas the 44 kDa protein did not show any activity during this time course. Transfer of suspension cells to 640 mOsm hyperosmotic medium (Hyper) resulted in an early and transient activation of 50 and 44 kDa protein kinases. For both proteins, the activation peaked at 2 min and decreased thereafter. Treatment of the cells with OGs resulted in a transient activation of 50 and 46 kDa kinases which peaked at 5 min and then decreased.

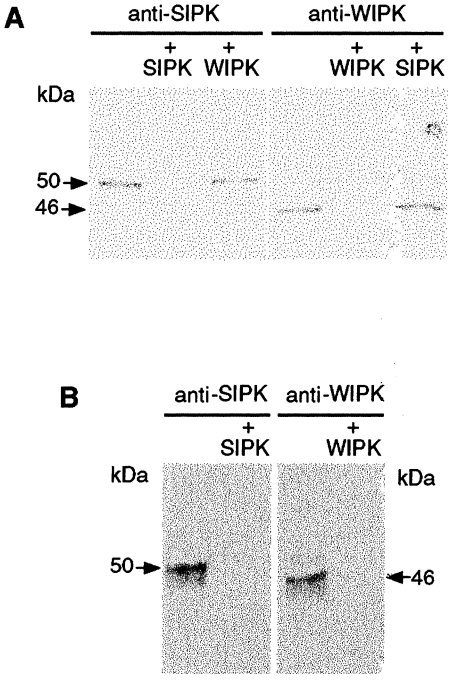

Fig. 2. Immunochemical detection of SIPK and WIPK using specific antibodies. Equilibrated cells were transferred for 5 min to hypoosmotic medium. Anti-SIPK and anti-WIPK antibodies were used for Western blotting (A) or immunoprecipitation (B), in the presence of N-terminal SIPK or WIPK peptides, as competitors, when indicated. In B, kinase activity of the immunocomplex was subsequently assayed with an MBP in-gel kinase assay.

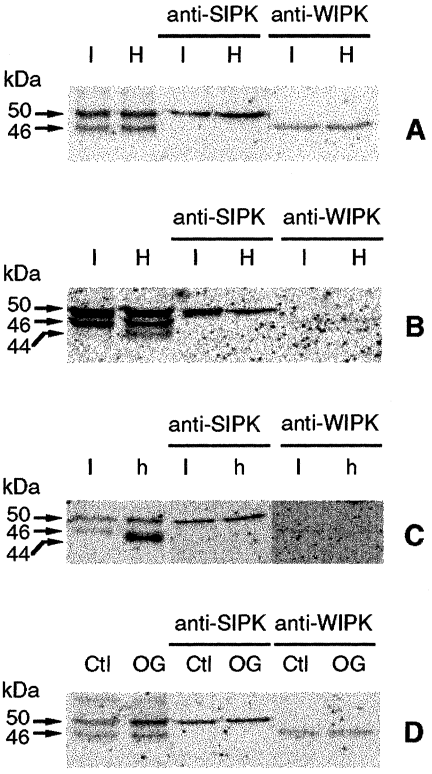

Fig. 3. Identification of SIPK and WIPK among the different kinases activated by osmotic or elicitor signals. In A and B, equilibrated cells were transferred to isoosmotic (I) or hypoosmotic (H) medium for 5 min (A) or 10 min (B). In C, equilibrated cells were transferred to isoosmotic (I) or hyperosmotic (h) medium for 2 min. In D, 15 μg ml^{-1} OGs was added for 5 min to the equilibrated cells, Ctl is the corresponding control without OG. When indicated, 5 μg anti-SIPK or 10 μg anti-WIPK antibodies were used to immunoprecipitate 100 μg of protein extract and kinase activity of the immunocomplex was subsequently assayed with an MBP in-gel kinase assay. Kinase activity of the corresponding initial extracts is presented in the left part of each gel.

3.2. The 50 kDa and 46 kDa protein kinases are identified as SIPK and WIPK, respectively

The hypoosmotically activated 50 and 46 kDa protein kinases were previously identified as MAPKs [24]. Using two-dimensional electrophoresis, the same kinases were shown to be induced by salicylic acid and OGs, suggesting that they could be related to the tobacco SIPK and WIPK. To test this hypothesis, antibodies specific for the unique N-terminal amino acid sequences of the two proteins were prepared. In Western blot analysis (Fig. 2A), the anti-SIPK antibody recognized a band with an apparent molecular mass of 50 kDa and the anti-WIPK antibody a band corresponding to 46 kDa. No binding was observed in the presence of the corresponding N-terminal peptide as competitor, while the use of the other peptide did not affect the detection. These two antibodies were able to immunoprecipitate (Fig. 2B) the two kinases, the 50 kDa kinase when anti-SIPK was used and the 46 kDa kinase with anti-WIPK antibody. No activity was detected when the corresponding peptide was added as competitor. The two antibodies, highly specific for WIPK and SIPK, were then used to identify these MAPKs among the kinases activated by the different signals (Fig. 3). The anti-SIPK antibody immunoprecipitated the 50 kDa kinase activated by hypoosmotic (Fig. 3A), hyperosmotic (Fig. 3C) and OG (Fig.

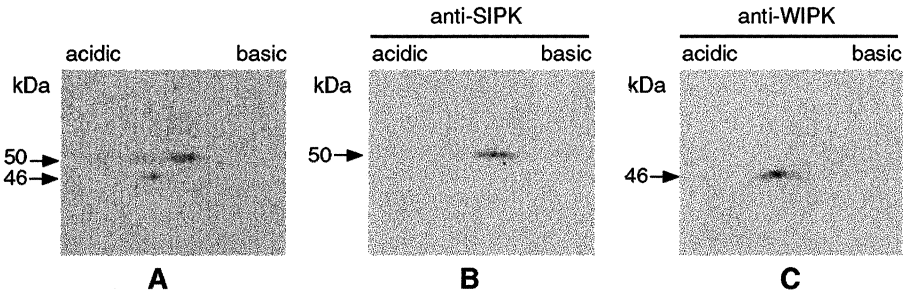

Fig. 4. Analysis of the polymorphism of the 50 and 46 kDa kinases by using anti-SIPK and anti-WIPK antibodies. Equilibrated cells were transferred to hypoosmotic medium for 5 min. Initial extract (50 μg, A) or immunocomplexes (B and C) were analyzed by two-dimensional in-gel kinase activity assay using MBP as a substrate. In B and C, 250 μg of protein extract was immunoprecipitated with either 12.5 μg anti-SIPK (B) or 25 μg anti-WIPK (C) antibodies.

3D) signals. The anti-WIPK antibody immunoprecipitated the 46 kDa protein activated by hypoosmotic (Fig. 3A) and OG (Fig. 3D) signals. It should be observed that in the illustrated experiment, the 50 and 46 kDa kinases, activated by 5 min hypoosmotic stress (Fig. 3A), have decreased to the corresponding isoosmotic level of activation after 10 min of the same stress (Fig. 3B). The 44 kDa protein slightly activated after 10 min of hypoosmotic stress (Fig. 3B) or early and strongly activated after 2 min of hyperosmotic stress (Fig. 3C) was not immunoprecipitated by any of these two antibodies. Thus, the 50 kDa kinase activated by osmotic and OG signals was identified as the SIPK and the 46 kDa activated by hypoosmotic and OG signals as the WIPK.

3.3. The three 50 kDa MAPKs induced by osmotic and elicitor treatments are identified as SIPK

Protein kinase activity resolved in an MBP two-dimensional in-gel assay revealed several 50 kDa spots and one 46 kDa spot activated by hypoosmotic or elicitor treatments [24]. In the present study, the 50 kDa kinase was identified as SIPK by a mono-dimensional electrophoresis technique (Fig. 3). The question thereafter was to determine if the three 50 kDa spots correspond to the same protein kinase or to three different kinases. Two-dimensional in-gel kinase assay (Fig. 4) shows that anti-SIPK antibody immunoprecipitates the three 50 kDa spots (Fig. 4B) observed in the initial extract (Fig. 4A). This result clearly indicates that the three 50 kDa kinases, differing by their isoelectrical points, are all identified as SIPK. The 46 kDa kinase spot was confirmed here (Fig. 4C) by immunoprecipitation and two-dimensional in-gel kinase assay to be a WIPK.

3.4. The hyperosmotically activated 44 kDa kinase is related to the hypoosmotically activated one and would not belong to the MAPK family

The question of the possible identity of the hypoosmotically and hyperosmotically activated 44 kDa kinases was investigated. In-gel kinase assays after two-dimensional electrophoresis of extracts from hyper- or hypoosmotically treated cells showed comparable patterns (Fig. 5A). In addition to the three SIPK spots and the WIPK spot, activity corresponding to the 44 kDa protein kinase was visualized. However, the 44 kDa protein was not resolved in distinct spots and showed an activity streak corresponding to a large pH range; lower loading on two-dimensional gel did not improve this separation (data not shown). The 44 kDa kinase activity was very high after hyperosmotic stress (Fig. 5A), confirming the results previously observed (Figs. 1 and 3C). When the mono-dimensional in-gel kinase assay was performed with casein as a substrate (Fig. 5B), only the 44 kDa protein kinase was activated, very strongly in 2 min hyperosmotically treated cells and slightly in 10 min hypoosmotically treated cells. Thus, this kinase phosphorylates MBP and casein as substrates. A 50 kDa band phosphorylated casein but did not show activation with osmotic stresses, as it was observed with this substrate in our previous paper [24]; therefore, this protein does not correspond to the activated 50 kDa SIPK observed in MBP gels. Anti-human ERK1/ERK2 antibody was previously shown to immunoprecipitate the hypoosmotically induced 50 and 46 kDa kinases [24]. Western blot analysis using this antibody did not allow the detection of any band with an apparent molecular mass lower than 46 kDa (Fig. 5C), indicating that the activated 44 kDa kinase would not belong to the ERK–MAPK family.

4. Discussion

In tobacco cell suspension submitted to osmotic stresses, several protein kinases with apparent molecular masses of 44, 46 and 50 kDa were activated. In contrast to the 46 and 50 kDa MAPKs, no activation of the 44 kDa protein kinase by OGs was observed (Fig. 1), suggesting that the 44 kDa protein(s) may be specific to osmoregulation. In hyperosmotically treated cells, a 44 kDa kinase was strongly and early activated, with a stimulation peak after 2 min. When hypoosmotic stress was applied, a 44 kDa kinase was also transiently activated, but slightly and later, with a maximal stimulation after 10 min. Thus, these two activations displayed very distinct time kinetics and intensity, depending on the nature of osmotic stress. Meanwhile, the 44 kDa kinase activities detected after hypo- or hyperosmotic stress displayed high similarities (Fig. 5): two-dimensional electrophoresis pattern, absence of labelling by anti-ERK1/ERK2 antibody and ability, in contrast to the 46 and 50 kDa MAPKs, to phosphorylate the casein substrate in addition to MBP. Thus they probably correspond to the same 44 kDa kinase, which would be stimulated during the early shrinking period of hyperosmotic signalling and also during the regulatory cell volume decrease occurring after hypoosmotic stress and following the early swelling step. The 44 kDa kinase, although active in the absence of calcium and able to phosphorylate MBP (Fig. 1), displayed several properties which are not shared by MAPKs, like the use of casein as a substrate and the lack of recognition by anti-mammalian MAPK antibody already mentioned. In

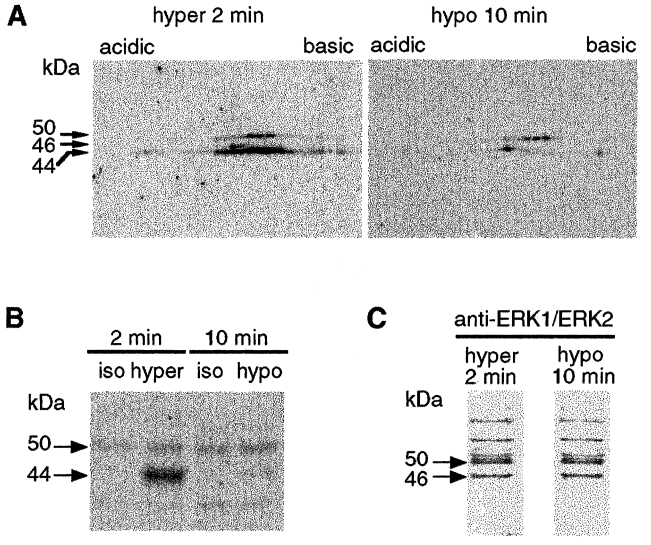

Fig. 5. Comparison of the 44 kDa kinases activated by hypoosmotic or hyperosmotic stresses. Equilibrated cells were transferred either for 2 min to hyperosmotic medium or 10 min in hypoosmotic medium. In A, kinase activity was determined with an MBP in-gel kinase assay after two-dimensional electrophoresis of 50 μg protein extract. In B, kinase activity was performed with an in-gel kinase assay using casein as a substrate. In C, immunoblot analysis was performed with 1:2000 dilution of the anti-human ERK1/ERK2 antibody.

addition, anti-phosphotyrosine antibody failed to immunoprecipitate the 44 kDa kinase strongly activated in hyperosmotically treated cells, although the 50 kDa kinase was specifically retained (data not shown). These data suggest that the 44 kDa kinase does not belong to the MAPK family and may be related to the 42 kDa kinase very recently shown to be activated by saline stress in BY2 tobacco cells [23]. According to these authors, the kinase requires serine/threonine, but not tyrosine phosphorylation for activation and would be a homolog of *Arabidopsis* serine/threonine kinase 1, a member of the SNF1 kinase family.

The 50 and 46 kDa MAPKs previously shown to be activated by hypoosmotic and OG signals in tobacco cells [24] were identified in the present study to the SIPK and WIPK, respectively, using specific antibodies which were raised against peptides corresponding to the N-terminus of these kinases. The difference in the estimated molecular masses of SIPK (48 kDa) and WIPK (44 kDa) reported by Zhang and Klessig [13] with the 50 and 46 kDa estimated masses observed here is likely to be due to the different pre-stained molecular weight markers used. In-gel two-dimensional kinase assay showed the activation, by hypoosmotic and OG signals, of three 50 kDa kinases distinct by their charge and one 46 kDa kinase [24]. In the present study, immunoprecipitation analysis confirms that the 46 kDa is the WIPK and indicates that all three 50 kDa kinases are recognized by the anti-SIPK antibody (Fig. 4). Although the hypothesis of three different kinase species may be discarded, it cannot be excluded that they are three isoforms with a very similar N-terminus sequence as it is for example the case for the tobacco MAPKs, Ntf4 and SIPK. It can also be proposed that phosphorylation events, additional to the dual activating phosphorylation, modify the isoelectric points of SIPK. Microsequencing and/or mass spectrometry would allow us to investigate these two hypotheses.

SIPK and WIPK have already been shown to be activated by various stimuli such as salicylic acid, tobacco mosaic virus, avirulence factor and wounding [10,13–15]. Thus our results enlarge the role of SIPK and WIPK, by their involvement in OG, osmotic as well as mechanical signalling, since mechanical induction of 50 and 46 kDa has previously been observed [24]. In contrast to WIPK, the SIPK was also activated when tobacco cells are transferred to hyperosmotic medium (Fig. 1). This result is in good agreement with the recently reported activation of SIPK, but not WIPK, by saline stress or high concentration of sorbitol in BY2 tobacco cells [23]. These results may be compared to the data concerning the alfalfa SIMK (originally named MsK7 and MMK1), highly related to the tobacco SIPK, which was activated by hyperosmotic stress [21], indicating that SIPK-type kinases respond in a similar way in different plants submitted to hyperosmotic stress. Munnik et al. [21] indicate that, in alfalfa, two distinct kinase pathways are able to distinguish between moderate and extreme hyperosmotic conditions, with the SIMK and an unidentified 38 kDa being activated respectively in these conditions. These two hyperosmolarity-sensing pathways were not observed in tobacco cells, where two kinases were activated, whatever the saline stress intensity [23]. For this comparison between two plants, it will be also interesting to know if the alfalfa SIMK, like the tobacco SIPK, may be induced by different signals, as it is likely, since mechanical stress activates the SIMK [21].

The tobacco WIPK is induced by hypo- but not by hyperosmolarity and this kinase can be compared to the yeast MAPK, MPK1, which is required for adaptation to hypoosmotic environment, while the other MAPK, HOG1, is activated under hyperosmolar conditions [28]. On the other hand, SIPK and WIPK appear located at the crossroad of a complex signalling network. It can be speculated that the duration and level of the activation of a multisignalling MAPK would regulate its output for cellular response. It is the case in mammalian cells, where the output of SAPK/JNK or p38 kinase has been shown to be regulated via the duration and the activation level of the respective MAPKs [29]. Hypoosmotic stress and elicitors of defence reactions, which both activate SIPK and WIPK with comparable kinetics, would probably share common transduction steps, like Ca^{2+} influx and phosphorylation events [7,14,30]. However, the MAPK activation by a defence reaction elicitor was shown to be independent of the oxidative burst induction [14], while activated MAPKs were suggested to be upstream of the burst response to hypoosmotic stress [24], underlying the presence of distinct signalling steps. In other respects, no oxidative burst was observed in hyperosmotically stressed tobacco cells [30] distinguishing once more that signal from defence and hypoosmotic stresses. Activations of SIPK and the 44 kDa kinase are induced both by hypoosmotic and hyperosmotic stresses, with comparable kinetics for the two enzymes in each condition. It would be very interesting to determine in future studies if the two enzymes are located within a same osmotic transduction pathway and how they are connected. More generally, the present results provided evidence on the importance in hyperosmotic and hypoosmotic signalling of respectively two and three protein kinases, which represent very useful elements in the identification of the partners of corresponding pathways.

References

[1] Jonak, C., Ligterink, W. and Hirt, H. (1999) Cell. Mol. Life Sci. 55, 204–213.
[2] Usami, S., Banno, H., Ito, Y., Nishihama, R. and Machida, Y. (1995) Proc. Natl. Acad. Sci. USA 92, 8660–8664.
[3] Stratmann, J.W. and Ryab, C.A. (1997) Proc. Natl. Acad. Sci. USA 94, 11085–11089.
[4] Stratmann, J.W., Stelmach, B.A., Weller, E.W. and Ryan, C.A. (2000) Photochem. Photobiol. 71, 116–123.
[5] Adam, A., Pike, S., Hoyos, E., Stone, J.M., Walker, J.C. and Novacky, A. (1997) Plant Physiol. 115, 853–861.
[6] Desikan, R., Clarke, A., Atherfold, P., Hancock, J.T. and Neil, S.J. (1999) Planta 210, 97–103.
[7] Lebrun-Garcia, A., Ouaked, F., Chiltz, A. and Pugin, A. (1998) Plant J. 15, 773–781.
[8] Bögre, L., Ligterink, W., Meskiene, I., Barker, P.J., Heberle-Bors, E., Huskisson, N.S. and Hirt, H. (1997) Plant Cell 9, 75–83.
[9] Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H. and Scheel, D. (1997) Science 276, 2054–2057.
[10] Zhang, S. and Klessig, D.F. (1997) Plant Cell 9, 809–824.
[11] Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. (1995) Science 270, 1988–1992.
[12] Zhang, S., Du, H. and Klessig, D.F. (1998) Plant Cell 10, 435–449.
[13] Zhang, S. and Klessig, D.F. (1998) Proc. Natl. Acad. Sci. USA 95, 7433–7438.
[14] Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H. and Jones, J.D. (1999) Plant Cell 11, 273–288.
[15] Seo, S., Sano, H. and Ohashi, Y. (1999) Plant Cell 11, 289–298.
[16] Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) Plant Cell 11, 1743–1754.
[17] Mizoguchi, T., Ichimura, K., Irie, K., Morris, P., Giraudat, J., Matsumoto, K. and Shinozaki, K. (1998) FEBS Lett. 437, 56–60.
[18] Knetsch, M.L.W. (1996) The Plant Cell 8, 1061–1067.
[19] Wilson, C., Voronin, V., Touraev, A., Vicente, O. and Heberle-Bors, E. (1997) Plant Cell 9, 2093–2100.
[20] Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N. and Hirt, H. (1996) Proc. Natl. Acad. Sci. USA 93, 11274–11279.
[21] Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Plant J. 20, 381–388.
[22] Takahashi, K., Isobe, M. and Muto, S. (1997) FEBS Lett. 401, 202–206.
[23] Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F. and Dobrowolska, G. (2000) Plant Cell 12, 165–178.
[24] Cazalé, A.-C., Droillard, M.J., Wilson, C., Barbier-Brygoo, H. and Laurière, C. (1999) Plant J. 19, 297–307.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Ramagli, L.S. and Rodriguez, L.V. (1985) Electrophoresis 6, 559–563.
[27] Zhang, S. and Klessig, D.F. (1998) Proc. Natl. Acad. Sci. USA 95, 7225–7230.
[28] Kültz, D. and Burg, M. (1998) J. Exp. Biol. 201, 3015–3021.
[29] Nishina, H. et al. (1997) Nature 385, 350–353.
[30] Cazalé, A.-C., Rouet-Mayer, M.-A., Barbier-Brygoo, H., Mathieu, Y. and Laurière, C. (1998) Plant Physiol. 116, 659–669.