FEBS Letters 574 (2004) 42–48 FEBS 28740

Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of *Arabidopsis thaliana*: activation by hypoosmolarity and negative role in hyperosmolarity tolerance

Marie-Jo Droillard, Marie Boudsocq, Hélène Barbier-Brygoo, Christiane Laurière*

Institut des Sciences du Végétal, UPR 2355, CNRS, 1 av. de la terrasse, 91198 Gif s/Yvette Cedex, France

Received 15 July 2004; revised 30 July 2004; accepted 3 August 2004

Available online 11 August 2004

Edited by Ulf-Ingo Flügge

Abstract Three of the protein kinases activated by hypoosmotic stress in *Arabidopsis thaliana* cell suspensions were previously characterized [FEBS, 2002, 527, 43–50] as mitogen-activated protein (MAP) kinases and two of them corresponded to *Arabidopsis* mitogen-activated protein kinase 6 (MPK6) (44 kDa) and MPK3 (39 kDa). The third MAP kinase was identified here to MPK4, using a corresponding specific antibody. Like MPK6 and MPK3, MPK4 activity is clearly inhibited by apigenin and MPK4 activation by hypoosmolarity needs upstream phosphorylation events. Activation of the 3 MAP kinases, MPK3, 4 and 6, was confirmed in plantlets submitted to hypoosmotic stress. The action of a biotic signal, flagellin, was also demonstrated to induce the activations of the 3 MAP kinases. Using the mutant displaying *MPK4* gene inactivation, the independence of the MPK3 and MPK6 activations towards the presence of MPK4 was demonstrated, both in hypoosmotic and flagellin signalling pathways. Although MPK4 was not activated by hyperosmolarity in cell suspensions nor in seedlings, a possible negative regulation of hyperosmolarity resistance by MPK4 is suggested, based both on phenotype and downstream gene expression studies.

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

1. Introduction

Mitogen-activated protein (MAP) kinases, through sequential activation of phosphotransfer reactions, play essential roles in the intracellular signal transduction processes of eukaryotes. The regulation of yeast and animal MAP kinases has been well characterized, but the analysis of plant MAP kinase cascades is slowed down by the larger number of MAP kinases identified in plants, partly due to important redundancy, and the low amount of known mutations affecting a plant MAP kinase. More than fifty terminal components of the MAP kinase module have been cloned from different plant species [1]. Among them, the twenty putative MAP kinases of *Arabidopsis thaliana* can be classified into four groups, the D group displaying the TDY phosphorylation motif instead of TEY for the other MAP kinases. The high degree of sequence similarity may be assumed to reflect a functional conservation between plant species.

Several works have suggested a role for MAP kinase cascades in disease resistance, partly based on the demonstration that specific MAP kinases become activated during plant responses to pathogens [2, for review]. Less information is available concerning a putative role of MAP kinase cascades in the perception of osmotic stresses. In plant stresses related to hyperosmolarity like drought or salinity, the involvement of several components of the MAP kinase module has however been suggested, based on transcript accumulation, functional complementation of yeast mutants or post-translational activation. The role in drought or salinity transduction was documented for the MAP kinase kinase kinase AtMEKK1 [3,4] and for several MAP kinases belonging to the A group, PsMAPK [5], MsMMK4 [6], MsMMK1 [7], NtSIPK [8–10], MPK3 [3], MPK6 [11], and OsMAPK5 [12]. For the perception of hypoosmolarity, activations of the two A group MAP kinases SIPK and WIPK from tobacco [8], MPK3 and MPK6 from *Arabidopsis* [11] were reported. However, the involvement of a more distant MAP kinase belonging to the B group, alfalfa MMK2, was also suggested by the specific replacement of the yeast MPK1 kinase, a MAP kinase belonging to the hypoosmolarity MAP kinase cascade [13]. Some information begins also to be available concerning other components of the osmosignalling MAP kinase cascades, underlying the complexity of the regulation process of MAP kinases by protein phosphatases or by other protein kinases. For example, the *Arabidopsis* MAP kinase MPK6 was demonstrated to be regulated in vivo by MAP kinase phosphatase 1, this protein phosphatase being itself involved in salt stress responses [14]. Overexpression of *Arabidopsis* nucleoside diphosphate (NDP) kinase 2 enhances the tolerance of *Arabidopsis* plants to salt or cold stresses and this kinase specifically interacts with MPK3 and MPK6 [15]. The MAP kinase kinase AtMEK1 from seedlings submitted to abiotic stresses like drought and high salt showed an increased kinase activity towards the substrate MPK4 [16].

Previous work in the laboratory has shown that several Ca^{2+}-independent protein kinases are rapidly activated by hypoosmotic stress, moderate or high hyperosmolarity

* Corresponding author. Fax: +33-1-69823768.
E-mail address: Christiane.Lauriere@isv.cnrs-gif.fr (C. Laurière).

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

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.001

induced by different osmolytes in cell suspensions [11]. Among them, some kinases do not belong to the MAP kinase family, but three were demonstrated to be MAP kinases. Two of them were previously identified to be MPK3 and MPK6 but the third MAP kinase, which represents a part of the protein kinase activity visualized around 37 kDa, remains to be identified. The hypothesis that MPK4, which belongs to the B group, may represent the third MAP kinase activated by hypoosmolarity was tested here both on cell suspensions and plantlets, using specific antibodies and a mutant showing *MPK4* inactivation, *mpk4*, [17]. Also based on the *mpk4* characterization, the independence of the MPK6 and MPK3 activations towards the presence of MPK4 is shown. Finally, a possible negative regulation of hyperosmolarity tolerance by MPK4 is suggested.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana cell suspensions (Columbia ecotype) cultured as previously described [11] were used after 5 days subculturing with 100 mg fresh weight/ml cell density. *Arabidopsis thaliana* seeds (Landsberg erecta ecotype), wild-type and homozygous mutant *mpk4*, were selected from the transposant line characterized by Petersen et al. [17] and provided by the Nottingham Stock Center. Seedlings were grown on solid medium containing 5 mM KNO_3, 2.5 mM K_2HPO_4/KH_2PO_4, pH 6, 2 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 mM MES, 50 μM Fe-EDTA, Murashige and Skoog micro-elements [18], 7 g/l agar and 30 or 130 mM sucrose depending on the type of stress applied. The culture conditions were as described by Frachisse et al. [19].

2.2. Cell suspension 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 or 1000 mM sucrose). In indicated cases, sucrose was replaced by 250 or 650 mM NaCl to get, respectively, similar moderate (500 mOsm) or high (1000 mOsm) hyperosmolarities. Osmolarity was monitored using a freezing point osmometer (Roebling, Berlin, Germany). For treatment with staurosporine, this inhibitor was added during the last 10 min of the equilibration time and during 2 min after transfer to hypoosmotic medium, at the concentration of 0.5 μM. For elicitor treatment, flagellin peptide (flg 22) synthesized by F. Fisher (Friedrich Miescher-Institute, Basel, Switzerland), was used at the concentration of 100 nM added to equilibrated cells during 2–60 min. To stop treatments at indicated times, cell suspensions were filtered, frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Seedling treatments

For osmotic or elicitor treatments, plantlets were cultured either for 9 days on the solid 130 mM sucrose medium for subsequent hypoosmotic stress or for 6 days on the solid 30 mM sucrose medium for subsequent hyperosmotic or elicitor treatments. They were then equilibrated on the corresponding liquid medium for 4 h. After equilibration, plantlets were transferred in a liquid medium deprived of sucrose (hypoosmotic stress), containing 500 or 1000 mM sucrose (hyperosmotic stresses) or containing 100 nM flg 22. In each case, control plantlets were prepared in the same way, except that they were transferred in the medium already used for equilibration. The osmotic treaments were performed during 10 min (for in gel kinase assays) or for 3 h (for Northern blot analysis). To stop treatments, seedlings were frozen in liquid nitrogen and stored at −80 °C until use.

For germination and growth studies on hyperosmotic medium, seeds were sown either on 30 (control), 130 or 200 mM sucrose medium. Part of *mpk4* seeds abnormally wrinkled, thus only swollen normal seeds were sown for that experiment. Three petri-dishes with 30 seeds each were prepared for each condition. The germination rate was evaluated after 2 days and the root length after 9 days.

2.4. Preparation of protein extracts

Cells or seedlings were ground in liquid nitrogen, homogenized at 4 °C in extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM *ortho*-vanadate, 10 mM NaF, 60 mM β-glycerophosphate, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin, and 5 μg/ml antipain) and centrifuged at $17\,600 \times g$ at 4 °C. Protein concentration of the supernatant was determined by the Bradford method with bovine serum albumin as standard [20].

2.5. SDS–PAGE in-gel kinase assay

Protein extracts (20 μg) in SDS–PAGE sample buffer were electrophoresed on 10% SDS–polyacrylamide gels embedded with 0.2 mg/ml myelin basic protein (MBP) as a substrate for the kinases. The gels were then treated for washing and renaturating steps as described by Zhang et al. [21]. For the activity, the gels were preincubated 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, and 0.1 mM *ortho*-vanadate), then incubated for 1 h in 8 ml of the same buffer added with 25 μM cold ATP and 2.9 MBq ^{33}P-ATP per gel. Then, the gels were washed extensively in 5% (w/v) TCA and 1% (w/v) disodium-pyrophosphate solution. The protein kinase activity was revealed on the dried gels by Storm imaging system (Molecular Dynamics). For treatments with kinase inhibitors, the activity buffer was completed with 500 μM apigenin or 1.5 μM staurosporine during pre-incubation and incubation steps.

2.6. Northern blot analysis

Total RNAs were extracted from seedlings using NucleoSpin RNA plant isolation kit (Macherey-Nagel). Total RNAs (20 μg/lane) were separated by 1.2% agarose formaldehyde gel electrophoresis and transferred to positive TM membrane (Q-BIOgene) in 10× SSC. The membranes were hybridized at 65 °C with specific cDNA region of actin probe to ensure equal loading, then reprobed with specific cDNA region of either RAB18 or RD29A. The probes were labelled with ^{32}P-dCTP using Megaprime DNA labelling system (Amersham Biosciences). The hybridization signals were revealed and quantified by Storm imaging system (Molecular Dynamics).

2.7. Immunoprecipitation

Immunoprecipitation assays were performed with three polyclonal *Arabidopsis* MAP kinase antibodies. They were raised against the 16 N-terminal amino acids of MPK4 (CFGSSGDQSSSKGVAT) and as previously described [11] for MPK3 and MPK6. Protein extract (400 μg for MPK4 antibody and 200 μg for the two others) was incubated with either 30 μg anti-MPK4, 10 μg anti-MPK3 or 10 μg anti-MPK6 in immunoprecipitation buffer [11] for 3 h. Then, the protein A–Sepharose immunoprecipitate was washed several times and resuspended in SDS–PAGE sample buffer before being electrophoresed in SDS–polyacrylamide gels embedded with MBP for in-gel kinase assay as earlier described.

3. Results

3.1. MPK4 is the third hypoosmotically activated MAP kinase in cell suspensions

When cells were transferred to hypoosmotic medium (Fig. 1A, lanes 1 and 2), three protein kinases with apparent molecular masses of 44, 39 and 37 kDa were activated. Using specific antibodies, two of these kinases have already been identified as the MAP kinases MPK6 and MPK3 corresponding, respectively, to the 44 and 39 kDa proteins [11]. The activation by hypoosmolarity of the 37 kDa kinase was partially prevented by the action of a tyrosine specific phosphatase, indicating that at least a part of this 37 kDa kinase corresponds to a MAP kinase. To identify the third activated MAP kinase visualized in gel, a specific antibody directed

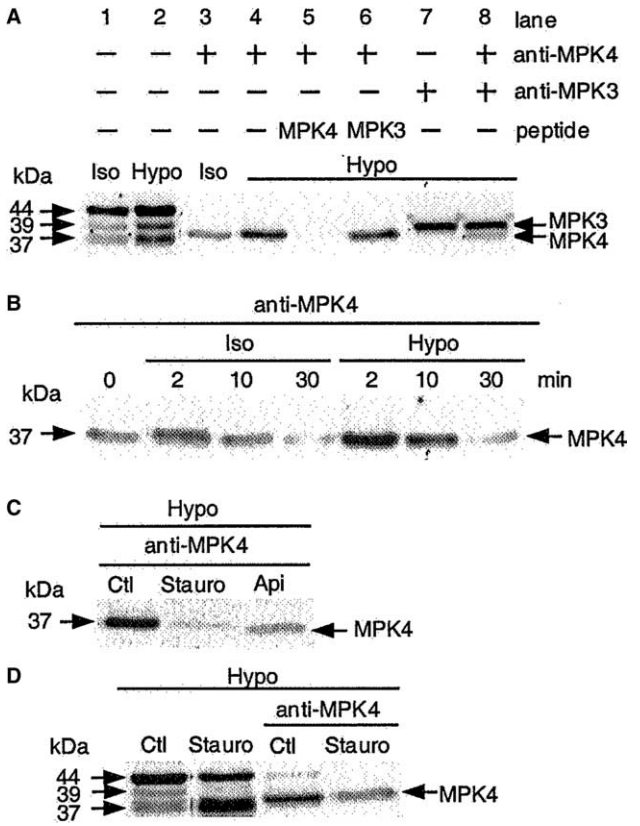

Fig. 1. Identification and characterization of MPK4 as the third hypoosmotically activated MAP kinase in cell suspensions. The specificity of the anti-MPK4 antibody was checked (A) and several characteristics of MPK4 were studied: time course activation (B), inhibition of the kinase activity in the gel (C) and in vivo effect of staurosporine (D). Equilibrated cells were transferred either to isoosmotic (Iso) or to hypoosmotic (Hypo) medium for 2 min (A, C, D) or for the indicated times (B). When indicated, anti-MPK4, anti-MPK3 or both antibodies were used to immunoprecipitate protein extracts. In A, immunoprecipitations using MPK4 antibody were also performed in the presence, as competitors, of the N-terminal MPK4 or MPK3 peptides used for immunizations. In B, the left lane (0) corresponds to untransferred control cells. In C, protein kinase inhibitors, 1.5 μM staurosporine (Stauro) or 500 μM apigenin (Api), were added during the in vitro radioactive labelling step, when indicated. In D, 0.5 μM staurosporine (Stauro) was added to cells during the last 10 min of the equilibration time and during 2 min after transfer, at the same concentration, when indicated. Ctl is the corresponding control without kinase inhibitors. Kinase activity of crude extracts and immunocomplexes was subsequently determined with an in-gel kinase assay using MBP as a substrate.

towards MPK4 was prepared and used here. This antibody immunoprecipitated an hypoosmotically activated kinase (Fig. 1A, lanes 3 and 4). The apparent molecular weight of the kinase in the immunocomplex was slightly higher than the corresponding molecular weight in the crude extract and this difference is due to the large amount of antibody heavy chains in this gel area. The specificity of the precipitation was shown using successfully the corresponding peptide (MPK4) as a competitor, while another unrelated peptide (MPK3) has no effect on the immunoprecipitation of MPK4 (Fig. 1A, lanes 5 and 6). The use of the anti-MPK3 antibody alone or together with anti-MPK4 antibody demonstrated that these two close kinases differ by their molecular masses (Fig. 1A, lanes 7 and 8).

Several characteristics of MPK4 were studied and compared with the 37 kDa kinase activity previously visualized in crude extracts [11], which may correspond to both MPK4 and a second 37 kDa kinase. In cells transferred to hypoosmotic medium, MPK4 was early (2 min) and transiently activated (Fig. 1B) as the 37 kDa kinase. However, when cells were transferred to isoosmotic medium (Fig. 1B), MPK4 was also, but slightly, activated by the mechanical stress due to transfer, while the 37 kDa protein did not show clear activation in crude extract, during this time course [11]. This difference is coherent with the previous suggestion that, in addition to MPK4, a second 37 kDa kinase is activated by hypoosmotic stress, which would not be activated by mechanical stress. When protein kinase inhibitors were added during the in-gel kinase activity assay (Fig. 1C), MPK4 activity was greatly reduced by staurosporine and partially inhibited by apigenin, similarly to the results obtained for 37 kDa inhibition from crude extract in the same conditions [11]. On the contrary, when the protein kinase inhibitor staurosporine was added in vivo to investigate transduction pathways leading to the kinase activations (Fig. 1D), results were opposite between a strong 37 kDa activation by staurosporine in crude extract and a reduced activation of MPK4 by staurosporine. These data clearly indicate that MPK4 corresponds only to a part of the 37 kDa activity, indicating that another kinase, displaying the same apparent molecular mass, remains to be identified. This result is coherent with the previous observation based on protein tyrosine phosphatase action [11] and suggests that the second kinase does not belong to the MAP kinase family.

3.2. The three MAP kinases MPK6, MPK3 and MPK4 are hypoosmotically activated in plantlets. Relation between MPK4 and the two other kinases

Three protein kinases (44, 39 and 37 kDa) were observed to be activated by hypoosmolarity in crude extracts from plantlets (Fig. 2A). Using the specific antibodies against MPK4, MPK3 and MPK6, the corresponding three MAP kinases were shown to be hypoosmotically activated in plantlets like in cell suspensions (Fig. 2A). To study the relation between MPK4 and the other two kinases, MPK3 and MPK6, the *mpk4* mutant was used. A Western blot using plantlets and anti-MPK4 displays a signal in wild-type but not in *mpk4* mutant plants (data not shown). Furthermore, the hypoosmotically activated 37 kDa kinase is present in wild-type but not in *mpk4* mutant (Fig. 2B), confirming that this kinase corresponds to MPK4. The same observation is visible after immunoprecipitation, again confirming the validity of MPK4 antibody (Fig. 2B). The levels of MPK6 and MPK3 activations observed in hypoosmotically treated wild-type (Fig. 2A) or *mpk4* (Fig. 2C) plantlets are comparable, indicating that MPK6 and MPK3 inductions are independent of the MPK4 activation in this stress signalling.

3.3. The three MAP kinases MPK6, MPK3 and MPK4 are also activated by the flagellin elicitor. Relation between MPK4 and the two other kinases in flagellin signalling

To test the possible activation of MPK4 by a biotic stimulus, the flagellin peptide, flg 22, previously shown active on *Arabidopsis* cell suspensions by Nühse et al. [22] was used. These authors have identified one kinase activated by that elicitor to

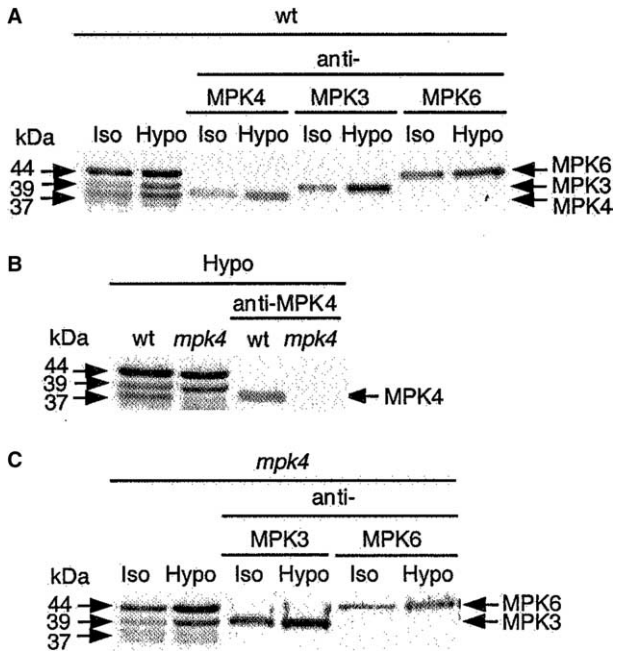

Fig. 2. Hypoosmotically activated MAP kinases in plantlets: MPK3 and MPK6 inductions are independent of the MPK4 induction in hypoosmotic signalling. In A, wild-type (wt) plantlets were transferred either to isoosmotic (Iso) or hypoosmotic (Hypo) medium for 10 min. When indicated, either anti-MPK4, anti-MPK3 or anti-MPK6 antibody was used to immunoprecipitate protein extracts. In B, wild-type (wt) and *mpk4* mutant seedlings were transferred in the hypoosmotic condition described in A and immunoprecipitation of MPK4 was performed, when indicated. In C, *mpk4* mutant plantlets were treated and analyzed as described in A. Kinase activity of crude extracts and immunocomplexes was determined with an in-gel kinase assay using MBP as a substrate.

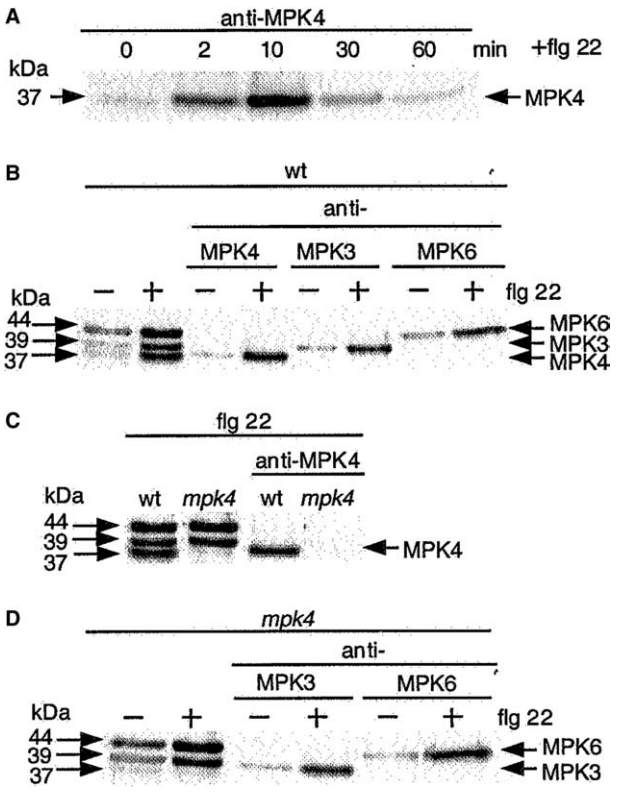

Fig. 3. Activation of MPK4 by the flagellin elicitor in cells and plantlets. MPK3 and MPK6 inductions are independent of the MPK4 induction in flagellin signalling. In A, equilibrated cell suspensions were treated with 100 nM flagellin peptide (flg 22) for the indicated times and anti-MPK4 antibody was used to immunoprecipitate protein extracts. In B, wild-type (wt) seedlings were treated or not for 15 min by 100 nM flagellin peptide and anti-MPK4, anti-MPK3 or anti-MPK6 antibody was used to immunoprecipitate protein extracts, when indicated. In C, wild-type (wt) and *mpk4* mutant seedlings were treated by flagellin in the conditions described in B and immunoprecipitation of MPK4 was performed when indicated. In D, *mpk4* mutant plantlets were treated and analyzed as described in B. Kinase activity of crude extracts and immunocomplexes was determined with an in-gel kinase assay using MBP as a substrate.

MPK6. When flg 22 was added to the cell culture medium (Fig. 3A), MPK4 was rapidly (2 min) and transiently activated, with a maximum at 10 min. The action of flagellin was also studied on plantlet crude extracts (Fig. 3B, left), showing the strong activations of three protein kinases, which can be identified as MPK4, MPK3, and MPK6, using the corresponding antibodies (Fig. 3B). Thus, the same MAP kinases were activated both by hypoosmotic and flagellin signals and the question of the relation between MPK4 and the other kinases was further analyzed using the *mpk4* mutant, in the flagellin signalling context. The flagellin activated 37 kDa kinase is present in wild-type but not in *mpk4* mutant and the same observation is visible after immunoprecipitation, clearly confirming the complete absence of the protein MPK4 in the mutant (Fig. 3C). The levels of MPK6 and MPK3 activations observed in wild-type (Fig. 3B) or *mpk4* (Fig. 3D) plantlets treated by flagellin are comparable, indicating that MPK6 and MPK3 inductions are also independent of the MPK4 activation in this biotic signalling.

3.4. *MPK4 is not activated by hyperosmotic stresses but plays a negative role in tolerance to hyperosmolarity*

When cell suspensions were hyperosmotically treated either by sucrose (500 or 1000 mM) or by NaCl (250 or 650 mM) (Fig. 4A), no MPK4 activation was observed, when compared with cells transferred to isoosmotic medium. Even, strong stresses such as 650 mM NaCl seem to inhibit the activity of MPK4. When plantlets were subjected to hyperosmotic stresses (Fig. 4B), as well, no MPK4 activation was shown. The *mpk4* mutant phenotype cultured in hyperosmotic conditions was then compared to the wild-type. Germination rate of wild-type and *mpk4* mutant seeds, after 2 days (Fig. 4C), was almost equal (94%) in the control, while it decreased when osmolarity of the medium increased. However, this loss of germination capability is clearly reduced in the mutant, particularly for the higher osmolarity (200 mM) for which germination rate reduction is half of the reduction observed in wild-type. In addition, after 9 days culturing (Fig. 4C), the reduction of root length due to increased osmolarity is clearly lower in *mpk4* in comparison to the wild-type and thus hyperosmolarity inhibited the root growth less strongly in the mutant. The inhibition of root growth is already significant with 130 mM sucrose, with a 43% inhibition in the wild-type and 23% in the mutant. However, it should be noticed that in the control conditions, the root length was slightly higher in *mpk4* (39 ± 2.2 mm) than in the wild-type (35 ± 1.4 mm). To visualize the effect of osmolarity alone, the root length in the control condition was

brought to 100% in each type of plantlets. These results indicate an increased tolerance of *mpk4* to hyperosmolarity and suggest a negative role of MPK4 in this tolerance. To further investigate this hypothesis, expression levels of two drought inducible genes, RD29A and RAB18, were quantified in plantlets 3 h after transfer in isoosmotic or hyperosmotic conditions (Fig. 4D). No significant difference in RD29A transcript levels was observed between the wild-type and mutant plantlets, either in isoosmotic or hyperosmotic condition. On the contrary, RAB18 levels were much more increased in *mpk4* than in the wild-type in response to hyperosmolarity (Fig. 4D). This increased response to hyperosmolarity of RAB18 expression in the plantlets deprived of the MAP kinase MPK4 is coherent with the increased tolerance of *mpk4* to hyperosmolarity reported above and both results argue for a negative role of MPK4 in the hyperosmolarity and drought tolerances.

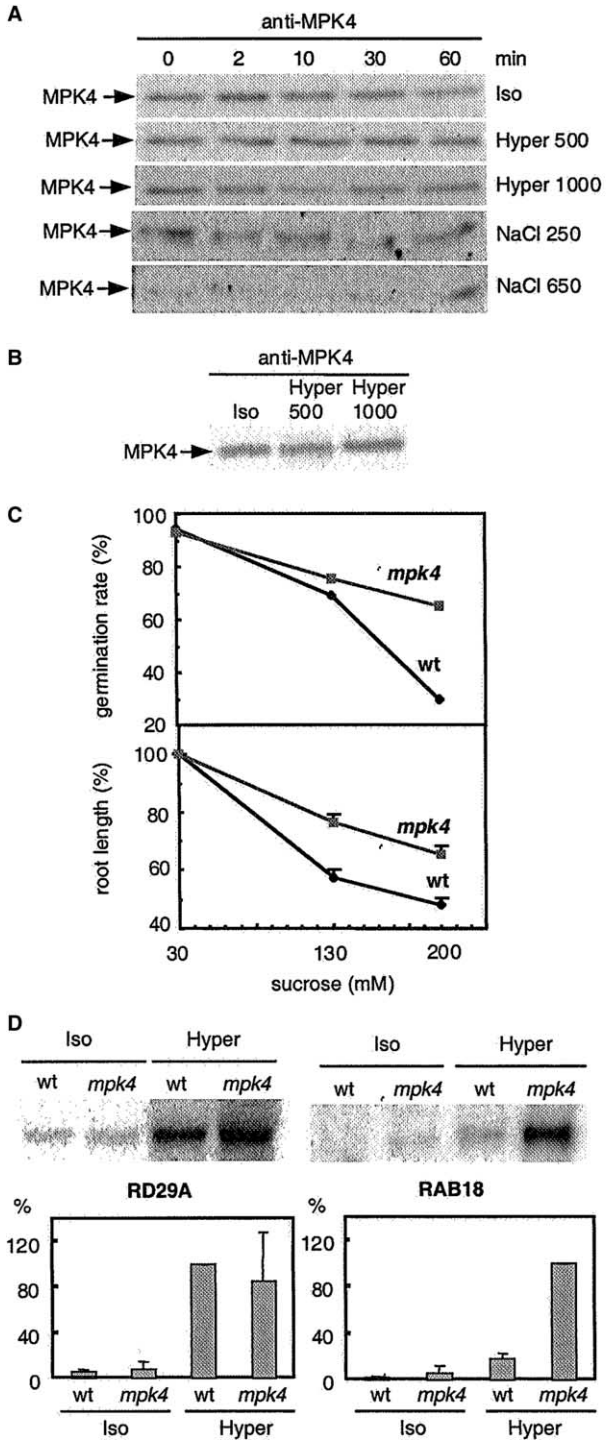

Fig. 4. MPK4 is not activated by hyperosmotic stresses in cells or plantlets, but decreased tolerance to hyperosmolarity. In A, cells were transferred either to isoosmotic (Iso) or to hyperosmotic media (Hyper, NaCl) for the indicated times. Hyperosmotic stresses were carried out with 500 mM sucrose (Hyper 500) or 250 mM NaCl (NaCl 250) medium for moderate stresses or with 1000 mM sucrose (Hyper 1000) or 650 mM NaCl (NaCl 650) for high hyperosmotic stresses. In B, plantlets were transferred either to isoosmotic (Iso), moderate (Hyper 500) or high (Hyper 1000) hyperosmotic medium for 10 min. In A and B, kinase activity of immunocomplexes was determined with an in-gel kinase assay using MBP as a substrate. In C, wild-type (wt) and *mpk4* seeds were allowed to germinate on media containing 130 or 200 mM sucrose or on the control medium (30 mM sucrose). The germination rate was evaluated after 2 days and the root length after 9 days. The root length in the control condition was brought to 100% for the wild-type and for the *mpk4* plantlets. Measurements were performed on 90 seeds (germination) or 90 plantlets (root length). In D, transcript levels of RD29A and RAB18 were evaluated by Northern blot in wild-type (wt) and *mpk4* mutant plantlets transferred either to isoosmotic (Iso) or 500 mM sucrose hyperosmotic (Hyper) medium for 3 h. The Northern signals were quantified and the ratio of each transcript level to the corresponding level of actin was reported in the lower part of the figure. The results are shown as means ± S.E. of 4 (RD29A) or 3 experiments (RAB18) and the highest value was called 100% in each case.

4. Discussion

Three protein kinases (44, 39 and 37 kDa), activated by hypoosmolarity in *Arabidopsis* cell suspensions, were previously shown to belong to the MAP kinase family [11]. This result was based on the higher efficiency of the 3 kinases to phosphorylate MBP rather than histone, their susceptibility to apigenin, a MAP kinase inhibitor, and the suppression of their activations observed after action of a protein tyrosine phosphatase. The 44 and 39 kDa kinases were identified to be MPK6 and MPK3 [11], two MAP kinases among the putative kinases corresponding to the 20 genes identified in the *Arabidopsis* genome [1]. MPK6 and MPK3 belong to the A group of plant MAP kinases, which also contains the two tobacco kinases activated by hypoosmolarity, SIPK and WIPK [8]. However, no other A group MAP kinase is present in the *Arabidopsis* genome and the hypothesis that the third hypoosmotically induced MAP kinase may correspond to MPK4, a B group MAP kinase, was tested here. A specific anti-MPK4 antibody was prepared, allowing the identification of MPK4 as the third MAP kinase activated by hypoosmolarity in cell suspensions (Fig. 1). Like for MPK3 and MPK6, MPK4 activity was shown to be clearly decreased by apigenin (Fig. 1C). In addition, the activations of the three kinases, MPK6, MPK3 and MPK4, were also evidenced in plantlets (Fig. 2A) like in cell suspensions.

MPK4 is also activated by isoosmotic transfer of the cells (Fig. 1B), which has been shown to induce a mechanical stress. This result is coherent with the previous observations that mechanical and hypoosmotic stresses present similarities in the

inductions of an extracellular oxidative burst [23] and the activations of the same kinases, WIPK and SIPK in tobacco [8], MPK3 and MPK6 in *Arabidopsis* [11].

Concerning biotic elicitors, MPK4, which was already shown to be activated by harpin in leaves and cell suspensions [24], is also activated by flagellin (Fig. 3A), a highly conserved component of bacterial flagella. The activation of MPK6 by flagellin was demonstrated by Nühse et al. [22] and, in the present study, the activations of 3 protein kinases with apparent molecular masses of 44, 39 and 37 kDa were observed in crude extracts of cells (data not shown) and plantlets (Fig. 3B). The three kinases were shown to correspond to MPK6, MPK3 and MPK4, respectively (Fig. 3B). Interestingly, a complete MAP kinase cascade functioning downstream the flagellin receptor and including MPK6 and MPK3 was recently demonstrated in mesophyll protoplasts [25]. The involvement of MPK4 in this flagellin signalling was not reported, but the 6 MAP kinases tested by transient expression in this study [25] did not include MPK4. More generally, the question of the respective roles of the three MAP kinases in a same signalling pathway may be asked. Very recently [26], the hypothesis that MPK6 and a 44 kDa activity, likely corresponding to MPK3, are partially redundant for mediating wounding responses was reported, based on the increase of the 44 kDa activity in MPK6 silenced plants.

Contrary to flagellin signal, none of the hyperosmotic or salt signal was able to activate MPK4 (Fig. 4A and B), although the study was performed with moderate or high stresses and both on cell suspensions and plantlets. The activity of MPK4 was lower after high hyperosmotic or salt stresses in comparison to moderate corresponding stresses (Fig. 4A). This result is consistent with the activation of MPK4 by mechanical stress, since this mechanical component is reduced in high hyperosmotic medium. However, MPK4 was shown to be slightly activated by hyperosmotic stress in *Arabidopsis* detached leaves [27]. This discrepancy may be likely explained by the differences in the models used, with a possible role of wounding in the case of detached leaves.

Despite the absence of MPK4 activation by hyperosmolarity or salinity, a possible role of this MAP kinase in hyperosmolarity tolerance is suggested here, based on the mutant exhibiting *MPK4* gene inactivation. Petersen et al. [17] have shown that *mpk4* displays constitutive systemic acquired resistance and increased resistance to virulent pathogens. The authors demonstrated that MPK4 kinase activity is required to suppress systemic acquired resistance, thus playing a role of negative regulator. The *mpk4* mutant exhibits dwarfism with decreased cell size at the two- to three-leaf stage, curled leaves and flowers with reduced fertility. However, *mpk4* is not significantly impaired in responses to desiccation or salt stresses [17]. When *mpk4* was compared here to the wild-type, higher germination rate was observed for the mutant after 2 days (Fig. 4C) when the osmoticum concentration was increased from 30 to 200 mM. This difference, which corresponds to a lower delay of germination in the mutant caused by the high osmolarity, was no longer observed after 3 days, since the germination rate reaches 100% at that time, both for the mutant and the wild-type (data not shown). Similar difference was observed concerning the root length, which was less decreased by hyperosmolarity in *mpk4* than in the wild-type (Fig. 4C). The difference, that was already observed after 3 days (data not shown), remains for at least 6 more days (Fig. 4C) before disappearing for subsequent growth stages, when the clear dwarfism of the mutant is expressed. These two observations suggest that MPK4 might negatively regulate the hyperosmolarity tolerance in addition to the systemic acquired resistance. In accord with this suggestion, the expression level of the drought-responsive gene, ABA-dependent RAB18, was much more increased in the *mpk4* plantlets than in the wild-type in response to hyperosmolarity. On the contrary, no significant difference was observed in the mutant for another drought-responsive gene, but ABA-independent, RD29A expression level. It is interesting to note that comparable independence between RD29A and RAB18 gene expressions was recently reported in a study showing that phospholipase D signalling for proline biosynthesis is similar to RD29A gene expression and different from the RAB18 gene expression [28]. This is the first report of a role of MPK4 in hyperosmolarity tolerance, but the other example of abiotic stress tolerance regulation by a MAP kinase of another group has been recently documented in rice by Xiong and Yang [12]. The authors demonstrated significant reductions in drought, salt and cold tolerance in transgenic plants silenced for OsMAPK5, an ortholog of the tobacco WIPK, although several pathogenesis related genes were constitutively expressed in these plants. Thus, contrary to *Arabidopsis* MPK4, disease resistance and abiotic stress tolerance appear inversely modulated by the rice OsMAPK5 [12].

The activations of MPK6 and MPK3 by hypoosmolarity and flagellin are shown here independent of the presence of MPK4 (Fig. 2C and 3D). It will be interesting to determine if MPK4 acts downstream of MPK3 and MPK6 or more probably, taking into account the early and simultaneous activations of the 3 MAP kinases, belongs to a different signalling cascade. Contrary to the 3 MAP kinases, the activation of a second 37 kDa kinase is repressed when another upstream kinase functions, as clearly shown by the reinforcement of this activity by staurosporine action (Fig. 1D). This result is consistent with the partial inhibition of the 37 kDa kinase activation by a tyrosine-specific protein phosphatase previously reported [11], suggesting that the second 37 kDa kinase does not belong to the MAP kinase family. The 37 kDa *Arabidopsis* kinase may correspond to the 44 kDa kinase already observed in tobacco [8] and it remains to be determined if the tobacco 44 kDa kinase and the 37 kDa *Arabidopsis* kinase activated by hypoosmolarity are identical to the corresponding kinases activated by hyperosmolarity. More generally, after the identification of the 3 *Arabidopsis* MAP kinases, the different other kinases already observed activated by osmotic stresses remain to be identified and are currently under investigation in the laboratory.

Acknowledgements: We thank Georg Felix, FMI, for gift of the flg 22 preparation.

References

[1] Ichimura, K. et al. (2002) Trends Plant Sci. 7, 301–308.
[2] Zhang, S.Q. and Klessig, D.F. (2001) Trends Plant Sci. 6, 520–527.
[3] Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. and Shinozaki, K. (1996) Proc. Natl. Acad. Sci. 93, 765–769.
[4] Covic, L., Silva, N.F. and Lew, R.R. (1999) Biochim. Biophys. Acta 1451, 242–254.
[5] Popping, B., Gibbons, T. and Watson, M.D. (1996) Plant. Mol. Biol. 31, 355–363.

[6] Jonak, C., Kiegerl, S., Ligterink, W., Barker, P.J., Huskisson, N. and Hirt, H. (1996) Proc. Natl. Acad. Sci. 93, 11274–11279.
[7] Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Plant J. 20, 381–388.
[8] Droillard, M.J., Thibivilliers, S., Cazalé, A.C., Barbier-Brygoo, H. and Laurière, C. (2000) FEBS Lett. 474, 217–222.
[9] Hoyos, M.E. and Zhang, S.Q. (2000) Plant Physiol. 122, 1355–1363.
[10] Mikolajczyk, M., Awotunde, O.S., Muszynska, G., Klessig, D.F. and Dobrowolska, G. (2000) Plant Cell 12, 165–178.
[11] Droillard, M.J., Boudsocq, M., Barbier-Brygoo, H. and Laurière, C. (2002) FEBS Lett. 527, 43–50.
[12] Xiong, L.Z. and Yang, Y.N. (2003) Plant Cell 15, 745–759.
[13] Jonak, C., Kiegerl, S., Lloyd, C., Chan, J. and Hirt, H. (1995) Mol. Gen. Genet. 248, 686–694.
[14] Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S.C., Zhu, T., Wang, X., Shinozaki, K. and Paszkowski, J. (2002) EMBO J. 21, 6483–6493.
[15] Moon, H. et al. (2003) Proc. Natl. Acad. Sci. 100, 358–363.
[16] Matsuoka, D., Nanmori, T., Sato, K., Fukami, Y., Kikkawa, U. and Yasuda, T. (2002) Plant J. 29, 637–647.
[17] Petersen, M. et al. (2000) Cell 103, 1111–1120.
[18] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–497.
[19] Frachisse, J.M., Colcombet, J., Guern, J. and Barbier-Brygoo, H. (2000) Plant J. 21, 361–371.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Zhang, S., Du, H. and Klessig, D.F. (1998) Plant Cell 10, 435–449.
[22] Nühse, T.S., Peck, S.C., Hirt, H. and Boller, T. (2000) J. Biol. Chem. 275, 7521–7526.
[23] Cazalé, A.C., Rouet-Mayer, M.A., Mathieu, Y., Barbier-Brygoo, H. and Laurière, C. (1998) Plant Physiol. 116, 659–669.
[24] Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K. and Neill, S.J. (2001) Plant Physiol. 126, 1579–1587.
[25] Asai, T. et al. (2002) Nature 415, 977–983.
[26] Menke, F.L.H., van Pelt, J.A., Pieterse, C.M.J. and Klessig, D.F. (2004) Plant Cell 16, 897–907.
[27] Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Plant J. 24, 655–665.
[28] Thiery, L., Leprince, A.-S., Lefebvre, D., Ali Ghars, M., Debardieux, E. and Savouré, A. (2004) J. Biol. Chem. 279, 14812–14818.