Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1

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The *Arabidopsis* genome contains 20 genes encoding mitogen-activated protein kinases (MAPKs), which drastically outnumber genes for their negative regulators, MAP kinase phosphatases (MKPs) (five at most). This contrasts sharply with genomes of other eukaryotes where the number of MAPKs and MKPs is approximately equal. MKPs may therefore play an important role in signal integration in plants, through concerted regulation of several MAPKs. Our previous studies identified *Arabidopsis* MKP1 and showed that its deficiency in the *mkp1* mutant results in plant hypersensitivity to genotoxic stress. Here, we identify a set of MAPKs that interact with MKP1, and show that the activity level of one of these, MPK6, is regulated by MKP1 *in vivo*. Moreover, using expression profiling, we identified a specific group of genes that probably represent targets of MKP1 regulation. Surprisingly, the identity of these genes and interacting MAPKs suggested involvement of MKP1 in salt stress responses. Indeed, *mkp1* plants have increased resistance to salinity. Thus MKP1 apparently plays a pivotal role in the integration and fine-tuning of plant responses to various environmental challenges.

Keywords: abiotic stress/*Arabidopsis*/MAP kinase/MAP kinase phosphatase/signaling

Introduction

Rapid adaptation to environmental challenges through the activation of complex signaling networks is a feature common to all organisms. A widespread mechanism to integrate and balance the various inputs involves cross-talk between signaling pathways through shared components. In eukaryotes, the mitogen-activated protein (MAP) kinase (MAPK) cascades constitute a prominent example for the cooperation of signaling pathways. Their core, which relays signals by sequential phosphorylation,

consists of three interlinked kinase components, a MAPK kinase kinase (MEKK), a MAPK kinase (MKK) and a terminal MAPK. Downstream targets of MAPKs in yeast and metazoans include other protein kinases, phosphatases, phospholipases, cytoskeleton-associated proteins and a number of transcription factors (e.g. reviewed by Widmann *et al.*, 1999). Phosphorylation of transcription factors leads to appropriate reprogramming of gene expression in response to activating stimuli leading to a particular functional readout of the MAPK cascade(s). Although MAPK signaling cascades exist in all eukaryotes, the composition and function of specific components may differ significantly.

In plants, several intra- and extracellular cues are known to activate MAPK pathways, and a number of possible components have been identified in the genomic sequence (Tena *et al.*, 2001; MAPK Group, 2002). In *Arabidopsis*, 20 genes (gene name: *MPK*) encode putative MAPKs (MAPK Group, 2002), more than in any other eukaryote. Surprisingly, however, all plant MAPKs are classified into the PERK subfamily (plant extracellular signal-regulated protein kinase; Kültz, 1998), and no other classes of stress-activated protein kinases [SAPKs; c-jun N-terminal kinase (JNK) or p38 kinases], present in other organisms, are discernible in the genomic sequence of *Arabidopsis*. This implies that the ancestor plant MAPKs evolved before differentiation of the SAPK subgroup in the animal/fungal lineage (Kültz, 1998).

At present, putative functions in *Arabidopsis* are assigned to only MPKs 3, 4 and 6 as deduced from their activation conditions (Tena *et al.*, 2001), and all three are linked to diverse stress responses (Mizoguchi *et al.*, 1996; Ichimura *et al.*, 2000; Kovtun *et al.*, 2000; Nühse *et al.*, 2000; Desikan *et al.*, 2001a,b; Asai *et al.*, 2002). However, genetic evidence supporting the deduced functions at the whole-organism level is rather limited. The only *Arabidopsis* MAPK mutant described so far, *mpk4*, suggests that MPK4 is a negative regulator of salicylic acid (SA) and systemic acquired resistance, while a positive regulator of jasmonate (JA)-activated gene expression (Petersen *et al.*, 2000). Thus, MPK4 has been postulated to be involved in the integration of SA- and JA-dependent signals to evoke the appropriate responses against pathogens and other stresses (Petersen *et al.*, 2000).

MAPK involvement in plant stress responses has usually been studied with isolated MAPKs, and information concerning the functional composition of the corresponding signaling pathways is incomplete (Tena *et al.*, 2001). Yeast two-hybrid analysis and complementation of yeast mutants using *Arabidopsis* components assembled a possible cascade composed of MEKK1, MKK1/MKK2 and MPK4 (Ichimura *et al.*, 1998; Mizoguchi *et al.*, 1998) and, accordingly, MPK4 is activated by MKK1 *in vitro*

Fig. 1. MKP1 function in genotoxic stress relief depends on its catalytic cysteine. Five-day-old seedlings were transferred to liquid medium supplemented with MMS at the concentration indicated. The picture of representative seedlings was taken after 2 weeks of further growth. Ws, Wassilewskija wild type; *mkp1*, *mkp1* mutant; line 6, *mkp1* complemented by ectopic expression of MKP1 (homozygous progeny); *mkp1* MKP1(C235S), representative line of *mkp1* with ectopic expression of MKP1(C235S) (heterozygous progeny); *mkp1* MKP1, representative line of *mkp1* complemented by ectopic expression of wild-type MKP1 (heterozygous progeny).

(Huang *et al.*, 2000). However, the physiological significance of these interactions was not verified *in planta*. Recently, a flagellin-responsive MAPK cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) was deduced using transient assays in *Arabidopsis* protoplasts (Asai *et al.*, 2002); however, this also awaits further studies at the organism level.

MAPKs are activated by upstream kinases and this activation can be reversed by phosphatases, including tyrosine-specific phosphatases, serine/threonine-specific phosphatases and also the dual-specificity MAPK phosphatases (MKPs), which are highly specific for MAPKs (Camps *et al.*, 2000; Keyse, 2000). Few negative regulators of plant MAPKs have been reported, and their characterization is limited mostly to molecular and biochemical studies. *In vitro* experiments identified the *Arabidopsis* protein tyrosine phosphatase PTP1 and the dual-specificity phosphatase DsPTP1 as potential MAPK-dephosphorylating enzymes (Gupta *et al.*, 1998; Huang *et al.*, 2000). PTP1 is transcriptionally activated under elevated salinity, but repressed by cold treatment (Xu *et al.*, 1998). However, for DsPTP1, no putative function could be deduced from gene expression analysis (Gupta *et al.*, 1998).

Interestingly, the 10 mammalian members of the MKP family identified so far appear to be selective in the inactivation of distinct MAPK isoforms and additionally exhibit individual properties in terms of subcellular localization, tissue distribution and transcriptional induction (Camps *et al.*, 2000; Tanoue *et al.*, 2001). These 10 known MKPs regulate 14 MAPKs presently identified in mammals. In organisms where the genome sequence is available, better estimates for the number of MAPK pathway elements have been obtained. *Saccharomyces cerevisiae* contains six MAPKs and four MKPs, *Caenorhabditis elegans* has 14 MAPKs and 10 MKPs, *Drosophila melanogaster* has five MAPKs and eight MKPs (Plowman *et al.*, 1999; and <http://www.kinase.com/>; Morrison *et al.*, 2000; and <http://intl.jcb.org/cgi/content/full/150/2/F57/T1/DC1>). In contrast, the *Arabidopsis* genome encodes 20 MAPKs (PERKs;

MAPK Group, 2002) and only five putative MKPs (<http://SMART.embl-heidelberg.de>), including the two previously described MKPs, DsPTP1 and MKP1 (Gupta *et al.*, 1998; Ulm *et al.*, 2001). This disproportion in the MKP/MAPK ratio in *Arabidopsis* in comparison with other eukaryotes may reflect principal differences in the integration and transmission of stress signals through plant or fungal/animal MAPK pathways.

Recent genetic studies in multicellular eukaryotes reinforced the biochemical evidence for the importance of MKPs in the precise regulation of MAPK activities (Martin-Blanco *et al.*, 1998; Berset *et al.*, 2001; Ulm *et al.*, 2001). Mutations in MKPs result in elevated MAPK activity *in vivo*, leading to embryonic lethality of the *puckered* mutant in *Drosophila* (Martin-Blanco *et al.*, 1998), visible effects of the *lip-1* mutation in *C.elegans* in a background of mutations affecting vulval development (Berset *et al.*, 2001), or genotoxic stress hypersensitivity in the *Arabidopsis* *mkp1* mutant (Ulm *et al.*, 2001). However, the only reported example of a mammalian MKP depletion (ERP/MKP-1 knockout in mouse) had no phenotypic consequences, most probably due to functional redundancy (Dorfman *et al.*, 1996). Importantly, the *Arabidopsis* *mkp1* mutant with its hypersensitivity to genotoxic stress but with normal development represents the only example of a genetically defined role for an MKP in stress signaling in multicellular organisms. However, the components of the MKP1-regulated pathway remain unknown.

Here, we identified MKP1-interacting MAPKs and genes with expression affected by MKP1 levels. Their identities reinforce the possibility that MKP1 acts as a cross-talk point of stress signaling pathways, a conclusion supported by the elevated salt resistance of the *mkp1* mutant.

Results

The conserved catalytic center is required for MKP1 function *in vivo*

The *mkp1* mutation resulted from the insertion of a T-DNA into the MKP1 gene close to the start ATG, leading to the

Fig. 2. MKP1 interacts with MPKs 3, 4 and 6. **(A)** MKP1 was expressed in yeast cells with the indicated MAPKs (MPK1–9) or with the empty vector pVP16. Each of the MKP1-interacting MAPKs was also expressed with the empty vector pBTM116 used to express MKP1. Qualitative interaction assays for His3 and lacZ were scored from no interaction (–) to strong interaction (++++). LacZ activity is shown as standard units $\times$ 1000. Values reported are the average of the assays of four independent transformants. **(B)** Co-pull-down of baculovirus-produced Py-MKP1 from insect cell lysate with GST-MPK6. The upper panel depicts immunoblot analysis of GST pull-downs with anti-Glu–Glu antibodies (detecting the Py epitope) and the lower panel a parallel Coomassie-stained SDS–polyacrylamide gel.

absence of its transcript, and consequently its protein (Ulm *et al.*, 2001). To determine if the phenotypes of the *mkp1* mutant can also occur through the specific loss of phosphatase activity, the catalytic center of MKP1 was modified by replacing the essential cysteine with serine (Cys235 in MKP1). This mutation is well known to abrogate fully the phosphatase activity of PTPases and DsPTPases (e.g. Sun *et al.*, 1993; Gupta *et al.*, 1998; Sugiura *et al.*, 1998; Xu *et al.*, 1998). Introduction of such a modified transgene, *MKP1(C235S)*, into the *mkp1* mutant, although resulting in the production of normal levels of its mRNA (data not shown), failed to complement the hypersensitivity to the genotoxic agent methyl methanesulfonate (MMS) in 38 independent transgenic lines tested (Figure 1). A control transgene containing the wild-type *MKP1* gene was able to complement the MMS sensitivity phenotype in 26 of 30 lines (Figure 1; Ulm *et al.*, 2001). These results demonstrate that the MKP1 phosphatase activity is essential for its function *in vivo* and that this enzymatic activity is responsible for MKP1 function in genotoxic stress signaling.

MKP1 interacts with MPKs 3, 4 and 6

To identify MAPKs interacting with full-length MKP1, nine *Arabidopsis* MAPK cDNAs (Mizoguchi *et al.*, 1997) were fused to the VP16 activation domain (AD), and their interaction with MKP1 fused to the LexA DNA-binding domain (DB) was examined in directed yeast two-hybrid assays. No activity of the reporter *His3* or *lacZ* gene products was detected when the LexA-MKP1 or VP16-MPK constructs were co-transformed with the corresponding empty vectors (Figure 2; data not shown). However, all three activity assays suggested interaction of MKP1 with MPKs 3, 4 and 6 (Figure 2A). Interaction with MPK6 was the most pronounced and was ~4-fold

stronger in the quantitative lacZ activity assay than the interaction with either MPK3 or 4 (Figure 2A). There was no indication of interaction with any of the six remaining MAPKs (Figure 2A). Thus, MKP1 apparently interacts with MPKs 3, 4 and 6 in the following rank order of binding strength: MPK6 >> MPK3 = MPK4. Interestingly, these three MKP1-interacting MAPKs have all been reported to be associated with abiotic and/or biotic stress responses (e.g. reviewed by Tena *et al.*, 2001). These data point toward multiple substrates of MKP1, with MPK6 as dominant and thus probably involved in genotoxic stress signaling.

To substantiate further the MKP1–MPK6 interaction, pull-down assays were carried out using both recombinant proteins. Polyoma (Py)-tagged MKP1 produced in baculovirus-infected Sf9 insect cells was pulled down specifically from crude cell lysate with GST–MPK6 made in *Escherichia coli* (Figure 2B), confirming the yeast two-hybrid data for their interaction.

Importantly, the mutated MKP1(C235S) interacted with MPK6 to a comparable level as the wild-type protein, in both the yeast two-hybrid and *in vitro* interaction assay (data not shown). Thus, the single amino acid exchange in the catalytic site does not alter the protein interaction properties, further strengthening the conclusion of the mutant complementation experiments showing that MKP1 phosphatase activity is required for genotoxic stress relief.

Activation of MPK6 in response to genotoxic stress

To examine whether MPK6 is activated by genotoxic stress *in vivo*, seedlings of transgenic *Arabidopsis* lines expressing MPK6 fused to green fluorescent protein (MPK6–GFP) were exposed to UV-C or treated with MMS. Cell extracts were used for in-gel kinase assays

Fig. 3. MPK6 is activated by genotoxic stress. (A) Nine-day-old seedlings of Columbia (Col) wild type or transgenic for GFP or MPK6-GFP were subjected to the indicated genotoxic stress treatment. Soluble protein extracts were used for MBP in-gel kinase assays or immunoblot analysis with anti-MPK6 (Ab6NT1) antibody. (B) The protein kinase activity of immunoprecipitated MPK4 (with Ab4CT1) and MPK6 (Ab6NT1) was assayed by MBP in-gel kinase assay. Nine-day-old seedlings were irradiated with 0, 1 or 2 kJ/m² UV-C as indicated and samples taken 10 min after treatment. (C) MPK6 protein kinase activity level in response to UV-C determined by MKP1 level. Ab6NT1-immunoprecipitated MPK6 from Ws, *mkp1* mutant and *MKP1*-overexpressing line 6 after UV-C irradiation, as indicated. An example of a control IP without antibody is shown (identical results were obtained for all other samples).

with myelin basic protein (MBP). Following stress treatments, the MPK6-GFP transgenics revealed the activation of the MPK6-GFP fusion protein of 76 kDa (i.e. of the predicted size, 49 kDa MPK6 + 27 kDa GFP) (Figure 3A, upper panel), while in both the wild-type and GFP transgenic line only an endogenous MAPK of 49 kDa apparent size was activated (Figure 3A). The protein gel blot probed with an antibody against MPK6 showed unchanged levels of both the MPK6-GFP and the endogenous 49 kDa MPK6 before and after genotoxic stress (Figure 3A, lower panel), providing evidence for their post-translational activation.

Moreover, we examined the activities of endogenous MPK4 and MPK6 in response to UV-C using immunoprecipitation with specific antibodies followed by an in-gel

Fig. 4. *MPK6* is constitutively transcribed in response to genotoxic stress, while *MPK3* is induced. RNA gel blot analysis of 10 µg of total RNA isolated from UV-C-treated (0.5 kJ/m²) 14-day-old seedlings. Blots were hybridized sequentially with specific probes for *MKP1*, *MPK3*, *MPK4* and *MPK6*. Radiant Red-stained rRNA is shown as loading control.

assay for MBP phosphorylation. The 49 kDa MPK6 was activated by UV-C, while the activity of MPK4 remained under the detection limit (Figure 3B). Furthermore, in response to UV-C treatment, MPK6 activity level was highest in the *mkp1* mutant, intermediary in the wild type and lowest in the *MKP1*-overexpressing line 6 (Figure 3C). Thus, the immunokinase assays show an inverse correlation of MPK6 activity and the level of MKP1.

The activation of both MPK6-GFP and endogenous MPK6 in response to genotoxic stress *in vivo* points to MPK6 as the 49 kDa genotoxic stress-responsive MAPK (Ulm *et al.*, 2001). Furthermore, we provide evidence that the level of MKP1 determines the activation level of MPK6, making MKP1 a regulator of MPK6 function in response to genotoxic stress *in planta*.

MPK3 and MPK4 transcripts accumulate in response to genotoxic stress but the level of MPK6 mRNA remains constant

Among the MKP1-interacting MAPKs, *MPK3* previously has been shown to be induced transcriptionally by various abiotic stresses (Mizoguchi *et al.*, 1996; Desikan *et al.*, 2001b). To determine if similar transcriptional activation may occur in response to genotoxic stress, we carried out RNA gel blot hybridizations. UV-C treatment strongly increased *MPK3* mRNA levels and to a lesser extent those of *MPK4* (Figure 4). The level of *MPK3* transcript had already increased 30 min after UV exposure, reaching a peak after ~1 h (~10-fold induction) followed by a return to the basal level 3 h after irradiation. In contrast, *MPK4* mRNA accumulated gradually through the 6 h following UV treatment, while levels of *MPK6* and *MKP1* mRNA remained unaffected (Figure 4). Interestingly, mRNA levels of *MPK3*, 4 and 6 in wild type, *mkp1* and the *MKP1*-overexpressing line 6 were similar (Figure 4), demonstrating that their transcriptional regulation is MKP1 independent.

Microarray analysis of mkp1

To identify genes acting downstream of MKP1 and representing a functional readout of its signaling, microarray analysis was used to compare transcript levels in *mkp1*, line 6 and wild-type seedlings. Considering differences in expression level ≥ 5 -fold as a conservative estimate of significant changes, among ~8300 genes represented on the array (Zhu *et al.*, 2001), 22 (~0.3%)

met this criterion, of which 21 were activated in *mkp1*, and only one was repressed (Figure 5). Importantly, the expression of all 22 genes was at the wild-type level in line 6, confirming functional complementation of the *mkp1* mutation not only at the phenotypic but also at the molecular level (Figure 5A). Of note is that overexpression of *MKP1* in line 6 just returns the expression of genes affected by the *mkp1* mutation to the wild-type level, when plants are not subjected to adverse environmental conditions (Figure 5A and B). To detect the possible involvement of MKP1 in regulating gene expression in response to genotoxic stress, we performed global expression analysis after UV-C treatment of *mkp1* and line 6, in comparison with the wild-type controls. Seedlings of the three genotypes were irradiated with 0.5 kJ/m² UV-C and harvested for RNA analysis at different time points after irradiation (0.5, 1 and 3 h). In wild-type, mRNA levels of 537 genes were found to be altered at least 3-fold after the UV-C treatment: 205 were repressed and 332 activated. Interestingly, from the 21 genes with elevated expression in the *mkp1* mutant without irradiation, 17 were transcriptionally activated in the wild type during the first 3 h after UV treatment (threshold 3-fold, Figure 5), suggesting that these genes indeed belong to a specific subset of UV-responsive genes. Importantly, comparison of the mRNA levels of these genes over the time course after UV-C treatment in wild type, *mkp1* and line 6 revealed a similar pattern for most of them: transcript levels were elevated in the *mkp1* mutant not treated with UV or shortly after irradiation (0.5 h), but had decreased by the later time points (1 and 3 h after irradiation). Furthermore, transcripts of these genes clearly accumulated in the wild type after UV-C treatment, but their transcriptional induction in line 6 was even more pronounced (Figure 5C). Thus, the identified genes are likely candidates for downstream effectors of UV-C-activated MAPK pathway(s). Their expression pattern strongly suggests a regulatory step in their transcriptional repression and activation, both involving MKP1.

The identities of a number of these genes (Figure 5B) suggest their involvement in combating various stresses, e.g. oxidative stress (peroxidases and GSTs), biotic stress (cinnamyl-alcohol dehydrogenase, pathogenesis-related proteins: chitinases, thaumatin-like protein) and abiotic stress (Na⁺/H⁺-exchanging protein). In addition, apart from proteins of unknown function, increased mRNA levels of putative signaling components, proteins involved in the tryptophan biosynthetic pathway (phosphoribosylanthranilate transferase, indole-3-glycerol phosphate synthase), cytochrome P450 and ethylene response factor 1 indicate links to systemic signaling with possible participation of plant hormones.

***mkp1* has elevated resistance to salt stress**

The three MKP1-interacting MAPKs have been reported to be involved in salt stress signaling (Mizoguchi *et al.*, 1996; Ichimura *et al.*, 2000), and the microarray analysis revealed an increased mRNA level of a Na⁺/H⁺-exchanger belonging to a family of proteins involved in salt stress tolerance (Apse *et al.*, 1999; Shi *et al.*, 2000). This indicates that the role of MKP1 may not be restricted to genotoxic stress signaling but could include other stresses, in particular salinity. We therefore examined salt

resistance of *mkp1* by transferring 2-week-old seedlings germinated under standard conditions onto media containing increasing NaCl concentrations. Indeed, *mkp1* exhibited elevated salt resistance in its early vegetative phase compared with the wild-type control and line 6 (Figure 6). Importantly, the wild-type level of salt sensitivity is restored in the complemented line 6 but not in lines expressing MKP1 with the modified catalytic center (C235S), indicating the involvement of MKP1 as a negative regulator of salt stress tolerance (Figure 6).

Discussion

An important determinant of the final biological response is the magnitude and duration of the activation of a set of MAPKs in a given stress situation, which is governed by the upstream activating MAPK kinases and the deactivating phosphatases. This concept is supported by a vast amount of biochemical data from mammalian cell cultures on the importance of MKPs in appropriate stress responses (e.g. Camps *et al.*, 2000), including those to genotoxic stress (Franklin *et al.*, 1998). However, genetic evidence in the entire organism of multicellular eukaryotes illustrating such regulation is scarce.

We showed recently that the MAPK phosphatase homolog MKP1 plays an important role in genotoxic stress relief in *Arabidopsis*. Depletion of MKP1 in the *mkp1* mutant results in its hypersensitivity to various genotoxic treatments (Ulm *et al.*, 2001). Here, we have characterized interaction partners of MKP1 among a comprehensive panel of the nine previously cloned and characterized *Arabidopsis* MAPKs (Mizoguchi *et al.*, 1997). We found that MKP1 can interact with a subset of these enzymes, namely the three stress-related MPKs 3, 4 and 6, and, of these, interaction of MKP1 was recognizably strongest with MPK6 (Figure 2). This is similar to the mammalian MKPs, some of which display differential specificity for MAPKs, e.g. mammalian MKP-1 acts preferentially on the stress-related JNK/SAPK and p38, but much less on ERK (reviewed in Camps *et al.*, 2000). As there are 20 potential MAPK genes present in the *Arabidopsis* genome, the panel of MKP1 interactors could be even broader.

The significance of MKP1 interactions with three *Arabidopsis* MAPKs was verified further under genotoxic stress conditions and related to the levels of MKP1 itself. Since MPK6 was the most prominent MKP1 interactor, we confirmed its involvement in response to UV-C and MMS using transgenic plants containing an MPK6-GFP fusion. MAPK in-gel activity assays clearly showed activation of the modified MPK6 under genotoxic stress. Moreover, immunokinase assays with antibodies against MPK6 showed activation of the 49 kDa MAPK, implicating MPK6 as the UV-responsive MAPK in *Arabidopsis* (Figure 3). Importantly, the activity level of MPK6 was highest in *mkp1*, intermediary in the wild type and lowest in the *MKP1*-overexpressing line 6 (Figure 3C), supporting the function of MKP1 as a regulator of genotoxic stress responses through inactivation of MPK6 *in planta*.

A subclass of plant MAPK genes, including *MPK3*, are activated transcriptionally by diverse stress treatments (e.g. Mizoguchi *et al.*, 1997). Interestingly, we found that *MPK3* and *MPK4* are also induced transcriptionally in

response to genotoxic stress in *Arabidopsis*, but independently of MKP1 (Figure 4). In contrast, neither *MPK6* nor *MKP1* is regulated transcriptionally (Figure 4). In addition to the apparent constitutive expression of *MKP1*, it is expressed equally in all plant tissues analyzed (cauline, rosette and senescent leaves, flowers and flower buds, root and stem; data not shown). All this points toward MKP1 as a pre-formed component to regulate stress responses, in contrast to the tissue-specific and stress-inducible expression of mammalian MKPs (e.g. Camps *et al.*, 2000). This difference in negative regulation of the MAPK pathway in the two kingdoms implies the likelihood of post-translational regulation of *Arabidopsis* MKP1.

The transcriptome analysis of *mkp1* compared with wild type and line 6 revealed mostly up-regulation of genes (21 out of 22), most of which were also induced by UV-C treatment in wild-type plants (17 of the 21) (Figure 5). Thus, this specific subset of coordinately regulated genes probably includes genes regulated by the UV-C-activated MAPK pathway. Furthermore, comparison of expression levels in the *mkp1* mutant grown under standard conditions and after UV treatment suggests a dual and contrasting regulatory role for MKP1 under stress and favorable

growth conditions. In a significant proportion of the 21 genes with an elevated transcript level in non-stressed *mkp1* mutant plants, UV irradiation reduced the mRNA levels compared with up-regulation in the wild type and 'hyper'-up-regulation in line 6 (Figure 5). This indicates that MKP1 is a negative regulator of these genes under standard growth conditions, while it is a positive regulator of gene induction in response to UV-C treatment. Thus, MKP1 presence and its level contribute to a particular UV-mediated transcriptional activation, implicating its involvement in regulating the timing and amplitude of the transcriptional response to genotoxic stress. Such specificity of the regulatory function of an MKP at the transcriptome level of a complex organism was not anticipated and has not been documented previously. In addition, genes encoding MKPs often are induced transcriptionally under stress conditions, suggesting that their regulatory function is executed by changes in the level of an MKP. MKP1 seems to be rather exceptional, performing its stress-signaling function without changes in its transcript level.

Recent work using transient expression has identified the first complete *Arabidopsis* MAPK pathway, activated

in response to the bacterial elicitor flagellin (Asai *et al.*, 2002). This signaling cascade consists of MEKK1, MKK4/MKK5 and MPK3/MPK6, which function together with the WRKY22/WRKY29 transcription factors downstream of the receptor-like kinase FLS2 (flagellin sensing). Furthermore, it was shown that the transcriptional induction of *WRKY22* and *WRKY29* is dependent on MAPK activation and forms a positive feedback control (Asai

et al., 2002). It is rather surprising that, given the effects of genotoxic stress on known defense genes (Figure 5; data not shown) and MPKs 3 and 6 (Figures 3 and 4), there was no transcriptional induction by UV-C (threshold 3-fold) of *WRKY22* and/or *WRKY29* (data not shown). Interestingly, however, we identified two other transcription factors of the WRKY family, *WRKY25* and *WRKY33*, which are clearly induced by UV-C (data not shown). In contrast to

Fig. 5. Oligonucleotide array comparing Ws and *mkp1*. (A) The 21 genes overexpressed (≥ 5 -fold) in *mkp1* are complemented in line 6. Black bars depict genes that are induced by UV-C (0.5 kJ/m²) during 3 h post-irradiation (≥ 3 -fold). (B) Genes with an altered basal expression level in *mkp1*. Bold font indicates genes that are induced by UV-C during the 3 h time course (≥ 3 -fold). The asterisk indicates that the expression level in wild type is very low and the signals are near background. Therefore, the fold changes of these genes are only approximate. (C) MKP1-dependent regulation of the 21 genes in *mkp1* overexpressed in response to UV-C treatment. Time points (x-axis; 0, 0.5, 1 and 3 h after irradiation) and expression levels (y-axis) are as indicated. Black bar, wild-type Ws; dark-gray bar, *mkp1*; light-gray bar, line 6.

Fig. 6. Elevated resistance of *mkp1* to salt stress. Two-week-old seedlings were transferred to plates containing the indicated concentration of NaCl (0–225 mM). Ws, Wassilewskija wild-type; *mkp1*, *mkp1* mutant; line 6, *mkp1* complemented by ectopic expression of *MKP1*; C235S-1/C235S-2, two independent lines of *mkp1* with ectopic expression of *MKP1*(C235S). Pictures were taken after 8 days of further growth, and representative plates from five replicates are shown.

WRKY22 and *WRKY29* that belong to subgroup IIe, both *WRKY25* and *WRKY33* belong to subgroup I (Eulgem *et al.*, 2000). Even though the link of MAPKs to the WRKY transcription factors in response to elicitor or genotoxic stress is not clear, the use of shared MAPKs but induction of divergent *WRKY* transcription factors seems to take place.

Among the genes deregulated in *mkp1*, a putative Na⁺/H⁺-exchanging protein showed an elevated transcript level in *mkp1* (Figure 5B). Overexpression of the vacuolar Na⁺/H⁺-exchanging protein NHX1 or the plasma membrane Na⁺/H⁺-exchanging protein SOS1 (salt overly sensitive) leads to elevated salt resistance (Apse *et al.*, 1999; Zhu, 2001), while mutation in *SOS1* results in salt hypersensitivity (Shi *et al.*, 2000). Thus, the microarray analysis suggested a link between *MKP1* function and salt stress responses. This hypothesis was substantiated further by the identities of *MKP1*-interacting MAPKs (MPK3, 4 and 6), which previously had been implicated in responses to elevated salinity conditions.

Re-examination of the *mkp1* phenotype under salt stress revealed that loss of *MKP1* or its phosphatase activity results in elevated tolerance to salinity, identifying *MKP1* as a negative regulator of salt resistance (Figure 6). This is contrasted by the positive regulator function in response to genotoxic stress (Ulm *et al.*, 2001). Thus, *MKP1* is involved in the regulation of the two stresses in opposing directions. However, since no difference in the activation level of either MPK4 or MPK6 was detected in *mkp1* compared with the wild type under salt stress (data not shown), the common determinant linking genotoxic/salinity signaling may be MPK3 or an as yet uncharacterized MAPK. *MKP1* function in balancing stress responses is rather subtle and, previously, using a different experimental set-up involving liquid cultures and different light conditions, the responses to elevated salinity of *mkp1* and

the wild type were similar (Ulm *et al.*, 2001). This suggests that integration of various stress signals through *MKP1* is connected intimately to the perception of other environmental factors influencing the final response at the organismal level.

UV-C activates MPK6 but not MPK4 (Figure 3B). This is in contrast to other abiotic stresses, including elevated salinity, osmolarity and cold treatment, which activate both MPK4 and MPK6 (Ichimura *et al.*, 2000), but is similar to the selective activation of MPK6 by oxidative stress (Yuasa *et al.*, 2001). Of note is that both salt stress and UV-C lead to production of active oxygen species, and recently the UV-C mediated activation of the tobacco MPK6 ortholog salicylic acid-induced protein kinase (SIPK) was found to be reliant on these (Miles *et al.*, 2002). Moreover, using protoplast transient expression assays, the *Arabidopsis* MAPKKK ANP1 was shown to be activated in response to oxidative stress and subsequently to initiate a signaling cascade involving MPK3 and 6. Interestingly, expression of a constitutively active form of the ANP1 ortholog NPK1 from tobacco in transgenic plants results in elevated resistance to multiple abiotic stresses, including cold, heat and salt (Kovtun *et al.*, 2000). Furthermore, *Arabidopsis* MKK1 is activated in seedlings by different abiotic stresses, including high salt (Matsuoka *et al.*, 2002). Thus a salt stress-responsive pathway conceivably involves ANP1, MKK1, MPK3, 4 and/or 6, and their negative regulator *MKP1*.

In addition, using genetic evidence, detoxification of active oxygen has been postulated to elevate salt tolerance, as illustrated by the *pst1* mutant (Tsugane *et al.*, 1999). *mkp1* and *pst1* are the only recessive mutations that increase salt resistance in the vegetative phase. The gene affected in *pst1* has not yet been identified, precluding clarification of their molecular relationship. However, unaltered sensitivity of *mkp1* in response to methyl

viologen (paraquat), H₂O₂ or Rose Bengal (Ulm *et al.*, 2001; data not shown) suggests that its salt tolerance does not involve enhanced detoxification of active oxygen species. In addition, *mkp1* seems to respond normally to freezing and drought stress; however, preliminary experiments indicate that *mkp1* may be slightly insensitive to abscisic acid (ABA), the plant stress hormone (data not shown). A connection between genotoxic stress resistance and salt/ABA responses had also been postulated based on phenotypes of the *uvs66* mutant (Albinsky *et al.*, 1999). However, since the molecular nature of the *uvs66* mutation is unknown, this phenotypic link again cannot be described at the molecular level.

In contrast to the single mutant with enhanced tolerance to elevated salinity levels during vegetative growth, a number of mutants exhibiting salt hypersensitivity have been identified (reviewed, for example, in Zhu, 2000, 2001). Interestingly, the gene affected in one of these mutants (*SOS3*) encodes a Ca²⁺-binding protein with three predicted EF-hands, and is most similar to the B-subunit of calcineurin and animal neuronal Ca²⁺ sensors. *SOS3* interacts with the serine/threonine protein kinase *SOS2*, defining the *SOS3*–*SOS2* regulatory complex (Zhu, 2000, 2001). Interestingly, the cross-talk of a calcineurin and a MAPK pathway in Cl[−] homeostasis in fission yeast has been described previously (Sugiura *et al.*, 1998). Overexpression of the MKP *Pmp1* suppressed the Cl[−] hypersensitivity of the calcineurin disruption mutant *ppb1*⁺ and deletion of *Pmp1* resulted in increased Cl[−] sensitivity (Sugiura *et al.*, 1998). No direct analogy is possible between this salinity stress signaling in *Schizosaccharomyces pombe* and *Arabidopsis* since the latter contains no obvious calcineurin homolog (Kerk *et al.*, 2002). Moreover, any relationship of the *SOS* pathway to the MAPK pathway that is activated by high salinity in *Arabidopsis* remains to be established. As the first genetically defined component of the MAPK pathway with an apparent link to salt stress perception, MKP1 may provide an entry point for further dissection of this signaling network.

Materials and methods

Plant material

Arabidopsis thaliana plants were grown under aseptic conditions and genotoxic stress treated as described previously (Ulm *et al.*, 2001). For salt resistance assays, the light intensity was 90 μmol photons/m²/s, and the light/dark cycle was 12 h/12 h at 22°C. Seeds were germinated aseptically on germination medium (GM; Van Valvekens *et al.*, 1988) with 0.8% agar and grown further for 2 weeks. Then seedlings were transferred to GM supplemented with NaCl at the indicated concentrations and grown further for another 8 days. The elevated salt resistance of *mkp1* was assayed and confirmed in five independent repetitions.

Arabidopsis thaliana was transformed by *Agrobacterium* using the floral dip method (Clough and Bent, 1998). To generate the MPK6–GFP transgenic plants, the *MPK6* coding region was fused in-frame to GFP driven by the cauliflower mosaic virus (CaMV) 35S promoter in the pCAMBIA1302 binary vector (EMBL/DDBJ/GenBank accession No. AF234298). Line 6 was selected during the genomic complementation of the *mkp1* mutant due to its higher accumulation of *MPK1* mRNA levels (~3-fold) compared with the wild-type (Ulm *et al.*, 2001). phygi5-MKP1(C235S) is the genomic, complementing construct described previously (Ulm *et al.*, 2001) modified using the QuikChange Site-Directed Mutagenesis kit (Stratagene) to introduce the single amino acid exchange cysteine to serine at position 235. The mutated construct was verified by sequencing and then introduced into the *mkp1* mutant. The resulting transgenic lines described in this work were genetically determined to have the transgene integrated at a single locus.

Yeast two-hybrid assay

The genotype of the *S.cerevisiae* reporter strain L40 is *MATa trp1 leu2 his3 ade2 LYS2::lexA-HIS3 URA3::lexA-lacZ GAL4* (Vojtek and Hollenberg, 1995). The plasmid vectors used for the yeast two-hybrid analysis were pVP16 and pBTM116 (Bartel and Fields, 1995; Vojtek and Hollenberg, 1995). Amplification by PCR was used to generate fragments of the coding sequences of *MPK1*–9 compatible for cloning into the pVP16 vector (Ichimura *et al.*, 1998; Mizoguchi *et al.*, 1998; this work), as well as *MPK1* into pBTM116. Transformation of lithium acetate-treated L40 yeast cells was carried out following the protocol described by Gietz *et al.* (1992). Qualitative and quantitative β-galactosidase assays were performed as described by Vojtek and Hollenberg (1995) and Bartel and Fields (1995), respectively. The activity is expressed in standard units multiplied by 1000.

Production of recombinant proteins and pull-down assay

The full-length *MPK6* was cloned in-frame with GST into the pGEX-3X vector (Pharmacia Biotech). Expression of the recombinant protein in *E.coli* was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3–4 h. Cells were harvested, resuspended in extraction buffer [1× phosphate-buffered saline (PBS), 50 mM EDTA and one tablet of Boehringer Complete per 50 ml] followed by sonication. Triton X-100 was added to 1% final concentration and the lysate was then clarified at 12 000 r.p.m. for 10 min at 4°C. GST fusion proteins were purified with glutathione–Sepharose 4B as described by the manufacturer (Pharmacia Biotech).

For baculovirus expression of Py-tagged MKP1 in insect cells, the coding region was amplified by PCR to generate a fragment compatible for cloning into a modified pAcSG2 vector (PharMingen), containing a linker with a double Py tag behind the start methionine. Production of recombinant protein in Sf9 insect cells was performed as described by Wirbelauer *et al.* (2000).

For pull-down assays, Sf9 cells were incubated in lysis buffer [50 mM Tris pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol (DTT) and one tablet of Boehringer Complete per 50 ml] for 30 min on ice. The lysate was then clarified for 10 min at 12 000 r.p.m. A 100 μl aliquot of supernatant containing ~2 μg of Py-MKP1 was combined with 2 μg of either GST alone or GST–MPK6 in 900 μl lysis buffer and incubated with head-over-head rotation for 2 h at 4°C. Then, 10 μl of 50% glutathione–Sepharose was added and incubated further for another 2 h at 4°C. Beads were collected by centrifugation and washed four times with lysis buffer before analysis on a protein gel blot.

Immunoblots and kinase assays

Antibodies raised against *Arabidopsis* MPK4 and 6 were described previously (Ab4CT1 and Ab6NT1; Ichimura *et al.*, 2000). Protein purification from plant tissue, immunoblot analysis and MBP in-gel kinase assays were carried out as described previously (Ulm *et al.*, 2001). Protein concentrations were determined with the Bio-Rad Protein Assay (Bio-Rad). Immunokinase assays were performed as described previously (Ichimura *et al.*, 2000).

RNA isolation, RNA gel blot analysis and profiling

Total RNA was isolated with the Trizol reagent following the supplier's instructions (Gibco-BRL) and RNA gel blot analysis was performed as described by Ulm *et al.* (2001). For UV-C-responsive gene expression analysis, 2-week-old *Arabidopsis* seedlings of Wassilewskija ecotype, *mkp1* and line 6 were irradiated with 0.5 kJ/m² UV-C as described previously (Ulm *et al.*, 2001), and samples were taken at the indicated time points. cRNA synthesis, hybridization to the Affymetrix AtGenome1 GeneChip, washing, staining and scanning were carried out according to the manufacturer's suggestions (Affymetrix), as described in Zhu *et al.* (2001).

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