Arabidopsis MAPK Phosphatase 2 (MKP2) Positively Regulates Oxidative Stress Tolerance and Inactivates the MPK3 and MPK6 MAPKs

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Two closely related Arabidopsis mitogen-activated protein kinases (MAPKs), MPK3 and MPK6, are rapidly but transiently activated in plants exposed to ozone. Although the contribution of these MAPKs to control of redox stress has been examined extensively, it remains unclear whether the dual-specificity MKPs play an essential role in the regulation of these processes. To explore this question, specific knockdown of each of the five putative MKPs in Arabidopsis was performed, and the ozone sensitivity phenotype of each MKP-suppressed line was assessed. Silencing of only one previously uncharacterized MKP, designated AtMKP2, rendered the plants hypersensitive to oxidative stress. AtMKP2-suppressed plants displayed significantly prolonged MPK3 and MPK6 activation during ozone treatment, and recombinant AtMKP2 was able to dephosphorylate both phospho-MPK3 and phospho-MPK6 in vitro, providing direct evidence that AtMKP2 may target these oxidant-activated MAPKs. In addition, the in vitro phosphatase activity of AtMKP2 was enhanced by co-incubation with either recombinant MPK3 or MPK6. In AtMKP2:YFP-expressing plants, the fusion protein was localized predominantly in the nucleus, the same compartment into which ozone-activated MPK3 and MPK6 have previously been shown to be translocated. Taken together, these data suggest that AtMKP2, a novel MKP protein in Arabidopsis, acts upon MPK3 and -6, and serves as a positive regulator of the cellular response to oxidant challenge.

To survive, plant cells must maintain redox homeostasis in the face of a range of oxidative challenges from both internal and external sources, including potentially damaging “reactive oxygen species” (ROS) generated by high energy electron transfer systems in the chloroplasts, mitochondria, and peroxisomes and by environmental insults such as UV and ozone (1–3). At the same time, there is good evidence that specific ROS can also act as signal transduction messengers, most notably in the detection and response processes by which plant cells deal with potential pathogens (4) and herbivores (5) but also in physiological processes such as control of stomatal aperture (6). These seemingly contrasting scenarios require the cell to manage ROS levels through temporally and spatially modulated mechanisms that allow suppression of undesirable ROS accumulation while still permitting intra- or intercellular transmission of ROS-encoded information. The first step in these redox homeostatic mechanisms is the detection and signaling of ROS levels through an orchestrated sequence of intracellular signaling events.

Genes encoding mitogen-activated protein kinases (MAPKs) and their upstream activators (MAPK kinases and MAPK kinase kinases) form highly conserved families in eukaryotes, including plants (7–9), and these kinase-based signal transduction modules are known to regulate a host of cellular processes, including responses to oxidant stress (10). In Arabidopsis, the MAPKs most implicated in oxidative stress signaling are MPK3 and MPK6. Suppression of the ozone-activated MAPK, MPK6, results in a marked ozone-hypersensitivity phenotype (11), as does loss of its close homologue, MPK3 (11), whereas constitutive activation of MPK6 in Arabidopsis by ectopic expression of a heterologous tobacco MAPKK, NtMEK, also induces rapid cell death (12). Thus, it appears that either loss or unregulated activation of MPK3 and/or MPK6 makes plant cells more vulnerable to oxidative stress.

The activation of MAPKs such as MPK3 and MPK6 is the result of MAPKK-catalyzed dual phosphorylation of a -TXY-motif in the activation loop near sub-domain VIII of the kinase domain (13). ROS-elicited activation of these MAPKs is normally transient (14), indicating that the -pTXXpY- phosphate groups are quickly removed, and the MAPK is deactivated, presumably through the action of phosphoprotein phosphatases. Although much is known about the activation and biological roles of MPK3 and MPK6 in plants (15), the process by which they are inactivated remains unclear. In mammalian systems, MAPK dephosphorylation is typically catalyzed by a group of specialized dual-specificity phosphotyrosine phosphatases known as MAPK phosphatases (MKPs) that regulate the activities of their MAPK targets through specific dephosphorylation.
of both phosphotyrosine and phosphothreonine residues (16). Because both the magnitude and duration of MAPK activity can dictate the outcome of physiological responses, MKPs play important roles in modulating MAPK signaling processes.

The 11 members of the mammalian MKP family exhibit differential specificities toward their MAPK substrates as well as distinct subcellular localization patterns (17), and they are collectively responsible for the regulated dephosphorylation and inactivation of the 14 presently identified mammalian MAPKs. In contrast, the Arabidopsis genome encodes five potential MKPs, based on the amino acid sequence similarity of the phosphatase catalytic domain to established animal MKPs (18). This five member family of putative AtMKPs includes the previously reported members, AtMKP1, DsPTP1, PHS1, and IBR5, but among these, only one candidate, DsPTP1, has been shown to possess dephosphorylation activity against an Arabidopsis MAPK (MPK4) (19). However, no physiological context has yet been defined for DsPTP1 activity. AtMKP1, on the other hand, was earlier reported to interact strongly with MAPK MPK6 in yeast two-hybrid assays, and the loss-of-function mkk1 mutant displayed mis-regulation of MPK6 activity specifically in response to genotoxic stress in planta (20, 21). Despite these indications of a functional relationship between AtMKP1 and MPK6, the ability of AtMKP1 to catalyze dephosphorylation of activated MAPKs has not been demonstrated experimentally.

Here, we describe genetic and biochemical studies showing that another putative MKP, AtMKP2 (At3g06110), participates in the regulation of cellular homeostasis in ozone-challenged tissue and can influence the activation state of MPK3 and MPK6. Suppression of AtMKP2 creates a marked ozone sensitivity phenotype in Arabidopsis plants, and this hypersensitivity is accompanied by prolonged activation of both MPK3 and MPK6 in vitro and that its catalytic activity is significantly increased by association with these MAPKs. Overall, these results demonstrate that AtMKP2, the fifth member of the putative MKP family in Arabidopsis, contributes to the survival of plant cells challenged by redox stress and that it may do so through its ability to specifically dephosphorylate the oxidant-activated MAPKs, MPK3 and -6.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Treatments**—Wild-type Arabidopsis (ecotype “Columbia-0”) and five MKP-RNAi lines (AtMKP1i, AtMKP2i, DsPTP1i, PHS1i, and IBR5i) were used in this study. After sterilization, all seeds were sown on agar-solidified MS medium containing 25 μg/ml gentamycin and 50 μg/ml kanamycin. Four-week-old Arabidopsis plants (Col-0) were transformed by the floral dip method (23).

**AtMKP2 and Oxidative Stress**

To analyze the level of gene expression by reverse transcriptase-mediated PCR, total RNA samples were prepared from 3-week-old plants using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. The concentration of RNA was determined by measuring the optical density at 260 nm. Reverse transcription was performed using a First-strand cDNA Synthesis Kit (Amersham Biosciences), and aliquots of the resulting RT reaction product were used as template for RT-PCR analysis. The following primers were used for RT-PCR: ACT8 forward, 5'-ATTAAAGGTCGTGGCA-3'; ACT8 reverse, 5'-TCCGAGTTTGAAGGGCTAC-3'; AtMKP1 forward, 5'-CCGGGATCCCGCGATGTGGAGAGGCAAAGTTTG-3'; AtMKP1 reverse, 5'-CCGGAAATTCGGTTTATA-CCGGCGTCAGCAGTGCTAGCA-3'; AtMKP2 forward, 5'-CCGGGATCCCGCGATGGAGAGGCAAAGTTTG-3'; AtMKP2 reverse, 5'-CCGGAAATTCGGCTTATACG-CTGCTTACGTTAAGT-3'; DsPTP1 forward; 5'-CCGGGATCCGGCGCTTTTCTTCTTAAAGGGTCTTC-3'; DsPTP1 reverse, 5'-CCGGAAATTCGGGCTTTTCTTCTTAAAGGGTCTTC-3'; PHS1 forward, 5'-CCGGCAGATCCCGCGATGGAGAGGCAAAGTTTG-3'; PHS1 reverse, 5'-TATAGGTCTTGGGATGTGACTCCCGGTTTACTCTCC-3'; IBR5 forward, 5'-CCGGGATCCCGCGATGGAGAGGCAAAGTTTG-3'; IBR5 reverse, 5'-CCGGGATCCCGCGCTTTTCTTCTTAAAGGGTCTTC-3'; and IBR5 reverse, 5'-CCGGGATCCCGCGCTTTTCTTCTTAAAGGGTCTTC-3'.

**Protein Extraction and Immunoblot Analysis**—The frozen tissues were ground in liquid nitrogen and homogenized in
Phosphatase Assay—Phosphatase activity of AtMKP2 was assayed at 22 °C in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM EGTA, 2 mM dithiothreitol, 10 mM NaF, 50 mM β-glycerolphosphate, 1 mM phenylmethylsulfonyl fluoride, 1 tablet/50 ml extraction buffer of proteinase inhibitor mixture (Roche Applied Science), 10% glycerol, 7.5% (w/v) polyvinylpyrrolidone. After centrifugation at 13,000 rpm for 30 min, aliquots of supernatant were frozen immediately in liquid nitrogen and stored at −20 °C. The protein concentration was determined using a Bradford assay (Bio-Rad) with bovine serum albumin as a standard. Immunoblot analysis was performed using anti-phospho-ERK (1:2000, New England Biolabs), anti-MPK3 (1:2000, Sigma), anti-MPK6 (1:5000, Sigma), or anti-GST (1:5000, Sigma) antibody as primary antibody, and peroxidase-conjugated goat anti-rabbit IgG (1:5000) or antimouse IgG (Dako) as secondary antibody.

Ion Leakage Assay—Ozone-induced cell death was quantified by measuring ion leakage with a Model 2052 digital conductivity meter (VWR) in whole rosette leaves after 4-h incubation in 5 ml of distilled water. Leakage was expressed as percentage of total ion release, quantified after killing the leaves by autoclaving. Fifteen leaves from five plants per genotype were assayed in each of the replicate experiments.

Construction of KIMPK3, KIMPK6, KIMPK12, CAMKK4, CAMKK9, and CIAtMKP2 Clones—Expression vectors were created for mutant forms of GST-MPK3-, GST-MPK6-, GST-MPK12-, GST-MK44-, GST-MK99-, and GST-AtMKP2-encoded proteins in which the ATP binding site was modified to block activity (K67R for KIMPK3, K92R for KIMPK6, and K70R for KIMPK12), or the MKK activation phosphorylation sites were replaced with acidic residues to create a constitutively active kinase (T224D and S230E for CAMKK4, and S195E and S201E for CAMKK9), or the catalytic active site cysteine residue was replaced by serine (C109S for CIAtMKP2). All such modifications were carried out by site-directed mutagenesis with the ExSite PCR-based site-directed mutagenesis kit (Stratagene), using the expression plasmid carrying a GST-MPK3, GST-MPK6, GST-MPK12, GST-MK44, GST-MK99, or GST-AtMKP2 cDNA insert, respectively, as template. The mutated constructs were sequenced to confirm the changes and the absence of mismatches.

Recombinant Protein Production—Full-length cDNAs corresponding to MPK3, MPK6, MPK12, MKK4, MKK9, AtMPK2, and DsPTP1 were amplified by PCR. The amplicons were purified and digested by the appropriate restriction enzymes and subcloned in either the pGEX 4T-2 or pDESTTM15 vector, which expresses the recombinant protein with a N-terminal GST tag, to yield the vectors pGEX-MPK3, pGEX-MPK6, pGEX-MPK12, pGEX-MK44, pGEX-MK99, pGEX-AtMPK2, and pGEX-DsPTP1. Wild-type and mutant expression vectors were subcloned in either the pGEX 4T-2 or pDESTTM15 vector, and the amplified fragment was introduced into the vector, generating the pGWB40 (Research Institute of Molecular Genetics, Matsue, Japan). To generate the ProAtMKP2:AtMKP2:YFP construct, a 1.5-kb AtMKP2 genomic fragment upstream of the open reading frame was amplified, and the amplified fragment was introduced into the vector pGWB40 (Research Institute of Molecular Genetics). Transgenic Arabidopsis seedlings expressing each AtMKP2:YFP fusion construct were grown in sterile agar culture for 5 days. DNA was stained with 1 mg/ml 4′,6-diamidino-2-phenylindole dihydrochloride for 10 min. Roots were washed three times for 10 min and then mounted in water under glass coverslips for microscopy. The mounted specimens were examined using a Zeiss Axioskop epifluorescence microscope. 514 nm was used for YFP excitation, along with a 63× numerical aperture 1.4 oil-immersion lens.

Recombinant Harpin—Recombinant harpin from Escherichia coli BL-21 cells harboring the pT7-7 plasmid containing the DNA fragment encoding harpin_pshp was purified essentially according to Lee et al. (24) except 40% saturation of ammonium sulfate was used for the precipitation, and desalting and concentration were achieved through dialysis (14- to 18-kDa cutoff).
RESULTS

RNAi Silencing Yields Gene-specific MKP-suppressed Genotypes—Arabidopsis thaliana plants of the Columbia ecotype are relatively ozone-tolerant and display no visible damage when exposed to ozone (500 ppb) for 24 h. Such continuous ozone treatment results in rapid activation of two MAPks, MPK3 and MPK6 (11, 25), which then return to basal levels of activity after ~2 h, but loss of function for either kinase blocks the deactivation of the other and is accompanied by increased ozone sensitivity (11). To determine whether any of the five putative Arabidopsis MKP candidates (AtMKP1, At3g06110, DsPTP1, PHS1, and IBR5) play a role in controlling this refractory behavior, or regulating the ozone tolerance phenotype, we examined the corresponding MKP loss-of-function genotypes.

We first sought T-DNA insertional mutant lines lacking each of these five genes but were not able to isolate homozygous lines with T-DNA insertions in all cases, either because of a lack of mutant stocks for a particular gene, or because homozygous mutant plants could not be recovered in progeny populations. Therefore, for consistency of genetic character, we designed gene-specific RNAi constructs that target the highly divergent 5′-region of each of the five potential MKP genes. To avoid potentially damaging effects of long term silencing, we also placed these RNAi constructs under the control of a DEX-inducible promoter.

A. thaliana plants were transformed with each of the DEX-inducible MKP-RNAi constructs, and multiple transgenic lines were selected for evaluation. In the absence of DEX induction, all of these lines grew normally and displayed the wild-type phenotype. A series of T2-generation plants carrying each MKP-RNAi construct were then treated with 30 μM DEX for 24 h, and the expression level of each MKP gene was assessed by RT-PCR. Suppression of transcripts from each of the five endogenous MKP genes was observed to degrees ranging from partial to complete reduction of detectable mRNA (Fig. 1A). From among the most strongly suppressed RNAi lines, two independent lines for each of the five MKP candidate genes were selected for confirmation of the specificity and effectiveness of the knockdown (Fig. 1B) and then used for further study. Because high levels of expression of the GVG transactivator in transgenic plants carrying the pTA7002 DEX-inducible cassette have been reported to sometimes display stress phenotypes (26), MKP-RNAi transgenic lines were screened by RT-PCR for GVG expression levels (data not shown), and only lines with moderate to low levels of GVG expression were carried forward.

Only At3g06110-silenced Plants Exhibit Enhanced Sensitivity to Ozone Stress—Simultaneous exposure of all five MKP-silenced genotypes to 500 ppb ozone resulted in tissue collapse across the leaf blade of At3g06110-silenced plants within 2 h, whereas no damage was observed on wild-type leaves, or on leaves of the other MKP-RNAi-suppressed genotypes, at this time point (Fig. 2A). Ozone-induced cellular damage can be quantitatively assessed by measurements of ion leakage, indicative of increased membrane permeability. Measurements over an 8-h ozone exposure period confirmed that At3g06110-silenced plants treated with acute ozone lost control of plasma membrane integrity far more rapidly than did wild-type plants (Fig. 2B). Leaves from wild-type plants did not display any visible tissue collapse by 8 h and exhibited only a small increase in ion leakage over this exposure period. DEX-treated plants carrying the empty pTA7002 vector also showed no signs of ozone damage under these treatment conditions (data not shown). Overall, these data indicate that loss of At3g06110 function severely compromises the ability of Arabidopsis plants to control redox stress.

MPK3 and MPK6 Deactivation Is Delayed in Ozone-treated At3g06110-RNAi Plants—If MPK3 and 6 deactivation in ozone-treated plants is dependent on MKP activity, we predicted that MKP silencing should prolong MPK3 and MPK6 activation profiles during acute ozone challenge. To test this, expression of each MKP-RNAi construct was induced by DEX treatment, and the induced plants were challenged 24 h later with 500 ppb ozone. Leaf samples were collected at times from 0 to 8 h after onset of the ozone exposure, and the state of activation of MPK3 and MPK6 was analyzed on Western blots of leaf extracts, using an anti-pERK antibody that recognizes only the doubly phosphorylated form of these MAPks. Uniformity of lane loading was monitored by subsequently stripping the blots and re-probing with anti-MPK6 antibodies, because MPK6 levels are known to be unaffected by stress treatments (25).
The ozone activation profiles of both MPK3 and MPK6 were broadly similar in wild-type and in AtMKP1-RNAi, DsPTP1-RNAi, PHS1-RNAi, and IBR5-RNAi plants (Fig. 2), but in ozone-treated At3g06110-RNAi plants (Fig. 2C), in which both kinases could be detected for up to ~8 h, well beyond the point at which both kinases had been largely inactivated in the other MKP-suppressed lines.

**Locus At3g06110 Encodes a Functional MKP**—Locus At3g06110 encodes a 18-kDa protein with a well conserved dual-specificity phosphatase (DSP) catalytic domain. DSPs act on both phospho-Tyr and phospho-Ser/Thr residues in protein substrates, and in animals and yeast the DSP sub-group of MKPs is specifically involved in regulating MAPK activity (27, 28). To determine whether the At3g06110 locus encodes a functional MKP enzyme, we examined the catalytic properties of the recombinant protein. Recombinant GST-chimeric protein was successfully expressed in E. coli and purified by affinity chromatography (Fig. 3A). When assayed against the synthetic phosphatase substrate, OMFP, the recombinant protein was shown to dephosphorylate OMFP in a concentration- and time-dependent manner (Fig. 3B and C).

It is well established that replacement of the conserved cysteine residue in the catalytic active site (VXCVXCVSSXAYLM) of canonical DSPs by serine eliminates enzyme activity (29). As expected, when the C109S mutant was expressed as a GST fusion protein (GST-CIAt3g06110) this form of the gene product was found to possess no phosphatase activity in the OMFP assay system. Taken together, our data demonstrate that the protein encoded by At3g06110 can be considered a bona fide member of the MKP sub-family of eukaryotic DSPs, and we therefore named it AtMKP2.

**Recombinant AtMKP2 Dephosphorylates Phospho-MPK3 and -MPK6 in Vitro**—The activity of a MAPK is dependent on the phosphorylation status of its -TXY-motif (30), and these phospho-amino acid residues are also the target of MKP activity. The observation that loss of AtMKP2 function in AtMKP2-RNAi plants is associated with delayed inactivation of MPK3 and MPK6 (Fig. 2C) suggested that the AtMKP2 phosphatase could be directly or indirectly responsible for dephosphorylating these two MAPKs. To test this hypothesis, we conducted in vitro MAPK dephosphorylation assays using dually phosphorylated recombinant MPK3 and MPK6 as substrates. To ensure that MAPK autophosphorylation activity would not interfere with the phosphatase assays, MPK3 and MPK6 were first mutagenized at their ATP binding site to silence kinase activity. These “kinase-inactive” products were expressed as recombinant fusion proteins (GST-KMPK3 and GST-KMPK6) and phosphorylated by preincubation with a recombinant constitutively activated form of the upstream cognate MAPKK, GST-CAMKK4 (Fig. 4B). The ability of AtMKP2 to dephosphorylate the -pTEpY- motif of MPK3 and MPK6 in...
vitro was tested by incubating different concentrations of recombinant GST-AtMKP2 with purified phospho-MPK3 or phospho-MPK6 and monitoring the disappearance of the -pTXpY- signal by immunoblot analysis using anti-pERK1/2 antibody. The phosphorylation of both MPK3 and MPK6 was decreased upon incubation with GST-AtMKP2, in a dose-dependent manner, whereas incubation of the phospho-MAPKs with GST alone had no effect on their phosphorylation state (Fig. 4, C and D). To ascertain whether the ability of GST-AtMKP2 to dephosphorylate phospho-MPK3 and -6 is a reflection of nonspecific phosphatase activity, another Arabidopsis MKP, DsPTP1, was expressed as a GST fusion and tested. Although DsPTP1 displays significant sequence similarity to AtMKP2, GST-DsPTP1 recombinant protein was unable to dephosphorylate either phospho-MPK3 or phospho-MPK6 (supplemental Fig. S1). Similarly, when recombinant GST-AtMKP2 was incubated with another phospho-MPK (pMPK12) belonging to the Arabidopsis -TEY- subclass of MAPKs, the phosphatase was unable to deactivate this MPK (supplemental Fig. S2). These results strongly support the idea that both MPK3 and MPK6 are direct and specific targets for AtMKP2.

AtMKP2 Catalysis Is Stimulated Specifically by Association with MPK3 and MPK6—It has been reported that the in vitro activity of some mammalian MKPs is increased in the presence of their substrate MAPK (31, 32). To examine the effect of Arabidopsis MAPKs on the catalytic activity of AtMKP2, we assayed recombinant GST-AtMKP2 against OMFP in the presence and absence of recombinant MPK3, MPK6, MPK4, or MPK12. The addition of either MPK3 or MPK6 significantly enhanced the phosphatase activity of GST-AtMKP2 (Fig. 5B), whereas addition of the same amount of either recombinant GST, or two other -TEY- class MAPKs, MPK4 and MPK12, had no effect on the enzyme activity. The observed activation of AtMKP2 by association with MPK3 or -6 reflected primarily a substantial increase in the affinity of AtMKP2 for its OMFP substrate (5-fold increase in $k_{cat}/K_m$) (Table 1). The stimulatory effect of association with MPK3 or -6 was unrelated to MAPK catalytic function, because co-incubation of GST-AtMKP2 with kinase-inactive mutant forms of MPK3 and -6 (GST-KIMPK3 and GST-KIMPK6) resulted in the same degree of enhancement of AtMKP2 phosphatase activity as had been observed with the catalytically active forms (Fig. 5C).

AtMKP2 Is Predominantly Localized in the Nucleus—Activation of MPK3 and MPK6 by ozone exposure was recently reported to trigger their translocation to the nucleus in Arabidopsis (25), a response that would place the activated MAPKs in close proximity to potential target nuclear proteins such as transcription factors (33). If AtMKP2 were actively involved in dephosphorylation of pMPK3 and pMPK6, we postulated that this phosphatase might be found in the same compartment. To examine this question, we generated transgenic plants expressing AtMKP2:YFP fusion products, using either a 390-bp native AtMKP2 promoter and the genomic AtMKP2 gene fragment fused to YFP (construct ProAtMKP2:AtMKP2:YFP), or the AtMKP2 cDNA fused to YFP under control of the cauliflower mosaic virus (CaMV) 35S promoter (construct 35S:AtMKP2:YFP).

Examination of transgenic seedling roots by epifluorescence microscopy showed that, with either construct, ectopically expressed AtMKP2-YFP accumulated predominantly in the nucleus, as assessed by co-localization with the 4'-6-diamidino-2-phenylindole dihydrochloride signal (Fig. 6A). No fluorescence signal was observed when untransformed wild-type plants were viewed with the same settings (data not shown), whereas plants expressing YFP alone (construct CaMV35S:YFP) displayed fluorescence throughout the cytoplasm and nucleus (Fig. 6A), consistent with the sub-
cellular localization pattern reported previously for YFP protein (34). These results demonstrate that AtMKP2 is a nuclear MKP in Arabidopsis, which places this negative regulator in the same subcellular location as ozone-activated MPK3 and MPK6.

AtMKP2-suppressed Plants Are Hypersensitive to an ROS-generating Biotic Stress—Challenge with the bacterial elicitor, harpin, has been shown to induce both ROS accumulation (35) and MPK3 and MPK6 activation (14) in plant tissues, ultimately leading to hypersensitive response-like cell death. Because AtMKP2 appears to be associated with the control of cellular redox stress management (Fig. 2, A and B) and suppression of AtMKP2 expression prolongs the activation of MPK3 and MPK6 in response to oxidative signals (Fig. 2C), we asked whether loss of AtMKP2 function might also influence the plant’s response to challenge with harpin. Topical application of low concentrations of recombinant harpin (0.5 μg/μl) to the leaves of wild-type plants did not induce any visible tissue response within 3 days, but when applied to the leaves of AtMKP2-RNAi plants, the same harpin concentration induced rapid cell death and tissue necrosis (Fig. 6B). Loss of AtMKP2 function thus compromises the ability of Arabidopsis plants to manage both abiotic oxidative stress (ozone) and biotic stress that is known to lead to increased ROS accumulation in the challenged tissues (harpin).

**DISCUSSION**

Transient activation of one or both of the MPK3/MPK6 dyad of MAPKs in plant cells is a consistent early response to a wide range of biotic and abiotic stresses that are also typically associated with ROS accumulation in the stressed cells (25, 36). However, despite this prominence it remains unclear how the activity of these two oxidant-responsive MAPKs is regulated in the context of orchestrating cellular responses. Indeed, the situation is further complicated by indications that, in addition to mediating various stress responses, MPK3 and/or MPK6 (or their putative orthologs) are also involved in plant developmental (37, 38) and hormone signaling (25) pathways.

MAPK inactivation in eukaryotes can be catalyzed by different classes of protein phosphatases, including serine/threonine protein phosphatases and phosphotyrosine phosphatases (PTPs) (27). Because MAPK activation involves dual phosphorylation on both threonine and tyrosine residues, the canonical MAPK deactivators are thought to be the dual-specificity subclass of the PTPs, which have been designated MKPs (16). The Arabidopsis genome encodes five candidate MKPs (AtMKP1, At3g06110, DsPTP1, PHS1, and IBR5), based on their catalytic domain sequence conservation (18), and several of these have been implicated in various biological scenarios. Mutants of AtMKP1 were reported to show hypersensitivity to genotoxic stress, but not to other oxidant stresses (20), and to also be less sensitive to elevated salt levels in the growth medium. Subsequent analysis revealed that AtMKP1 interacts with MPK6 and, to a lesser extent, with MPK3 and MPK4 in yeast two-hybrid screens (21). Loss-of-function mutants of PHS1 displayed impaired microtubule organization (39) and abscisic acid hypersensitivity (40), whereas ibr5 mutants showed reduced responsiveness to auxin and abscisic acid, compared with wild-type plants (41). Only DsPTP1 has been directly demonstrated to be capable of dephosphorylating an Arabidopsis MAPK (MPK4), but no in vivo role for DsPTP1 has yet been established. Indeed, it remains unknown whether the biological impacts of loss-of-function in the PHS1 and IBR5 genes are in any way related to an ability of the respective gene products to act as canonical MKPs and dephosphorylate specific MAPKs. In the present report, we use in vivo functional screening of all five putative Arabidopsis MKPs to demonstrate...
that the fifth family member, which we have designated AtMKP2, deactivates both MPK3 and MPK6, and positively influences the ability of the plant to withstand oxidative stress.

The AtMKP2 gene (At3g06110) appears to fit the model of a cellular “housekeeping gene,” because it is expressed at moderate levels in all Arabidopsis tissues and developmental stages, and that expression is essentially unaffected by either ozone exposure (data not shown) or any of the 87 other treatments recorded in the Genevestigator microarray data base. The encoded protein is relatively small (18 kDa), containing little more than the essential phosphatase catalytic domain. Although AtMKP2 is phylogenetically most closely related to DsPTP1 (64% amino acid sequence identity within the catalytic domain), the genes encoding these two proteins appear to have diverged functionally. Unlike AtMKP2, DsPTP1 expression is largely restricted to the male reproductive organs (stamen and pollen) in Arabidopsis (Genevestigator analysis). In addition, the DsPTP protein is predicted to possess an N-terminal transit peptide that would direct the protein to the chloroplast compartment, and recombinant DsPTP1 dephosphorylates MPK4 in vitro (19), whereas AtMKP2 is localized to the nucleus (Fig. 6A), and recombinant AtMKP2 is able to dephosphorylate two other MAPKs, MPK3 and MPK6, but not MPK4 (Fig. 4C and D).

However, although both our in vivo and in vitro data indicate that AtMKP2 could contribute to regulation of the MPK3 and
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MPK6 activation cycle, it is not clear that the extended activation of these MAPKs in ozone-treated AtMKP2-suppressed plants is necessarily related to the enhanced sensitivity of plants to oxidant. Prolonged activation of ERK1/2 in mammalian cells has been reported to trigger cell death (42) and sustained activation of MPK3 and/or MPK6 also has been shown to be associated with stress-induced cell death in plants (11), but the loss of membrane integrity in ozone-challenged AtMKP2-suppressed plants is detected even before MPK3/6 deactivation might normally begin to take effect in wild-type plants (Fig. 2B). It therefore seems very likely that loss of AtMKP2 function is also affecting other cellular targets, in addition to MPK3 and MPK6 deactivation. Because no T-DNA insertional mutants have been identified for this gene, and seedlings in which RNAi-mediated AtMKP2 suppression has been induced show severe developmental defects (data not shown), it is possible that AtMKP2 activity is crucial for regulation of early development, either directly or through the modulation of intracellular ROS pools.

Although the deactivation of MPK3 and MPK6 is substantially delayed in ozone-treated AtMKP2-suppressed plants, it is clear that the inactivation process is not completely blocked. This might indicate that other protein phosphatases, presumably belonging to functional classes other than the MKPs, can participate in dephosphorylation of these two MAPKs. In this context, it is noteworthy that loss of AtMKP1 function in Arabidopsis not only resulted in increased genotoxic stress sensitivity but also reduced the level of MPK6 activation induced by genotoxic agents (21), consistent with the idea that MKP1 may act as a negative regulator of MPK activity but also reduced the level of MPK6 activation induced by genotoxic agents (21), consistent with the idea that MKP1 may act as a negative regulator of MPK activity.

In conclusion, we have shown that AtMKP2, the fifth member of the putative MKP gene family in Arabidopsis, is a functional MAPK phosphatase that possesses the ability to specifically deactivate the Arabidopsis MAPKs, MPK3 and MPK6. The catalytic activity of AtMKP2 is enhanced in vitro by association with its physiological targets, MPK3 and MPK6, and our genetic evidence strongly suggests that AtMKP2 function helps control the outcome of the cellular response to oxidant challenge in Arabidopsis.

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