Phosphorylation and Stabilization of Arabidopsis MAP Kinase Phosphatase 1 in Response to UV-B Stress

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Background: Arabidopsis MKP1 regulates MPK3 and MPK6 and has a crucial role in the UV-B stress response. MKP1 is a phosphoprotein that is stabilized in response to UV-B.

Results: Plant treatment with UV-B results in a gel mobility shift and accumulation of MKP1.

Conclusion: MKP1 phosphorylation and stabilization identifies novel post-translational regulation of a plant MAP kinase phosphatase in vivo.

Significance: MKP1 phosphorylation and stabilization identifies novel post-translational regulation of a plant MAP kinase

MAP kinase phosphatases (MKPs) are important regulators of the activation levels and kinetics of MAP kinases. This is crucial for a large number of physiological processes during development and growth, as well as interactions with the environment, including the response to ultraviolet-B (UV-B) stress. Arabidopsis MKP1 is a key regulator of MAP kinases MPK3 and MPK6 in response to UV-B stress. However, virtually nothing is presently known about the post-translational regulation of plant MKPs in vivo. Here, we provide evidence that MKP1 is a phosphoprotein in vivo and that it accumulates in response to UV-B stress. Moreover, proteasome inhibitor experiments suggest that MKP1 is constantly turned-over under non-stress conditions and that it is stabilized upon stress treatment. Stress-responsive phosphorylation and stabilization of MKP1 demonstrate the post-translational regulation of a plant MKP in vivo, adding an additional regulatory layer to MAP kinase signaling in plants.

Signaling through mitogen-activated protein kinases (MAPKs) regulates a vast array of cellular responses in all eukaryotes. In plants, MAPKs are involved in developmental processes including cytokinesis, cell differentiation and senescence, and in response to a broad range of environmental stresses, such as heavy metals, cold, pathogens, salinity, and UV-B radiation (1–3). Typically, MAPKs become active after dual phosphorylation of the TXY motif in their activation loop by MAPK kinases (MAPKKs) that were themselves phosphorylated and activated by upstream MAPKK kinases (MAPKKKs). Activated MAPKs then phosphorylate specific downstream targets to generate appropriate physiological responses (2–5). Therefore, the cellular outcome of a MAPK cascade largely depends on tight regulatory mechanisms acting on MAPKs. This includes the action of specific dual-specificity phosphatases (MKPs) that are able to dephosphorylate MAPKs, thereby ensuring adequate intensity and duration of MAPK activation (6–9). Work with non-plant organisms has shown that MAPKs control their own negative regulators, establishing negative feedback loops to attenuate the response (10–13).

In plants, MKP regulation is much less understood. Arabidopsis MKP1 was shown to be phosphorylated in vitro by MPK6 (14). Moreover, its tobacco ortholog NtMKP1 is catalytically activated in vitro by interaction with the MAP kinase SIPK (15). Other modes of MKP regulation include transcriptional control and regulation by calmodulin. Rice OsMKP1, wheat TMKP1 and NtMKP1 are transcriptionally activated upon diverse stress treatments (7, 16–18). Similar to Arabidopsis DsPTP1, tobacco NtMKP1 and rice OsMKP1, Arabidopsis MKP1 was shown to bind calmodulin in vitro (20), indicating the possibility of MKP regulation by calcium in plants. However, the physiological relevance of the different regulatory mechanisms suggested by in vitro experiments remains to be determined.

Arabidopsis mkp1 mutants are hypersensitive to methyl methanesulfonate (MMS) and UV-B stress (1, 21–23). On the other hand, MKP1 is a negative regulator of MPK6-mediated PAMP responses and resistance against bacteria (24, 25). UV-B stress and PAMPs activate MAPKs, including Arabidopsis MPK3 and MPK6 (1–3). Indeed, the UV-B hypersensitivity and Pseudomonas resistance phenotypes of the mkp1 mutant have been attributed to MPK3 and/or MPK6 hyperactivation (1, 24, 25). However, if and how Arabidopsis MKP1 itself is regulated in response to UV-B or other stress signaling is unknown. Here, we provide evidence that MKP1 is continuously turned over under non-stress conditions and that it is phosphorylated and stabilized in response to UV-B stress.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—mkp1(Col), mkp1/Pro35S:Pyo-MKP1 and mkp1 mpk6/Pro35S:Pyo-MKP1 are in the Columbia wild-type accession (Col) (24). The mkp1/ProMkp1:HA-MKP1S342D line is in the Ws background.
Plants were grown under aseptic conditions or on soil as described previously (24). Plant Treatments—UV-B sensitivity assays and broadband UV-B irradiations using Philips TL40W/12RS tubes were performed as described (1, 26). For UV-B-induced MAP kinase activation and gene expression assays, 7-day-old aseptically grown seedlings were used, unless otherwise indicated. For analysis of MKP1 protein, 6-day-old seedlings were transferred to ddH₂O overnight before treatment.

**Protein Extraction, α-Phosphatase Treatment, and Immunoblot Analysis**—Proteins to be treated with α-phosphatase were extracted according to Ref. 27. Incubation with α-phosphatase (NEB) was at 30 °C for 2 min, in the presence or absence of a phosphatase inhibitor mix (50 mM NaF, 20 mM NaVO₃, 5 mM EDTA, and 5 mM EGTA). Otherwise, proteins were extracted exactly as described before (1). For detection of MAP kinases, 15 μg of total protein extract were separated by electrophoresis in 10% SDS-polyacrylamide gels. For detection of tagged MKP1, total cellular proteins or α-phosphatase-treated extracts were separated in 6% SDS-polyacrylamide gels. For detection of endogenous MKP1, proteins were concentrated by Amicon Ultra 3K Centrifugal Filter Devices (Millipore) and 80 μg of the eluate were used for electrophoresis. Transfer to PVDF membranes was performed according to the manufacturer’s instructions (Bio-Rad).

Rabbit polyclonal antibodies were generated against a synthetic peptide derived from the MKP1 protein sequence (amino acids 755–770: CQMDLPKDTPIKIVRE) and were affinity purified against the peptide (Eurogentec). We used the primary antibodies anti-MKP1, anti-Glu-Glu (against Polyoma tag), anti-HA.11, anti-myc (Covance), anti-actin (Sigma-Aldrich), anti-MKP3, anti-MKP6 (24), and anti-phospho-p44/42 MAP kinase (Cell Signaling Technologies) with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulins (DAKO) as secondary antibodies, as required. Signal detection was performed using the ECL Plus Western Detection Kit (GE Healthcare). Data shown are representative for at least two independent experiments.

Quantitative Real-time PCR—RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and treated with DNaseI according to the manufacturer’s instructions. cDNA synthesis and quantitative RT-PCR were performed as previously described (24) using a 7900HT real-time PCR system (Applied Biosystems). cDNA concentrations were normalized to the 18S rRNA transcript levels as standard using the Eukaryotic 18S rRNA kit (Applied Biosystems).

**Site-directed Mutagenesis and Generation of Transgenic Plants**—Point mutations were introduced with the Quick-Change II Site-Directed Mutagenesis Kit (Stratagene) using the primers listed in supplemental Table S1. Gateway-based cloning was used to insert the different versions of MKP1 into the binary vector pGWB518 (28). Arabidopsis plants were transformed by Agrobacterium tumefaciens using the floral dip method (29). Experiments were performed with homozygous seeds in the T3 generation.

**Multiple Alignment**—The multiple sequence alignment was generated by ClustalW2 using a BLOSUM matrix (www.ebi.ac.uk) and edited by Jalview (www.jalview.org).

**RESULTS**

**MKP1 Is Phosphorylated in Response to UV-B Stress**—To understand how MKP1 is regulated at the post-translational level, we used Arabidopsis seedlings expressing Polyoma epitope-tagged MKP1 (Pyo-MKP1) driven by the constitutive CaMV 35S promoter (24). Treatment with UV-B resulted in a gel mobility shift of Pyo-MKP1, as detected by immunoblot analysis (Fig. 1A). Consistently, UV-B exposure altered the mobility of endogenous MKP1 and of C-terminally truncated HA-MKP1ΔC243 (driven by the endogenous promoter) (supplemental Fig. S1, A and B). Electrophoretic mobility shifts are indicative of post-translational modifications and some MAP kinase phosphatases were shown to be phosphorylated in non-plant organisms (10, 11, 30). Therefore, we examined whether MKP1 was indeed phosphorylated upon UV-B exposure. In vitro dephosphorylation of protein extracts by α-phosphatase resulted in the elimination of the UV-B-dependent slow
PHOSPHORYLATED MKP1 ACCUMULATES IN RESPONSE TO UV-B STRESS — In addition to MKP1 phosphorylation, higher levels of the Pyo-MKP1 protein were detected upon UV-B stress treatment within few minutes (Fig. 1A, supplemental Fig. S1B). As the Pyo-MKP1 was expressed using the constitutive CaMV 35S promoter (not responsive to UV-B; Ref. 31), we examined the stability of the MKP1 protein by applying proteasome inhibitors in planta. In comparison to the DMSO mock control, combinatorial treatment with MG132 and ALLN resulted in accumulation of Pyo-MKP1, readily detectable under electrophoresis conditions that do not separate the differentially phosphorylated forms of Pyo-MKP1 (Fig. 2A; short run using 8% SDS-PAGE). This indicates an involvement of the proteasome in the constant degradation of Pyo-MKP1 under non-stress conditions. It is of note that both the faster and slower migrating forms of MKP1 were stabilized upon proteasome inhibitor treatment, suggesting that the accumulation of phosphorylated Pyo-MKP1 resulted in part from its reduced degradation (i.e. p-Pyo-MKP1 detectable in proteasome inhibitor but not DMSO control samples in -UV-B controls) (Fig. 2, B and C; 6% SDS-PAGE). In contrast to Pyo-MKP1, the protein levels of MPK3 and MPK6 were not affected by proteasome inhibition (Fig. 2C).

A longer time course on MKP1 protein stability after UV-B stress release revealed that phospho-Pyo-MKP1 levels decreased more rapidly than those of the constitutively phosphorylated variant. Indeed Pyo-MKP1 remained in high amounts for at least 24 h after UV-B radiation (Fig. 2D). We performed qRT-PCR on UV-B-treated mkp1/Pro35S:Pyo-MKP1 plants to exclude regulation at the transcriptional or mRNA stability level. Similar to endogenous MKP1, Pyo-MKP1 mRNA levels remained unchanged after 20 min exposure to...
UV-B (supplemental Fig. S2), supporting that the immediate UV-B-induced accumulation of Pyo-MKP1 is a post-translational event. Surprisingly however, the stabilization of Pyo-MKP1 after return to non-stress conditions was associated with increased transcript levels (supplemental Fig. S2). Therefore, as the 35S promoter is not affected by UV-B (31), MKP1 mRNA stabilization could partly account for the protein accumulation observed after release from UV-B stress. However, such a mechanism remains to be unequivocally established.

**MPK6 Is Not the Only Kinase Phosphorylating MKP1**—The mammalian MKP-1 and yeast Msg5 MAPK phosphatases were shown to be phosphorylated by their substrate MAPKs (10, 30). In addition, Arabidopsis MPK6 was found to phosphorylate MKP1 in vitro (14). To investigate whether this feedback regulation takes place in vivo, we generated mpk6 mutants containing the Pro35S-Pyo-MKP1 transgene. In the absence of MPK6, Pyo-MKP1 was still phosphorylated in response to UV-B (Fig. 3, A and B). Moreover, MPK6 was dispensable for UV-B-induced phospho-Pyo-MKP1 stabilization (Fig. 3, A and B). However, out of presently unknown reason, the 35S-driven Pyo-MKP1 was less expressed and Pyo-MKP1 accumulated less in the mpk6 background (Fig. 3, B and C). Notwithstanding this, our data indicate that if MPK6 phosphorylates Pyo-MKP1 in vivo, additional kinase(s) may compensate for the loss of MPK6. Besides MPK6, MPK3 was shown to function in MKP1-regulated UV-B stress signaling (1); however, MPK3-mediated phosphorylation of MKP1 remains to be demonstrated. Our data suggest that MKP1 is not (exclusively) phosphorylated by MPK6, indicating that MPK3 and MPK6 could redundantly phosphorylate MKP1 in vivo or that the phosphorylation resulting in the mobility shift is independent of MPKs 3 and 6 or MAP kinases altogether.

Several Potential MAPK Phosphorylation Sites of MKP1 Are Conserved in Plants—Arabidopsis MKP1 is a protein of 784 amino acids with 111 serine and 30 threonine residues, of which 13 have a proline at position H11001 (Fig. 4A and supplemental Table S2), which is common among MAP kinase targets (32, 33). This high number of potential serine/threonine phosphorylation sites suggests high complexity in MKP1 phosphorylation and in its experimental analysis. Nonetheless, to better predict functionally important phosphosites, we aligned MKP1 sequences from different plant species and focused on potential MAP kinase target sites. Four PX_XX(S/T)P high stringency (Thr-64, Thr-109, Ser-295, Ser-309) and three XX(S/T)P low stringency (Ser-504, Ser-509, Ser-598) sites were conserved among the different MKP1 sequences (supplemental Fig. S3). Among these residues, in vivo phosphorylation of Ser-295 was previously identified by mass spectrometry in two different phospho-
teomic approaches (34, 35). Thr-64, Thr-109, and Ser-558 were shown to be phosphorylated by MPK6 in vitro (14). However, Ser-558 and other C-terminal phosphosites identified by proteomic approaches (36, 37) are less likely of functional importance, since transgensics expressing HA-MKP1<sup>AC243</sup> (C-terminal 243 amino acids missing) complemented the UV-B phenotype of <i>mkp1</i>(Ws) and HA-MKP1<sup>AC243</sup> showed a UV-B-induced mobility shift (supplemental Fig. S1, B and C). No previous evidence supports Ser-309 as a phosphosite, but its high stringency motif, proximity to Ser-295 and evolutionary conservation implies possible phosphorylation.

**MKP1<sup>AAAA</sup>** and **MKP1<sup>DDDD</sup>** Complement the <i>mkp1</i>(Col) Growth Phenotype, but Not UV-B Stress Hypersensitivity—To get initial insight into the contribution of potential MAP kinase target sites among the 141 possible serine/threonine phosphosites, we chose T64, T109, S295, and S309 for mutagenesis to alanine or aspartate to avoid and mimic phosphorylation, respectively (Fig. 4B). To study the contribution of these residues to MKP1 function, we introduced myc epitope-tagged MKP1<sup>WT</sup>, MKP1<sup>AAAA</sup>, and MKP1<sup>DDDD</sup> into <i>mkp1</i>(Col) mutants.

First we tested expression and protein accumulation in these lines to identify comparable material. Actually, the levels of mRNAs coding for MKP1<sup>WT</sup>, MKP1<sup>AAAA</sup>, and MKP1<sup>DDDD</sup> correlated with the respective protein amounts, indicating that there is no alteration in protein stability (supplemental Fig. S4) and thus that potential phosphorylation of the analyzed four sites does not influence MKP1 stability.

When grown on soil, <i>mkp1</i>(Col) displays growth defects associated with constitutive defense responses and elevated levels of the phytohormone salicylic acid (SA) (24). This phenotype is associated with elevated expression of the <i>SID2</i> gene, encoding the isochorismate synthase of the major SA biosynthetic pathway (38) and the <i>PR5</i> marker gene (24). Interestingly, both MKP1<sup>AAAA</sup> and MKP1<sup>DDDD</sup> plants could complement the <i>mkp1</i>(Col) growth phenotype and largely suppress <i>SID2</i> and <i>PR5</i> overexpression (Fig. 5, A–C), indicating that they are functional proteins able to repress aberrant defense-related signaling.

<i>mkp1</i>(Col) mutants are hypersensitive to UV-B stress, clearly independent of SA and the defense-related phenotype (1). Thus, MKP1 phosphorylation may have a differential impact on UV-B stress and defense-related responses. Indeed, in contrast to the <i>mkp1</i>(Col) growth phenotype, both MKP1<sup>AAAA</sup> and MKP1<sup>DDDD</sup> failed to complement the UV-B hypersensitivity phenotype, albeit protein levels were similar to those of the complementing MKP1<sup>WT</sup> lines (supplemental Fig. S4). Consistent with their hypersensitive phenotype, MKP1<sup>AAAA</sup> and MKP1<sup>DDDD</sup> lines showed enhanced UV-B-mediated MPK3 and MPK6 activation, similar to <i>mkp1</i>(Col) (supplemental Fig. S5). Altogether, our data suggests that proper phosphorylation among residues Thr-64, Thr-109, Ser-295, and Ser-309 is of importance for MKP1 function in the UV-B stress response, but not under standard growth conditions.

**FIGURE 5.** MKP1<sup>AAAA</sup> and MKP1<sup>DDDD</sup> complement <i>mkp1</i>(Col) growth phenotype but not its UV-B hypersensitivity. <i>A</i>, constitutive defense-related phenotype of <i>mkp1</i>(Col) compared with plants carrying myc-tagged MKP1<sup>AAAA</sup> or MKP1<sup>DDDD</sup> mutated variants of the protein. Photographs of 23-day-old plants grown on soil under standard conditions. Scale bar = 3 cm. <i>B</i> and <i>C</i>, quantitative RT-PCR analysis of <i>PR5</i> (<i>B</i>) and <i>SID2</i> (<i>C</i>) expression levels in 22-day-old plants compared with wild-type Col. Error bars represent S.D. of three biological replicates. <i>D</i>, UV-B stress tolerance of <i>mkp1</i>(Col) compared with plants carrying MKP1<sup>AAAA</sup> or MKP1<sup>DDDD</sup> mutated variants of the protein. Seven-day-old seedlings were irradiated for 3.5 h with broadband UV-B under a WG305 cutoff filter and allowed to recover for 9 days.
DISCUSSION

Abiotic and biotic stresses initiate MAPK cascades that drive the corresponding physiological response. Although it is well established that MKP1 controls these responses by regulating MPK3 and MPK6 (1, 23–25), virtually nothing is known about its own regulation in vivo. This study demonstrates that (i) MKP1 exists in at least two phosphorylation states in vivo, (ii) MKP1 is continuously turned over under standard growth conditions, which can be blocked using proteasome inhibitor, (iii) upon UV-B stress phospho-MKP1 is very rapidly stabilized, likely independently of Thr-64, Thr-109, Ser-295, and/or Ser-309 phosphorylation, and (iv) proper phosphorylation at these residues is yet likely required for MKP1 function in response to UV-B stress but not to repress the defense-related growth phenotype of mkp1(Col).

The expression of MAPK phosphatases is often induced by the same stimuli that trigger MAPK activation, forming negative feedback loops (8). Consistently, the activation of the p42/p44 MAPK cascade in hamster fibroblasts results in MKP-1 phosphorylation, followed by reduced proteasomal degradation and thus protein stabilization (10). A similar mechanism was also reported in Xenopus oocytes (11). In Arabidopsis, MPK6 was found to phosphorylate MKP1 in vitro (14). We have observed a correlation between MAPK activation and phospho-MKP1 stabilization upon UV-B stress in vivo. However, redundancy in MPK3 and MPK6, and the fact that mpk3 mpk6 double null mutants are embryo-lethal (39, 40), prevented conclusive analysis of in vivo MKP1 phosphorylation by these MAPKs. Independent of this, our data do not support stress-dependent MKP1 stabilization solely due to MPK3- and MPK6-mediated phosphorylation: proteasome inhibitor treatment revealed that phosphorylated MKP1 exists as a labile protein under standard growth conditions, hence in the absence of detectable active MPK3 and MPK6. We thus conclude that, in addition to UV-B stress-responsive phosphorylation, UV-B also impinges directly on the degradation pathway for MKP1, which remains to be identified.

It was proposed that MPK6-dependent phosphorylation of MKP1 may stimulate its phosphatase activity (14). However, it was previously demonstrated that interaction between SIPK and NtMKP1 per se may result in enhanced phosphatase activity (15), and such substrate-triggered activation was also shown for mammalian MKPs (9). Phosphorylation of the yeast MPK Msg5 was shown to affect its protein-protein interaction properties (30). The exact molecular effect of MKP1 phosphorylation may involve one or a combination of these (reduced degradation, catalytic activation, protein-protein interaction), or yet unknown mechanisms.

The modification of the putative MAPK phosphorylation sites T64, T109, S295, and S309 in MKP1 affected the UV-B response of the transgenic lines. Since both non-phosphorylatable MKP1AAAA and phosphomimetic MKP1DDDD were not able to complement the mkp1 UV-B phenotype, we propose that proper (e.g. transient) MKP1 phosphorylation is required to fulfill its function in UV-B signaling. Another possible explanation may be that MKP1DDDD does not actually mimic phosphorylation. Indeed, although often used (27), substitutions with aspartate or glutamic acid do not always act like a “phosphorylated residue” (41, 42). Moreover, the residues chosen for mutagenesis could have opposing or divergent functions. The detailed analysis of the contribution of MKP1 phosphorylation to its function is complicated by the presence of 141 Ser/Thr residues that are potential phosphorylation targets. Our preliminary analysis of MKP1AAAA and MKP1DDDD already indicate the importance of phosphorylation among the four targeted residues, but more detailed understanding will require extensive investigation of the contribution of each single residue to plant UV-B tolerance, which will be a challenging task. Moreover, all the in vivo UV-B-responsive phosphosites remain to be identified and their effects on MKP1 function needs to be analyzed. Nonetheless, due to the facts that both MKP1AAAA and MKP1DDDD complemented the mkp1(Col) defense-related growth phenotype (this work) and that MKP1 function in vivo depends on its phosphatase activity (23), the failure to complement the UV-B phenotype is intriguing.

We conclude that phospho-MKP1 undergoes preferential UV-B stress-induced stabilization in comparison to constitutively phosphorylated MKP1. In addition, MKP1 phosphorylation by stress-activated MPK6 (14), and possibly MPK3, could play an important role in determining UV-B tolerance, independent of MKP1 accumulation. Our data provide a first insight into post-translational regulation of a plant MKP in vivo. It will be an interesting task to elucidate how differential MKP regulation in plants contributes to MAPK signaling specificity and physiological responses.

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