

## Stress-induced Protein Phosphatase 2C Is a Negative Regulator of a Mitogen-activated Protein Kinase\*

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Protein phosphatases of type 2C (PP2Cs) play important roles in eukaryotic signal transduction. In contrast to other eukaryotes, plants such as *Arabidopsis* have an unusually large group of 69 different PP2C genes. At present, little is known about the functions and substrates of plant PP2Cs. We have previously shown that MP2C, a wound-induced alfalfa PP2C, is a negative regulator of mitogen-activated protein kinase (MAPK) pathways in yeast and plants. In this report, we provide evidence that alfalfa salt stress-inducible MAPK (SIMK) and stress-activated MAPK (SAMK) are activated by wounding and that MP2C is a MAPK phosphatase that directly inactivates SIMK but not the wound-activated MAPK, SAMK. SIMK is inactivated through threonine dephosphorylation of the pTEpY motif, which is essential for MAPK activity. Mutant analysis indicated that inactivation of SIMK depends on the catalytic activity of MP2C. A comparison of MP2C with two other PP2Cs, ABI2 and AtP2CHA, revealed that although all three phosphatases have similar activities toward casein as a substrate, only MP2C is able to dephosphorylate and inactivate SIMK. In agreement with the notion that MP2C interacts directly with SIMK, the MAPK was identified as an interacting partner of MP2C in a yeast two-hybrid screen. MP2C can be immunoprecipitated with SIMK in a complex *in vivo* and shows direct binding to SIMK *in vitro* in protein interaction assays. Wound-induced MP2C expression correlates with the time window when SIMK is inactivated, corroborating the notion that MP2C is involved in resetting the SIMK signaling pathway.

Reversible protein phosphorylation catalyzed by protein kinases and phosphoprotein phosphatases regulates numerous biological processes. Whereas all protein kinases share extensive similarities in both primary and three-dimensional structures, protein phosphatases constitute a diverse family of enzymes that can be divided into two major classes: protein

serine/threonine phosphatases and protein tyrosine phosphatases. Protein serine/threonine phosphatases are further categorized into four groups according to their substrate specificities, divalent cation requirements, and sensitivity to inhibitors: type 1 (PP1),<sup>1</sup> 2A (PP2A), 2B (PP2B), and 2C (PP2C) (1). Whereas PP1, PP2A, and PP2B share ~40% identity in their catalytic domains, PP2C proteins have no apparent sequence homology to the other classes of phosphatases (2). PP2C enzymes also have the unique feature of functioning as monomers, whereas all other classes of serine/threonine phosphatases are found in oligomer complexes with regulatory subunits, that are thought to regulate the activity, substrate specificity, and/or cellular localization of the phosphatases (1, 2). PP2Cs also differ from other serine/threonine phosphatases in that they require Mn<sup>2+</sup> and/or Mg<sup>2+</sup> and are insensitive to the phosphatase inhibitor okadaic acid (1). Recent interest in PP2Cs was aroused when it was found that PP2Cs are involved in regulation of eukaryotic signaling pathways.

In yeast, mammals, and plants, regulation of MAPK cascades has been assigned as a PP2C function (3–5). Multiple MAPK pathways are differentially activated by a variety of external stimuli, including growth factors and biotic and abiotic stresses. The core of the MAPK-signaling module is composed of three protein kinases. MAPKs become activated exclusively by the phosphorylation of threonine and tyrosine residues at the highly conserved TXY motif close to subdomain VIII, which is carried out by a specific MAPK kinase (MAPKK). Further on, MAPKK is activated by a specific MAPKK kinase (MAPKKK) through phosphorylation of conserved threonine and/or serine residues (6). On the contrary, inactivation of MAPK pathways can be mediated by dephosphorylation of the TXY motif by a number of phosphoprotein phosphatases, including dual specificity phosphatases (7, 8), tyrosine phosphatases (9, 10), or serine/threonine phosphatases (3, 11, 12). PP2C-type phosphatases inactivate MAPKs in yeast and mammalian cells (4, 13–15).

In contrast to other eukaryotes, plant genomes such as *Arabidopsis* contain a large family of PP2C genes (16). Most plant PP2Cs possess a conserved catalytic domain but highly divergent N-terminal extensions. Studies on plant PP2Cs thus far suggest that these phosphatases are involved in the regulation of diverse signaling pathways. MP2C, an alfalfa PP2C, was shown to be involved in regulating wound-activated MAPK

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<sup>1</sup> The abbreviations used are: PP, protein phosphatase; MP2C, *Medicago sativa* protein phosphatase 2C; MMK2, *Medicago* MAPK 2; PRKK, pathogen-responsive MAPK; MAPK, mitogen-activated protein kinase; SIMK, salt stress-inducible MAPK; MAPKK, MAPK kinase; SAMK, stress-activated MAPK; HA, hemagglutinin; GST, glutathione S-transferase; MBP, myelin basic protein.

pathways (5). The *ABI1* and *ABI2* genes encode PP2Cs that have been isolated in a screen for abscisic acid-insensitive *Arabidopsis* mutants (17, 18, 46). Two other *Arabidopsis* PP2Cs, AtP2CHA and AthPP2CA, were also isolated (19, 20) and implicated in abscisic acid signaling (21). A transcription factor, ATHB6, was identified as a protein interacting with ABI1 (22). It was shown that kinase-associated protein phosphatase KAPP (23) interacts with CLAVATA1, a receptor-like kinase involved in the development of vegetative and floral meristems (24). In most cases, however, the targets of the various PP2Cs are still unknown. Other unresolved issues relate to questions of how substrate specificity is determined and how much functional redundancy exists among different members of this large family of plant PP2Cs.

To identify the molecular targets of MP2C, we performed a yeast two-hybrid interaction screen and identified the stress-induced MAP kinase SIMK as a specific interactor of MP2C. We found that MP2C is an extremely potent inactivator of SIMK as compared with two other PP2Cs, ABI2 and AtP2CHA. MP2C shows high substrate specificity to SIMK when compared with the related stress-activated MAP kinase, SAMK.

#### EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screen**—A yeast two-hybrid *Medicago* cDNA library (Hybri-ZAP, Stratagene) was screened using full-length MP2C in the pBD-Gal4 Cam vector (Stratagene) as bait. Yeast colonies of strain PJ69-4A (25) were selected for Ade<sup>+</sup> on medium lacking leucine, tryptophan, and adenine.

**Immunocomplex Kinase Assays**—For immunoprecipitation of SIMK and SAMK, crude M23 and M24 antibody was used, respectively (26, 27). For immunoprecipitation of HA-tagged kinases a commercial HA antibody (Babco) was used. Protein extracts were prepared as described (28) in protein extraction buffer (25 mM Tris, pH 7.8, 75 mM NaCl, 10 mM MgCl<sub>2</sub>, 15 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM NaVO<sub>3</sub>, 15 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 0.1% Tween 20, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin). After centrifugation at 20,000 × *g* for 45 min, equal protein amounts from the cleared supernatant were subjected to a 2-h preincubation in the presence of mixed protein G and protein A-Sepharose beads (20 μl, 1:1; pre-clearance step for HA-tagged kinases only). The supernatant was then incubated for 2 h with protein G beads for HA antibody and protein A beads for M23 and M24 antibodies. The beads were washed three times with wash buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% Tween 20) and once with kinase buffer (50 mM Tris, pH 7.5, 1 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 0.1 mM ATP). Kinase reactions with the immunoprecipitated MAPKs were performed for 30 min at room temperature in 20 μl of kinase buffer containing 5 μg of MBP or SIMK(K84R) where applicable and 2 μCi of [ $\gamma$ -<sup>32</sup>P]ATP. The reaction was stopped by adding SDS-PAGE loading buffer, and the phosphorylation of MBP was analyzed by autoradiography after SDS-PAGE.

**Immunoblots**—For Western analysis, recombinant proteins or protein extracts from 10,000 protoplasts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting. Membranes were probed with either monoclonal HA or Myc antibodies (1:5000, Santa Cruz A-14) or with a polyclonal phospho-MAPK antibody, p44/42 (New England Biolabs) (1:1000 dilution). Membranes were developed by enhanced chemiluminescence as recommended by the manufacturer (Gene Image, Amersham Biosciences).

**Expression and Purification of Recombinant Proteins**—SIMK, kinase-deficient mutant SIMK(K84R), SIMKK, and MP2C were expressed as GST fusion proteins as described previously (29). Recombinant AtP2CHA and ABI2 were expressed as maltose-binding fusion proteins according to the manufacturer's instructions (New England Biolabs). The protein concentrations of the recombinant proteins were determined with the Bio-Rad detection system (Bio-Rad) using BSA as a standard, and the purity of the protein fractions was determined by Coomassie staining after 10% SDS-PAGE.

**Measurement of Phosphatase Activity**—Phosphatase activity was measured using <sup>32</sup>P-labeled casein as a substrate.<sup>2</sup> Briefly, dephosphorylated casein was <sup>32</sup>P-labeled with bovine heart cAMP-dependent pro-

tein kinase (Sigma). The radiolabeled protein was precipitated with 20% trichloroacetic acid. After exhaustive washing with 10% trichloroacetic acid, the casein was dissolved in 0.5 M Tris-HCl, pH 8. Phosphatase assays were performed in a total volume of 100 μl of phosphatase buffer (50 mM Tris-HCl, pH 8, 0.1 mM EGTA, 1 mM dithiothreitol, 100 nM okadaic acid, 10 mM MgCl<sub>2</sub>). The reaction was started by the addition of 5 μg of <sup>32</sup>P-labeled casein (~200,000 cpm) or SIMK(K84R) (~100,000 cpm). After incubation for 30 min at 30 °C, the reaction was stopped by the addition of 700 μl of an acidic charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, 4% (v/v) Norit A). Samples were centrifuged (10 min, 10,000 × *g*), and the release of P<sub>i</sub> in 600 μl of supernatant was determined by scintillation counting (including replicates for each sample).

**MAPK Dephosphorylation and Phosphoamino Acid Analysis**—For the phosphorylation of SIMK, recombinant GST-SIMKK was added to GST-SIMK(K84R) at a 1:15 ratio in 100 μl of phosphatase buffer in the presence of 1 μCi of [ $\gamma$ -<sup>32</sup>P]ATP (specific radioactivity 3000 Ci/mmol) and incubated for 1 h at room temperature. The reaction was aliquoted and stopped by boiling the samples at 95 °C for 10 min. After cooling, the dephosphorylation of SIMK was carried out at room temperature in the presence of MP2C (10:1 ratio) for 0, 5, 15, 30, and 60 min. The reaction was stopped by the addition of SDS sample buffer, and the phosphorylation of SIMK was analyzed by autoradiography after SDS-PAGE.

Alternatively, when phosphoamino acids were analyzed, the reaction was stopped by protein precipitation with 20% trichloroacetic acid in the presence of 50 μg of bovine serum albumin. After exhaustive washing with 10% trichloroacetic acid, the proteins were hydrolyzed under argon with 6 N HCl at 110 °C for 1 h. The HCl was eliminated by evaporation under vacuum and successive washes with water. Amino acids were resuspended in chromatography solvent (isobutyric acid-0.5 M NH<sub>4</sub>OH, 5:3 v/v), and the phosphoamino acid composition was resolved by autoradiography after one-dimensional chromatography on cellulose plates.

**In Vitro Binding Assay**—SIMK was *in vitro* translated using TnT-coupled reticulocyte lysate system in the presence of [<sup>35</sup>S]methionine according to the manufacturer's instructions (Promega). 5 μl of *in vitro* translated SIMK was incubated overnight at 4 °C in 100 μl of phosphatase buffer in the presence of 1 μg of GST, GST-MP2C, GST-MP2C(G165D), or GST-MP2C(R289A), respectively. Protein complexes were washed four times with 50 mM Tris-HCl, pH 8, buffer containing 1% Nonidet P-40 and 150 mM NaCl and denatured with SDS sample buffer. The proteins were analyzed by autoradiography after SDS-PAGE.

**Transient Expression Assay**—Transient expression assay was performed with isolated protoplasts from cultured parsley cell suspension (*Petroselinum crispum*). The isolation, transformation, and cultivation of protoplasts were described earlier (30). The open reading frames of MP2C, MP2C(G165D), MP2C(R289A), and SIMKK were cloned into the plant expression vector pRT101 under the control of the 35S promoter (29, 31). SIMKK, SAMK, and SIMK were fused at the C terminus to a triple HA epitope. ABI2 and AtP2CHA were expressed under the control of the 35S promoter (21). Parsley protoplasts were transiently transformed via polyethylene glycol with 5 μg each of the plasmids pSH9-SIMK-HA, pRT101-SAMK-HA, pRT101-SIMKK-HA, or pRT101-SIMKK and the indicated concentrations of pRT101-MP2C-Myc, pRT101-MP2C(G165D)-Myc, or pRT101-MP2C(R289A)-Myc. Eight hours after transformation protoplasts were subjected to biochemical analysis. For the co-immunoprecipitation assay, transiently coexpressed proteins were immunoprecipitated from 300,000 protoplasts with HA or Myc antibody as described above for kinase assays. After SDS-PAGE, immunocomplexes were analyzed by Western blot with Myc or HA antibody, respectively (1:5000 dilution).

#### RESULTS

**Wounding Transiently Activates SIMK and SAMK**—Several reports have shown that wounding causes activation of MAP kinase pathways (32, 33). In alfalfa, we have shown that wounding of leaves results in activation of SAMK (32). In tobacco (33–35), evidence was provided that wound-induced protein kinase (WIPK), a close relative of alfalfa SAMK, is involved in wounding. In another study, tobacco salicylic acid-induced protein kinase (SIPK) was reported to be activated by wounding (35).

In an attempt to clarify the situation in alfalfa, activation of MAP kinases was investigated in wounded leaves. Cell extracts

<sup>2</sup> H. Saito, personal communication.

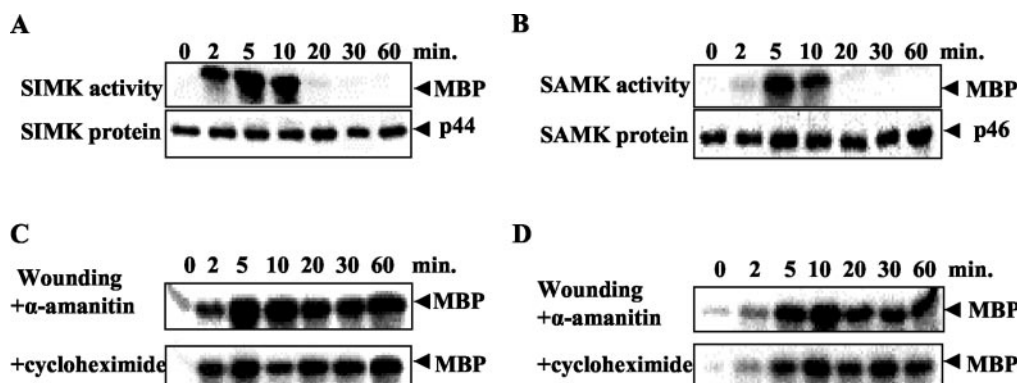


FIG. 1. Post-translational activation of SIMK and SAMK by wounding. SIMK (A) and SAMK (B) activities before and after wounding of leaves as determined by immunocomplex kinase assays (upper panels). Leaf extracts containing 100  $\mu$ g of total protein were immunoprecipitated with SIMK- or SAMK-specific antibodies. Kinase reactions of immunoprecipitated MAPKs were performed using [ $\gamma$ - $^{32}$ P]ATP and MBP as a substrate. MBP phosphorylation was visualized by autoradiography after SDS-PAGE. SIMK (A) and SAMK (B) protein levels before and after wounding determined by immunoblotting of leaf extracts containing 40  $\mu$ g of total protein with SIMK and SAMK antibodies (lower panels). SIMK (C) and SAMK (D) activity determined by immunocomplex kinase assays before and after wounding of leaves, preincubated with either  $\alpha$ -amanitin to block transcription or cycloheximide to block protein translation.

of wounded leaves were immunoprecipitated with four different antibodies, M23, M11, M14, and M24, which were produced against synthetic peptides encoding the C terminus of the alfalfa MAP kinases SIMK, MMK2, MMK3, and SAMK, respectively. These antibodies were shown to react monospecifically and immunoprecipitate the respective MAP kinases in an active state (26, 27). Immunokinase assays of the MAPKs reflect the *in vivo* state of the kinases at different times after wounding. As shown in Fig. 1A, SIMK was found to be activated at 2 min after wounding. The activity of SIMK showed a transient profile, disappearing at around 20–30 min after wounding. As reported previously (32), wounding resulted in strong and transient activation of SAMK (Fig. 1B) but not of MMK2 and MMK3 (data not shown). These data show that in alfalfa, leaf wounding activates SIMK and SAMK.

Wounding induces accumulation of SAMK transcripts, but no changes of mRNA amounts were observed for SIMK (32). These results suggest that the two MAP kinases are regulated in different ways. However, when the same leaf extracts used for the immunokinase assays were immunoblotted with the specific SIMK and SAMK antibodies, no changes in protein amounts were observed for either SIMK (Fig. 1A) or SAMK (Fig. 1B). These data are consistent with the model that activation of the MAPKs occurs through MAPKK-mediated Thr and Tyr phosphorylation of the TEY activation loop, whereas inactivation occurs through dephosphorylation of these sites.

**Inactivation of SIMK and SAMK Is Mediated by *de Novo* Synthesized Protein Factor(s)**—Whereas MAP kinases are activated by dual specificity MAPKKs, inactivation can occur by various phosphatases through removal of any of the phosphate groups of the pTEpY motif (36). To investigate which type of phosphatases are responsible for inactivation of wound-activated SIMK and SAMK, we analyzed leaf extracts after wounding in the presence of various phosphatase inhibitors. When leaf extracts were treated with 500 nM okadaic acid, a blocker of PP1 and PP2A phosphatases, and 10 mM EDTA, blocking PP2C activity, phosphotyrosine phosphatase activity measured as *p*-nitrophenyl phosphate hydrolysis was found to be induced by 1.2-fold at 10 min after wounding. When leaf extracts were treated with 1 mM vanadate and 10 mM EDTA, no significant change in PP1/PP2A phosphatase activity measured as phosphocasein dephosphorylation could be detected. In contrast, when vanadate- and okadaic acid-treated leaf extracts were analyzed, a 1.8-fold induction of PP2C activity was observed after wounding, indicating that wounding activates phosphatases of type PP2C and possibly, to a minor degree, phosphotyrosine phosphatase.

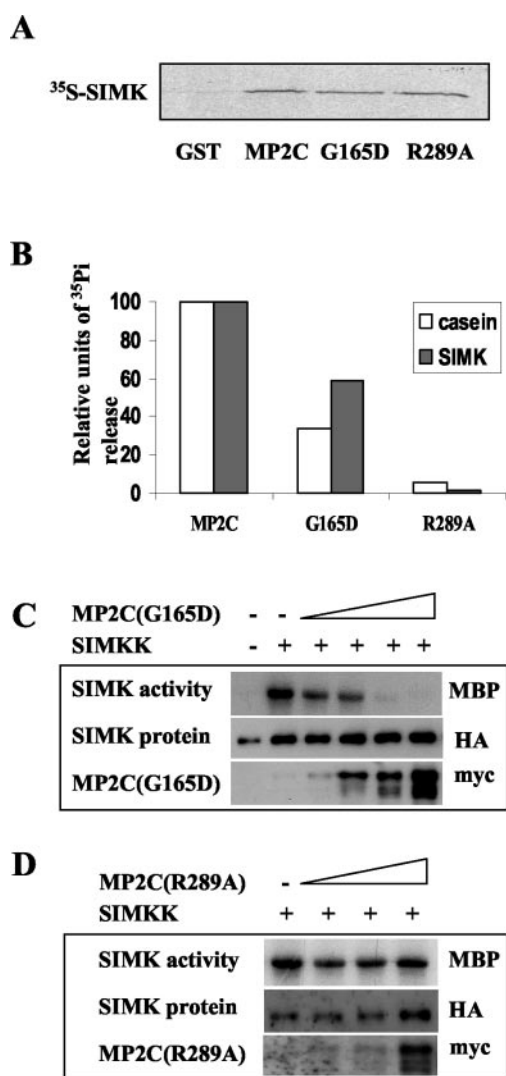
Depending on the biological system, the phosphatases that inactivate MAPKs can be under post-translational or transcriptional control (36). To investigate whether inactivation of SIMK and SAMK depends on *de novo* synthesized phosphatases, detached leaves were preincubated with  $\alpha$ -amanitin before wounding. Under conditions in which *de novo* transcription of genes was completely inhibited (32), wound activation of SIMK and SAMK was comparable with untreated leaves but showed complete lack of inactivation (Fig. 1, A and B). Therefore,  $\alpha$ -amanitin locked SIMK and SAMK in a constitutively active state (Fig. 1, A and B), suggesting that *de novo* transcribed phosphatases are required for the inactivation of wound-induced MAP kinases.

To substantiate the idea that *de novo* synthesis of phosphatases is required for inactivation of wound-induced MAPKs, detached leaves were incubated with cycloheximide before wounding. Under conditions that inhibited more than 95% incorporation of [ $^{35}$ S]methionine into proteins, SIMK and SAMK remained fully activable by wounding (Fig. 1, C and D) indicating that wound-activation of both MAP kinases occurs by a post-translational mechanism. As seen with  $\alpha$ -amanitin, cycloheximide almost completely inhibited the inactivation of SIMK and SAMK. To test the possibility that transcriptional or translational inhibitors can activate protein kinase pathways in some cellular systems, leaves were also treated with either  $\alpha$ -amanitin or cycloheximide in the absence of wounding. Under these conditions, no activation of either SIMK or SAMK was observed (data not shown). Taken together, these data support the model that inactivation of wound-induced MAPKs is mediated by *de novo* synthesized phosphatases.

**MP2C Inactivates SIMK in Plant Protoplasts**—Previously, we have shown that MP2C is a wound-induced gene, the expression of which correlates with the inactivation of SAMK (32). Although SAMK was found to be inactivated *in vitro* by the addition of recombinant MP2C protein to plant cell extracts, SIMK was inactivated under these conditions already at a 10-fold lower concentration of MP2C protein (data not shown.)

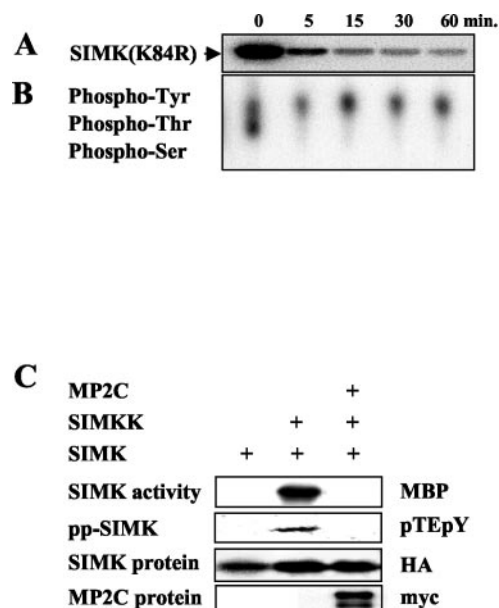
To test MP2C for substrate specificity *in vivo*, we coexpressed different amounts of MP2C (0.05, 0.1, 0.5, and 1  $\mu$ g of plasmid) with each of the two MAPKs in protoplasts. Both MAP kinases can be activated by the constitutively active MAPKK SIMKK in the absence of external stimuli (29, 37). As shown in Fig. 2, A and B, SIMK and SAMK were activated by SIMKK, but only SIMK was inactivated by MP2C. Increased amounts of MP2C resulted in a dose-dependent inactivation of SIMK (Fig. 2A), whereas SAMK remained unaffected even at the highest





**FIG. 3. Analysis of MP2C mutations *in vitro* and *in vivo*.** *A*, *in vitro* interaction of GST only, GST-MP2C, GST-MP2C(G165D), or GST-MP2C(R289A) with [<sup>35</sup>S]methionine-labeled *in vitro* translated SIMK. The physical interaction of the proteins was detected by autoradiography after SDS-PAGE. *B*, phosphatase activity measured as released <sup>32</sup>P<sub>i</sub> from <sup>32</sup>P-labeled casein or <sup>32</sup>P-GST-SIMK(K84R) substrates. 1 μg of GST-MP2C, GST-MP2C(G165D), or GST-MP2C(R289A) was incubated with 5 μg of substrate for 30 min. Released <sup>32</sup>P<sub>i</sub> was calculated after scintillation counting of three independent measurements. *C*, MP2C(G165D) co-transfection with SIMK. Increasing amounts of MP2C(G165D) plasmid (0.05, 0.1, 0.5, 1 μg) were used with 5 μg of SIMK. SIMK activity was detected after immunoprecipitation with HA antibody. SIMK-HA protein levels were determined on total protein extracts with HA antibody, and MP2C(G165D)-Myc protein amounts were determined with Myc antibody by Western blotting. *D*, MP2C(R289A) co-transfection with SIMK. Increasing amounts of MP2C(R289A) plasmid (0.1, 0.5, 1 μg) were used with 5 μg of SIMK. SIMK activity was detected after immunoprecipitation with HA antibody. SIMK-HA protein levels were determined on total protein extracts with HA antibody, and MP2C(R289A)-Myc protein amounts were determined with Myc antibody by Western blotting.

bated with recombinant GST-MP2C, the level of <sup>32</sup>P-labeled GST-SIMK(K84R) decreased rapidly (Fig. 4A). In comparison, casein was dephosphorylated with 20-fold lower efficiency (data not shown). However, even after prolonged incubation of SIMK with MP2C, some <sup>32</sup>P label remained (Fig. 4A). When phosphoamino acid analysis of <sup>32</sup>P-labeled SIMK(K84R) was performed before and after incubation with MP2C, it was verified that SIMKK phosphorylated GST-SIMK(K84R) preferentially on threonine and to a lesser extent on tyrosine residues (Fig. 4B, at 0 min). After dephosphorylation by MP2C, the



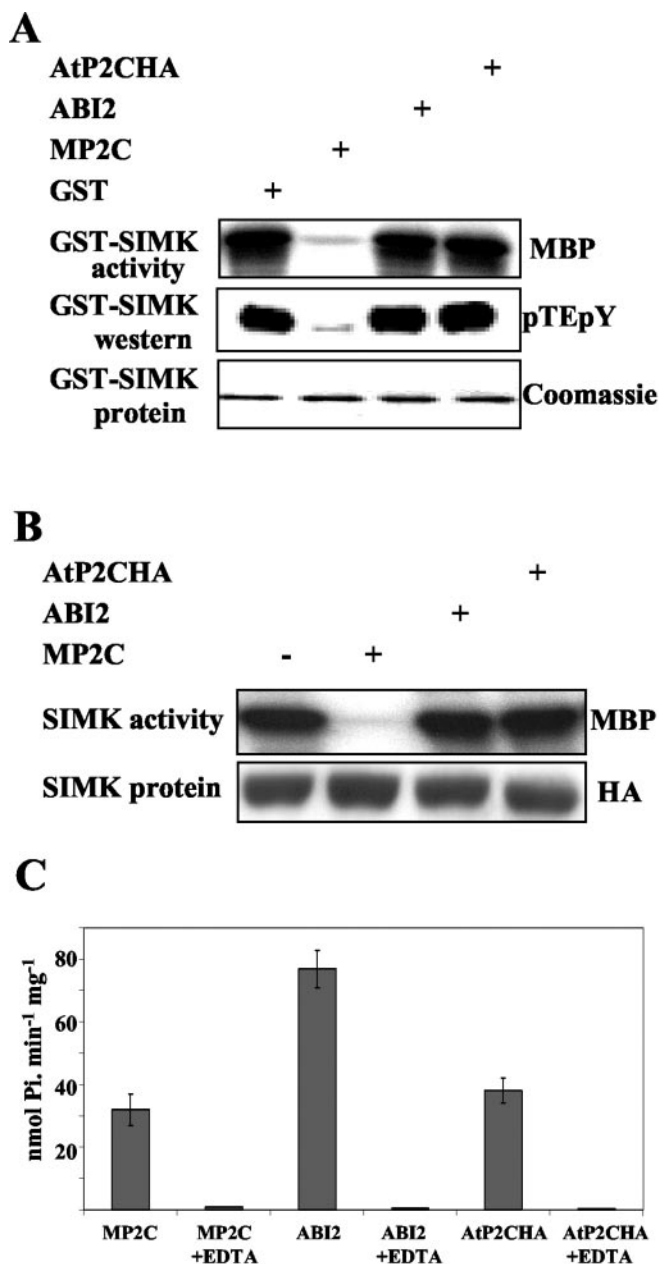
**FIG. 4. MP2C dephosphorylates SIMK on the threonine residue of the pTEpY motif.** *A*, dephosphorylation of <sup>32</sup>P-labeled kinase-deficient SIMK(K84R) by MP2C. GST-SIMK(K84R) was <sup>32</sup>P-phosphorylated by recombinant constitutively active SIMKK. Following incubation of <sup>32</sup>P-labeled SIMK(K84R) with GST-MP2C for the indicated times, the proteins were separated by SDS-PAGE. Dephosphorylation of GST-SIMK(K84R) was detected by autoradiography. *B*, the same samples were subjected to acid hydrolysis. Phosphorylated amino acids were resolved by one-dimensional chromatography and detected by autoradiography. *C*, plant protoplasts were transfected with SIMK alone or with its activator SIMKK and with or without MP2C, respectively. Protein extracts were analyzed for SIMK activity after immunoprecipitation with HA antibody. Phosphorylation of SIMK and SIMK protein levels were detected by immunoblotting with p44/42 phospho-MAPK antibody and HA antibody, respectively. MP2C-Myc protein amounts were determined with Myc antibody.

phosphate label on the threonine residue was completely removed, whereas tyrosine phosphorylation remained unaffected (Fig. 4B, 5–60 min). These data are in agreement with the notion that MP2C is a serine/threonine phosphatase that acts as a MAPK phosphatase by dephosphorylating threonine 213 of SIMK.

To show that MP2C dephosphorylated SIMK at the phosphothreonine residue 213 of the essential TEY motif *in vivo*, a pTEpY-specific antibody was used that exclusively reacts with the dual-phosphorylated activated form of the MAPK but does not recognize MAPKs when only one or none of the residues of the TEY motif are phosphorylated. SIMK was activated in protoplasts by coexpression of SIMKK (Fig. 4C). SIMK activity correlated with threonine 213 and tyrosine 215 dual phosphorylation as detected by immunoblotting with anti-pTEpY antibody (Fig. 4C). However, both SIMK activity and dual phosphorylation vanished when MP2C was coexpressed (Fig. 4C).

Next we tested whether MP2C retains its specificity to dephosphorylate SIMK on the pTEpY motif *in vitro*. GST-SIMK was recognized by the anti-pTEpY antibody after phosphorylation by SIMKK (Fig. 5A). After incubation with MP2C, the anti-pTEpY antibody was unable to detect dual-phosphorylated pTEpY, demonstrating that MP2C is a phosphatase that directly dephosphorylates the pTEpY motif on SIMK.

Plants contain very large families of PP2Cs. Analysis of the *Arabidopsis* genome revealed the presence of at least 69 distinct PP2C genes. To assess the possibility that the plant PP2Cs all act as MAPK phosphatases, we compared MP2C specificity with two other plant PP2Cs, ABI2 and AtP2CHA (19, 40). By *in vitro* phosphatase assays, all three PP2Cs showed considerable phosphatase activities toward casein as a



**FIG. 5. MP2C, but not ABI2 or AtP2CHA, inactivates and dephosphorylates SIMK.** *A*, *in vitro*, 1  $\mu$ g of GST-SIMK was phosphorylated by the constitutively active SIMKK and incubated with 0.1  $\mu$ g of each recombinant protein (GST only or recombinant MP2C, ABI2, or AtP2CHA) for 30 min and then subjected to kinase assay with MBP as a substrate. GST-SIMK kinase activity is shown as phosphorylation of MBP. pTepY dephosphorylation of GST-SIMK by GST-MP2C was analyzed by Western blotting with p44/42 phospho-MAPK antibody. Coomassie staining shows equal loading of the SIMK protein substrate. *B*, *in vivo*, SIMK was expressed transiently alone or with different PP2Cs: MP2C, ABI2, or AtP2CHA. SIMK was immunoprecipitated with HA antibody, and kinase activity was determined on MBP as a substrate. Western blot with HA antibody shows equal protein amounts of SIMK. *C*, recombinant MP2C, ABI2, and AtP2CHA are active phosphatases that dephosphorylate casein.

substrate, although ABI2 was more than twice as active as MP2C or AtP2CHA (Fig. 5C). In contrast, dephosphorylation of GST-SIMK was observed only for recombinant MP2C but not for ABI2 or AtP2CHA (Fig. 5A). To analyze the properties of the three PP2Cs also *in vivo*, MP2C, ABI2, and AtP2CHA were coexpressed with SIMK in plant protoplasts, where SIMK was activated by coexpression of SIMKK (Fig. 5B). SIMK activation was prevented by MP2C but not by the other two PP2Cs,

confirming that MP2C alone can inactivate SIMK (Fig. 5B).

Taken together, these results indicate that MP2C, but not two other PP2Cs, ABI2 and AtP2CHA, can inactivate SIMK. Interestingly, the N terminus of MP2C revealed the presence of a potential MAP kinase interaction motif (KIM) that is conserved in the N termini of the SIMK-activating MAPKKs (29, 37) and in a subfamily of four *Arabidopsis* MP2C homologs (see below). It is tempting to speculate that PP2C members containing KIM motifs all act as MAPK phosphatases, whereas other PP2Cs might contain other substrate interaction motifs for targeting other classes of substrates.

#### DISCUSSION

All available evidence indicates that distinct MAPK pathways are involved in mediating different external stimuli. MAPK activation was observed to rely on MAPKK-dependent dual phosphorylation of the threonine and tyrosine residues of the highly conserved TXY motif. In most cases, stimulation of MAPKs is followed by inactivation, giving rise to the transient nature of the signaling process. Inactivation of MAPKs can occur through dephosphorylation of any of the phosphorylated amino acid residues of the pTXYpY motif. A number of phosphatases, including dual specificity tyrosine and serine/threonine phosphatases, can carry out this reaction and thus can act as negative regulators of MAPKs (41). Depending on the biological system and pathway, different MAPK cascades were found to be regulated by distinct phosphatases, reflecting the complexity of signaling networks.

In this study we have identified a potential substrate for MP2C as the stress-induced MAPK SIMK. A number of stresses can activate SIMK, including salt stress, microbial elicitors, and wounding (26, 27, 32). Salt activation of SIMK is mediated by the MAPKK SIMKK (29), whereas the elicitors activate SIMK through SIMKK and/or PRKK, another MAPKK (37). Remarkably, our results show that MP2C inactivates SIMK, but not the stress-activated MAPK SAMK, as was suggested by previous *in vitro* experiments (5). However, as observed for unspecific substrate phosphorylation with protein kinases, our data indicate that substrate discrimination is also lost at high concentrations of protein phosphatases. This appears to be true specifically under *in vitro* conditions when competition between different substrates is omitted. In contrast, highly specific enzymatic reactions are observed at low phosphatase concentrations and when various related phosphatases are compared. In the case of MP2C, dose-response curves *in vivo* with transiently expressed phosphatase indicated that SIMK is inactivated at 20-fold lower concentrations of MP2C than SAMK or SIMKK. When the ability of MP2C to dephosphorylate SIMK was compared with that of two related PP2Cs, ABI2 and AtP2CHA, the latter enzymes were unable to accept SIMK as a substrate. These data show that measurements on artificial substrates can give comparable activities, which are far from representing activities on specific substrates.

PP2Cs are protein phosphatases that should only be able to dephosphorylate phosphoserine and phosphothreonine residues. Accordingly, it was expected that inactivation of SIMK by MP2C should be achieved through dephosphorylation of pT213 in the pTepY motif of SIMK. By phosphoamino acid analysis and immunoblotting with phospho-specific pTepY antibodies, we were able to show that MP2C directly inactivates SIMK by dephosphorylation of pT213.

Theoretically, MP2C could also have inactivated SIMK by inactivating its upstream activator, SIMKK. However, under the *in vivo* conditions used, MP2C had no inhibitory effect on SIMKK, suggesting that the main action of MP2C is exerted at the level of the MAPK and not the MAPKK. These observations

recall the situation in various other systems, such as yeast, where the PP2C Ptc1 inactivates the HOG1 MAPK pathway by dephosphorylating Hog1 but has little effect on the MAPKK Pbs2 (13). Similarly, the mammalian PP2C-type phosphatase Wip1 also inactivates the p38 MAPK but displays little activity toward MAPKKs (15).

The notion that MP2C interacts selectively with SIMK not only rests on activity measurements *in vitro* and *in vivo* but also on the identification of SIMK as an interacting partner in a yeast two-hybrid screen with MP2C as bait. Moreover, two-hybrid interaction assays of MP2C with several MAPKs and MAPKKs revealed no interaction with the MAPKs SAMK or MMK2, or the MAPKKs, SIMKK and PRKK (data not shown).

The PP2C family of phosphatases shows extraordinary complexity in plants (16). However, information about the targets and specificities of these PP2Cs is still limited. Our data show that MP2C can inactivate SIMK, but inactivation of this MAPK cannot be carried out by two other members of the plant PP2C family, such as ABI2 or AtP2CHA. Because PP2Cs function as monomers, information for substrate recognition should reside in their particular protein structures. With the exception of kinase-associated protein phosphatase, the majority of plant PP2Cs, including MP2C, ABI1, and ABI2, have a common structural organization, displaying a highly conserved catalytic domain but unique noncatalytic N-terminal extensions. Analysis of the primary structure of the N terminus of MP2C revealed the presence of a potential MAP kinase interaction motif (KIM) that is conserved in mammalian and yeast MAPK-interacting proteins (42, 43) and also in the N termini of the SIMK-activating MAPKKs, SIMKK and PRKK (29, 37). The closest *Arabidopsis* homolog of MP2C is AP2C. AP2C belongs to a six-member subfamily of *Arabidopsis* PP2Cs, four of which contain a KIM domain in their N terminus. Preliminary results indicate that at least one of the members of the AP2C group functions in MAPK docking,<sup>3</sup> suggesting that specificity of this PP2C subfamily might be attained through an N-terminal substrate docking domain. ABI2 and AtP2CHA lack this motif at the noncatalytic N terminus, which possibly explains the inability of these phosphatases to inactivate SIMK. Although domain swapping experiments between the N-terminal domains of MP2C and ABI2 confirmed this notion,<sup>3</sup> there must be additional structural elements required for substrate recognition; otherwise, SIMK and SAMK both should be targeted by MP2C.

In an effort to understand ABI1 action, the transcription factor ATHB6 was identified as an interacting protein (22). It was shown that the interaction of ABI1 with ATHB6 was dependent on its phosphatase activity. A similar conclusion was drawn from a study of PP2CA interaction with AKT2, a K<sup>+</sup> channel (44). Contrary to this finding, introducing a point mutation that renders MP2C completely inactive did not impair MP2C interaction with SIMK, suggesting that distinct mechanisms could function in different plant PP2Cs for substrate selection.

*abi1-1* was identified genetically as a dominant ABA-insensitive mutation. The nature of this mutation is still not completely understood, but it was shown to result in a reduced AtPP2C activity as determined by dephosphorylation of casein as an artificial substrate (38). Taking advantage of the fact that we have identified a potential substrate for MP2C, we tested the mechanism whereby the *abi1-1* mutation might affect PP2C action. The introduction of an equivalent mutation reduced the phosphatase activity of MP2C(G165D) toward casein

and to a lesser degree toward SIMK *in vitro*. The interaction of SIMK and MP2C was not affected by the mutation. However, MP2C(G165D) was still able to inactivate SIMK even at low concentrations *in vivo*. Possibly the decreased phosphatase activity can be compensated by the KIM substrate interaction domain. Because abscisic acid-insensitive mutation ABI2 and AtP2CHA do not contain KIM domains, the G165D mutation might have stronger effects on substrate interaction specificity in these PP2Cs. Nonetheless, our data do not exclude the possibility that the catalytic parts of the PP2Cs are involved in determining substrate specificity; further investigations will be necessary to clarify this issue.

Transcriptome analysis of *Arabidopsis* leaves after wounding revealed strong up-regulation of *PP2C* genes, which are closely related to alfalfa *MP2C*, but no dual specificity or other phosphatases were induced, suggesting that these *PP2C* genes are the only transcriptionally regulated phosphatases involved in wound signal transduction (45). In agreement with these data, transcript analysis of wounded alfalfa leaves also revealed strong induction of *MP2C* expression after wounding (5). The timing of *MP2C* expression upon wounding corresponds to the inactivation of SIMK and SAMK in leaves. Wound-induced SIMK and SAMK activity in leaves is sustained at high levels by treatment with transcription or translation inhibitors, indicating that *de novo* protein synthesis is required for MAPK inactivation (Fig. 1). These results and our previous data from experiments *in vitro* with recombinant MP2C protein (5) suggest that MP2C should down-regulate both SIMK and SAMK. However, coexpression of MP2C with SIMK and SAMK in protoplasts revealed that SAMK cannot be inactivated by MP2C. It is presently unclear which phosphatase regulates SAMK activity, but besides dual specificity phosphatases, obvious candidates could be found among the other members of the PP2C subfamily. Nevertheless, the finding that coexpression of MP2C efficiently blocks the SIMK pathway and that *MP2C* expression closely correlates with the inactivation of SIMK following wounding strongly suggests that down-regulation of the SIMK pathway relies at least in part on transcriptional activation of *MP2C*. This negative feedback mechanism is not unique in plants but is also found in other systems, where MAPK inactivation is dependent on *de novo* transcription of the respective phosphatases (36).

Overall, our results suggest that at least a subset of plant PP2Cs is involved in the negative regulation of specific MAPK pathways. Analysis of knock-out mutants in the putative *Arabidopsis* MP2C homologs should give more definitive answers concerning the function of this subfamily of PP2Cs. Together with functional studies of the involved MAPKs, this approach should help to clarify the role of these PP2Cs in MAPK signaling.

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