ATMPK4, an Arabidopsis Homolog of Mitogen-Activated Protein Kinase, Is Activated in Vitro by AtMEK1 through Threonine Phosphorylation[1]

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The modulation of mitogen-activated protein kinase (MAPK) activity regulates many intracellular signaling processes. In animal and yeast cells, MAP kinases are activated via phosphorylation by the dual-specificity kinase MEK (MAP kinase kinase). Several plant homologs of MEK and MAPK have been identified, but the biochemical events underlying the activation of plant MAPKs remain unknown. We describe the in vitro activation of an Arabidopsis homolog of MAP kinase, ATMPK4. ATMPK4 was phosphorylated in vitro by an Arabidopsis MEK homolog, AtMEK1. This phosphorylation occurred principally on threonine (Thr) residues and resulted in elevated ATMPK4 kinase activity. A second Arabidopsis MEK isoform, ATMAP2Kα, failed to phosphorylate ATMPK4 in vitro. Tyr dephosphorylation by the Arabidopsis Tyr-specific phosphatase AtPTP1 resulted in an almost complete loss of ATMPK4 activity. Immunoprecipitates of Arabidopsis extracts with anti-ATMPK4 antibodies displayed myelin basic protein kinase activity that was sensitive to treatment with AtPTP1. These results demonstrate that a plant MEK can phosphorylate and activate MAPK, and that Tyr phosphorylation is critical for the catalytic activity of MAPK in plants. Surprisingly, in contrast to the animal enzymes, AtMEK1 may not be a dual-specificity kinase but, rather, the required Tyr phosphorylation on ATMPK4 may result from autophosphorylation.

The mitogen-activated protein kinase (MAPK) signal transduction cascade is utilized by eukaryotic cells to transduce a wide variety of extracellular signals such as growth factors, hormones, and stress stimuli (Seger and Krebs, 1995; Robinson and Cobb, 1997; Lewis et al., 1998). This cascade typically consists of three functionally interlinked protein kinases: Raf/MEKK (MAP kinase kinase kinase), MEK (MAP kinase kinase), and MAPK. In this phosphorylation module, either a Raf or a MEKK phosphorylates and activates a particular MEK, which in turn phosphorylates and activates a MAPK, which is also referred to as ERK in mammalian systems. Activated MAPK is often imported into the nucleus, where it phosphorylates specific transcription factors (Chen et al., 1992; Lenormand et al., 1993; Khokhlatchev et al., 1998).

The regulation of yeast and animal MAPK has been well characterized. In these systems, activation of MAPK requires dual phosphorylation of Thr and Tyr residues in the invariant TXY motif by the upstream dual-specificity protein kinase MEK (Payne et al., 1991). The stoichiometry of MAPK phosphorylation on Thr and Tyr residues by MEK is 1:1, and phosphorylation on both residues is required for full enzymatic activity (Anderson et al., 1990; Payne et al., 1991). The phosphorylation of Tyr generally precedes that of Thr (Haystead et al., 1992), and MAPK is thought to dissociate from MEK following the first phosphorylation (Ferrell and Bhatt, 1997). This Tyr is also the major site of MAPK autophosphorylation, but autophosphorylation is not sufficient to activate the kinase fully.

The process of inactivating MAPKs is also important in regulating cell growth and development. MAPKs are dephosphorylated and inactivated by several routes that involve distinct types of protein phosphatases (Cobb and Goldsmith, 1995; Keyse, 1998). Because MAPKs are phosphorylated on both Thr and Tyr by MEKs, they may be regulated by Tyr-specific, Ser/Thr-specific, and/or dual-specificity protein phosphatases. The activation of plant MAPKs has been correlated with Tyr phosphorylation (Seo et al., 1995; Usami et al., 1995; Knetsch et al., 1996; Ádám et al., 1997; Zhang and Klessig, 1997), and a cDNA encoding a Tyr-specific protein phosphatase, AtPTP1, has been cloned from Arabidopsis (Xu et al., 1998).

Elevated MAPK activities, assayed using myelin basic protein as a substrate, are observed when plant cells are stimulated by wounding (Usami et al., 1995; Börge et al., 1997; Zhang and Klessig, 1998; Seo et al., 1999), pathogen elicitors (Suzuki and Shinshi, 1995; Ligterink et al., 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1997; Zhang et al., 1998; Romeis et al., 1999), or extracellular stresses (Jonak et al., 1996; Mizoguchi et al., 1996). MAPKs are also postulated to act in the signaling pathways for the hormones auxin, abscisic acid (ABA), and ethylene (Mizoguchi et al., 1994; Knetsch et al., 1996; Kieber, 1997; Kovtun et al., 1998).

Several plant homologs of MEKs and MAPKs have been identified based on sequence similarity to the yeast and animal enzymes (Hirt, 1997; Mizoguchi et al., 1997). A plant MEK homolog was first identified in tobacco (Shibata et al., 1995), and in Arabidopsis, five MEK homologs have been identified: AtMEK1, ATMKK2, ATMKK3, ATMKK4, and MBP-ATMAP2Kα (Jouannic et al., 1996; Mizoguchi et al., 1997; Morris et al., 1997; Ichimura et al., 1998a). Phylogenetic analysis indicates that these five MEK homologs belong to three subgroups. A family of MAPKs, consisting of nine members (ATMPK1–9), which can be categorized into four subgroups, has been isolated from Arabidopsis (Mizoguchi et al., 1993, 1997). The interaction of the Arabidopsis MEKs and MAPKs has been examined (Ichimura et al., 1998b; Mizoguchi et al., 1998). AtMEK1 and ATMPK4, which are the subject of this report, were found to specifically interact using both two-hybrid analysis and functional complementation in yeast. It has been suggested that this pair along with ATMEKK1 form a functional kinase cascade (Ichimura et al., 1998b; Mizoguchi et al., 1998).

To understand how plant MAPKs are regulated, we set out to examine the biochemical interactions of the proteins encoded by *AtMEK1*, *ATMPK4*, and *AtPTP1*. We describe the activation of ATMPK4 by AtMEK1 and its inactivation by AtPTP1. ATMPK4 was activated by AtMEK1 in vitro through Thr phosphorylation. Notably, we failed to detect Tyr phosphorylation of ATMPK4 by AtMEK1 using recombinant enzymes. Tyr dephosphorylation by AtPTP1 results in almost complete loss of ATMPK4 enzymatic activity, suggesting that ATMPK4 activation requires Tyr phosphorylation, which appears to occur in vitro primarily by autophosphorylation. These results implicate Tyr phosphorylation in the activation of plant MAPKs, which are similar to animal MAPKs except the source of the Tyr phosphorylation of ATMPK4 may be distinct.

MATERIALS AND METHODS

Materials

Arabidopsis ecotype Wassilewskija was used in this study. Plants were grown as described previously (Vogel et al., 1998). Myelin basic protein was purchased from Gibco/BRL (Cleveland), [γ-^{32}P]ATP (6,000 Ci/mmol) was from Amersham Pharmacia (Piscataway, NJ), and ATP was from Boehringer Mannheim/Roche (Basel). Leupeptin and pepstatin A were from Sigma-Aldrich (St. Louis).

Expression of Arabidopsis AtPTP1, AtMEK1, ATMAP2Kα, and ATMPK4 in *Escherichia coli*

Arabidopsis Tyr-specific protein phosphatase AtPTP1 was expressed in *E. coli* and purified to homogeneity as described previously (Xu et al., 1998). cDNA fragments containing the *AtMEK1* (from a cDNA clone), *ATMAP2Kα* or the *ATMPK4* (both obtained by RT-PCR) coding regions were cloned into the *E. coli* expression vector pMAL-c2 (New England Biolabs, Beverly, MA). The resultant plasmids directed the expression of a fusion of each open reading frame to the maltose-binding protein: MBP-MEK1,

MBP-ATMAP2Kα, and MBP-MPK4, respectively. The junctions of each plasmid, as well as the entire *ATMAP2Kα* and *ATMPK4* coding regions, were verified using the automated sequencing facilities at the University of Illinois (Chicago). The fusion proteins were expressed and purified using amylose-affinity chromatography as described by the manufacturer (New England Biolabs). Fractions containing the fusion proteins were pooled and dialyzed overnight against 4 L of column buffer (25 mM Tris-HCl, pH 7.5, and 1 mM dithiothreitol [DTT]). The dialyzed sample was loaded onto a Q-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) column (1.5 × 15 cm) previously equilibrated with column buffer, and the proteins eluted with a 200-mL linear gradient of 0 to 0.5 M NaCl in column buffer. Fractions containing purified MBP-MEK1, MBP-ATMAP2Kα, or MBP-MPK4 were pooled, desalted, and concentrated (Centriprep-30 concentrator, Amicon). All purification steps were carried out at 4°C.

PCR Site-Directed Mutagenesis of ATMPK4

A mutagenic oligonucleotide primer (CTGAATGCAAA-TTGTGATCTAAAGCTTGGGG**GC**TTTCG) containing a single base change (from GAT to **GC**T) that converted Asp-187 to Ala (D187A), together with a downstream primer that included a *Pst*I cloning site (aactgcagTCAAATTACAG-ACATATTATCAAACTTATC) were used to amplify the *ATMPK4* gene from the wild-type cloned *ATMPK4* cDNA using PCR. The PCR product was digested with *Bsm*I (contained within the mutagenic oligonucleotide) and *Pst*I, and the resultant fragment ligated with the plasmid used for expressing the MBP-MPK4 fusion. Plasmids containing the correct mutation were identified by DNA sequencing. The D187A MBP-MPK4 fusion protein was expressed in *E. coli* and purified as described above for the wild-type MBP-MPK4.

Preparation of Antiserum to ATMPK4

Purified MBP-MPK4 was separated by SDS-PAGE and the Coomassie Blue-visualized band corresponding to MBP-MPK4 was excised. The protein was eluted from the gel slice by electroelution and then emulsified in adjuvant (Ribi Immunochem Research, Hamilton, MT) to a final volume of 1 mL. MBP-MPK4 (250 μg) was injected into a 3-kg New Zealand rabbit on d 1 and booster injections given on d 21 and d 35 with 200 μg of the protein. High-titer antiserum was obtained 1 week after the final injection.

5′*p*-Flurosulfonylbenzoyl Adenosine (FSBA) Treatment of MBP-MPK4

Recombinant AtMPK4 was treated with FSBA under conditions similar to those reported for the mammalian p38 MAP kinase (Young et al., 1997). Purified MBP-MPK4 (0.2 mg/mL) was incubated in the dark for 30 min at room temperature with 100 mM MgCl$_2$ and 1 mM FSBA (Sigma-Aldrich), followed by overnight incubation at 4°C. To remove the unbound FSBA from MBP-MPK4, the reaction

mixture was passed through a PD-10 column (Pharmacia Biotech).

Kinase Assays

The autophosphorylation assay mixtures (30 μL) contained kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$ and 10 mM MnCl$_2$), 1 μCi of [γ-^{32}P]ATP, and 0.5 μM MBP-MEK1, MBP-MAP2Kα, MBP-MPK4, or D187A MAPK. For the phosphorylation assay of ATMPK4 by At-MEK1 or ATMAP2Kα, 0.5 μM MBP-MEK or MBP-MAP2Kα was incubated with 0.5 μM D187A MBP-MPK4 in reaction buffer containing 1 μCi of [γ-^{32}P]ATP. The reactions were started by the addition of the enzymes. After incubation at 30°C for 30 min, the reactions were terminated by the addition of 30 μL of Laemmli sample buffer (Laemmli, 1970). The samples were heated at 95°C for 5 min and then loaded on a SDS-polyacrylamide gel (7.5% acrylamide [w/v]). The gels were stained with Coomassie Blue R-250, and then destained and dried. The ^{32}P-labeled bands were detected using X-Omat AR film (Eastman-Kodak, Rochester, NY).

Activation of ATMPK4 by AtMEK1

Assay mixtures (60 μL) contained kinase reaction buffer plus 50 μM ATP, 50 mM sodium ortho-vanadate, 1 μM okadaic acid, purified MBP-MPK4 (6 μg), and various amounts of MBP-MEK1 (0, 0.2, 0.6, 1, 2, and 3 μg). After incubation at 30°C for 30 min, 1 μL of anti-MBP-MPK4 antiserum was added to each reaction. The reaction mixtures were incubated at 15°C for 30 min, followed by the addition of 20 μL of protein A agarose beads (Boehringer Mannheim/Roche). After further incubation at 15°C for 30 min, the reaction mixtures were centrifuged at 10,000g for 1 min. The pellets were washed three times with kinase reaction buffer at 4°C, and then resuspended in 50 μL of kinase reaction buffer. A 10-μL aliquot of the resuspended immunoprecipitate was added to 20 μL of kinase reaction buffer plus 10 μM ATP, myelin basic protein (3 μg), and 1 μCi of [γ-^{32}P]ATP. The reactions were incubated at 30°C for 30 min and then analyzed by SDS-PAGE (15% acrylamide [w/v]) as described above, followed by autoradiography.

Dephosphorylation of ATMPK4 by AtPTP1

Dephosphorylation of ATMPK4 by AtPTP1 was performed as follows. Assay mixtures (30 μL) contained kinase reaction buffer plus 1 mM DTT, 1 μCi of [γ-^{32}P]ATP, and 5 μg of purified MBP-MPK4, or 5 μg of D187A MBP-MAPK that had been previously phosphorylated by MBP-MEK1 (2.5 μg). The assay mixtures were incubated at 30°C for 30 min, followed by the addition of 50 ng of purified AtPTP1 or a buffer control. The mixtures were further incubated at 30°C for 30 min and then terminated by the addition of Laemmli sample buffer. Aliquots of the above reactions were analyzed by SDS-PAGE (7.5% acrylamide [w/v]) followed by autoradiography. For the western analysis, purified MBP-MPK4 (2 μg), D187A MBP-MPK4 (2

μg), and MBP-MEK1 (1 μg) were mixed in various combinations in kinase reaction buffer containing cold ATP for 30 min at 30°C. AtPTP1 (50 ng) was then added to each reaction and the mixtures incubated a further 30 min at 30°C. The samples were then separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Micron Separations, Westborough, MA). The blot was then probed with an anti-phospho-Tyr-specific mouse monoclonal antibody (clone 4G10 from Upstate Biotechnology, Lake Placid, NY) using an enhanced chemoluminescence detection system as described by the manufacturer (Amersham).

Inactivation of ATMPK4 by AtPTP1

To determine the effect of Tyr dephosphorylation on ATMPK4 activity, MBP-MPK4 (3 μg) was phosphorylated by MBP-MEK1 (1 μg) as described above, except that the ATP concentration was 100 μM and there was no [γ-^{32}P]ATP. After phosphorylation, MBP-MPK4 was immunoprecipitated by the addition of 1 μL of anti-ATMPK4 antibody, and the immunoprecipitate was treated with AtPTP1 (50 ng) or a control containing buffer alone as described above. The AtPTP1-treated sample was washed three times to remove the AtPTP1 and then resuspended in 50 μL of 50 mM Tris-HCl, pH 7.5. A 10-μL aliquot was added to the assay mixture (20 μL) containing kinase reaction buffer plus 2 mM DTT, 20 μM ATP, 3 μg of myelin basic protein, and 1 μCi of [γ-^{32}P]ATP. After incubation at 30°C for 30 min, the reaction was terminated by the addition of Laemmeli sample buffer and analyzed by SDS-PAGE.

Phosphoamino Acid Analysis

Phosphoproteins were first separated by SDS-PAGE in a mini-gel apparatus (Bio-Rad Laboratories, Hercules, CA), then electroblotted onto a PVDF membrane with 10 mM 3-(cyclohexylamino)propanesulfonic acid (CAPS), pH 10.6, and 10% (v/v) methanol. The ^{32}P-labeled proteins were excised and hydrolyzed in 6 N HCl at 110°C for 1 h. The hydrolyzed samples were subjected to two-dimensional phosphoamino acid analysis, as described previously (Kamps and Sefton, 1989), using a Hunter apparatus (model HTLE 7000, VWR Scientific, S. Plainfield, NJ).

Inactivation of Immunoprecipitated MAPK from Arabidopsis Leaves by AtPTP1

Fully expanded adult Arabidopsis leaves (1 g) were ground with a mortar and pestle in 2 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 10 μg/mL PMSF, and 5% [w/w] Polyclar AT) in the presence of acid-washed glass beads (150–200 μm; Sigma-Aldrich). The extract was centrifuged at 8,000g for 30 min at 4°C, and the supernatant used for immunoprecipitation. MBP-MPK4-depleted serum was prepared by incubating 4 μL of anti-MPK4 antiserum with 5 μg of purified D187A MBP-MPK4 for 30 min at 16°C. For the controls, 500 μg of protein extract was mixed with 4 μL

of preimmune serum or 4 μL of MBP-MPK4-depleted serum. Various amounts of the extracts (125, 250, or 500 μg) were also mixed with 4 μL of anti-MPK4 antiserum. Immunoprecipitation was carried out as above. The immunoprecipitate was washed three times with 1 mL of buffer A (50 mM Tris-HCl, pH 7.5) and then resuspended in 100 μL of buffer A. Aliquots (50 μL) of immunoprecipitate were incubated with AtPTP1 (500 ng) or a buffer alone control for 20 min at 30°C. The samples were centrifuged, washed three times with buffer A, and resuspended in 50 μL of buffer A. The samples (10 μL) were assayed for kinase activity using myelin basic protein as a substrate (as described above).

RESULTS

Expression and Purification of AtMEK1, ATMAP2Kα, ATMPK4, and D187A ATMPK4

To determine if AtMEK1 can activate ATMPK4 in vitro, and to delineate the biochemical properties of this interaction, we expressed ATMPK4, AtMEK1, and ATMAP2Kα in *E. coli* as fusions to the maltose-binding protein. The fusion proteins were present predominantly in the soluble portion of the *E. coli* extract (not shown). Using amylose-affinity chromatography followed by ion-exchange chromatography with Q-Sepharose, MBP-MEK1, MBP-MPK4, and a mutant, ATMPK4 (D187A MBP-MPK4; see below), were purified to apparent homogeneity as determined by Coomassie Blue staining of SDS-PAGE gels (Fig. 1). The MBP-ATMAP2Kα was purified solely by amylose-affinity chromatography. The apparent molecular masses of MBP-MEK1, MBP-ATMAP2Kα, MBP-MPK4, and D187A MBP-MPK4 on SDS-
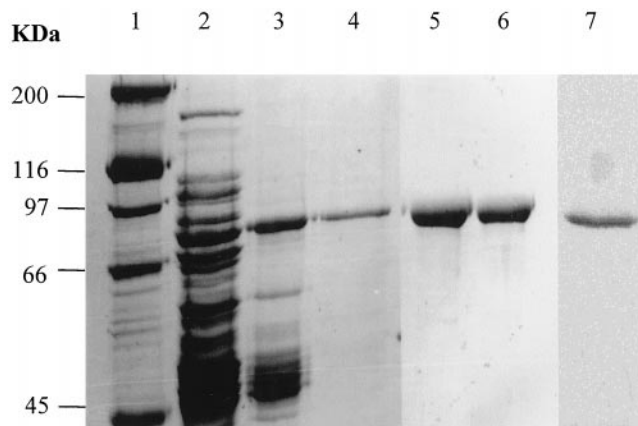
Figure 2. Kinase assays of AtMEK1 and ATMPK4. A, The products of in vitro autophosphorylation from purified MBP-MEK1, MBP-MAP2Kα, MBP-MPK4, and D187A MBP-MPK4, or the products of phosphorylation of D187A MBP-MPK4 by MBP-MEK1 or MBP-MAP2Kα were separated by SDS-PAGE subjected to autoradiography. B, The products of in vitro kinase assays from purified MBP-MEK1, untreated MBP-MPK4, FSBA-treated MBP-MPK4, D187A MBP-MPK4, or FSBA-treated MBP-MPK4 plus MBP-MEK1, as indicated above each lane. The products were separated by SDS-PAGE and analyzed by autoradiography.

PAGE are in good agreement with those calculated from the predicted amino acid sequences.

Autophosphorylation of AtMEK1, ATMAP2Kα, and ATMPK4

To determine whether the AtMEK1, ATMAP2Kα, and ATMPK4 fusion proteins purified from *E. coli* are catalytically active, the enzymes were subjected to in vitro autophosphorylation assays. Purified MBP-MEK1, MBP-ATMAP2Kα, or MBP-MPK4 was incubated in reaction buffer in the presence of [γ-^{32}P]ATP, and the products analyzed by SDS-PAGE. Autoradiography of the gel revealed a single labeled band in each of the MBP-MEK1, MBP-ATMAP2Kα, and MBP-MPK4 lanes (Fig. 2), and the position of this labeled band was indistinguishable from that of the respective Coomassie Blue-stained protein bands. This suggests that the three recombinant enzymes are catalytically active protein kinases.

A mutant form of ATMPK4, D187A MPK4, was generated by site-directed mutagenesis. The mutated Asp is

Figure 1. SDS-PAGE analysis of purification of MBP-MEK1, MBP-MPK4, MBP-MAP2Kα, and D187A MBP-MPK4 from *E. coli*. Lanes 2 to 4 contain various steps of the MBP-MEK1 purification: the soluble fraction of the *E. coli* cells expressing MBP-MEK1 (lane 2), the pooled fractions from the amylose-agarose column (lane 3), and the pooled fractions of the Q-Sepharose column containing MBP-MEK1(1 μg, lane 4). Lanes 5, 6, and 7 contain purified MBP-MPK4 (5 μg), D187A MBP-MPK4 (4 μg), and MBP-MAP2Kα (1 μg), respectively. The gel (7.5% arcylamide [w/v]) was stained with Coomassie Blue R-250, and molecular mass markers as indicated were applied to lane 1.
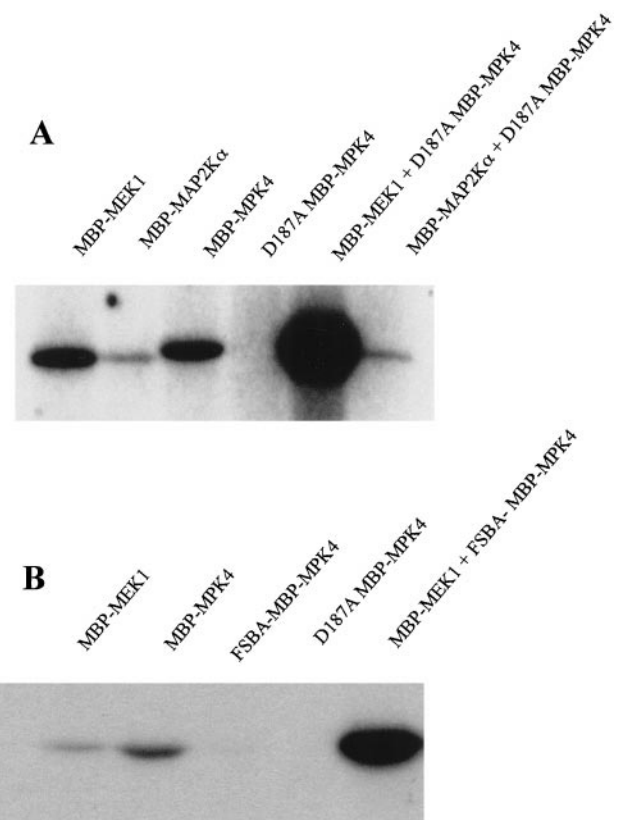
located in the DFG motif (kinase subdomain VII) of the kinase, a motif absolutely conserved in all protein kinases, and the invariant Asp residue is responsible for base-catalyzed transfer of the phosphate in catalysis (Taylor, 1989). Substitution of the negatively charged Asp with an aliphatic Ala is predicted to abolish the kinase activity. As shown in Figure 2, no phosphate was incorporated into the mutant fusion protein, indicating that D187A ATMPK4 is indeed catalytically inactive.

ATMPK4 Is Phosphorylated and Activated by AtMEK1 in Vitro

To determine if AtMEK1 could phosphorylate ATMPK4, the inactive D187A MBP-MPK4 was incubated with MBP-MEK1 in a kinase reaction. The intensity of the band resulting from the reaction containing both MBP-MEK1 and D187A MBP-MPK4 is much stronger than the MBP-MEK1 autophosphorylation band (Fig. 2), indicating that the MBP-MPK4 is indeed phosphorylated by AtMEK1. MBP-MEK1 did not phosphorylate purified maltose binding protein in an in vitro kinase assay (data not shown), which indicates that AtMEK1 phosphorylates the ATMPK4 portion of the MBP-MPK4. A second MEK homolog, ATMAP2Kα, which is from a distinct Arabidopsis MEK subfamily, was tested for its ability to phosphorylate D187A MBP-MPK4. Using purified components, we failed to detect any significant phosphorylation of D187A MBP-MPK4 by MBP-ATMAP2Kα (Fig. 2).

To determine if phosphorylation of ATMPK4 by AtMEK1 leads to its activation, we performed a two-step in vitro kinase assay using purified recombinant MBP-MEK1, MBP-MPK4, and myelin basic protein as the end substrate. Myelin basic protein is an excellent substrate for MAPKs, and is widely used for MAPK assays. MBP-MPK4 was first incubated with MBP-MEK1 in the presence of ATP in kinase buffer (see "Materials and Methods). An aliquot was then added to a reaction cocktail containing myelin basic protein and [γ-^{32}P]ATP. As shown in Figure 3A, MBP-MPK4 activity, as measured by the incorporation of label

Figure 3. Activation of ATMPK4 by AtMEK1. A, Purified MBP-MEK1 (1 μg) and/or MBP-MPK4 (as indicated) were incubated at 30°C for 30 min, and the products assayed for myelin basic protein (MBP) kinase activity. B, Purified MBP-MPK4 (6 μg) was incubated with the indicated amounts of MBP-MEK1 at 30°C for 30 min in the presence of ATP in kinase buffer, and the activity of the MBP-MPK4 was subsequently measured by phosphorylation of myelin basic protein.

into myelin basic protein, was greatly stimulated by prior treatment with MBP-MEK1. In the absence of MBP-MPK4, no phosphorylation was detected, suggesting that myelin basic protein is not a substrate of AtMEK1. However, a faint ^{32}P signal was detected in the control with untreated MBP-MPK4, indicating that the purified MAPK possesses some low basal activity. The activity of ATMPK4 increased with increasing amounts of MBP-MEK1, up to a ratio of 0.5 mol of MBP-MEK1 to 1 mol of MBP-MPK4 (Fig. 3B).

Activation of ATMPK4 Is Accompanied by Thr and Tyr Phosphorylation

To begin to unravel the activation mechanism of ATMPK4, we performed phosphoamino acid analysis on the products of in vitro kinase reactions (Fig. 4). AtMEK1 autophosphorylated principally on Ser(s), and slightly on Thr(s) (Fig. 4A). Notably, we did not detect any phospho-Tyr from the AtMEK1 autophosphorylation reaction. This autophosphorylation property is different from that of animal MEKs, which autophosphorylate on Ser, Thr, and Tyr residues (Crews and Erikson, 1992; Seger et al., 1992; Resing et al., 1995). ATMPK4 autophosphorylated predominately on Tyr; small amounts of phospho-Ser were also detected (Fig. 4B), although the level was variable. Analysis of wild-type MBP-MPK4 phosphorylated by MBP-MEK1 revealed predominately Thr and Tyr phosphorylation, with variable levels of phospho-Ser (Fig. 4C). Activated ATMPK4 phosphorylated myelin basic protein almost exclusively on Thr residue(s) (Fig. 4D).

To determine if phospho-Tyr was the result of MBP-MPK4 autophosphorylation or due to phosphorylation by MBP-MEK1, we analyzed the phosphoamino acids that resulted from phosphorylation of FSBA-treated MBP-MPK4. FSBA is an ATP analog that covalently binds to the ATP-binding site of protein kinases, which results in inactivation of many protein kinases, including MAPK (Young et al., 1997). As shown in Figure 2B, treatment of MBP-MPK4 with FSBA greatly reduced its autophosphorylation activity. The reduction in autophosphorylation activity is approximately equal to the effect of the D187A mutation, which also disrupts the ATP binding site of ATMPK4. The FSBA-treated MBP-MPK4 was phosphorylated by MBP-MEK1, and the phosphorylation occurred predominately on Thr residue(s); only minute amount of phospho-Tyr were detected (Fig. 4F). To further confirm that AtMEK1 does not phosphorylate ATMPK4 on Tyr, we examined the phosphorylation of the catalytically inactive D187A MBP-MAPK4 by MBP-MEK1. Consistent with the results obtained with the FSBA-treated ATMPK4, phosphorylation of the D187A mutant occurred predominately on Thr residue(s), and little or no phospho-Tyr was detected (Fig. 4E). This is also consistent with the lack of phospho-Tyr in the AtMEK1 autophosphorylation reaction.

Dephosphorylation of Tyr Results in Complete Loss of ATMPK4 Activity

To determine if Tyr phosphorylation plays a role in the regulation of ATMPK4, we utilized the Arabidopsis phos-
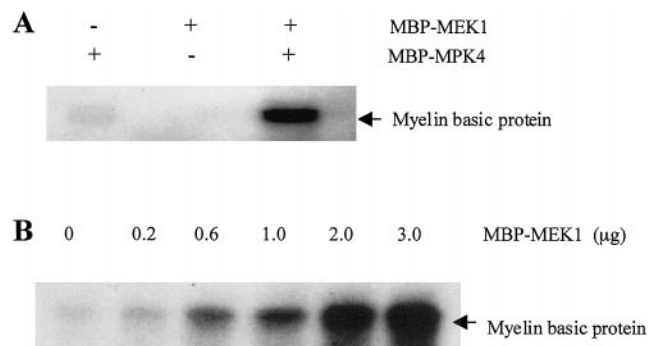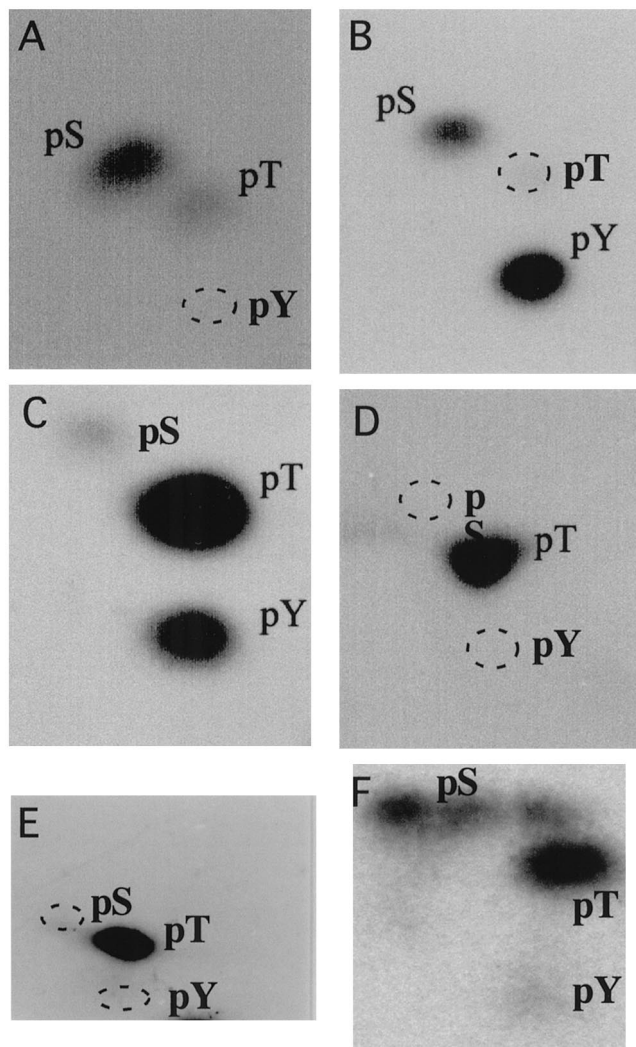
Figure 4. Phosphoamino acid analysis of AtMEK1 and ATMPK4. Following in vitro kinase assays, the ^{32}P-labeled proteins were hydrolyzed with 6 N HCl and the phosphoamino acids were separated by two-dimensional thin layer electrophoresis. The phosphoamino acids were detected by staining with 0.25% (w/v) ninhydrin (for the unlabeled standards) and autoradiography. The position of the phosphoamino acid standards is indicated by dashed ovals. pS, Phospho-Ser; pT, phospho-Thr; and pY, phospho-Tyr. Phosphoamino acid analysis of autophosphorylated AtMEK1 (A), autophosphorylated ATMPK4 (B), ATMPK4 phosphorylated by AtMEK1 (C), myelin basic protein phosphorylated by AtMEK1-activated ATMPK4 (D), D187A MBP-MPK4 phosphorylated by AtMEK1 (E), FSBA-treated MBP-MPK4 phosphorylated by AtMEK1 (F).

phatase AtPTP1. Purified, recombinant AtPTP1 specifically hydrolyzes phospho-Tyr from artificial substrates (Xu et al., 1998). We examined the ability of purified AtPTP1 to dephosphorylate ATMPK4 in vitro. Treatment of autophosphorylated ATMPK4 with purified AtPTP1 results in the removal of most of the incorporated ^{32}P label (Fig. 5A), and because the majority of this label is on Tyr, it indicates that AtPTP1 efficiently dephosphorylates phospho-Tyr. Specific Tyr dephosphorylation of ATMPK4 by PTP1 was demonstrated further by our phosphoamino acid analysis,

which showed specific removal of phosphate from the phospho-Tyr of ATMPK4 upon the treatment of AtPTP1, and little or no dephosphorylation on Ser or Thr (Fig. 5C). AtPTP1 treatment also results in the loss of immunoreactivity with a phospho-Tyr-specific antibody (Fig. 5B), confirming that the residual phosphorylation is not on Tyr. However, treatment of MBP-D187A MPK4, which is phosphorylated predominately on Thr residues (Fig. 4E), with AtPTP1 did not result in significant hydrolysis of the phosphate, as would be expected if AtPTP1 is specific for Tyr residues (Fig. 5A).

We examined the effect of Tyr dephosphorylation on the activity of AtMEK1-activated MBP-MPK4. As shown in Figure 5D, treatment of MEK-activated MBP-MPK4 by AtPTP1 resulted in a near complete loss of ATMPK4 activity, suggesting that Tyr phosphorylation is essential for high ATMPK4 activity.

Tyr Phosphorylation of MAPKs in Vivo

To determine if the phosphorylation of Tyr plays a role in the activity of MAPK in vivo, we examined the effect of AtPTP1 treatment on myelin basic protein kinase activity measured from extracts of Arabidopsis leaves that had been immunoprecipitated with an anti-ATMPK4 polyclonal antibody (Fig. 6). This polyclonal antibody was raised against the entire ATMPK4 protein and, given the similarity of the Arabidopsis MAPK gene family, it likely recognizes multiple MAPK isoforms. This antibody immunoprecipitated MBP-MPK4 purified from *E. coli*, but the preimmune serum or antibody that had been pretreated with purified MBP-D187A-MPK4 failed to do so (Fig. 6A). Treatment of Arabidopsis leaf extracts with the anti-MPK4 antibody immunoprecipitated a level of myelin basic protein kinase activity proportional to the amount of extract analyzed (Fig. 6B, lanes 3–5), as would be expected if the antibody were in excess. Arabidopsis extracts treated with either preimmune serum (controls) or with the MBP-D187A-MPK4-depleted serum failed to precipitate myelin basic protein kinase activity (Fig. 6B, lanes 1 and 2). Treatment of the immunoprecipitated protein with AtPTP1 resulted in a substantial reduction in kinase activity (Fig. 6B, lanes 6–8). These results suggest that Arabidopsis MAPKs are phosphorylated on Tyr in vivo, and that this phosphorylation is required for high activity.

DISCUSSION

We have expressed Arabidopsis homologs of MEK and MAPK in *E. coli* and purified them to apparent homogeneity using affinity chromatography. Consistent with their similarity to other protein kinases, both possess intrinsic protein kinase activity, as determined by in vitro phosphorylation assays. Purified, recombinant AtMEK1 was able to phosphorylate and activate ATMPK4 in vitro, which indicates that a plant MEK homolog is able to phosphorylate and activate a MAPK from plants.

Activation of animal MAPK is achieved by phosphorylation of both Tyr and Thr by the dual-specificity kinase MEK. Similarly, in order for ATMPK4 to be highly active
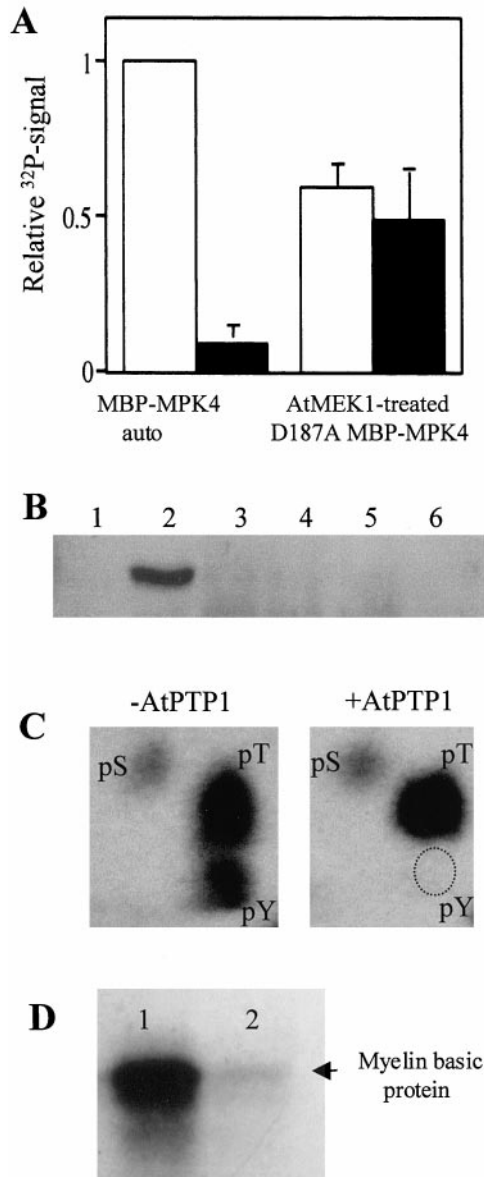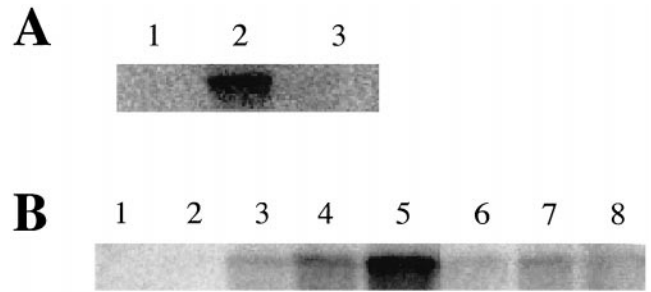
Figure 6. Inactivation of Arabidopsis MBP kinase activity by AtPTP1. A, In vitro kinase assay of immunoprecipitated MBP-MAPK4. Purified MBP-MAPK4 was treated with preimmune serum (lane 1), anti-MAPK4 serum (lane 2), or ATMPK4-depleted anti-MAPK4 serum (lane 3). The immunoprecipitates were then mixed with myelin basic protein in kinase buffer containing of [γ-^{32}P]ATP and the products analyzed by SDS-PAGE followed by autoradiography. B, Effect of PTP treatment on myelin basic protein kinase activity from Arabidopsis leaf extracts. Fifty microliters (lanes 3 and 6), 100 μL (lanes 4 and 7), or 200 μL (lanes 1, 2, 5, and 8) of soluble Arabidopsis leaf extracts were incubated with preimmune (lane 1), ATMPK4-depleted (lane 2), or anti-MAPK4 serum (lanes 3–8). The immunoprecipitates were then treated with 500 ng of AtPTP1 (lanes 6–8) or a buffer alone control (lanes 1–5) for 30 min at 30°C. The products were then assayed in vitro for myelin basic protein kinase activity as described in "Materials and Methods" and the products analyzed by SDS-PAGE followed by autoradiography.

requires phosphorylation of both Tyr and Thr residues, as demonstrated using phospho-Tyr-specific phosphatases and antibodies. This is consistent with the findings of Ádám et al. (1997), who utilized an animal Tyr phosphatase to demonstrate that Tyr phosphorylation is required for full activity of a myelin basic protein kinase from harpin-treated tobacco leaves. However, phosphoamino acid analysis of catalytically inactive ATMPK4 phosphorylated by AtMEK1 indicated that there was almost no phosphorylation of Tyr residues. Additionally, analysis of AtMEK1 autophosphorylation revealed that it did not autophosphorylate on Tyr residues, which is distinct from the animal enzymes. These results suggest that, in contrast to animal MEKs, AtMEK1 may not be a dual-specificity kinase, but rather a Ser/Thr specific kinase. Alternatively, it is possible that the lack of Tyr phosphorylation by AtMEK1 in vitro is an artifact of the recombinant enzyme, although this is unlikely because other recombinant MEKs from animal and fungal sources have phosphorylation profiles similar to the native enzymes.

Tyr phosphorylation is clearly required for full ATMPK4 activity, and this, at least in vitro, results from ATMPK4 autophosphorylation activity. Animal MAPKs autophosphorylate, via an intramolecular reaction, on the Tyr and Thr residues of the TXY motif, with the Tyr phosphorylation being stronger (Seger et al., 1991; Wu et al., 1991;

Figure 5. Tyr dephosphorylation and inactivation of ATMPK4 by AtPTP1. A, Specificity of AtPTP1. Purified MBP-MPK4 (auto, 5 μg) and MBP-MEK1 (2.5 μg) plus D187A MBP-MPK4 (MEK-treated, 5 μg) were incubated in kinase buffer in the presence of [γ-^{32}P]ATP for 30 min at 30°C. Either 50 ng of purified AtPTP1 or a buffer alone control was then added and the reaction was incubated an additional 30 min at 30°C. The products were then separated by SDS-PAGE, and the incorporated ^{32}P was quantified with a phosphor imager. The highest signal was assigned a value of 1, and the other signals normalized to it. The values represent the means ± SD from two replicates. B, Western-blot analysis of the products of in vitro kinase assays using cold ATP. The western blots were probed with an anti-phospho-Tyr antibody. The lanes contain the products of the following reactions: Lane 1, MBP-MEK1; lane 2, MBP-MPK4; lane 3, D187A MBP-MPK4; lane 4, MBP-MEK1 plus D187A MBP-MPK4; lane 5, MBP-MPK4 plus AtPTP1; and lane 6, MBP-MEK1 plus D187A MBP-MPK4 plus AtPTP1. C, Phosphoamino acid analysis of wild-type MBP-ATMPK4 phosphorylated by MBP-AtMEK1 and either untreated or treated with 50 ng of AtPTP1 as indicated. D, Effect of Tyr dephosphorylation on MBP-MPK4 activity. MBP-MPK4 (3 μg) was incubated with MBP-MEK1 (1 μg) in the presence of 100 μM ATP, then purified by

immunoprecipitation with anti-MPK4 antibody. The immunoprecipitate was washed and then treated with 50 ng of AtPTP1 (right lane) or a buffer alone control (left lane). The beads were washed to remove the AtPTP1, and the activity of ATMPK4 was then measured by the phosphorylation of myelin basic protein.

Robbins and Cobb, 1992; Her et al., 1993; Robbins et al., 1993). The rate of autophosphorylation varies widely among MAP kinases, and this variance has been linked to differences in the loop between the kinase subdomains VII and VIII (Jiang et al., 1997). Interestingly, this loop is very divergent in the Arabidopsis MAP kinase family (Mizoguchi et al., 1993). The Tyr autophosphorylation of ATMPK4 may play an important role in its activation, although it is not yet known if this occurs on the Tyr residue of the TEY motif. However, the observation that removal of this autophosphorylated Tyr by AtPTP1 decreased the activity of ATMAPK4 indicates that it occurs on a regulatory Tyr. Kinetic analysis indicates that animal MEK exhibits a 10-fold increase in apparent affinity for the Tyr phosphorylated MAPK (Haystead et al., 1992), and perhaps Tyr autophosphorylation of AtMPK4 is a prerequisite for its activation and may obviate the requirement for phosphorylation of this residue by AtMEK1.

An alternative to autophosphorylation being the in vivo source of phospho-Tyr in ATMPK4 is that a second MEK catalyzes the Tyr phosphorylation. There is some precedent for this mode of activation for MAP kinases. The SAPK1/JNK1 MAP kinase is phosphorylated by two MEKs synergistically, one of which phosphorylates the Tyr residue and one of which phosphorylates the Thr residue within the TXY motif (Lawler et al., 1998). There are multiple MEK genes in Arabidopsis, and it may be that AtMEK1 phosphorylates ATMPK4 on Thr and a distinct isoform phosphorylates the Tyr residue of the TXY motif. Nevertheless, results presented here indicate that the autophosphorylation of ATMPK4 on Tyr is sufficient to activate, at least partially, the enzyme in vitro in combination with the Thr phosphorylation catalyzed by AtMEK1.

The activation of ATMPK4 by AtMEK was close to linear up to a 1:1 ratio. This is similar to animal systems in which high ratios of MEK:MAPK (40:1) are required to fully phosphorylate MAPK in vitro (Scott et al., 1995). However, addition of a MEK-enhancing factor results in full phosphorylation of MAPK by MEK at equal molar concentrations in vitro (Scott et al., 1995). Interestingly, MEK-enhancing factor also greatly stimulates MAPK autophosphorylation activity in vitro. Consistent with the relatively poor phosphorylation, MAPK and MEK are present at roughly equal concentrations in vivo, and is some cases MEK is even in excess (Ferrell, 1996). This suggests that little or no amplification occurs when a signal is passed from MEK to MAPK. It has been postulated that the dual phosphorylation of MAPK by MEK may play a role in converting graded inputs into a switch-like output (Ferrell, 1996). If autophosphorylation of ATMPK4 is indeed the in vivo source of Tyr phosphorylation, then it may be that it acts less switch-like and perhaps produces a more graded signaling output.

ATMPK4 is efficiently dephosphorylated by AtPTP1 in vitro, but it is unclear whether AtPTP1 interacts with ATMPK4 in vivo. Animal and yeast MAPKs are deactivated by dual specificity protein phosphatases, or by the combination of a Ser/Thr protein phosphatase and a Tyr-specific phosphatase (Cobb and Goldsmith, 1995; Keyse, 1998). A dual-specificity protein phosphatase, AtDsPTP1, has also been identified from Arabidopsis (Gupta et al.,

1998). AtDsPTP1 is able to hydrolyze both phospho-Ser and phospho-Tyr using artificial protein substrates. Furthermore, this enzyme is capable of dephosphorylating and inactivating ATMPK4; however, the catalytic efficiency is at least 30-fold lower than that of AtPTP1 (data not shown). Thus, AtDsPTP1 is unlikely to deactivate ATMPK4 in vivo. Arabidopsis homologs of the Ser/Thr phosphatase PP2C have also been identified and have been implicated in the repression of MAPK cascades (Luan, 1998; Meskiene et al., 1998).

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