



# Identification of tyrosine autophosphorylation sites of Arabidopsis MEKK1 and their involvement in the regulation of kinase activity

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Title: Identification of tyrosine autophosphorylation sites of *Arabidopsis* MEKK1 and their involvement in the regulation of kinase activity

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## **Abstract**

The MEKK1 kinase is a key regulator of stress signaling in *Arabidopsis*; however, little is known about the regulation of its kinase activity. Here, we found that recombinant MEKK1, expressed in both mammalian HEK293 cells and *E. coli*, shows a mobility shift in SDS-PAGE, and immunoblotting detected phosphorylation of serine, threonine, and tyrosine residues.

N-terminal deletions, site-directed mutagenesis, and protein phosphatase treatment revealed that

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the mobility shift results from autophosphorylation of the kinase domain. We identified the tyrosine autophosphorylation sites in the N-terminal region of MEKK1. Tyrosine to phenylalanine mutations decrease phosphorylation of the substrate MKK1, suggesting the important role of this residue in the regulation of MEKK1 kinase activity. The present study is the first to show that plant MAPKKs are regulated by tyrosine phosphorylation.

## **1. Introduction**

Mitogen-activated protein kinase (MAPK) cascades are universal signaling modules in eukaryotes, including yeasts, animals and plants. These cascades serve as central regulators of growth, death, differentiation, proliferation and stress responses [1-4]. In plants, MAPK cascades are involved in responses to various biotic and abiotic stresses, hormones, cell division and developmental processes [4-6]. MAPK cascades comprise three classes of protein kinases, MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), and each of these classes is a family with multiple subtypes. Generally, MAPK is activated by phosphorylation from an upstream MAPKK on the Thr and Tyr residues in the activation loop that contains the consensus TxY motif. MAPKK is also activated by the phosphorylation of Ser and Thr residues

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in its consensus S/T-X<sub>3-5</sub>-S/T motif, which is catalyzed by MAPKKKs. According to upstream signals, such as various biotic and abiotic stresses or the perception of phytohormones, MAPKKKs are activated and subsequently phosphorylate downstream MAPKKs. However, little is known about the regulation of MAPKKK activity and the signaling pathways between receptors and MAPKKKs in plants.

*Arabidopsis* MEKK1 is one of the most thoroughly characterized MAPKKKs in plants. Its transcripts have been found in vascular tissues and guard cells [7] and accumulate in response to touch, cold, and salt stresses [8]. Based on pair-wise yeast two-hybrid analysis and functional complementation tests of yeast mutants, the MEKK1-MKK1/2-MPK4 cascade was the first cascade identified in plants [9]. It was reported that MEKK1 activated MKK2-MPK4/6 during cold and salt stresses [10]. In a previous study, we also reported that MKK1 was activated by wounding stimuli and phosphorylated downstream MPK4 [11]. MEKK1 was also activated by the same stimuli and phosphorylated MKK1 [12], indicating the MEKK1-MEK1-MPK4 cascade as a signal transducer of wounding. Moreover, MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1 [13]. Similar results have been reported by other groups, showing that MKK1 and MKK2 function redundantly in

innate immunity signaling [14] and that the interaction between MEKK1 and MKK1 or MKK2 occurs at the plasma membrane [14]. With respect to the upstream pathways and regulation of MEKK1, transient expression analysis with biochemical and genetic approaches showed that the flagellin receptor FLS2, a leucine-rich-repeat receptor kinase, functions upstream of MEKK1 [15]. Additionally, MEKK1 kinase activity and protein stability is regulated by H<sub>2</sub>O<sub>2</sub> in a proteasome-dependent manner [16]. A recent study showed the phosphorylation of MEKK1 via Ca<sup>2+</sup> signaling during the cold response [17] and membrane rigidification functions upstream of the MEKK1-MKK2-MPK4 cascade during cold acclimation [18]. Moreover, the Ca<sup>2+</sup>/calmodulin (CaM)-regulated receptor-like kinase, CRLK1, a positive regulator of the response to freezing temperatures that is activated in the presence of Ca<sup>2+</sup>/CaM, can phosphorylate MEKK1 [17, 19, 20]. However, little is known about the regulatory mechanism of MEKK1 by these upstream components. In the present study, we prepared recombinant MEKK1 protein by using mammalian and bacterial expression systems and identified the tyrosine autophosphorylation site in the N-terminal region of MEKK1. The tyrosine-phenylalanine mutant of MEKK1 reduced phosphorylation of its substrate MKK1,

indicating the important roles of these tyrosine residues for the regulation of MEKK1. These findings help to clarify the MEKK1 signaling pathways in detail.

## **2. Materials and Methods**

### *2.1. Construction of the mammalian expression vector for 3xFlag Tag fused Arabidopsis*

#### *MEKK1*

cDNA for the *Arabidopsis* MEKK1 gene was isolated by RT-PCR as previously described [17]. Full-length MEKK1 was cloned into the p3xFLAG-CMVTM-10 mammalian expression vector (Sigma, St. Louis, Mo, USA) for translational fusion of the 3xFLAG tag to the N-terminus.

### *2.2. Transfection of HEK293 cells and preparation of whole cell extracts*

HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells at 20–30% confluence in 100 mm dishes were transfected with 2 µg of plasmid DNA/dish by using Effectene™ reagent (Qiagen, Hilden, Germany) according to the manufacturer's standard protocol. The transfection treatment proceeded for 24 h at 37°C, and the resulting transfected cells were serum-starved in Dulbecco's modified Eagle's medium for 18 h prior to cell extraction. After incubation for 24 h at 37 °C, the transfectants were washed twice with ice-cold phosphate-buffered saline and lysed in

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extraction buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM  $\beta$ -mercaptoethanol, 1 mM  $\text{Na}_3\text{VO}_4$ , 10  $\mu\text{g}/\text{ml}$  leupeptin, and 20  $\mu\text{M}$  APMSF. The cell lysates were vortexed (10 s, 2 times), sonicated (30 s, 2 times), clarified by centrifugation ( $15,000 \times g$ , 10 min), diluted to a protein concentration of 1 mg/ml with the extraction buffer, and used as Triton X-100-solubilized whole cell extracts.

### *2.3. Immunoprecipitation of 3xFlag MEKK1*

Whole cell extracts (50  $\mu\text{g}$  of protein per lane) were immunoprecipitated with 10  $\mu\text{g}/\text{ml}$  anti-FLAG antibody at 4°C overnight. Immunocomplexes were collected with 25  $\mu\text{l}$  per sample of protein A-Sepharose by 30-min rocking at 4°C and then washed three times with extraction buffer as described above.

### *2.4. Immunoblot analysis*

Immunoblot analysis was performed as previously described [11]. Aliquots of the immunoprecipitates were separated by SDS-PAGE on a 10% gel. After SDS-PAGE, the proteins were electro-blotted onto an Immobilon-P membrane (Millipore, Boston, MA, USA). An anti-Flag antibody (Sigma, St. Louis, Mo, USA, diluted 10,000-fold), anti-phospho-serine antibody (ENZO LIFE SCIENCES INTERNATIONAL, Plymouth Meeting, PA, USA, diluted 1,000-fold), anti-phospho-threonine antibody (Cell Signaling Technology, Beverly, MA, USA, diluted 1,000-fold), and anti-phospho-tyrosine antibody (PY-99, Santa Cruz Biotechnology,



Santa Cruz, CA, USA, diluted 200-fold) were used as the primary antibodies. After extensive washing of the membrane with TBS-T buffer, an alkaline phosphatase-conjugated goat anti-rabbit IgG second antibody (Chemicon International, Inc., Temecula, CA, USA) was applied for analysis with anti-phospho-serine and threonine antibodies, and an alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Chemicon International, Inc., Temecula, CA, USA) was applied for analysis with anti-FLAG and phospho-tyrosine antibodies, respectively. The color reaction was performed by using 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium as substrates. The whole cell extracts (15  $\mu$ g/lane) were also separated by SDS-PAGE on 10% gels. Immunoblot analysis was performed as described above. An anti-Flag antibody, anti-MEKK1 serum (raised against the synthetic peptide corresponding to the carboxyl terminal of MEKK1[12], diluted 1,000-fold), anti-p44/42 MAP kinase antibody (Cell Signaling Technology, Beverly, MA, USA, diluted 500-fold) and anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology, Beverly, MA, USA, diluted 1,000-fold) were used as the primary antibodies, and an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody was applied for analysis with the latter two antibodies.

### 2.3. Expression and purification of recombinant proteins in *E. coli*

Full-length MEKK1, a kinase-negative form of full-length MEKK1 (MEKK1 KN), and N-terminal deletions of MEKK1 ( $\Delta$ 83,  $\Delta$ 166, and KD) were cloned into a pGEX4T-1 vector (GE Healthcare, Waukesha, WI, USA) for translational fusion to glutathione S-transferase (GST) as previously described [17]. The tyrosine – phenylalanine mutants of MEKK1 (Y38F, Y84F, Y204F, Y323F, and Y204F/Y323F) were created by using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions, and verified by DNA sequencing. The kinase-negative mutant of MKK1 (K97R) was created as previously described [11]. Each vector was transformed into *E. coli* strain JM109. The recombinant GST-fusion proteins were purified as previously described [11].

### 2.5. Protein Phosphatase treatments

Bacterially expressed MEKK1 Full and KN (0.5  $\mu$ g) were incubated with 400 units of Lamda protein phosphatase (New England BioLab, Ipswich, MA, USA) for 20 min at 30°C according to the manufacturer's instructions. After the phosphatase reaction, the samples were separated via SDS-PAGE and subjected to immunoblot analysis with an anti-GST antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA), anti-phospho-Serine antibody, anti-phospho-Threonine antibody and anti-phospho-Tyrosine antibody as described above. An alkaline phosphatase-conjugated rabbit anti-goat IgG second antibody was applied for analysis with an anti-GST antibody.

## 2.5. Kinase assays

Bacterially expressed GST-fusion proteins, MEKK1 Full, KN and YF mutants (Y38F, Y84F, Y204F, Y323F, and Y204F/Y323F) were incubated with MKK1 KN in a kinase reaction mixture containing 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl<sub>2</sub>, 50 μM ATP and [ $\gamma$ -<sup>32</sup>P] ATP (37 kBq) at 30°C for 20 minutes. The reaction mixtures were separated by SDS-PAGE on 10% gels, and the phosphorylation of MKK1 KN was visualized and quantified using a Bioimaging Analyzer BAS2500 (Fuji Film Co., Tokyo, Japan).

## 3. Results

### 3.1. Expression of Arabidopsis MEKK1 in Human Embryonic Kidney (HEK) 293 cells

To characterize the protein function of *Arabidopsis* MEKK1, we used a mammalian expression system. We constructed the expression vector, in which 3xFlag tag was fused to the N-terminus of MEKK1. 3xFlag-tagged MEKK1 (designated as FLAG-MEKK1) was transiently expressed in HEK293 cells. Triton X-100-soluble extracts were analyzed by western blot by using an anti-Flag antibody (Fig. 1A). Two bands were clearly detected between a molecular mass of 75 and 100 kDa, which were both higher than the calculated molecular mass of FLAG-MEKK1 (70.6 kDa). These bands were also detected by anti-MEKK1 rabbit antiserum

[12], which was produced using the synthetic peptide (Cys-Val-Gly-Ser-Gly-Gly-Ser-Gly-Ala-Ser-Pro-Leu-Leu-Arg-Arg) corresponding to the carboxyl terminal of MEKK1 (MEKK1CT). These results indicate that the two bands are the products of full-length MEKK1 and the lower band is not a degradative product, suggesting that FLAG-MEKK1 can assume various conformations in cells. Some phosphorylated protein bands change mobility on SDS-PAGE gels. We analyzed the phosphorylation status of the recombinant MEKK1. FLAG-MEKK1 was immunoprecipitated from whole cell extracts and separated by SDS-PAGE, and immunoblot analysis was performed using anti-phospho amino acid-specific antibodies (anti-pSer, pThr, and pTyr, respectively). As shown in Fig. 1A, the two bands were detected using anti-pSer, pThr, and pTyr antibodies, suggesting the possible involvement of these phosphorylation in the mobility shift of the Flag-MEKK1 on SDS-PAGE.

Overexpression of the catalytic domain of *Arabidopsis* MEKK1 suppressed the *ste11Δ* mutation of *S. cerevisiae*, indicating that *Arabidopsis* MEKK1 can function as the MAP3K of the Ste11-Ste7- Fus3/Kss1 cascade in the mating pheromone response pathway in yeast cells [8]. Fus3/Kss1 is a homolog of mammalian Erk1/2 MAPK. To analyze the effects of the MEKK1 transient expression in HEK293 cells, whole cell extracts were analyzed by

immunoblotting using anti-Erk1/2 and anti-phospho-Erk1/2 antibodies. Phosphorylated Erk1/2 bands were clearly detected in the FLAG-MEKK1 lane without a change in the protein amounts of Erk1/2 (Fig. 1B). These results suggest that *Arabidopsis* MEKK1 may function as an upstream MAP3K of Erk1/2, indicating the highly conserved signaling system of this MAPK cascade from yeasts to mammals and plants.

### 3.2. Autophosphorylation of bacterially expressed MEKK1

As described above, we detected the phosphorylation of transiently expressed MEKK1 in mammalian HEK293 cells. These phosphorylation reactions were potentially caused by the autophosphorylation of MEKK1 or phosphorylation from other protein kinases, as there are many protein kinases in mammalian cells. To clarify MEKK1 phosphorylation, full-length MEKK1 (MEKK1 Full) and its kinase negative form (MEKK1 KN, K361R) were expressed as GST fusion proteins in *E. coli* (Fig. 2A). The recombinant proteins were affinity purified by Glutathione-Sepharose resin and separated by SDS-PAGE, followed by CBB staining. MEKK1 Full has two CBB staining bands of approximately 130 kDa, which is higher than the predicted molecular mass of GST-MEKK1 Full (92 kDa) (Fig. 2B). However, MEKK1 KN has a single

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band of approximately 105 kDa. Next, we analyzed the phosphorylation status of MEKK1 Full and KN by immunoblotting with anti-phospho-amino acid-specific antibodies (pSer, pThr, and pTyr). The two bands of MEKK1 Full were detected with all of the antibodies, whereas phosphorylation of MEKK1 KN was not detected (Fig. 2C). To analyze the relationship between the phosphorylation status and mobility of the GST-MEKK1 protein via SDS-PAGE, MEKK1 Full and KN were treated with Lambda protein phosphatase. The samples were separated by SDS-PAGE, and the phosphorylation status was monitored by immunoblotting. Via phosphatase treatment, the two bands of MEKK1 full became a single band, which was not detected by any phospho-amino acid specific antibodies (Fig. 2C). The mobility of MEKK1 Full in SDS-PAGE was accelerated by de-phosphorylation and migrated to an equal position with MEKK1 KN. These results indicated that MEKK1 FL undergoes autophosphorylation of Ser, Thr, and Tyr residues, which causes the mobility shift on SDS-PAGE. To analyze the autophosphorylation of MEKK1 in detail, truncated versions of MEKK1 were constructed (Fig. 3A) and expressed as GST-fusion proteins in *E. coli*. MEKK1 Full and N-terminal deletions ( $\Delta 83$ ,  $\Delta 166$ , and KD) were separated via SDS-PAGE. The gel was stained by CBB and immunoblotted with an anti-MEKK1CT antibody (Fig. 3B). N-terminal deletions ( $\Delta 83$ ,  $\Delta 166$ ,

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and KD) also had two bands of undegraded products because these proteins were purified using Glutathione-Sepharose resin and detected by an anti-MEKK1CT antibody. Next, we performed immunoblot analysis of these proteins with anti-phospho amino acid-specific antibodies (Fig. 3B). The two bands of the N-terminal deletions were detected using anti-pSer and pThr antibodies. Interestingly, tyrosine phosphorylation was not detected in MEKK1KD, indicating that tyrosine phosphorylation of MEKK1 occurred in the N-terminal region, in particular, from amino acid residues 166 to 332. The mobility shift of MEKK1 in SDS-PAGE resulted from serine and/or threonine phosphorylation of the kinase domain because the mobility shift was also detected in MEKK1KD without tyrosine phosphorylation.

### *3.3. Identification of the tyrosine autophosphorylation site of MEKK1 and its regulatory function for substrate phosphorylation*

As shown Fig. 4A, MEKK1 has four tyrosine residues in its N-terminal region (1-332). To identify the tyrosine phosphorylation site of MEKK1, we generated the tyrosine-phenylalanine mutants Y38F, Y84F, Y204F, Y323F, and Y204F/Y323F. These mutants were also expressed as GST-fusion proteins in *E. coli*, and tyrosine phosphorylation was monitored. Tyrosine

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phosphorylation was detected in Y38F, Y84F, and Y323F, but was dramatically decreased in Y204F and almost not detected in Y204F/Y323F (Fig. 4B). These results indicate that Y204 is the major tyrosine phosphorylation site and that Y323 is also a potential phosphorylation site. Furthermore, phosphorylation of Y204 was directly detected by LC-MS/MS analysis (Fig. 1S). To analyze the role of tyrosine phosphorylation of MEKK1, we monitored the kinase activity of YF mutants by using the recombinant protein of kinase-negative MKK1 (MKK1 KN) as a substrate. Phosphorylation of MKK1 KN was detected in MEKK1 Full, Y38F, Y84F, and Y323F (Fig. 4C). Interestingly, phosphorylation of MKK1 KN by Y204F was slightly decreased and further decreased in Y204F/Y323F. These results suggest that autophosphorylation of the tyrosine residue is involved in the regulation of substrate phosphorylation of MEKK1.



#### 4. Discussion

MEKK1 functions as a key regulator of stress signaling in *Arabidopsis*; however, little is known about its upstream pathway and regulation of kinase activity. To characterize the MEKK1 protein, we used a mammalian expression system. The 3xFlag tag was fused to the N-terminus of MEKK1 and expressed in HEK293 cells. We detected the recombinant MEKK1 protein by immunoblotting (Fig. 1A). Interestingly, endogenous Erk1/2, a mammalian MAPK, was activated by MEKK1 expression (Fig. 1B), suggesting a potential function of MEKK1 as a MAPKKK in HEK293 cells. Overexpression of the catalytic domain of *Arabidopsis* MEKK1 suppressed the *ste11Δ* mutation of *S. cerevisiae*, indicating that *Arabidopsis* MEKK1 can function as a MAP3K of the Ste11-Ste7- Fus3/Kss1 cascade in the mating pheromone response pathway in yeast cells [8]. These results suggest that the highly conserved system of this MAPK cascade is present in eukaryotic cells. FLAG-MEKK1 showed a mobility shift in SDS-PAGE, and two bands were clearly detected between the molecular masses of 75 and 100 kDa, which were both higher than the calculated molecular mass of FLAG-MEKK1 (70.6 kDa) (Fig. 1A). The results of the bacterially expressed MEKK1 showed that GST-MEKK1 has Ser, Thr, and Tyr autophosphorylation activity and undergoes a mobility shift in SDS-PAGE; therefore, it is

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likely that the phosphorylation of Ser, Thr, and Tyr residues detected in FLAG-MEKK1 was also caused by autophosphorylation. In a previous study, transiently expressed Myc-tagged MEKK1 was detected to have low protein and kinase activity in untreated *Arabidopsis* protoplasts, and upon H<sub>2</sub>O<sub>2</sub> treatment, the amount of MEKK1 protein and kinase activity greatly increased [16]. Treatment with the proteasome inhibitor MG115 increased the amount of Myc-tagged MEKK1 [16]. In this study, we tried to generate the transgenic *Arabidopsis* plants overexpressing *3xFLAG-MEKK1 Full and KN*, respectively. We obtained the several lines of each transgenic plant that detected the transgene expression and also the 3xFLAG-MEKK1 KN protein was detected in several lines, however, we could not detect the 3xFLAG-MEKK1 Full protein in each line (Fig S2). The kinase activity of MEKK1 may affect the protein amounts of MEKK1 in plant cells. In the present study, we identified the tyrosine autophosphorylation site of bacterially expressed MEKK1. The tyrosine non-phosphorylation mutant Y204F reduced the substrate phosphorylation activity toward MKK1, and the Y204F/Y323F double mutant further decreased the substrate phosphorylation activity, indicating that these two tyrosine residues are important for the regulation of MEKK1 activity (Fig. 4C). To analyze the role of tyrosine autophosphorylation, the interaction of MEKK1 and downstream MKKs was monitored by

using yeast two-hybrid system. The effect of kinase activity of MEKK1 was detected in the interaction between the kinase domain of MEKK1 and MKK1, however, the effect of the YF mutations was not detected (Fig. S3). Further analysis is needed to clarify the role of tyrosine phosphorylation, such as *in vitro* and *in planta* binding assays with MKK1. MAPK is activated by the phosphorylation of Thr and Tyr residues in the TxY motif by its upstream MAPKK. We previously reported that the bacterially expressed *Arabidopsis* MAPK, MPK4, has autophosphorylation activity of this Tyr residue; however, this Tyr residue is also phosphorylated by its upstream MAPKK, MKK1 [11]. It is likely that the Tyr residues of MEKK1 are also phosphorylated by other kinases. The Y341 residue of c-Raf, a mammalian MAP3K, is phosphorylated by the tyrosine protein kinase Src, and its kinase activity is regulated by this phosphorylation [21]. The flagellin receptor FLS2, a leucine-rich-repeat receptor kinase, functions upstream of MEKK1 by using transient expression analysis with biochemical and genetic approaches [15]. We previously reported that the Ca<sup>2+</sup>/calmodulin (CaM)-regulated receptor-like kinase CRLK1, a positive regulator of the response to freezing temperatures, phosphorylated recombinant MEKK1 *in vitro*. Analyzing the phosphorylation of MEKK1 from these upstream kinases in detail may clarify the regulation of MEKK1 activity.

In conclusion, we used mammalian and bacterial expression systems to generate recombinant MEKK1 proteins. Recombinant MEKK1 had autophosphorylation activity for Ser, Thr, and Tyr residues. We showed the Tyr autophosphorylation site of the N-terminus of MEKK1 and that Tyr residues play important roles in regulating the substrate phosphorylation activity. The present study is the first to show that plant MAPKKs are regulated by tyrosine phosphorylation, and this finding will help to clarify the MEKK1 signaling pathways in detail.

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### Figure legends

**Fig. 1.** Expression of *Arabidopsis* MEKK1 in Human Embryonic Kidney (HEK) 293 cells. (A) Triton X-100 solubilized extracts (whole cell extracts, WCL) were prepared from mock-transfected HEK293 cells (Mock) and from cells expressing 3xFLAG-tagged *Arabidopsis* MEKK1 (FLAG-MEKK1), as described in the Materials and Methods. WCL were separated via SDS-PAGE and analyzed by immunoblotting with an anti-FLAG antibody (IB: anti-FLAG) and anti-MEKK1 antiserum (IB: anti-MEKK1CT). WCL were immunoprecipitated (IP) with an anti-Flag antibody, and the immunoprecipitates were separated via SDS-PAGE and analyzed by immunoblotting with an anti-FLAG antibody (IB: anti-FLAG), anti-phospho-Serine antibody (IB: anti-pSer), anti-phospho-Threonine antibody (IB: anti-pThr) and anti-phospho-Tyrosine antibody (IB: anti-pTyr). (B) Activation of endogenous MAPKs (Erk1/2) in HEK293 cells expressing *Arabidopsis* MEKK1. WCL were separated via SDS-PAGE and analyzed by immunoblotting with an anti-Erk1/2 antibody (IB: anti-Erk) and anti-phospho-Erk1/2 antibody (IB: anti-pErk).

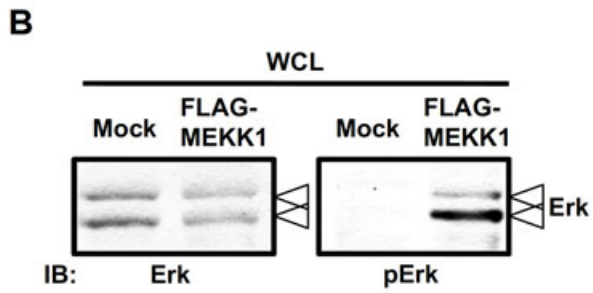
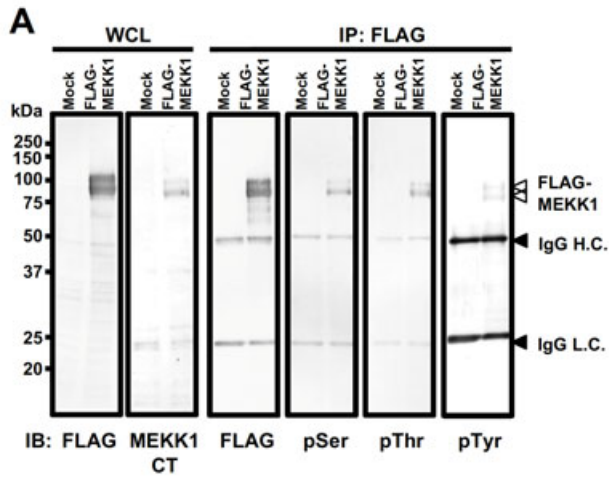
**Fig. 2.** Expression of MEKK1 and its kinase-negative mutants in *E. coli*. (A) Schematic representation of the MEKK1 constructs used for the expression in *E. coli*. The kinase negative (KN) construct (MEKK1 KN) was produced by site-directed mutagenesis of the consensus ATP-binding Lys residue to Arg (K361R) using the full-length of MEKK1 (Full) as a template. The kinase domain is indicated with gray boxes. The mutation site for the kinase negative is indicated as a bold black line. (B) MEKK1 Full and KN were expressed as GST-fusion proteins in *E. coli* and purified by affinity chromatography. These proteins were separated via SDS-PAGE and stained with CBB. (C) Bacterially expressed MEKK1 Full and KN were incubated with protein phosphatase, as described in the Materials and Methods. After the phosphatase reaction, the samples were separated via SDS-PAGE and subjected to immunoblot analysis with an anti-GST antibody (IB: anti-GST), anti-phospho-Serine antibody (IB: anti-pSer), anti-phospho-Threonine antibody (IB: anti-pThr) and anti-phospho-Tyrosine antibody (IB: anti-pTyr).

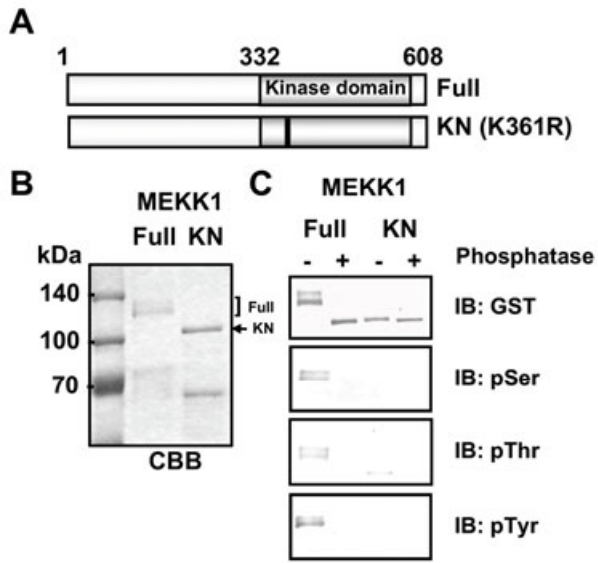
**Fig. 3.** Autophosphorylation of bacterially expressed MEKK1 and its N-terminal deletion mutants. (A) Schematic representation of MEKK1 constructs:  $\Delta 83$  (N-terminal 1-83 amino acid deletion),  $\Delta 166$  (1-166 deletion) and KD (1-332 deletion, kinase domain), were generated using PCR. (B) MEKK1 Full and N-terminal deletions ( $\Delta 83$ ,  $\Delta 166$ , and KD) were expressed as GST-fusion proteins in *E. coli* and purified by affinity chromatography. These proteins were separated via SDS-PAGE and stained with CBB. Equal amounts of the samples were separated via SDS-PAGE and subjected to immunoblot analysis with anti-MEKK1 antiserum (IB:

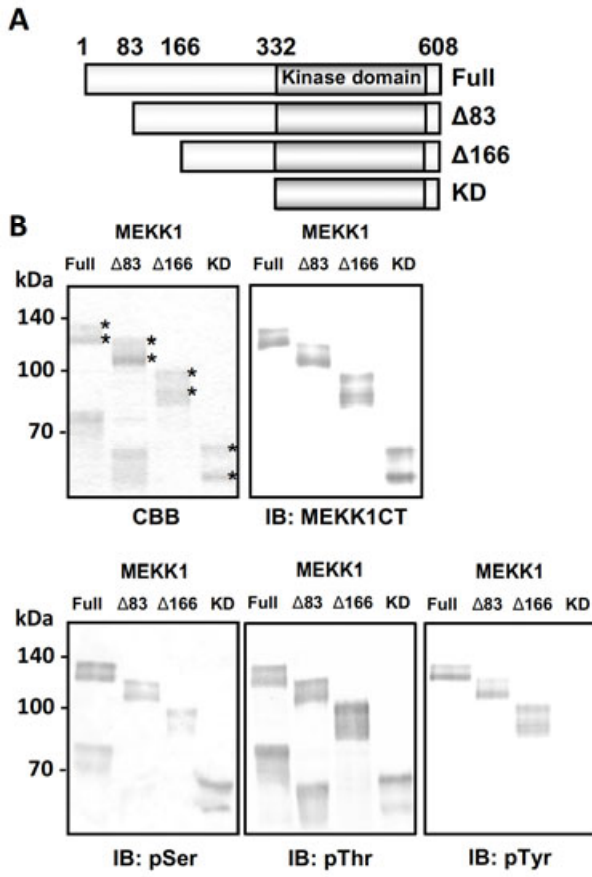
anti-MEKK1CT), anti-phospho-Serine antibody (IB: anti-pS), anti-phospho-Threonine antibody (IB: anti-pT) and anti-phospho-Tyrosine antibody (IB: anti-pTyr).

**Fig. 4.** Identification of the tyrosine autophosphorylation sites of MEKK1. (A) The amino acid sequence of the N-terminal region of MEKK1. The tyrosine residues are highlighted by black boxes. Four tyrosine residues (38, 84, 204, and 323) are located in the N-terminal region of MEKK1.

(B) Bacterially expressed MEKK1 Full, Y38F, Y84F, Y204F, Y323F, Y204F/Y323F and KN were separated via SDS-PAGE stained with CBB. Equal amounts of the samples were separated via SDS-PAGE and subjected to immunoblot analysis with an anti-phospho-Tyrosine antibody (IB: anti-pTyr). (C) The kinase activity of bacterially expressed MEKK1 Full, Y38F, Y84F, Y204F, Y323F, Y204F/Y323F and KN were measured by phosphorylation using kinase negative MKK1 as a substrate. Phosphorylation and CBB staining of MKK1 are shown in the upper and lower panels, respectively. The phosphorylation of MKK1 was quantified and relative amounts of each phosphorylation were calculated when the MKK1 phosphorylation by MEKK1 Full was 100. *Vertical lines* on each bar indicate  $\pm$ SD (n=3).







**A**

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1 MDRILARMKK STGRRGGDKN ITPVRRLEER DAARNIN38DA
41 ASCSSSSAED LSVSTSSLMT RSLEFFPEPTS FRIGGGVGEM
81 DRIR84SLGVS GPDDLAI84SFD AWEACKKRSS SDVVNRFKSF
121 DLDKVRDQDL SEEGPSGVVV GSDSMNHKVQ GQDLSEAGPS
161 GGI204VT204ELSEI GNLITPV204DRL VADGVVENRR VMERTPTIVK
201 SKG204LV204PNNV VAVGVGVGGG IKGLRPPVLK PPPAMKR204PPI
241 DHRGSSWDFL THFAPSETVK RPSSSSSSSE DGCDEE204E204GKE
281 EEAEAEEMGA RFIQLGDTAD ETC204SFTTNEG DSSSTVSNTS
321 P323IPDGG323AII TS
    
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