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# Identification of a possible MAP kinase cascade in *Arabidopsis thaliana* based on pairwise yeast two-hybrid analysis and functional complementation tests of yeast mutants

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**Abstract** **A possible MAP kinase (MAPK) cascade of *Arabidopsis thaliana* was identified on the basis of both yeast 2-hybrid analysis and complementation analysis of yeast mutants. Specific protein-protein interactions between ATMPK4 (a MAPK) and MEK1 (a MAPKK) and interactions between MEK1 and ATMEKK1 (a MAPKKK) were detected by using the 2-hybrid system. A growth defect of the yeast *mpk1*Δ mutant was reversed by coexpression of ATMPK4 and MEK1. Coexpression of the N-terminal deletion form of ATMEKK1 increased the ability of MEK1 to suppress a growth defect of the yeast *pbs2*Δ mutant. These results suggest that ATMPK4, MEK1, and ATMEKK1 may interact with each other and constitute a specific MAPK cascade in *Arabidopsis*. This is the first demonstration of a possible MAPK cascade in plants.**



*Key words:* Mitogen-activated protein kinase cascade; Yeast two-hybrid; *trans*-Complementation; *Arabidopsis thaliana*

## 1. Introduction

Mitogen-activated protein kinases (MAPKs) were initially identified as serine/threonine kinases that were activated by various growth factors and by differentiation factors during mitosis in animals (for reviews, see [1–4]). MAPK cascades are composed of 3 protein kinases: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). MAPKs are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by MAPKKs. MAPKKs are activated when serine and serine/threonine residues in the SXXXS/T motif are phosphorylated by MAPKKKs. MAPK cascades have been shown to function in various signal transduction pathways, including stress responses in animals and yeasts (for reviews, see [1–7]).

A number of genes for MAPKs and several genes for MAPKKs and MAPKKKs have been reported in higher plants [5–10]. Not only are activities regulated but the mRNA levels of MAPKs and MAPK-like kinases are also regulated by plant hormones and environmental stresses in plants [5–7,9,10]. However, there have been no reports of the demonstration in plants of direct interactions between a MAPK and a MAPKK or between a MAPKK and a MAPKKK, or of direct phosphorylation and activation of either a MAPK by a MAPKK or a MAPKK by a MAPKKK (for reviews, see [5–7]).

Recently, yeast 2-hybrid analyses have revealed physical interactions among components of animal and yeast MAPK cascades whose functional interactions had been identified by biochemical and genetic analyses [11–16]. In the sexual response pathway of *Schizosaccharomyces pombe*, byr1 (a MAPKK) interacts with spk1 (a MAPK) [15]. In the growth-factor-mediated pathway of animal cells, Raf (a MAPKKK) interacts with MEK (a MAPKK) [11,15]. No positive interaction was detected, however, between byr2 (a MAPKKK from *S. pombe*) and MEK (a MAPKK from animal cells), which function in different signal transduction pathways of distinct organisms [11]. Therefore, it appears that the components of one MAPK cascade physically interact with each other but not with members of other MAPK cascades that function in different signal transduction pathways. Conversely, if positive protein-protein interactions are detected among MAPK, MAPKK, and MAPKKK homologues of plants whose biochemical and genetic relations have not yet been studied, these kinases may constitute a MAPK cascade in vivo, like the components of the well-characterized MAPK cascades in yeasts and animals.

Yeast cells can be used to examine not only physical but also functional interactions among components of MAPK cascades. Hughes et al. showed that expression of a mammalian MAPKK alone failed to complement a yeast *byr1* mutant (MAPKK-deficient) [17]. When it was coexpressed with Raf (an activator of the MAPKK), however, the MAPKK was activated to suppress the mating defect of the *byr1* mutant [17]. Therefore, coexpression of a MAPK with its activator MAPKK and coexpression of a MAPKK with its activator MAPKKK can complement MAPK- and MAPKK-deficient yeast mutants, respectively. These characteristics are used to identify possible MAPK cascades in yeast mutants.

In this paper, we show both physical and functional interactions among specific MAPK, MAPKK, and MAPKKK homologues of *Arabidopsis thaliana* based on pairwise yeast 2-hybrid analysis and functional complementation tests of *Saccharomyces cerevisiae* mutants, and suggest that the ATMEKK1 MAPKKK [10], MEK1 MAPKK [18], and

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ATMPK4 MAPK homologues [8] may constitute a MAPK cascade in *Arabidopsis*.

## 2. Materials and methods

### 2.1. Strains, media, and yeast transformation

The *S. cerevisiae* strains used in this study were L40 [12], 1788 [19], DL456 [19], TM222 [20], and TM334 [20]. *Escherichia coli* strains XL-1 and JM109 were used for propagation of plasmid DNAs. Yeast strains were grown on YPD medium containing 2% glucose, 2% Bacto Peptone (Difco, Detroit, MI, USA), 1% Bacto Yeast Extract (Difco), and 0.04% adenine sulfate. Yeast transformations were done by the lithium acetate method [21]. Transformants were selected on SD medium containing 2% glucose and 0.7% yeast nitrogen base without amino acids (Difco), supplemented with amino acids when required. Standard yeast genetic manipulations were performed as described previously [21].

### 2.2. Molecular biological techniques

Standard molecular biological techniques were used for plasmid construction, polymerase chain reaction (PCR), and DNA sequencing [25]. The plasmid vectors used for the yeast 2-hybrid analyses were pVP16 [12], pBTM116 [12], and pGAD424 (Clontech). PCR was used to generate fragments of the coding sequences of ATMPK1 [9], ATMPK3 [8], ATMPK4 [8], ATMPK8 [6], MEK1 [18], ATMEKK1 [10], Fus3 [26], Ste7 [27], and *Xenopus* MPK1 [28] compatible for cloning into the vectors. The plasmid vectors used for complementation analyses were YEpGAP112 [22], pNV7 [29], and YEp51 [30]. The DNA fragments of the coding sequences of ATMPK1 [9], ATMPK3 [8], ATMPK4 [8], ATMPK8 [6], MEK1 [18], ATMEKK1 [10], and ATMEKK1ΔN (encoding residues 288–608) were subcloned into the vectors.

### 2.3. Two-hybrid assays

For qualitative assays, cotransformed yeast colonies were plated on the same selective medium to test for protein interaction. The test used a β-galactosidase activity assay on a filter with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) or growth in the absence of histidine in medium containing 5–30 mM 3-aminotriazole (3-AT). For quantitative assays, cells of each transformant were cultured in medium lacking tryptophan and leucine, and β-galactosidase activity was measured by the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay method [31].

### 2.4. Complementation of growth defects of the mpk1Δ and pbs2Δ mutants

The yeast *mpk1*Δ mutant DL456 [19] was transformed with 2 plasmids, one containing MAPKs (ATMPKs [6,8,9]), the other containing a MAPKK (MEK1 [18]), and transformants carrying both plasmids were selected. Complementation analyses were performed as described previously [19,24] with minor modifications. The *pbs2*Δ mutant TM334 [20] was transformed with 2 plasmids, one containing MEK1 MAPKK [18], the other containing ATMEKK1 MAPKKK [10], and transformants carrying both plasmids were selected. Complementation analyses were performed as described previously [32] with minor modifications.

## 3. Results

### 3.1. Analysis of a protein-protein interaction between ATMPK4 (MAPK) and MEK1 (MAPKK) using the yeast 2-hybrid system

We have already isolated 9 cDNAs for *Arabidopsis* MAPKs [8,9]. Based on their amino acid sequences, they can be classified into 4 subgroups: group 1 (ATMPK1, ATMPK2, and ATMPK7), group 2 (ATMPK3 and ATMPK6), group 3 (ATMPK4 and ATMPK5) and group 4 (ATMPK8 and ATMPK9) [6,8,9]. For this study we chose 4 MAP kinases, ATMPK1, ATMPK3, ATMPK4, and ATMPK8, one from each subfamily. To test which ATMPKs interact with MEK1, we constructed vectors to express ATMPKs and

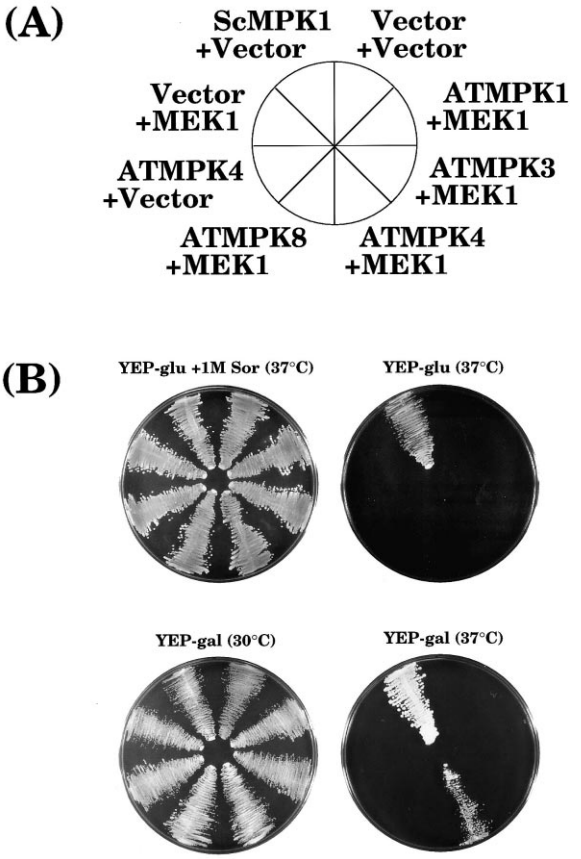


Fig. 1. Suppression of the temperature-sensitive growth defect of the yeast *mpk1* mutant by coexpression of ATMPK4 and MEK1. A: Plasmid combinations are (clockwise from top) pNV7 [29] plus YEp51 [30] (vector+vector), pNV7-ATMPK1 plus YEp51-MEK1 (ATMPK1+MEK1), pNV7-ATMPK3 plus YEp51-MEK1 (ATMPK3+MEK1), pNV7-ATMPK4 plus YEp51-MEK1 (ATMPK4+MEK1), pNV7-ATMPK8 plus YEp51-MEK1 (ATMPK8+MEK1), pNV7-ATMPK4 plus YEp51 (ATMPK4+vector), pNV7 plus YEp51-MEK1 (vector+MEK1), and YCp50-MPK1 plus YEp51 (ScMPK1+vector). B: The *mpk1* mutant (DL456) transformed with different combinations of plasmids was streaked onto YEP-glu medium supplemented with 1 M sorbitol (upper left), YEP-glu medium (upper right), or YEP-gal medium (lower right) and then incubated for 3 days at 37°C, or streaked onto YEP-gal medium and then incubated for 3 days at 30°C (lower left).

MEK1 fused to the LexA binding domain (LBD), VP16 activation domain (VAD), and Gal4 activation domain (GAD) in the tester strain, L40. Table 1 summarizes the protein-protein interactions between MEK1 and the ATMPKs tested in this study.

As shown in Table 1, interaction between ATMPK4 and MEK1 was detected, based on both growth on His-lacking medium and β-galactosidase activity. Although the LBD-ATMPK4 itself had relatively high background activity of both growth on His-lacking medium and β-galactosidase activity, when coexpressed with MEK1 this background activity decreased. A protein-protein interaction between ATMPK4 and MEK1 might cause conformational change of the LBD-ATMPK4 fusion, and might result in a decrease in the background activity. We also detected positive interaction between GAD-ATMPK4 and LBD-MEK1 (Table 1). We did not detect interactions between MEK1 and other *Arabidopsis* ATMPKs (ATMPK1, ATMPK3, and ATMPK8) or *X. laevis*

Table 1
Two-hybrid interactions between MAPKs and MAPKKs, and between MAPKKs and MAPKKKs

| AD fusion | LexA-BD fusion | β-Gal filter assay[a] | Growth on His− media[b] | β-Gal activity[c] |
|---|---|---|---|---|
| VP16-AD fusion | | | | |
| ATMPK1 | MEK1 | − | − | < 0.1 |
| ATMPK3 | MEK1 | − | − | < 0.1 |
| ATMPK4 | MEK1 | ++ | ++ | 120.6 ± 11.5 |
| ATMPK8 | MEK1 | − | − | < 0.1 |
| ATMPK4 | Ste7 | − | − | ND |
| XlMPK1 | MEK1 | − | − | ND |
| ATMPK4 | Vector | − | − | < 0.1 |
| Vector | MEK1 | − | − | < 0.1 |
| MEK1 | ATMPK1 | − | − | < 0.1 |
| MEK1 | ATMPK3 | − | − | < 0.1 |
| MEK1 | ATMPK4 | − | − | < 0.1 |
| MEK1 | ATMPK8 | − | − | < 0.1 |
| MEK1 | Vector | − | − | < 0.1 |
| Vector | ATMPK4 | + | + | 1.5 ± 1.0 |
| Vector | Vector | − | − | < 0.1 |
| MEK1 | ATMEKK1 | ++ | ++ | 38.5 ± 2.7 |
| Vector | ATMEKK1 | + | − | < 0.1 |
| ATMEKK1 | MEK1 | ++ | ++ | 52.6 ± 3.1 |
| ATMEKK1 | Ste7 | − | − | ND |
| Raf | MEK1 | − | − | ND |
| ATMEKK1 | Vector | − | − | < 0.1 |
| Gal4-AD fusion | | | | |
| ATMPK1 | MEK1 | − | − | ND |
| ATMPK3 | MEK1 | − | − | ND |
| ATMPK4 | MEK1 | ++ | ++ | ND |
| ATMPK8 | MEK1 | − | − | ND |
| ATMPK4 | Vector | − | − | ND |
| Vector | MEK1 | − | − | ND |
| Vector | Vector | − | − | ND |
| ATMEKK1 | MEK1 | ++ | ++ | ND |
| ATMEKK1 | Vector | − | − | ND |

[a]β-Galactosidase activity was determined by a filter assay for the yeast strains containing the indicated plasmids. ++, + and − represent a strong positive (blue), a weak positive (pale blue) and no (white) indication, respectively, of β-galactosidase activity in filter assays. Essentially identical results were obtained in more than 3 independent experiments.
[b]Transformants containing the indicated plasmids were streaked onto synthetic medium plates lacking tryptophan, leucine and histidine. The plates were incubated at 30°C for 3 days. ++, + and − represent rapid, slow and no growth, respectively, on the medium. Essentially identical results were obtained in more than 3 independent experiments.
[c]β-Galactosidase activity was determined 3 times in 3 independent transformants. The mean is expressed in Millar units ± standard deviation.

MPK1 [28]. These results suggest that MEK1 interacts specifically with ATMPK4.

### 3.2. Detection of a protein-protein interaction between MEK1 (MAPKK) and ATMEKK1 (MAPKKK) using the yeast 2-hybrid system

Interaction between ATMEKK1 and MEK1 was detected, based on both growth on His-lacking medium and β-galactosidase activity (Table 1). Neither MEK1 nor ATMEKK1 fusion proteins interacted with VAD or LBD proteins, which were used as controls. We did not detect interactions between MEK1 and a mammalian MAPKKK, Raf, or between ATMEKK1 and a yeast MAPKK, Ste7. These results suggest that ATMEKK1 may interact specifically with MEK1.

### 3.3. Coexpression of MEK1 (MAPKK) and ATMPK4 (MAPK) complements the mpk1Δ defect

*Arabidopsis* MAPKs (ATMPKs) are 40–46% identical in amino acid sequences to *S. cerevisiae* MPK1 [8,9,19]. Expression of *Xenopus* MAPK suppressed the defect associated with loss of *S. cerevisiae* MPK1 [19]. This suggests that ATMPKs might be able to complement the yeast *mpk1* mutant. To test this, cDNAs for ATMPK1, ATMPK3, ATMPK4, and ATMPK8 were transformed into the *mpk1* mutant (DL456). We constructed high-copy-number plasmids (pNV7-ATMPKs) in which cDNAs encoding ATMPKs are expressed under the control of the galactose-inducible yeast *GAL7* promoter [29]. The *mpk1* mutant cannot grow at 37°C without 1 M sorbitol [19]. Therefore, the ability of ATMPKs to complement the growth defect associated with the *mpk1*Δ::*TRP1* allele (in strain DL456) was tested on galactose-containing medium at 37°C in the presence or absence of 1 M sorbitol. Expression of the ATMPKs did not complement the growth defect (data not shown). Two-hybrid analysis suggested that MEK1 (MAPKK) may interact with ATMPK4 (MAPK). Therefore we next examined whether MEK1 can activate ATMPK4 in *S. cerevisiae* (Fig. 1). When the *mpk1* mutant cells were transformed with both ATMPK4 and MEK1, they were able to grow at the restrictive temperature. However, coexpression of MEK1 and 3 other ATMPKs (ATMPK1, ATMPK3, and ATMPK8) did not complement this defect. These results suggest that MEK1 not only interacts with but also may activate ATMPK4, at least in *S. cerevisiae* [17].

### 3.4. Expression of ATMEKK1 (MAPKKK) increases activity of MEK1 (MAPKK) to complement the pbs2Δ defect

*Arabidopsis* MEK1 is 36–41% identical in amino acid se-

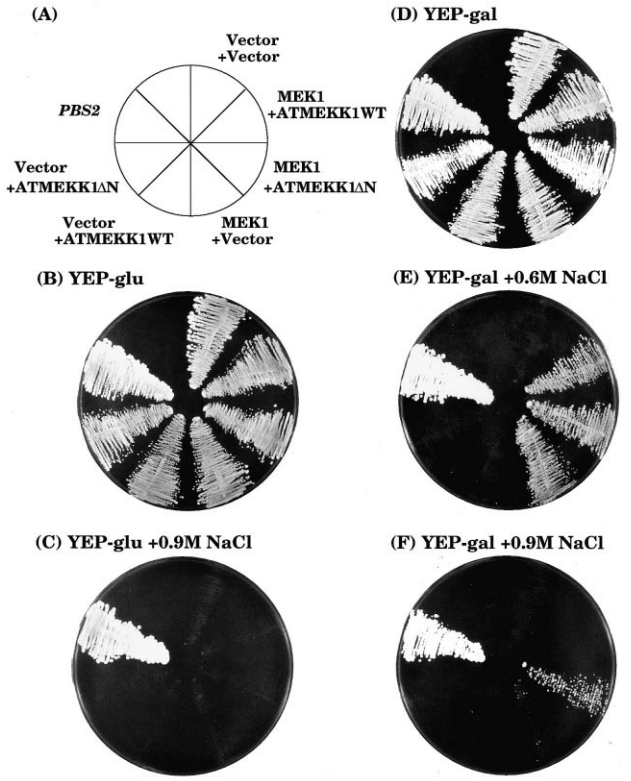


Fig. 2. Suppression of the growth defect of a *pbs2* mutant on medium supplemented with NaCl by coexpression of MEK1 (MAPKK) and ATMEKK1 (MAPKKK). A: Plasmid combinations are (clockwise from top) YEp51 plus YEpGAP112 (vector+vector), YEp51-MEK1 plus YEpGAP112-ATMEKK1WT (MEK1+ATMEKKK1WT), YEp51-MEK1 plus YEpGAP112-ATMEKK1ΔN (MEK1+ATMEKKK1ΔN), YEp51-MEK1 plus YEpGAP112 (MEK1+vector), YEp51 plus YEpGAP112-ATMEKK1WT (vector+ATMEKKK1WT), YEp51 plus YEpGAP112-ATMEKK1ΔN (vector+ATMEKKK1ΔN). ATMEKK1WT and ATMEKK1ΔN encode residues 1–608 and 288–608 of ATMEKK1, respectively. *PBS2* indicates the wild-type strain (TM222), used as a positive control. The *pbs2* mutant (TM334), transformed with different combinations of plasmids, and the wild-type strain (TM222) were streaked onto YEP-glu medium (B), YEP-glu medium supplemented with 0.9 M NaCl (C), YEP-gal medium (D), or YEP-gal medium supplemented with 0.6 M NaCl (E) or 0.9 M NaCl (F) and incubated for 3 days at 30°C.

quence to members of the MAPKK family from yeasts and animals [18]. Expression of rat SEK (MAPKK) suppressed the defect associated with the loss of *S. cerevisiae* PBS2 [32]. This suggests that MEK1 might be able to complement defects in yeast MAPKK mutants, such as *ste7*, *mkk1/mkk2*, and *pbs2*. To test this, MEK1 cDNA was transformed into the *pbs2* mutant (TM334) (Fig. 2). We constructed high-copy-number plasmids (YEp51-MEK1) in which cDNA encoding MEK1 is expressed under the control of the *GAL7* promoter [30]. The mutant cannot grow at 30°C in the presence of 0.6 M NaCl. Therefore the ability of MEK1 to complement the growth defect of the *pbs2*Δ cells was tested on galactose-containing medium in the presence of 0.6 M NaCl. The *pbs2* mutant cells expressing MEK1 could grow in the presence of 0.6 M NaCl (Fig. 2E) but not in the presence of 0.9 M NaCl (Fig. 2F). Two-hybrid analysis suggested that ATMEKK1 may interact with and activate MEK1 (Table 1). Therefore, we then examined whether ATMEKK1 could increase the ability of MEK1 to complement the *pbs2* defect. When the *pbs2* mutant cells were transformed with both MEK1 and ATMEKK1ΔN (the N-terminal deletion form of ATMEKK1), they could grow even in the presence of 0.9 M NaCl (Fig. 2F). ATMEKK1WT (the full-length form of ATMEKK1) did not increase the ability of MEK1 to complement the *pbs2* defect under the same conditions (Fig. 2F). Coexpression of MEK1 and ATMEKK1 (or ATMEKK1ΔN) did not complement the *ste7* or *mkk1/mkk2* mutant defects (data not shown). These results suggest that ATMEKK1 not only interacts with but also may activate MEK1, at least in *S. cerevisiae* [17], and that deletion of the N-terminal regulatory domain of ATMEKK1 may activate kinase activity, like other MAPKKKs such as Ste11, Bck1, Raf, and Ssk2 [1–4,33].

## 4. Discussion

To analyze a possible MAPK cascade in *Arabidopsis*, we first examined the protein-protein interactions among MAPK, MAPKK, and MAPKKK homologues of *Arabidopsis* by using the yeast 2-hybrid system. Table 1 shows the protein-protein interaction between ATMPK4 (MAPK) and MEK1 (MAPKK) of *Arabidopsis*. When MEK1 and ATMPK4 were coexpressed in yeast cells, the cells could grow on histidine-minus medium and possessed significant β-galactosidase activity. MEK1 did not interact with other MAPKs, such as ATMPK1, -3, -8 and *Xenopus* MPK1. ATMPK4 did not interact with yeast MAPKK Ste7. These results suggest that MEK1 specifically interacts with ATMPK4. Then we examined the protein-protein interactions between MAPKKs and MAPKKKs of *Arabidopsis* (Table 1). Interaction was observed only when MEK1 and ATMEKK1 (MAPKKK) were coexpressed. MEK1 did not interact with other MAPKKKs, such as mammalian Raf or *Arabidopsis* NPK1 homologue ANP4 ([23]; data not shown). These results suggest that MEK1 specifically interacts with ATMEKK1. We examined all combinations but we did not detect any positive interactions in other combinations.

To analyze the functional relationship between ATMPK4 and MEK1 in *Arabidopsis*, we used the yeast *mpk1* deletion mutant (DL456) for complementation analysis. Bck1, Mkk1/2, and Mpk1 are MAPKKK, MAPKK, and MAPK homologues, respectively, of budding yeast [4,19,24,34]. The *mpk1* deletion mutant exhibits temperature- and caffeine-sensitive phenotypes. ATMPK4 alone did not complement this mutant (data not shown). However, when ATMPK4 was coexpressed with MEK1, the *mpk1* cells could grow at the restrictive temperature (Fig. 1) and on medium containing caffeine (data not shown). By contrast, other combinations could not suppress these phenotypes. Essentially the same results were obtained by using the yeast *bck1* mutant (data not shown). Bck1 is an upstream kinase of Mpk1 and encodes a MAPKKK. These results suggest that MEK1 not only interacts with but also activates ATMPK4 in yeast cells. To examine the functional relationship between MEK1 and ATMEKK1, we did complementation analysis using the *pbs2* deletion mutant (TM334) (Fig. 2). Pbs2 is a MAPKK involved in high-osmolarity signal transduction in budding yeast [4,24,33]. The *pbs2* mutant cannot grow in high osmotic strength media. MEK1 alone weakly suppressed this phenotype. However, when coexpressed with ATMEKK1ΔN, MEK1 suppressed this phenotype clearly. These results suggest that ATMEKK1 not only interacts

with but also activates MEK1. Kinases in the MAPKKK family, such as Ste11, Bck1, Ssk2, Raf, and MEKK, can be activated by eliminating their N-terminal non-catalytic domains [1–7,33,35]. ATMEKK1 seems to be activated by deletion of its N-terminal regulatory domain. Taken together, our results from both the yeast 2-hybrid system and the complementation analyses strongly suggest that at least 3 protein kinases, ATMEKK1 (MAPKKK), MEK1 (MAPKK), and ATMPK4 (MAPK), may constitute a MAP kinase cascade in *Arabidopsis*.

To confirm this possibility, we took two other approaches. One is the isolation of additional MAPKK and MAPKKK homologues and analyses of their physical and functional interaction with ATMPK4 and ATMEKK1; the other is the 2-hybrid screening of ATMEKK1-interacting proteins. In this screening, we isolated a MAPKK homologue, ATMKK2 [6], which is closely related to *Arabidopsis* MEK1 [18], and a MAPK homologue, ATMPK4. We will report elsewhere more details of physical and functional interactions among these MAPK, MAPKK, MAPKKK homologues in *Arabidopsis*. Application of our molecular approach using the yeast 2-hybrid system in combination with complementation analysis of yeast mutants is valuable for identifying other possible MAP kinase cascades not only in plants but also in other multicellular organisms.

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