The *Arabidopsis thaliana* MEK AtMKK6 activates the MAP kinase AtMPK13

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Abstract Mitogen-activated protein (MAP) kinases mediate cellular responses to a wide variety of stimuli. Activation of a MAP kinase occurs after phosphorylation by an upstream dual-specificity protein kinase, known as a MAP kinase kinase or MEK. The *Arabidopsis thaliana* genome encodes 10 MEKs but few of these have been shown directly to activate any of the 20 Arabidopsis MAP kinases. We show here that functional complementation of the cell lysis phenotype of a mutant yeast strain depends on the co-expression of the Arabidopsis MEK AtMKK6 and the MAP kinase AtMPK13. The kinase activity of AtMPK13 is stimulated in the presence of AtMKK6 in yeast cells. RT-PCR analysis showed the co-expression of these two genes in diverse plant tissues. These data show that AtMKK6 can functionally activate the MAP kinase AtMPK13.

Keywords: Arabidopsis; Mitogen-activated protein kinase; MAP kinase kinase

1. Introduction

Mitogen-activated protein (MAP) kinase cascades contain a central kinase module composed of three kinases (a MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MEK), and a MAP kinase) that are sequentially activated following the perception of various stimuli by a cell [1]. The three-component MAP kinase module is found in all eukaryotes and any given organism encodes a family of proteins at all three levels of the module. One challenge in elucidating the function(s) of these signalling pathways is to determine which of these kinases interact and form part of a particular module. MEKs are dual-specificity protein kinases that phosphorylate MAP kinases on the conserved threonine and tyrosine residues of the amino acid triplet TXY located before subdomain VIII, a signature feature of MAP kinases. MEKs show stringent specificity for MAP kinases, but are capable of activating more than one MAP kinase [2]. Docking domains in MAP kinases and MEKs provide some of the specificity required to ensure that catalytic activation of the MAP kinase occurs [3].

The completed sequence of the *Arabidopsis thaliana* genome shows the presence of 10 MEK genes and 20 MAP kinase genes [4], suggesting that any given MEK can activate multiple MAP kinases. Only a few of these kinases have been linked together: AtMKK1 and AtMKK2 can activate AtMPK4 [5,6], while AtMKK4 and AtMKK5 can activate AtMPK3 and AtMPK6 [7]. Interactions between the remaining Arabidopsis MEKs and MAP kinases have not been described so far.

Functional complementation of yeast mutant phenotypes and two-hybrid analysis provides a readily accessible system to isolate new genes or to study protein–protein interactions. This approach has been used successfully in many instances, including the interaction of MAP kinase pathway components [8–11]. In the present report, we have used a yeast mutant phenotype complementation assay and kinase assays to show that the MEK AtMKK6 can functionally activate the MAP kinase AtMPK13.

2. Materials and methods

2.1. Yeast strains and plasmids

The yeast strain DL456 (Δ*mpk1*::TRP1, *leu2-3*, *ura3-52*, *trpl-1*, *his4*, *can*^{r}) contains a disrupted MPK1 MAP kinase gene leading to a temperature-sensitive cell lysis phenotype [12]. The yeast expression vector pVT103U [13] contains a URA3 selection marker, a 2 μ origin of replication, and the expression of cloned inserts is driven by the constitutive ADH (alcohol dehydrogenase) promoter. The yeast expression vector pUG34 [14] is a low copy centromeric plasmid and contains the methionine-regulatable promoter MET25 and a HIS3 selectable marker. The pUG34 plasmid was modified for use in the DL456 strain, first by deleting the GFP gene by digesting with *Xba*I followed by self-ligation to produce vector pUM34 and then replacing the HIS3 gene with the HIS4 gene. The HIS4 gene was obtained by PCR from yeast genomic DNA using the oligonucleotides 5′ AATCAAACATATGGAACAGGCTCAAGCACAAAC 3′ (*Nde*I underlined) and 5′ ATTTAACTGCCGGCGTTCGAAGTGATGACAATAAG 3′ (*Ngo*MIV underlined), and included the promoter (446 bp upstream of the ATG) and terminator (228 bp downstream of the stop codon) sequences. The vector pUM34 was digested *Nde*I/*Ngo*MIV, which removes most of the HIS3 gene (*Nde*I cuts at nucleotide 191 of the HIS3 gene), and the HIS4 gene was cloned in its place to produce pUM34(H4). The functionality of the HIS4 gene was confirmed by complementation of the *his4* deficiency of the strain DL456.

Complementation tests of yeast transformants were performed by streaking the cells on YPD medium (2% glucose, 2% peptone, 1% yeast extract, and 2% agar) and on YPD medium containing 1 M sorbitol, and incubating the plates at 37 °C. The pVT-AtMPK13 or pVT-ntf6

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Abbreviations: HIS, histidine; MAP kinase, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; RT-PCR, reverse transcription polymerase chain reaction; URA, uracil

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plasmid (see below) was lost from the double transformants by two passages in liquid minimal medium (2% glucose, 0.66% yeast nitrogen base) containing uracil (the selectable marker of the pVT103U plasmid), plating on the same solid medium, and then replica plating to solid minimal medium lacking uracil. The colonies that did not grow after replica plating were selected from the master plate to test for pUM(H4)-AtMKK6-dependent complementation (see below) of the *mpk1* mutant. To repress the MET25 promoter in pUM34(H4)-containing double transformants, cells were plated on minimal medium (2% glucose, 0.66% yeast nitrogen base, and 2% agar) containing 1 mM methionine.

2.2. Arabidopsis *cDNA isolation*

The AtMPK13 cDNA was isolated from an *A. thaliana* cDNA library (provided by Markus Teige, Vienna Biocenter) by PCR and cloned *Bam*HI/*Xho*I into the yeast expression vector pVT103U to produce pVT-AtMPK13. Attempts to isolate the AtMKK6 cDNA by reverse transcription-polymerase chain reaction (RT-PCR) resulted in two full-length but partially spliced products, one containing the first 3 introns and the other only intron 3 (the AtMKK6 gene contains seven introns). Splicing by overlap extension PCR [15] was used to in vitro splice the single intron-containing cDNA to produce the full-length intron-less cDNA. The AtMKK6 cDNA was cloned *Bam*HI/*Sal*I into pUG34 and from there recloned *Bam*HI/*Kpn*I into pUM34(H4) to produce pUM34(H4)-AtMKK6. The sequences and integrity of the ATMPK13 (At5g56580) and AtMKK6 (Atlg07880) cDNAs were confirmed by sequencing. The tobacco MAP kinase *ntf6* cDNA, a putative orthologue of AtMPK13, cloned in the yeast expression vector pVT103U (pVT-ntf6), and the yeast MPK1 gene in the vector pFL44 have been described previously [16].

2.3. Reverse transcription-polymerase chain reaction

RT-PCR was performed on RNA isolated from *A. thaliana* Col-0 tissues using the Qiagen RNeasy Plant Mini Kit. The RT-PCR was performed using the Sigma Enhanced Avian HS RT-PCR Kit according to the manufacturer's instructions. The oligonucleotides used were: AtMPK13, 5′ ATTTCCCCAATGGCTCTAGATCTT 3′ and 5′ ATTATGAAGAGAGTGAGAATTTGGGAG 3′: AtMKK6, 5′ TAGAGAATCCACCACCAACTGCTC 3′ and 5′ GGCTTACTTATCTAAGGTAGTTAACAGGTGG 3′: actin 8 (Atlg49240), 5′ ATGAAGATTAAGGTCGTGGCACCACCC 3′ and 5′ TTTTTATCCGAGTTTGAAGAGGCTACAAAC 3′. The parameters for the PCRs were: 95 °C 15 min, then 94 °C 30 s, 57 °C 40 s, 72 °C 1 min, for 32 cycles, and 72 °C 10 min. Arabidopsis genomic DNA was amplified with the same primers and parameters. All PCRs used the Hotstart *Taq* Polymerase from Qiagen.

2.4. Kinase assays

Proteins were extracted from yeast cells by centrifuging aliquots of yeast cultures at 5000 rpm, 5 min, 4 °C, resuspending the cell pellet in an equal volume of lysis buffer (0.1 M Tris–HCl, pH 8.0, 20% glycerol, 1 mM DTT, 5 mM sodium fluoride, 15 mM β-glycerophosphate, 1 mM PMSF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, and 5 μg/ml antipain, chymoslatin, and pepstatin), adding one volume of glass beads and vortexing for 45 min, 4 °C, followed by two centrifugations at 13 000 rpm. Immunoprecipitation of protein extracts (100 μg yeast protein) and kinase assays was as described previously [17]. The anti-AtMPK13 antibody was raised against the amino acid sequence MEKREDGGILTYDG representing the first 14 amino acids of the AtMPK13 protein.

3. Results

3.1. Co-expression of AtMPK13 and AtMKK6 is required for complementation of the Δmpk1 cell lysis defect

Yeast DL456 (Δ*mpk1*) cells are temperature-sensitive due to the disruption of the MAP kinase gene MPK1(slt2) [12]. They grow at 28 °C but not at 37 °C unless an osmotic stabiliser, such as 1 M sorbitol, is added to the medium [12]. We used complementation of this mutant phenotype as an assay to determine if the MEK AtMKK6 could activate the AtMPK13 MAP kinase. DL456 cells were transformed with the plasmids pVT-AtMPK13, pVT-Ntf6, or pUM(H4)-AtMKK6 (see Section 2), either as single transformants or as double transformants, or with the yeast MPK1 gene as a control. All transformants could grow at 37 °C in the presence of sorbitol, but only the double transformants and the MPK1 control grew when sorbitol was omitted from the medium (Fig. 1A). Weak growth of cells transformed with AtMKK6 alone was observed on occasions (Fig. 1A). However, the fact that significant growth similar to that observed after transformation with the yeast MPK1 gene requires the presence of both AtMPK13 and AtMKK6 indicates that the expression of both genes is necessary for efficient complementation of the lysis defect. The complementation of the lysis defect occurred with both AtMPK13 and its putative tobacco orthologue ntf6 in the presence of AtMKK6.

To confirm that the complementation indeed required the expression of the MEK, and as the pUG34 vector is a low copy centromeric plasmid and thus quite stably maintained even in the absence of selection, we used the ability to regulate the MET25 promoter by the addition of 1 mM methionine to the medium to repress AtMKK6 expression. Under these conditions, the pUM(H4)-AtMKK6/pVTAtMPK13 and pUM-(H4)-AtMKK6/pVTNtf6 double transformants could not complement the lysis defect of DL456 cells (Fig. 1B), showing that expression of AtMKK6 was necessary for the complementation. To show that the MAP kinase (either AtMPK13 or Ntf6) was required for the complementation, plasmid-losing experiments were performed (see Section 2). Cells that had lost either MPK13

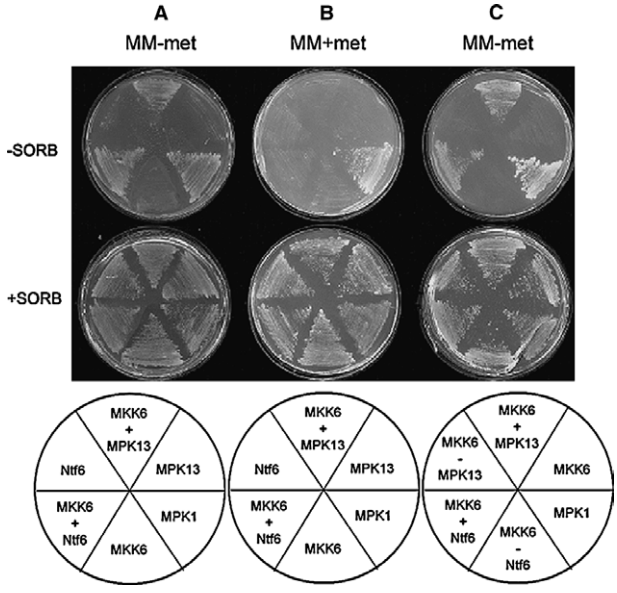

Fig. 1. Co-expression of AtMKK6 and AtMPK13 is required for complementation of the cell lysis defect of Δ*mpk1* cells. (A) Double but not single transformants grow at 37 °C in the absence of sorbitol. (B) No complementation occurs after repression of AtMKK6 expression by plating on medium containing 1 mM methionine. (C) No complementation occurs after plasmid losing of AtMPK13 or ntf6 (MKK6-AtMPK13 and MKK6-Ntf6, respectively). Single or double yeast transformants (as indicated) were plated with (+SORB) or without (−SORB) 1 M sorbitol and incubated at 37 °C. The cells were plated on minimal medium either with (MM + Met) or without (MM − Met) 1 mM methionine. MPK1 represents Δ*mpk1* cells transformed with the yeast MPK1 gene as a control.

or Ntf6, but retained AtMKK6, were unable to grow at 37 °C in the absence of sorbitol (Fig. 1C). Therefore, the co-expression of MKK6 and MPK13 is required for complementation of the DL456 lysis defect, suggesting that MKK6 can activate MPK13.

3.2. AtMPK13 activity is higher in the presence of AtMKK6

To further confirm these data, immunokinase assays were performed using protein extracts from yeast cells expressing AtMKK6 alone, AtMPK13 alone, co-expressing AtMKK6 and AtMPK13, or transformed with the pVT103 empty vector. An anti-AtMPK13 antibody, raised against a peptide corresponding to the first 14 amino acids of the AtMPK13 protein (see Section 2), was used to immunoprecipitate the AtMPK13 protein from the protein extracts of the various transformants, and kinase activity was measured by incubating the immunoprecipitate in kinase buffer containing myelin basic protein (MBP), a standard substrate for measuring MAP kinase activity. Strong phosphorylation of MBP was observed after immunoprecipitating protein extracts prepared from cells co-expressing AtMKK6 and AtMPK13, but not from any of the other transformants (Fig. 2). Therefore, MKK6 activates the kinase activity of MPK13 and this activation can functionally activate MPK13 as shown by the complementation assay described above.

3.3. Expression analysis of AtMPK13 and AtMKK6 in Arabidopsis tissues

The TAIR (http://www.arabidopsis.org/) and TIGR (http://www.tigr.org/tdb/e2kl/athl/) database entries for AtMPK13 (Atlg07880) show a cDNA of 765 bp encoding a protein of 254 amino acids. However, in silico analyses of the MAP kinase gene family have predicted an open reading frame of 1092 bp encoding a protein of 363 amino acids [4,18], in keeping with the gene and protein structure of the MAP kinase family, and as annotated in the MIPS (http://mips.gsf.de/proj/thal/) and PlantsP (http://plantsp.sdsc.edu/) databases. The AtMPK13 cDNA sequence determined in this study was in agreement with the MIPS annotation, which, together with the expression analysis described below, provides experimental evidence for the correct annotation of AtMPK13 as a 363 amino acid protein.

We examined the expression profile of AtMKK6 and AtMPK13 in various Arabidopsis tissues using RT-PCR analysis. The oligonucleotides used for the analysis were located on either side of the last intron of each gene, as were the oligonucleotides for the amplification of the actin 8 gene used as a control (Fig. 3). Amplification of genomic DNA was compared to the cDNA amplifications to ensure that there was no DNA contamination of the samples (Fig. 3). The RT-PCR analyses showed co-expression of AtMKK6 and AtMPK13 in roots, flower buds, and stems (Fig. 3). Expression of AtMKK6 was also observed in cauline and rosette leaves and in siliques, but no expression of AtMPK13 was detectable in these tissues. Either AtMPK13 is not expressed in these tissues or it is undetectable due to a low level of expression or because it is only expressed in a small number of cells. As mentioned above, a MEK may activate multiple MAP kinases so that AtMKK6 might have other substrates in those tissues in which

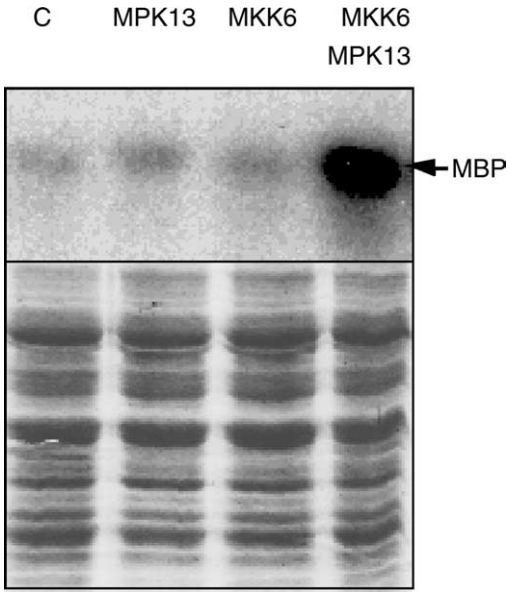

Fig. 2. AtMPK13 activity is higher in the presence of AtMKK6. Immunokinase assays of extracts from cells transformed with the pVT103U empty vector (C), pUM(H4)-AtMKK6 (MKK6), pVT-AtMPK13 (MPK13), or with both pUM(H4)-AtMKK6 and VT-AIMPK13 (MKK6, MPK13). The phosphorylation of MBP is shown (top panel) and Coomassie staining of the protein extracts as a loading control (bottom panel).

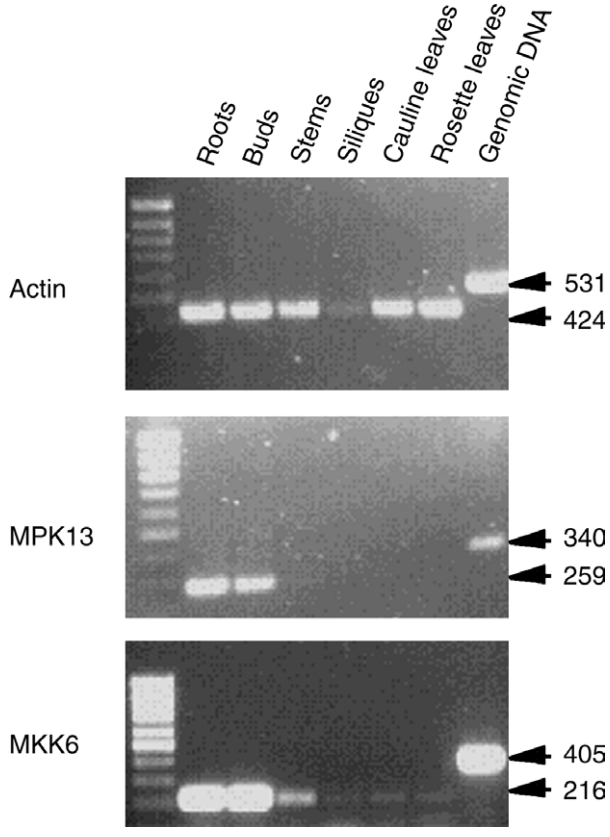

Fig. 3. Expression analysis of AtMPK13 and AtMKK6 in Arabidopsis tissues using RT-PCR. Amplification of the actin 8 gene was used as a control, while amplification of genomic DNA was used to demonstrate the absence of DNA contamination in the samples. The sizes of the amplicons are shown on the right. A 100 bp DNA ladder from Fermentas, shown on the left, was used as a molecular size marker.

AtMPK13 is not expressed. Although the RT-PCR analyses are not quantitative, the much stronger amplification obtained for both AtMKK6 and AtMPK13 in roots and flower buds relative to the other tissues, and compared to the control amplifications of actin, suggests that they are expressed at a higher level in these two tissues.

4. Discussion

There are 10 MEK genes and 20 MAP kinase genes in the Arabidopsis genome [4], but the majority of the data published to date concerns a relatively small number of these kinases (see [19] for review). Interactions between the components of a cascade are fundamental for their function, and various interaction domains have been identified in MAP kinases and in their activators, inactivators, and substrates [3,20]. Even though two such kinases may physically interact, however, it does not necessarily mean that catalytic activation occurs. For example, NtMEK1 from tobacco can physically interact with the MAP kinases Ntf4 and Ntf6, but functionally activates only Ntf6 [16]. The tobacco MAP kinase SIPK physically interacts with the MEK SIPKK, but does not seem to be phosphorylated by SIPKK [21]. Instead, SIPK is activated by another MEK, NtMEK2 [22]. This implies that these proteins contain characteristics that not only determine protein–protein interaction, but also the ability to activate the kinase. Indeed, distinct domains may operate independently in protein binding and protein phosphorylation/activation [20]. Therefore, it is important to show that a MEK and a MAP kinase interact in a manner that leads to the functional activation of the MAP kinase, as shown in this work for AtMPK13.

It was shown recently that mutation of the AtMKK6 (ANQ1) gene causes cytokinesis defects in Arabidopsis [23]. AtMKK6 is the putative orthologue of NtMEK1 from tobacco, which forms part of the NPK1-NtMEK1-Ntf6 MAP kinase signalling pathway that is involved in cytokinesis [16,23]. The putative orthologues of NPK1 in Arabidopsis are ANP1, ANP2, and ANP3, mutation of which also results in cytokinesis defects [24]. To date, there have been no data to directly link the components of the proposed ANPl/2/3-AtMKK6-AtMPK13 pathway. The requirement of AtMKK6 for the functional complementation of the yeast *mpk1* mutant by AtMPK13 and the increase in AtMPK13 kinase activity in the presence of AtMKK6 show that AtMKK6 functionally activates AtMPK13. These data are supported by the expression analysis, which indicates a high level of co-expression of the two genes in roots and flower buds. In summary, the present report provides evidence that AtMKK6 and AtMPK13 form part of the Arabidopsis ANP1/2/3-AtMKK6-AtMPK13 MAP kinase signalling pathway.

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