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Short Communication

Comprehensive analysis of protein-protein interactions between Arabidopsis MAPKs and MAPK kinases helps define potential MAPK signalling modules

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Abbreviations: MAPK, mitogen-activated protein kinase; MPK, plant MAPK; MAPKK, mitogen-activated protein kinase kinase; MKK, plant MAPKK; Y2H, yeast two-hybrid

Key words: mitogen-activated protein kinase, mitogen-activated protein kinase kinase, yeast two-hybrid, phosphorylation, protein-protein interaction, Arabidopsis

The Arabidopsis genome encodes a 20-member gene family of mitogen-activated protein kinases (MPKs) but biological roles have only been identified for a small subset of these crucial signalling components. In particular, it is unclear how the MPKs may be organized into functional modules within the cell. To gain insight into their potential relationships, we used the yeast two-hybrid system to conduct a directed protein-protein interaction screen between all the Arabidopsis MPKs and their upstream activators (MAPK kinases; MKK). Novel interactions were also tested in vitro for enzyme-substrate functionality, using recombinant proteins. The resulting data confirm a number of earlier reported MKK-MPK relationships, but also reveal a more extensive pattern of interactions that should help to guide future analyses of MAPK signalling in plants.

Plant genomes are notably rich in the number of protein kinase signalling components they encode[1-3] and it can therefore be anticipated that the associated signal transduction networks will be highly specialized and complex. Within the plant protein kinase superfamily, the highly conserved Arabidopsis mitogen-activated protein kinases (MAPKs; MPKs) are represented by a 20-member family that is most closely related to the ERK class of metazoan MAPKs.[4] This family includes three sub-families of MPKs whose activation domain carries a -TEY- motif, as well as a fourth, evolutionarily distinct -TDY- sub-family.[4,5] Dual-specificity MAPK kinases (MKK) serve as the canonical activators of MPKs through phosphorylation of both the threonine and tyrosine residues within the MPK activation loop -TXY- motif. The Arabidopsis genome encodes ten members of the *MKK* gene family, among which one (MKK10) lacks the fully conserved -S/T-X_{3-5}-S/T- motif that typifies eukaryotic MAPK kinases.[5]

Numerous reports have provided evidence for the involvement of plant MPKs in a wide range of biotic and abiotic stress responses, as well as phytohormone signalling and developmental patterning, as recently reviewed in.[6] However, defining functional MKK-MPK module combinations by connecting a particular activated MPK to a specific upstream MKK remains a challenge. Since there are precedents for activation of multiple MPKs by one MKK, as well as evidence for more than one MKK having the capability of activating a given MPK, there are many possible ways in which MKK-MPK signalling modules might potentially be configured. Phenotype-based forward genetic screens in Arabidopsis have provided relatively little insight into these relationships, with only one MPK (MPK4) being recovered as a loss-of-function mutant.[7] The failure to recover mutations in the other MPK loci, or in any of the MKKs, in such screens could indicate that there is considerable functional redundancy within the MAPK signalling network, that the phenotypic consequences of a loss-of-function mutation are subtle or conditional, or that loss-of-function genotypes are non-viable.

Since the nature of protein kinase/phosphatase activities depends on direct physical encounters between the enzyme and its target protein, we hypothesized that the ability of particular proteins to interact effectively with each other would define one level of specificity within the global Arabidopsis MKK/MPK network. To test this idea, we conducted a comprehensive directed yeast two-hybrid screen using the ten Arabidopsis MKKs as individual bait proteins, and each of the twenty MPKs as prey proteins. Several of the protein-protein interactions detected in this Y2H screen were also tested in direct phosphorylation assays in vitro, using recombinant proteins.

Nine of the ten Arabidopsis MKK proteins were found to interact with at least one MPK protein in our Y2H assays (Table 1). The only non-interacting MKK family member (MKK8) appears to be structurally intact, in terms of its kinase domain and specific MAPK kinase sequence motifs, but the *MKK8* gene is expressed at only very low levels in any of the >3000 experiments represented in

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Table 1 **Full-length cDNA clones corresponding to the open reading frame of each of the ten Arabidopsis MKK and twenty distinct MAPKs were isolated from Arabidopsis cDNA and cloned into a Gateway™ entry vector, either pENTR (Invitrogen) or pCR8 (Invitrogen)**

	MKK1	MKK2	MKK3	MKK4	MKK5	MKK6	MKK7	MKK8	MKK9	MKK10
MPK1	-	-	++	-	-	-	-	-	-	-
MPK2	-	-	++	-	-	-	+	-	-	-
MPK3	-	-	-	+	-	-	-	-	-	-
MPK4	+++	+++	-	-	-	+++	-	-	-	-
MPK5	-	-	-	-	-	-	-	-	-	-
MPK6	-	+++	-	+++	+++	++	-	-	-	-
MPK7	-	-	+++	-	-	-	-	-	-	-
MPK8	-	-	-	-	-	-	-	-	-	-
MPK9	-	-	-	-	-	-	-	-	-	-
MPK10	-	+++	-	-	-	-	-	-	+	-
MPK11	+++	+++	-	-	-	+++	-	-	-	-
MPK12	-	-	-	-	-	-	-	-	-	-
MPK13	-	+	-	-	-	+	-	-	-	-
MPK14	-	-	++	-	-	-	-	-	-	-
MPK15	-	-	-	-	-	-	++	-	-	-
MPK16	-	-	-	-	-	-	-	-	-	-
MPK17	-	-	-	-	-	-	-	-	+	+
MPK18	-	-	-	-	-	-	-	-	-	-
MPK19	-	-	-	-	-	-	-	-	-	-
MPK20	-	-	-	-	-	-	-	-	+	-

Each cloned MKK and MAPK was sequence-verified to ensure integrity of the cloned gene prior to its transfer into Gateway™ compatible yeast two-hybrid bait and prey vectors (pDEST32 (Invitrogen) and pDEST22 (Invitrogen), respectively). Each MKK (in pDEST32 vector) and MPK (in pDEST22 vector) was introduced pairwise into the yeast strain, MaV203. Positive clones were isolated on the basis of their ability to activate HIS3 or URA3, according to the manufacturer's instructions (ProQuest; Invitrogen). Interaction strength for HIS3 and URA3 activation assays was scored visually, from no interaction (-) to strong interaction (+++).

the *Genevestigator* microarray database (https://www.genevestigator.ethz.ch/gv/index.jsp). Its putative orthologue in *Populus trichocarpa* is similarly silent,[5,8] consistent with a gene that may be losing its biological functionality. Most other MKKs were found to interact with two or more MPK targets, and in several cases these results confirmed earlier reports of MKK-MPK interactions. For example, we found that MKK1 and MKK2, two closely related MAPKKs, both interacted with MPK 4 and with MPK11, a pair of paralogous MPKs. Interaction between MKK1 (MEK1) and MPK4 had already been observed in one of the first studies of plant MKK-MPK relationships,[9] while a later study also found that MKK2 could interact with MPK4, among twelve MPKs surveyed.[10] However, neither of these reports had examined MPK11. We could confirm by in vitro phosphorylation assays using "constitutively active" (CA) forms of recombinant MKK1 and MKK2 that both of these MKKs can phosphorylate recombinant MPK4, but, in contrast to the Y2H interaction pattern, both CAMKKs showed only very weak activity with MPK11 as a substrate (Fig. 2A and B).

MKK2 appears to have a wider range of interactions than its paralogue since, in addition to MPK4 and MPK11, it can bind with MPK6, MPK10, and considerably more weakly with MPK13 (Fig. 1, Table 1). The interaction with MPK6 had been observed previously,[10] and the quantitative Y2H assay in that study also detected a weak interaction between MKK2 and MPK13. However, we also observed a clear MKK2-MPK10 interaction, a combination which Teige et al., (2004) had not tested, whereas that previous study reported a MKK2-MPK5 interaction, which we do not see in our assays. In vitro phosphorylation assays demonstrated that, in addition to strongly phosphorylating MPK4 and MPK10, recombinant CAMKK2 displayed very weak activity against MPK6, MPK11 and MPK13 (Fig. 2B).

MKK3 is a distinctive monophyletic plant MAPKK whose extended C-terminal region displays homology to yeast NTF2 proteins. It was recently reported that MKK3 could interact with MPKs1, 2, 7 and 14 in Y2H assays,[11] and our survey fully confirmed this pattern (Table 1). That earlier report also demonstrated that MKK3 is capable of using the same four MPKs as substrates, although the functional relationships displayed *in planta* appear to depend on the nature of the upstream stimuli being processed.[11] Interestingly, it has also been proposed that MKK3 forms a functional signalling pathway with a different MPK (MPK6), in the context of jasmonic acid signal transduction.[12] Although this model appeared to be supported by genetic evidence, the ability of MKK3 to use MPK6 as a direct substrate was not demonstrated.

MKK4 and MKK5 are paralogous Group C MAPKKs that appear to be important to the ability of plants to respond to a range of environmental stresses. Based on several in vitro and in vivo studies, the downstream targets of MKK4 are believed to be MPK3 and MPK6, and consistent with this model, MKK4 was found to interact only with MPK3 and MPK6 among the 20 MPKs tested in the Y2H screen (Table 1). MKK5, on the other hand, interacted only with MPK6. Although these two MKKs are sometimes regarded

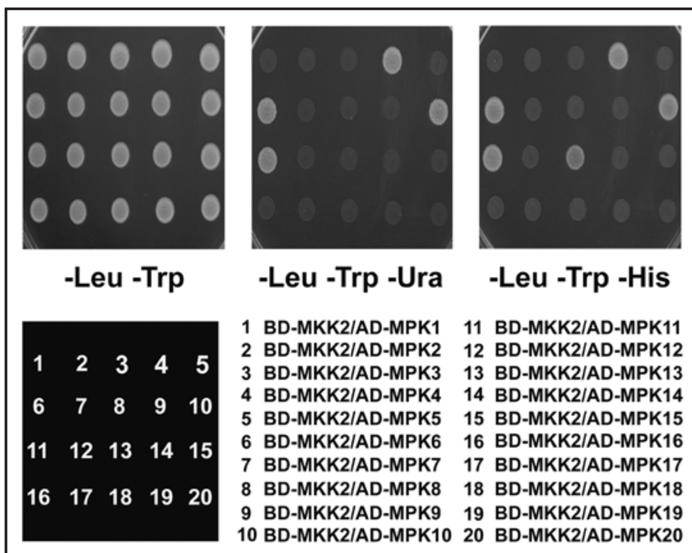

Figure 1. MKK2 interaction with Arabidopsis MAPKs in Yeast. MKK2 interacts specifically with MPK4, 6, 10, 11 and 13. The Y2H screen of MKK2 against each of the twenty Arabidopsis MPKs was conducted using bait and prey constructs prepared as described in the Table legend.

as redundant, this result suggests that while MKK4 and MKK5 do have overlapping functions, they may also play distinct roles in some contexts.

MKK6 has been proposed to use MPK13 as a substrate in a signal transduction pathway involved in regulation of cytokinesis.[13] This pathway has been investigated most extensively in *Nicotiana*, where the putative orthologues of MKK6 and MPK13 have been named NQK1 and NRK1, respectively.[13,14] Most of the evidence for the functionality of this pathway has come from genetic analysis, but combined ectopic expression of both Arabidopsis MKK6 and MPK13 in an *mpk1* mutant yeast background was able to complement the mutant's signal transduction deficiency, and MPK13 activation could be detected in the MKK6-expressing yeast.[15] Interestingly, while MKK6 was found to interact with MPK13 in our Y2H screen (Table 1), it did so only weakly, whereas it bound strongly with MPK4 and MPK11. An intermediate level of interaction was also detected between MKK6 and MPK6. However, attempts to demonstrate substrate functionality by use of in vitro phosphorylation assays produced an unusual pattern in which the autophosphorylation activity of MPK4, 6 and 13 was strongly suppressed by co-incubation with recombinant CAMKK6, although no direct phosphorylation of the MPKs could be detected (Fig. 2C). While no direct phosphorylation of MPK11 by CAMKK6 was observed, autophosphorylation suppression could not be assessed because recombinant MPK11 displays no autophosphorylation activity in vitro (Fig. 2C). Since the same recombinant CAMKK6 was found to be able to phosphorylate MPK12 in vitro (Lee JS and Ellis BE, unpublished data), the lack of direct kinase activity against MPK4, 6, 11 and 13 can presumably not be attributed to defective MKK6 protein.

The phenotypes of MKK7-suppressed and overexpression mutants indicate that signalling through this MKK contributes to both disease resistance[16] and polar auxin transport,[17] but to date no MPK substrate has been reported for MKK7. In our Y2H screen, MKK7 was found to interact with both MPK2 and MPK15, but when using recombinant proteins we could detect no in vitro activity of CAMKK7 against MPK2 (Fig. 2D), although the CAMKK7, like CAMKK6, was able to phosphorylate MPK12 (Lee JS and Ellis BE, unpublished data). Our inability to produce high quality recombinant MPK15 precluded any test of the ability of CAMKK7 to phosphorylate MPK15.

MKK9 was recently reported to play a role in regulation of ethylene signalling, where it operates downstream of the CTR1 MAPKKK, and upstream of MPK6.[18] Curiously, unlike canonical MAPK cascades based on sequential activation events, this proposed signalling module appears to involve CTR1 inactivation of MKK9 through an undefined mechanism. However, in our Y2H screen, MKK9 did not interact with MPK6, but rather with MPK10, MPK17 and MPK20, three MPKs whose biological roles have yet to be determined. In vitro phosphorylation assays revealed that recombinant CAMKK9 can also phosphorylate MPK10 and MPK20, and that MPK6 serves as a substrate, as well, (Fig. 2E) in keeping with the previous report.[18] However, CAMKK9 also catalyzed in vitro phosphorylation of MPK12 (Lee JS and Ellis BE, unpublished data), a MPK family member with which MKK9 did not interact in the Y2H system.

Finally, MKK10, a family member that lacks part of the MKK consensus motif and may not be biologically functional,[5,6] was found to interact with MPK17 in the Y2H screen; this potential relationship was not tested for in vitro activity.

While most Arabidopsis MKKs could be shown to interact with and/or phosphorylate one or more putative target MPKs, it is notable that, for several of the 20 MPKs (MPK5, MPK8, MPK9, MPK16, MPK18 and MPK19), there were neither MKK interactions nor substrate relationships detected. While the yeast two-hybrid system is a powerful tool for exploring protein-protein interactions, both false positive and false negative results can be generated, and different Y2H formats can also result in different outcomes. Thus, although we failed to detect a MKK2-MPK5 interaction in the present work, that specific interaction was reported in another study[10] in which a different version of the Y2H assay was used. Overall, however, the results of this comprehensive screen are very consistent with the data obtained in previous smaller studies, which gives confidence that the interactions reported here are reproducible.

The biological interpretation of such interactions is, of course, ultimately reliant on additional information, such as demonstration of enzyme-substrate activity relationships and characterization of the molecular phenotypes of appropriate gain-of-function and loss-of-function genotypes. Our in vitro phosphorylation assays, together with other literature reports, confirm that some of the putative modular relationships defined by the Y2H data probably represent genuine kinase-substrate relationships. Interestingly, in other cases, our CAMKK constructs were able to phosphorylate MPK substrates that had failed to display corresponding Y2H interactions. This is most striking in the case of MPK12, which did not interact with any of the MKKs in our screen, but proved to be an in vitro substrate for four members of the family (MKK1, MKK6, MKK7 and MKK9) (Lee JS and Ellis BE, unpublished data).

There are two caveats to be attached to this dataset. Since we did not attempt to assay all CAMKK proteins in vitro against all MPKs, there may well be other enzyme-substrate relationships that remain to be defined within the overall Arabidopsis MKK-MPK matrix.

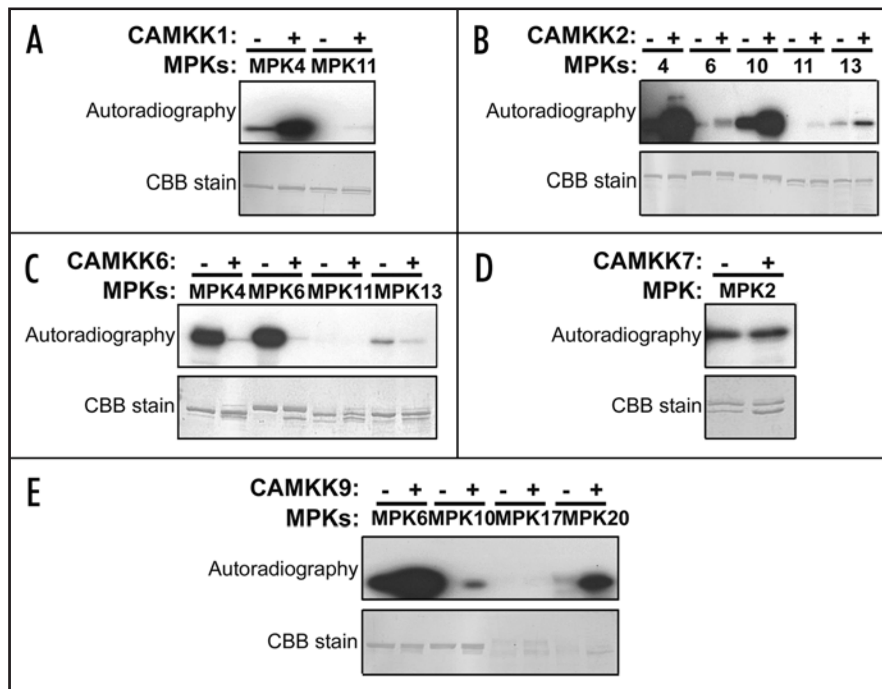

Figure 2. Effects of incubation with recombinant GST-CAMKK proteins on protein phosphorylation of recombinant GST-MPKs. The constitutively active mutant forms of the MKKs were generated by QuickChange site-directed mutagenesis (Stratagene) and confirmed by sequencing. The conserved Ser or Thr residues in the activation loop in MKKs were replaced with acidic residues to create a "constitutively active" kinase (T218E and S224D for CAMKK1, T220D and T226E for CAMKK2, S221D and T227E for CAMKK6, S193E and S199D for CAMKK7, and S195E and S201E for CAMKK9). PCR amplicons of the full-length cDNAs corresponding to MPK2 (At1g59580), MPK4 (At4g01370), MPK6 (At2g43790), MPK10 (At3g59790), MPK11 (At1g01560), MPK13 (At1g07880), MPK17 (At2g01450), MPK20 (At2g42880), MKK1 (At4g26070), MKK2 (At4g29810), MKK6 (At5g56580), MKK7 (At1g18350) and MKK9 (At1g73500) were purified, digested with the appropriate restriction enzymes and subcloned in either the pGEX 4T-2 or pDEST15 vector, which expresses the recombinant protein with a N-terminal GST tag. Each of wild-type MAPK and mutant recombinant CAMKK1, CAMKK2, CAMKK6, CAMKK7 and CAMKK9 were expressed as glutathione S-transferase (GST) fusion proteins as previously described.[19] For the in vitro phosphorylation assays, each GST-MPK (1 μg) was incubated in 25 μL of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.1 mM ATP and 3 μCi of [γ-^{32}P] ATP) either with or without constitutively active GST-MKKs (0.3 μg) at 30°C for 30 min. The reaction was terminated by addition of concentrated SDS-PAGE sample buffer followed by boiling for 5 min. Reaction products were analyzed using SDS-PAGE, autoradiography, and CBB staining. (A) Phosphorylation of MPKs by incubation with CAMKK1. (B) Phosphorylation of MPKs by incubation with CAMKK2. (C) Phosphorylation of MPKs by incubation with CAMKK6. (D) Phosphorylation of MPK2 by incubation with CAMKK7. (E) Phosphorylation of MPKs by incubation with CAMKK9.

Second, we selected MPK cDNA clones for expression that were consistent with the current TAIR/NCBI reference sequences, but for several MPK genes we have observed that multiple splice forms are being expressed in Arabidopsis tissues, and the biological relevance of these has yet to be defined. If translated, such MPK isoforms could conceivably play important roles in modulating the structure and activity of Arabidopsis MAPK signalling modules, in part through their differential ability to interact with, and/or serve as substrates for, upstream MKKs.

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