AtMKK6 and AtMPK13 are required for lateral root formation in Arabidopsis

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The mitogen-activated protein (MAP) kinase cascades are important signaling components that mediate various biological pathways in all eukaryotic cells. In our recent publication,[1] we identified AtMPK4 as one of the downstream targets of AtMKK6 that is required for executing male-specific meiotic cytokinesis. Here we provide evidence that another target, AtMPK13, is developmentally co-expressed with AtMKK6 in Arabidopsis, and both *AtMPK13* and *AtMKK6* display high *Promoter::GUS* activity in the primary root tips and at the lateral root primordia. Partial suppression of either *AtMKK6* or *AtMPK13* expression significantly reduces the number of lateral roots in the transgenic lines, suggesting that the AtMKK6-AtMPK13 module positively regulates lateral root formation.

The Proposed AtMKK6-AtMPK13 Module

The dual-specificity MAP kinase kinases reside in the center of the MAP kinase signaling module and transmit signals by interacting with and phosphorylating MAP kinases on threonine and tyrosine residues of the conserved -TXY- motif. All plant phyla examined seem to possess fewer MKKs than either MAPKKKs or MPKs,[2] suggesting a common theme that one MKK might be capable of phosphorylating several MPKs. In fact, Arabidopsis makes efficient use of MKKs by recruiting different MPKs to the same MKK in different biological contexts.[3] Among the ten Arabidopsis MPK kinases, AtMKK6 has long been proposed to specifically regulate phragmoplast expansion during cytokinesis, with AtMPK13 as its downstream target, based on homology between AtMPK13 and NtNRK1 in tobacco.[4] NtNRK1/NtNTF6, as well as its alfalfa ortholog, MsMMK3, has been functionally characterized, and both of them are regulated in a cell cycle dependent manner.[5-7] Consistent with such a model, heterologously-expressed AtMKK6 was shown to activate AtMPK13 in yeast.[8] Meanwhile, AtMKK6 was also shown to phosphorylate AtMPK18 when transiently co-expressed with this AtMPK in *Nicotiana benthamiana* leaves.[9] In a large scale protein microarray screen, AtMKK6 was reported to activate AtMPK3, AtMPK6 and AtMPK8, but in this study only 10 AtMPKs were assayed, and these did not include AtMPK13.[10] A directed yeast two-hybrid assay using the full set of AtMKKs and AtMPKs revealed that AtMKK6 can interact with AtMPK4, AtMPK11, AtMPK6 and AtMPK13.[11] Recently, AtMPK4 was demonstrated to serve as a target of AtMKK6 in processing somatic cytokinesis in the root,[12] as well as in male-specific meiotic cytokinesis.[1] By contrast, no cytokinesis defect was

observed for the homozygous *mpk13* T-DNA insertion mutant (SALK_130193).[12] However, two variants of the *AtMPK13* mRNA are expressed in Arabidopsis and this insertion event disrupts only the long coding sequence (CDS) of *AtMPK13*, and not the short CDS.[1] Therefore, a clear picture of the biological function of the proposed AtMKK6-AtMPK13 cascade is still lacking.

AtMKK6 and *AtMPK13* are Co-Expressed Developmentally

A comprehensive co-transcriptional analysis of 114 Arabidopsis MAP kinases was conducted earlier, using publicly available microarray data.[13] In this study, *AtMPK4*, *AtMPK13* and *AtMPK20* are the MPKs whose expression is most closely correlated with that of *AtMKK6* during plant development, and together they were assigned to the "shoot apex" group. In addition, *AtMKK6* and *AtMPK13* expression patterns are associated with mitotic activity, as are two MAPKKKs, *AtMAPKKK2* and *AtMAPKKK12*. In silico analysis using the AtGenExpress Visualization Tool (AVT) supported the idea that *AtMPK13* is co-expressed with *AtMKK6* during different developmental stages (jsp.weigelworld.org/expviz/expviz.jsp?experiment=development&normalization=absolute&probesetcsv=At5g56580%2Cat1g07880&action=Run). We confirmed this co-expression pattern experimentally by RT-PCR analysis, using RNA samples from 15 different plant tissues/organs, where we compared the *AtMKK6* and *ATMPK13* patterns to the ubiquitous expression patterns of *AtMPK4* and *AtMPK6*, and the restricted pattern of *AtMPK11* (**Fig. 1A**).

To obtain a more fine-grained view of these gene expression patterns, we examined $Pro_{MKK6}::GUS$ and $Pro_{MPK13}::GUS$
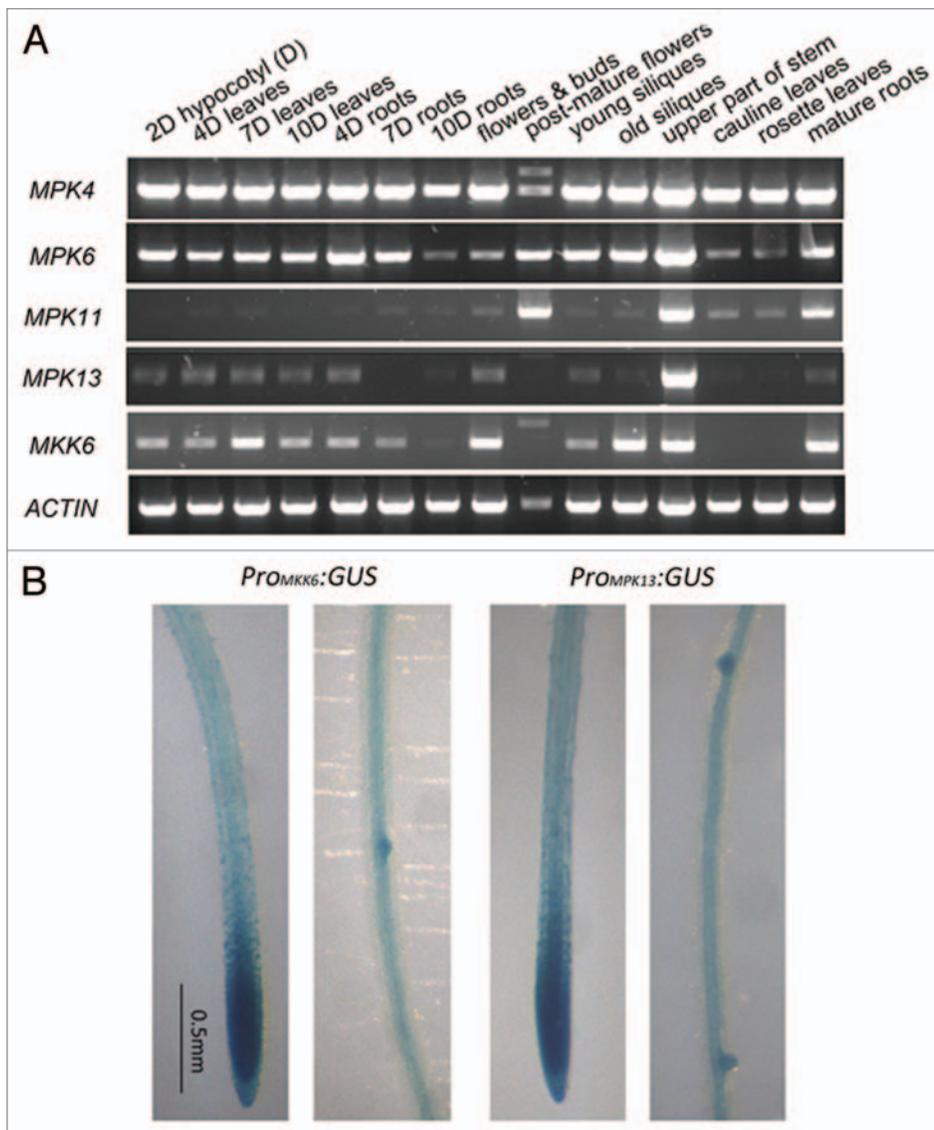
Figure 1. Expression patterns of *AtMKK6* and related *AtMPKs*. (A) Comparison of expression patterns of *AtMKK6* and *AtMPK4, AtMPK6, AtMPK11* and *AtMPK13* by RT-PCR. The (D) after "2D hypocotyls" indicates that the seedlings were germinated and grown in darkness before sample collection. Slower mobility bands observed in the "post-mature flowers" sample are from the genomic DNA fragment. (B) Histochemical analysis of Promoter~MKK6~::*GUS* and Promoter~MPK13~::*GUS* lines.

Conditional Knockdown Alleles of *AtMKK6* and *AtMPK13* Produce Fewer Lateral Roots

Since the *AtMKK6* knockout allele is severely stressed and sterile, and there is no null knockout allele of *MPK13*,[1] we generated dexamethasone (Dex)-inducible RNA interference lines for each kinase. Multiple independent T3 transgenic lines were selected that showed reduced accumulation of either *AtMKK6* mRNA or *AtMPK13* mRNA, respectively, 24 h after Dex-treatment (**Fig. 2A**).

When germinated on ½ MS media and then transferred to Dex-containing plates 5 d after germination (DAG), both the *AtMKK6 RNAi* and *AtMPK13 RNAi* genotypes produced far fewer lateral roots (LRs) following Dex induction than did the untreated plants, while Dex treatment of pTA7002 empty vector lines grown in the same manner had no effect on LR formation (**Fig. 2B**). Quantitative analysis indicated that the reduction in LR formation was statistically significant (**Fig. 2C**). In contrast, primary root (PR) growth of *AtMKK6 RNAi* or *AtMPK13 RNAi* genotypes was largely unaffected by Dex induction under these growth conditions, indicating that partial suppression of either *AtMKK6* or *AtMPK13* results specifically in suppression of LR formation (**Fig. 2D**). Interestingly, the residual LRs formed in the Dex-treated transgenic plants are mostly located at the basal region of the PRs (**Fig. 2B**). Since these plants were transferred to Dex-containing medium 5 DAG, when the seedlings had already formed their initial lateral root primordia (LRP), this distribution of LRs in the older seedlings suggests that LRP formed earlier ("pre-Dex") can continue to develop into mature lateral roots, whereas Dex treatment, and the associated suppression of *AtMPK13* or *AtMKK6* expression, block the formation of new LRP.

transgenic lines. The result showed that both *AtMKK6* promoter activity and *AtMPK13* promoter activity were concentrated in the root tip and in the sites of incipient lateral root emergence (**Fig. 1B**). In addition, GUS activity was only observed within the stele and pericycle cells, but not in the cortex or the epidermal cells. Taken together, these data suggest that the expression pattern of *AtMPK13* is broadly similar to that of *AtMKK6*, and provide initial insight into the relevant biological function.

Figure 2 (See next page). Phenotypic analysis of the Dex-inducible *AtMKK6* and *AtMPK13 RNAi* lines. (A) RT-PCR examination of *AtMKK6* and *AtMPK13* transcripts in the *AtMKK6 RNAi* lines and the *AtMPK13 RNAi* lines, respectively, 24 h after Dex-treatment. Both transcript variants for *AtMPK13* can be seen in the PCR products. (B) Lateral root reduction phenotype of the RNAi lines. Seedlings were germinated and grown on ½ MS for 5 days before being transferred to ½ MS plates with or without 1 μM dexamethasone. (C) Quantitative measurement of the lateral root (LR) number in the RNAi transgenic lines. (D) Quantitative measurement of the primary root (PR) length of the RNAi lines.
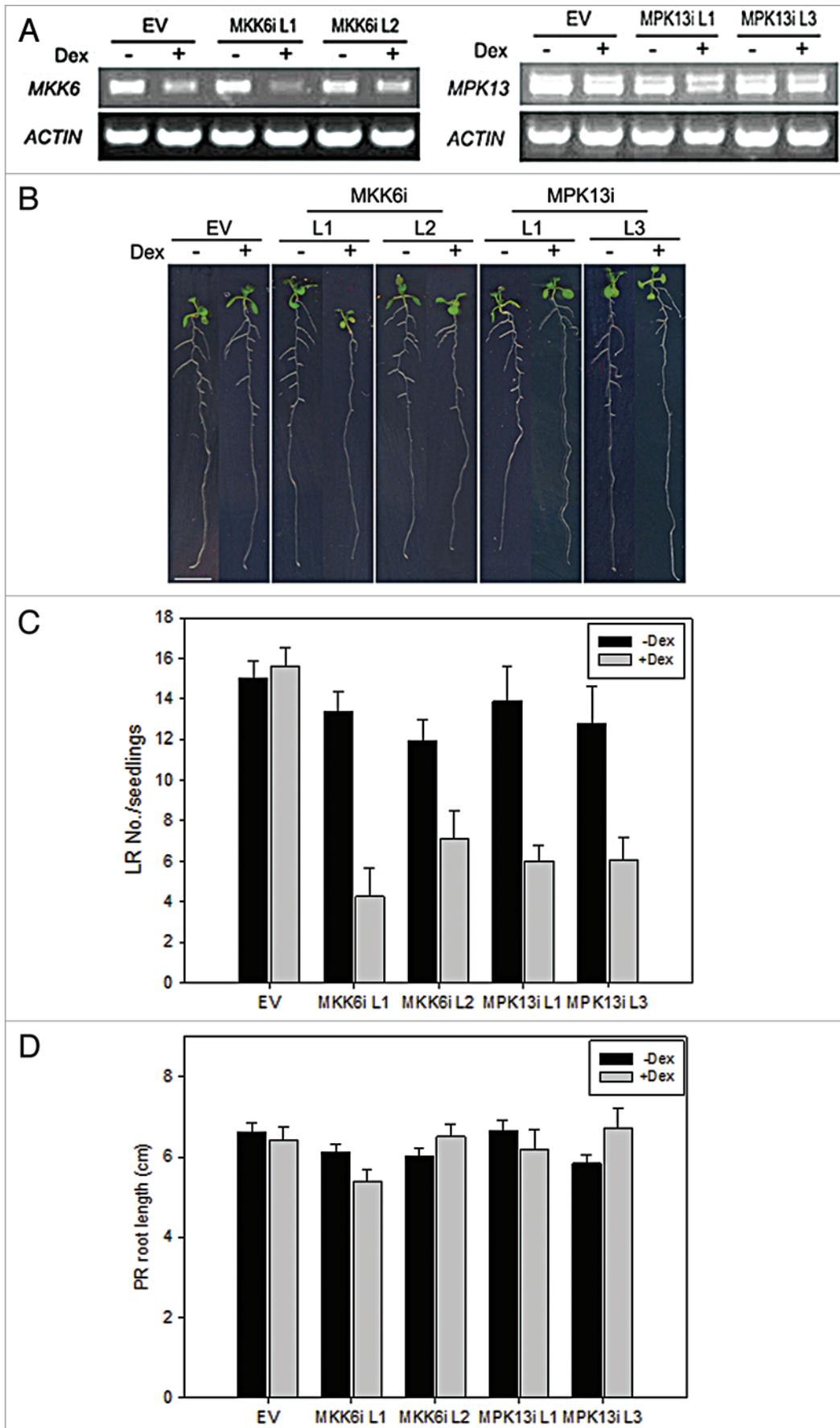
Taken together, our results suggest that the AtMKK6-AtMPK13 module is required for initiating and/or sustaining pericycle cell division during lateral root initiation in Arabidopsis. The observation that partial suppression of either *AtMKK6* or *AtMPK13* gene expression resulted in formation of far fewer lateral roots without affecting primary root growth, provides evidence for this developmental specificity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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