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Original Paper

MAP3Kδ4, an *Arabidopsis* Raf-like MAP3K, regulates plant growth and shoot branching

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Abstract MAP3Kδ4 (At4g23050) is a Raf-like mitogen-activated protein kinase kinase kinase (MAP3K) in *Arabidopsis thaliana*, classified as subgroup B2. The B2 subgroup MAP3Ks have a kinase domain in the C-terminus and a PAS domain in the N-terminal region. PAS domains are found in a variety of proteins and are reported to mediate protein–protein interactions and to sense environmental stimuli. However, the function of MAP3Kδ4 has not yet been determined. We generated transgenic plants constitutively expressing *MAP3Kδ4* or its kinase-negative mutant (*MAP3Kδ4KN*) and characterized their physiological traits. The transgenic plants overexpressing *MAP3Kδ4* showed earlier bolting than wild-type plants. They also showed more vigorous growth by both fresh weight and stem length. In contrast, the transgenic plants overexpressing *MAP3Kδ4KN* showed a highly branched phenotype; *MAP3Kδ4* overexpression had no effect on branch number. These results indicated that MAP3Kδ4 is crucial to regulating both plant growth and shoot branching. In addition, *MAP3Kδ4* transcripts were found in all *Arabidopsis* tissues examined and upregulated by auxin treatment in seedlings, suggesting that MAP3Kδ4 functions in an auxin-dependent manner.

Key words: *Arabidopsis thaliana*, auxin, MAP3Kδ4, Raf-like MAP3K, shoot branching.

Mitogen-activated protein kinase (MAPK) signaling cascades have important functions in plants and are reported to be involved in growth and development, cell division, and responses to biotic and abiotic stresses and plant hormones (Andreasson and Ellis 2010; MAPK Group 2002; Sharma et al. 2005; Tena et al. 2001). A classical MAPK pathway consists of three classes of protein kinases: MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAP3K or MAPKKK). Upstream signals activate the MAP3K, which phosphorylates and activates the MAPKK. The activated MAPKK in turn phosphorylates the specific MAPK and causes its activation, which leads to regulation of growth, development and stress responses.

In *Arabidopsis thaliana*, 20 MAPK, 10 MAPKK and about 80 MAP3K genes have been found (Colcombet and Hirt 2008). *Arabidopsis* MAP3Ks are divided into two subfamilies: MEKK-like and Raf-like MAP3Ks (Colcombet and Hirt 2008; Jouannic et al. 1999a; Jouannic et al. 1999b; MAPK Group 2002). Some of the MEKK-like MAP3Ks have been characterized in *Arabidopsis*. We previously reported that the *Arabidopsis* MEKK1-MKK1-MPK4 cascade is stimulated upon wounding stress (Hadiarto et al. 2006; Matsuoka et al. 2002). MEKK1 has been shown to function in various stress signaling pathways, the MEKK1-MKK2-MPK4/MPK6 cascade in salt and cold stress signaling (Teige et al. 2004), and the MEKK1-MKK4/MKK5-MPK3/MPK6 cascade in pathogen infection (Asai et al. 2002). *Arabidopsis* ANP1, ANP2, ANP3, the other MEKK-like MAP3Ks, are involved in plant cell division (Krysan et al. 2002) and ANP1 responds to oxidative stress and blocks auxin action (Kovtun et al. 2000). However, few Raf-like MAP3Ks have been studied, even though they are a multigene family. *Arabidopsis* CTR1 acts as a negative regulator of ethylene signaling (Clark et al. 1998; Hahn and Harter 2009; Huang et al. 2003; Kieber et al. 1993; Yoo et al. 2008). *Arabidpsis* EDR1 negatively

Abbreviations: BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; KN, kinase negative; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAP3K, MAPKK kinase; PAS, Per-Arnt-Sim.

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regulates the pathogen defense response to powdery mildew disease caused by the fungus *Erysiphe cichoracearum* (Frye et al. 2001) and At1g73660 encodes a putative MAP3K that negatively regulates salt stress responses (Gao and Xiang 2008). The functions of these Raf-like MAP3Ks have been proposed mainly based on analyses with knockout mutants, but evidence that they act as typical MAP3Ks is still lacking.

Arabidopsis MAP3Kδ4 (At4g23050) is a member of the RAF-like MAP3Ks, classified as subgroup B2. The B2 subgroup consists of six *Arabidopsis* MAP3K members, with *MAP3Kδ4* the outgroup gene to the other five members (MAPK Group 2002), implying a unique function for MAP3Kδ4. MAP3Kδ4 has 736 amino acids with two typical domains: a PAS domain in the N-terminal region and a kinase domain in the C-terminus. The PAS domain was named after the three proteins in which it occurs: period circadian protein, aryl hydrocarbon receptor nuclear translocator protein, and single-minded protein. Although many PAS domains are reported to bind cofactors or ligands to detect sensory signals and many PAS-containing proteins are involved in the sensing of environmental stimuli such as oxygen, redox or light (Hefti et al. 2004; Zhulin et al. 1997), the function of PAS-containing MAP3Kδ4 has not been reported. To clarify the function of MAP3Kδ4, we generated transgenic *Arabidopsis* plants constitutively expressing *MAP3Kδ4* or its kinase-negative form (*MAP3Kδ4KN*) and characterized their physiological traits. We describe here a new finding that MAP3Kδ4 promotes plant growth and regulates shoot branching. We also show that the expression of the *MAP3Kδ4* gene is correlated to auxin response.

Materials and methods

Plant materials

Seeds of *Arabidopsis thaliana* (Columbia ecotype) were surface-sterilized with 70% (v/v) ethanol for 3 min and a solution of NaClO (1% w/v) and Triton X-100 (0.1% v/v) for 7 min followed by washing with sterile water five times. They were placed on Gamborg's B5 agar (0.8% w/v) medium and incubated for 2 days at 4°C before transfer to 22°C for germination. After 20 days of growth on the plates at 22°C under continuous light, the seedlings were transplanted to vermiculite and grown at 22°C under continuous light.

For plant hormone treatment, 20-day-old seedlings were removed from agar plates and placed in distilled water for 24 h prior to treatment. Then, seedlings were treated with 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 10 μM 6-benzylaminopurine (BA) or distilled water for 2 h. Seedlings were frozen in liquid nitrogen and stored at −80°C until use.

RT-PCR and genomic PCR

For RT-PCR analysis, total RNA was extracted from *Arabidopsis* plants using an RNeasy Plant Mini Kit (Qiagen) and treated with DNaseI (Invitrogen) to remove residual DNA contamination. The cDNA was synthesized from 0.5 μg of *Arabidopsis* total RNA using a PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). *MAP3Kδ4* transcripts were amplified using forward primer 5′-GGGAGTCTCTTCAA-AATACTTCAT-3′ and reverse primer 5′-TGCGATCCGGGG-ATTTAATCCTTC-3′. *IAA1* (At4g14560) transcripts were amplified using forward primer 5′-TAAGGACACAGAG-CTTCGTTTG-3′ and reverse primer 5′-GACCAACAT-CCAATCTCCATCT-3′. *ARR5* (At3g48100) transcripts were amplified using forward primer 5′-TCTTGAAGAAGGA-GCTGAAGATTTC-3′ and reverse primer 5′-TAGCTTCA-AGCTCTCTCTTGTGCAT-3′. *Actin8* (At1g49420) transcripts served as a control and were amplified by forward primer 5′-GAAGGACCTTTACGGTAACA-3′ and reverse primer 5′-CCAATCCAGACACTGTACTT-3′. PCR was performed with *TaKaRa Ex Taq* HS (TAKARA) at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min with final extension at 72°C for 10 min. PCR products were separated on 1.6% agarose gels and visualized under UV light.

For genomic PCR analysis, genomic DNA was extracted from 7-day-old *Arabidopsis* seedlings using a DNeasy Plant Mini Kit (Qiagen). Genomic PCR used the conditions described above.

Plasmid construction and Arabidopsis transformation

To create transgenic *Arabidopsis* plant lines overexpressing *MAP3Kδ4*, the full-length *MAP3Kδ4* cDNA (2208 bp) was amplified by RT-PCR using total RNA from *Arabidopsis* seedlings. PCR was performed with *PfuUltra* High-Fidelity DNA Polymerase (Stratagene) using forward primer 5′-GAAAGGATCCATGGCCGGAAACAACTCGGA-3′ (*Bam*HI site underlined) and reverse primer 5′-GTTTGAGCTCTCAA-TCGTCTTCTTCTTGCGC-3′ (*Sac*I site underlined). The PCR product was cloned into the pT7Blue-2 vector (Novagen) and verified by DNA sequencing with an ABI3100 DNA sequence analyzer (Applied Biosystems). Using the cloned *MAP3Kδ4* cDNA as a template, the kinase-negative *MAP3Kδ4* mutant (designated *MAP3Kδ4KN*) was generated by QuikChange II Site-Directed Mutagenesis Kit (Stratagene) with forward primer 5′-GATGTTGCTATTAGGGTTTACTTCGAT-3′ and reverse primer 5′-ATCGAAGTAAACCCTAATAGCAACATC-3′ to replace Lys-495 in the ATP-binding site to Arg (replaced nucleotide underlined). The mutation was confirmed by DNA sequencing.

After digestion with *BamH*I and *Sac*I, the *MAP3Kδ4* and *MAP3Kδ4KN* fragments were inserted between the CaMV 35S promoter and the NOS terminator of plant expression vector pBI121 (Clontech). The resulting constructs were introduced into *Agrobacterium tumefaciens* (strain C58) by triparental mating with *Escherichia coli* (strain DH5α) containing pRK2013 (Wise et al. 2006) and transferred into wild-type *Arabidopsis* (Columbia ecotype) by the vacuum infiltration method (Bechtold et al. 1993). Transgenic plants were germinated on 0.5×Murashige and Skoog medium with 20 μg ml^{-1} kanamycin. All transgenic lines used in this study were T_3 homozygous plants.

Growth measurements

Wild-type and transgenic plants were germinated and grown as described above. Bolting rates were monitored from 20 days after germination in 17 plants and mean values calculated from three replicate measurements. Fresh weights, inflorescence stem lengths and branch numbers were measured every five days from 25, 30 and 45 days after germination. Branches arising from the base of the inflorescence stems were defined as first-order branches, branches from the stems and the first-order branches were defined as second-order branches, and branches from the second-order branches were defined as third-order branches. To measure flowering days, flowering was monitored using visible petals as an indicator. To analyze seed productivity, at 60 days after germination, plants were harvested and dried, and seed weights and seed numbers were measured.

Results

Expression analysis of MAP3Kδ4

Transcripts of *MAP3Kδ4* were analyzed by RT-PCR in *Arabidopsis* flowers, siliques, cauline and rosette leaves, inflorescence stems, and roots from mature plants and 7-day-old seedlings grown at 22°C under continuous light. *MAP3Kδ4* was expressed in all tissues examined, with relatively higher levels in stems, and lower levels in siliques (Figure 1), indicating that MAP3Kδ4 may function throughout all developmental stages in *Arabidopsis* plants. These data are largely consistent with publicly available microarray data (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007).

Transgenic Arabidopsis plants with higher expression of MAP3Kδ4

To clarify the physiological role of MAP3Kδ4 in *Arabidopsis*, the full-length cDNA (2208 bp) for *MAP3Kδ4*, under the control of the CaMV 35S promoter, was introduced into *Arabidopsis* cells. Eight independent lines of transgenic plants were generated and one overexpression line, FL2-5, was chosen for further experiments (Supplementary Figure 1).

To examine the role of kinase activity, we also created transgenic lines that overexpressed *MAP3Kδ4* kinase-negative, mutated at Lys495 in the ATP-binding site to Arg (designated *MAP3Kδ4KN*). Ten independent lines were generated and one overexpression line, KN2-2, was chosen for further experiments (Supplementary Figure 2).

Transgenic and wild-type *Arabidopsis* plants were grown at 22°C under continuous light for 60 days. Representative plants are in Figure 2A. Transgenic *35S:MAP3Kδ4* plants exhibited more vigorous growth than wild-type plants. However, *35S:MAP3Kδ4KN* plants showed a highly branched phenotype. These phenotypes were also observed in other *35S:MAP3Kδ4* lines and *35S:MAP3Kδ4KN* lines (data not shown).

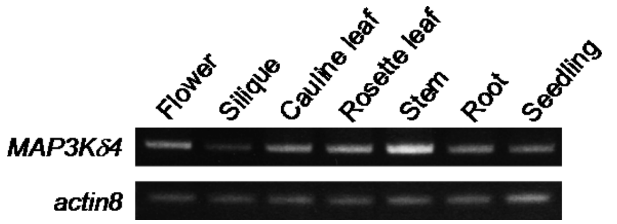

Figure 1. Tissue-specific transcription of the *MAP3Kδ4* gene analyzed by RT-PCR. Total RNA was extracted from flowers, siliques, cauline and rosette leaves, inflorescence stems, and roots of *Arabidopsis* mature plants, and whole 7-day-old seedlings. *Actin8* expression was used as a control.

Overexpression was confirmed by RT-PCR (Figure 2B). To characterize the growth of transgenic plants in detail, we continuously scored fresh weight as a growth indicator. As shown in Figure 2C, the fresh weights were 24 ± 1.5 mg for wild-type, 43 ± 2.0 mg for *35S:MAP3Kδ4,* and 41 ± 1.2 mg for *35S:MAP3Kδ4KN* plants at 25 days after germination. Throughout the experiment, *35S:MAP3Kδ4* plants grew the most rapidly and *35S:MAP3Kδ4KN* plants showed an intermediate value between wild-type and *35S:MAP3Kδ4* plants. At the end of the experiment (60 days after germination), *35S:MAP3Kδ4* plants weighed 367 ± 56.1 mg, almost twice as much as wild-type plants (186 ± 13.9 mg), whereas *35S:MAP3Kδ4KN* plants reached 232 ± 11.5 mg.

Compared to wild-type and *35S:MAP3Kδ4* plants, shoot branching was more pronounced in *35S:MAP3Kδ4KN* plants (Figure 2A). Transgenic *35S:MAP3Kδ4KN* plants produced more shoot branches (16 ± 1.2 mg) than wild-type (7.5 ± 0.98 mg) and *35S:MAP3Kδ4* plants (6.2 ± 0.37 mg) at 60 days after germination (Figure 3A). The branching phenotype of *35S:MAP3Kδ4KN* plants was mainly attributed to the increased first-order branches and third-order branches (Figure 3B–D).

Constitutive expression of MAP3Kδ4 modified inflorescence stem development and seed productivity in Arabidopsis

We next compared the bolting rate, growth of inflorescence stems, and flowering days among wild-type and transgenic plants. The bolting of *35S:MAP3Kδ4* plants preceded that of wild-type plants by approximately 4–6 days (Figure 4A). *35S:MAP3Kδ4KN* plants also bolted slightly earlier than wild-type plants. At 30 days after germination, the stem length of *35S:MAP3Kδ4* plants was 77.6 ± 3.15 mm, with 56.5 ± 0.947 mm for *35S:MAP3Kδ4KN* plants and 32.3 ± 2.32 mm for wild-type plants (Figure 4B). In all plants, the inflorescence stems elongated at similar rates through the experiment. For flowering day measurement, *35S:MAP3Kδ4* plants flowered at 44 ± 0.63 days after

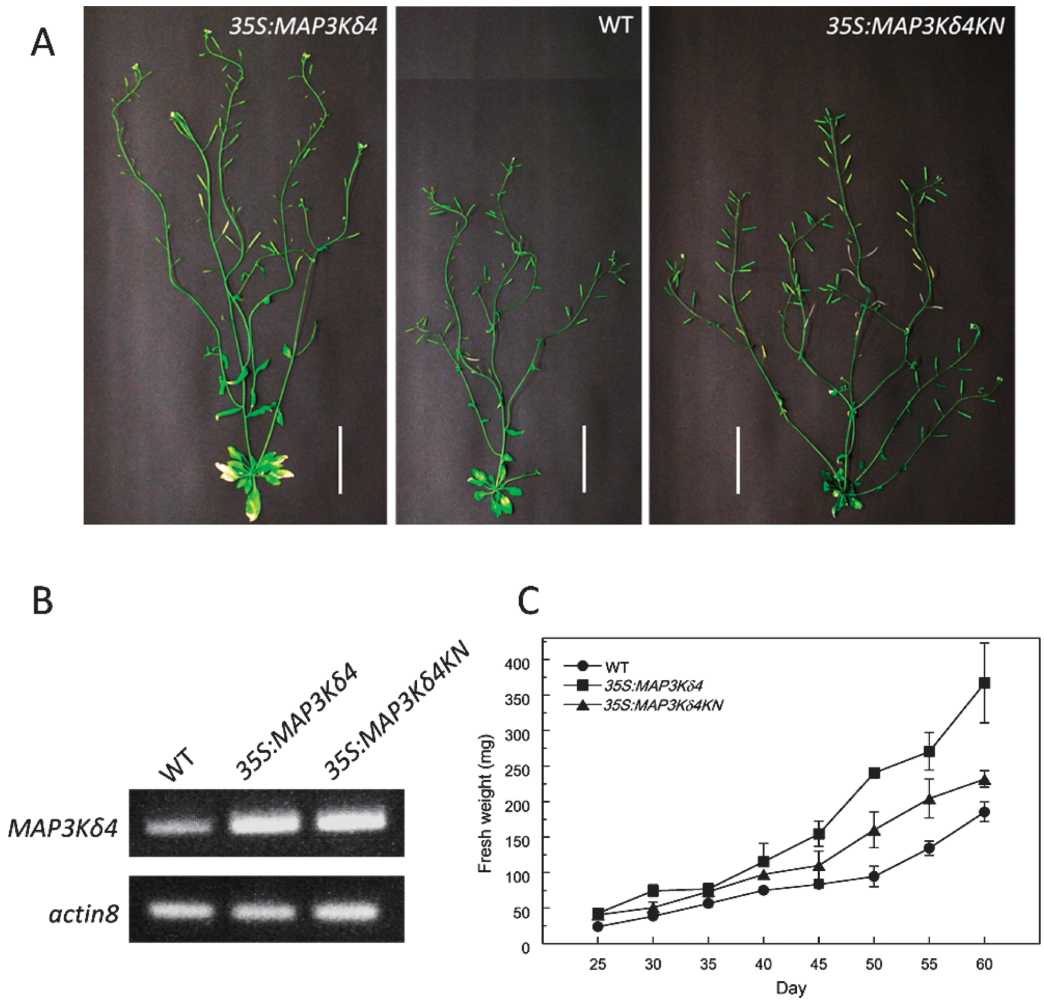

Figure 2. Growth characteristics of transgenic *Arabidopsis* plants overexpressing *MAP3Kδ4* and *MAP3Kδ4KN*. (A) *35S:MAP3Kδ4*, wild-type Columbia ecotype and *35S:MAP3Kδ4KN* plants, 50 days old. Scale bars=50 mm. (B) Overexpression of *MAP3Kδ4* and *MAP3Kδ4KN* in transgenic plants confirmed by RT-PCR. Total RNA was extracted from 7-day-old seedlings. *Actin8* expression was used as a control. (B) Kinetic changes in fresh weights of wild-type (circle), *35S:MAP3Kδ4* (square) and *35S:MAP3Kδ4KN* plants (triangle). From 25 days after germination, fresh weights were measured every five days. Results are means and standard error (SE) of three replicates.

germination (Figure 4C), significantly earlier than wild-type plants (51 ± 1.0 days; $p<0.05$). Transgenic *35S:MAP3Kδ4KN* plants flowered at 47 ± 0.79 days after germination, which was significantly different from wild-type plants.

We also measured seed weights and numbers in wild-type and the transgenic plants. Seed weights were 1.8 ± 0.10 mg/100 seeds for wild-type, 2.3 ± 0.11 mg/100 seeds for *35S:MAP3Kδ4* and 2.0 ± 0.088 mg/100 seeds for *35S:MAP3Kδ4KN* plants (Figure 5A), with a significant difference between wild-type and *35S:MAP3Kδ4* plants ($p<0.05$). In contrast, the seed numbers per plant of wild-type and *35S:MAP3Kδ4* plants were not significantly different, whereas those of *35S:MAP3Kδ4KN* plants were 2.1 times higher than wild-type plants (Figure 5B).

Auxin induces MAP3Kδ4 expression

As described above, overexpression of *MAP3Kδ4* resulted in pleiotropic effects on growth and development in *Arabidopsis*. In addition, *35S:MAP3Kδ4KN* plants branched more than wild-type and *35S:MAP3Kδ4* plants (Figures 2A, 3A). These traits in the transgenic plants may be related to plant hormone activity. Shoot branching is known to be controlled by the plant hormones auxin and cytokinin (Domagalska and Leyser 2011; Shimizu-Sato et al. 2009). Auxin promotes apical dominance, and cytokinin promotes lateral shoot outgrowth. Therefore, we examined expression levels of *MAP3Kδ4* in response to auxin (2,4-D) and cytokinin (BA) in wild-type plants. As shown in Figure 6A, *MAP3Kδ4* expression was upregulated in plants treated with 0.5 μM 2,4-D, but not in plants treated with 10 μM BA. Those treatments were validated by upregulation of the auxin-responsive gene *IAA1* and the cytokinin-responsive gene *ARR5* (Figure 6B, C).

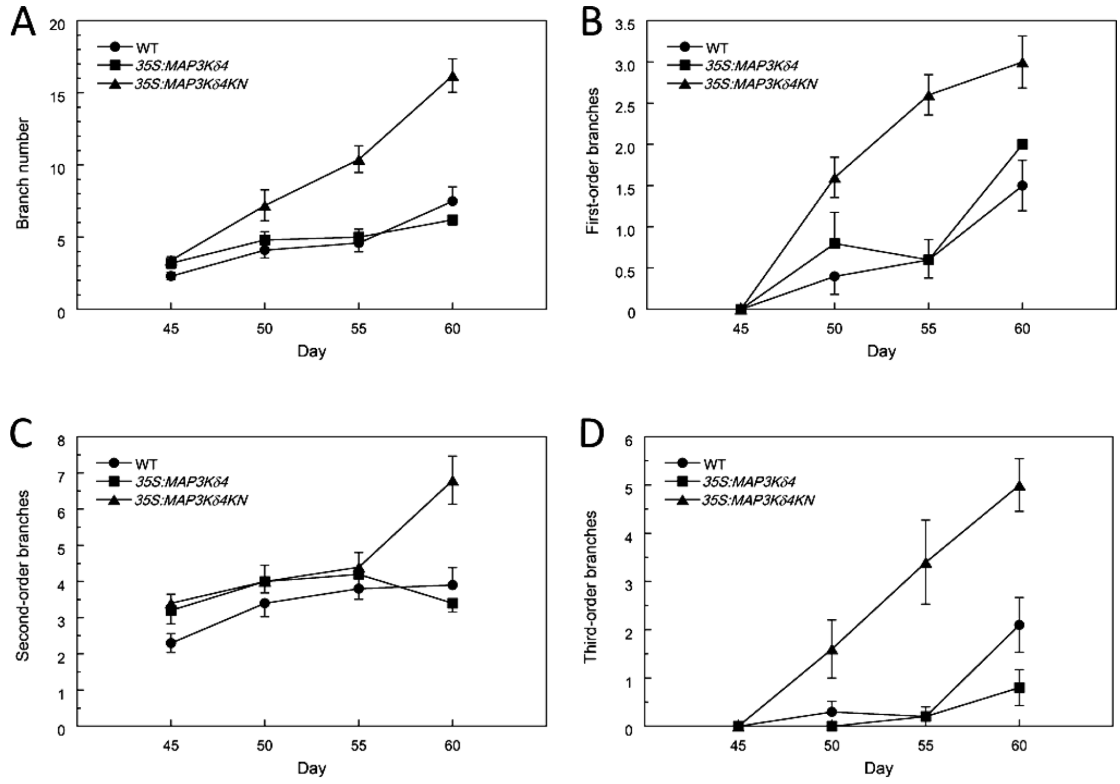

Figure 3. Branching phenotypes of transgenic plants. Numbers of total branches (A), first-order branches (B), second-order branches (C) and third-order branches (D) in whole plants. From 45 days after germination, branch numbers were counted every five days. Results are means and SE of five replicates.

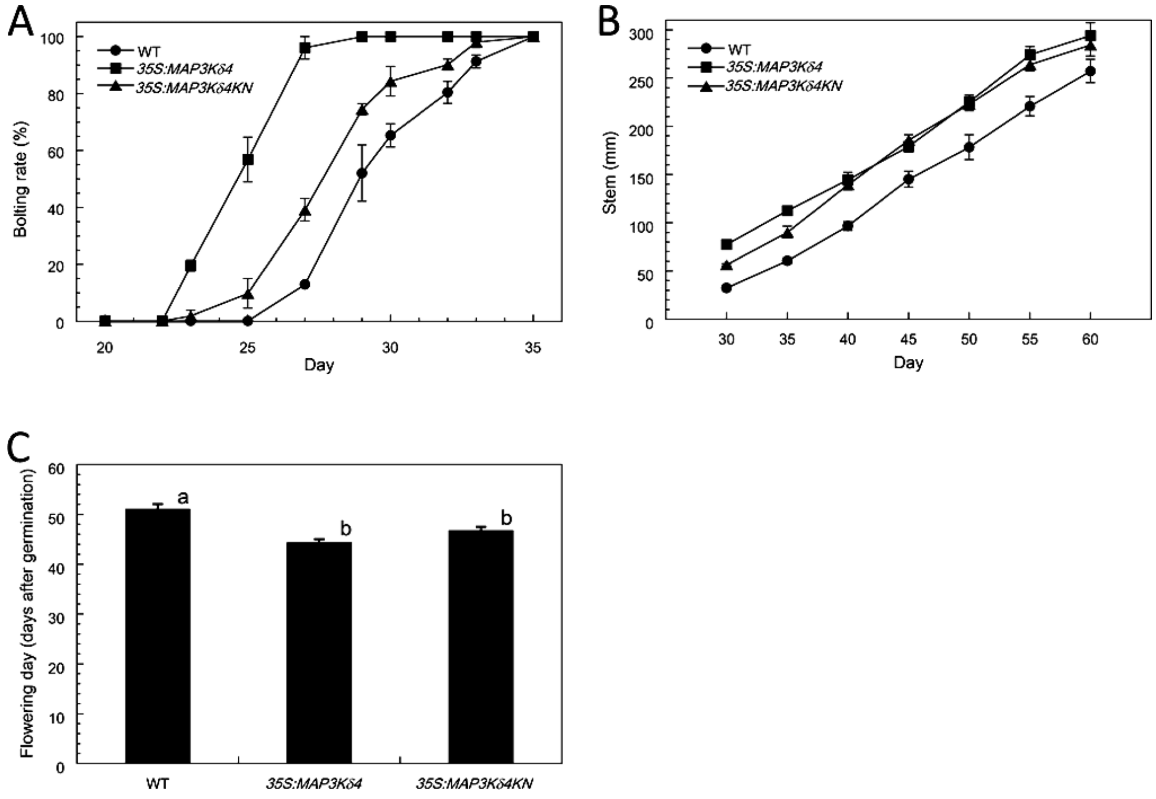

Figure 4. Characterization of inflorescence stem development. (A) Bolting rates of wild-type and transgenic plants. From 20 days after germination, bolting rates were monitored in three replicates of 17 plants. Results are means and SE. (B) Kinetic changes in inflorescence stem lengths of wild-type and transgenic plants. From 30 days after germination, stem lengths were measured every five days. Results are means and SE of 10 replicates. (C) Flowering days of wild-type and transgenic plants. Flowering was monitored using visible petals as an indicator. Results are means and SE of 26 replicates. Significantly different values at $p<0.05$ are marked with different letters (Tukey-HSD test).

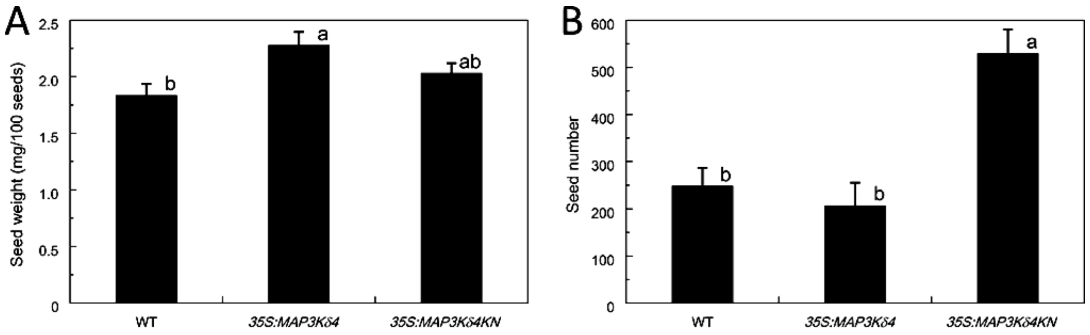

Figure 5. Characterization of seed productivity. At 60 days after germination, wild-type and transgenic plants were harvested and dried. Seed weights (A) and seed numbers per plant (B) were measured. Results are means and SE of seven replicates. Significantly different values at $p<0.05$ are marked with different letters (Tukey-HSD test).

Discussion

In this study, we clarified the physiological role of *MAP3Kδ4* by generating transgenic *Arabidopsis* plants that constitutively express *MAP3Kδ4* under control of CaMV 35S promoter. Overexpression of *MAP3Kδ4* caused more vigorous growth and increased the fresh weight of adult *Arabidopsis* plants (Figure 2). The transgenic *35S:MAP3Kδ4* plants also showed pleiotropic phenotypes such as earlier bolting (Figure 4A), faster growth of inflorescence stems (Figure 4B), earlier flowering (Figure 4C), and increased seed weights (Figure 5A). Similar to *35S:MAP3Kδ4* plants, Kim et al. (2010) reported that transgenic *Arabidopsis* plants overexpressing *CaSRP1* (*Capsicum annuum* stress-related protein 1) from hot pepper also bolted earlier with inflorescence stems that elongated faster than wild-type plants, and found that these physiological traits were linked to enhanced cell cycle progression. In this context, more research is needed to elucidate whether cell cycle progression was enhanced in *35S:MAP3Kδ4* plants.

We report here a highly branched phenotype with increased seed numbers observed in *35S:MAP3Kδ4KN* plants (Figures 2A, 3), suggesting that MAP3Kδ4 suppresses shoot branching and that MAP3Kδ4 kinase activity is essential for biological function. In contrast to *35S:MAP3Kδ4KN* plants, *35S:MAP3Kδ4* plants showed a branching phenotype similar to wild-type plants (Figures 2A, 3). This observation is consistent with the results described above, assuming that MAP3Kδ4 fully exerts an inhibitory effect on shoot branching in wild-type plants so that overexpression of *MAP3Kδ4* had little effect on the branching phenotype in transgenic *35S:MAP3Kδ4* plants. It seems that over expression of *MAP3Kδ4KN* gene directs to a dominant negative effect on the transgenic plant. The observed phenotypes other than branching and seed numbers of *35S:MAP3Kδ4KN* showed in-between with those of *35S:MAP3Kδ4* transgenic plant and wild type plant, suggesting that highly expression of the PAS domain on MAP3Kδ4 is concerned with those. It is expectable to examine phenotypes of MAP3Kδ4 knockout plant in order to show further roles of the MAP3K.

The role of auxin in the regulation of shoot branching has been studied extensively (Domagalska and Leyser 2011; Shimizu-Sato et al. 2009). Previous studies showed that many aberrant phenotypes in shoot branching are correlated with auxin. In *Arabidopsis*, loss of *AXR1* function, an auxin signaling component, results in increased shoot branching (Lincoln et al. 1990; Stirnberg et al. 1999). Auxin-overproducing *ycc1* mutants produced fewer shoot branches relative to wild-type plant (Aguilar-Martínez et al. 2007) and this phenotype was rescued by overexpression of the bacterial *iaaL* gene, which encodes an enzyme that conjugates free indole acetic acid to lysine (Zhao et al. 2001). *Arabidopsis* MAP kinase kinase 7 (MKK7) negatively regulates polar auxin transport. Its overexpression results in loss of apical dominance (Dai et al. 2006). In this study, we report the enhanced expression of *MAP3Kδ4* by treatment with 2,4-D (Figure 6A), suggesting that MAP3Kδ4 may act downstream of auxin signaling to suppress shoot branching. Our data was obtained by PCR amplification of the *MAP3Kδ4* gene. But according to the micro-array database on Arabidopsis (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007), *MAP3Kδ4* gene expression is constant under auxin treatment. Further quantitative analysis is needed on auxin dependent gene expression of *MAP3Kδ4* in detail.

As described above, *Arabidopsis* MKK7 negatively regulates polar auxin transport, and MKK7 overexpression caused a highly branched phenotype. However, transgenic *Arabidopsis* plants containing the MKK7 kinase-inactive mutant showed the same branching phenotype as wild-type plants (Dai et al. 2006). In contrast, overexpression of *MAP3Kδ4* and *MAP3Kδ4KN* led to normal and highly branched phenotypes, respectively (Figure 3). *MAP3Kδ4* may function to suppress shoot branching through the inactivation of an MKK7-containing signaling module that positively regulates shoot branching.

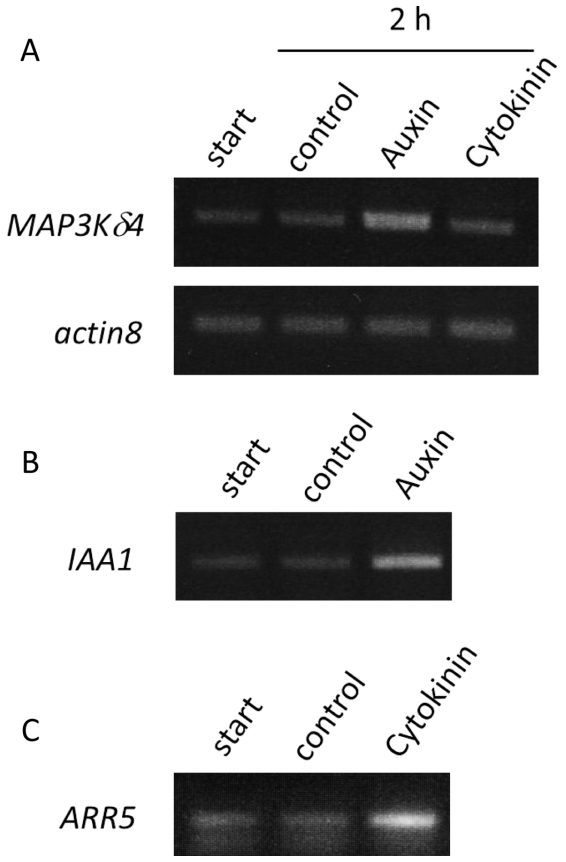

Figure 6. Changes in expression of the *MAP3Kδ4* gene after auxin and cytokinin treatments. *Arabidopsis* 20-day-old seedlings were preincubated in distilled water for 24 h and treated with 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 10 μM 6-benzylaminopurine (BA) or distilled water for 2 h. Total RNA was extracted from whole seedlings and expression of *MAP3Kδ4* (A), the auxin-responsive gene *IAA1* (B) and the cytokinin-responsive gene *ARR5* (C) were analyzed by RT-PCR.

The Raf-like MAP3K CTR1 functions as a negative regulator of the MKK9-MPK3/6 cascade that positively regulates ethylene signaling (Hahn and Harter 2009; Yoo et al. 2008). *Arabidpsis* EDR1, another Raf-like MAP3K, is responsible for negative regulation of pathogen defense responses (Frye et al. 2001) and a putative Raf-like MAP3K, At1g73660, is reported to act as a negative regulator in salt stress responses (Gao and Xiang 2008). This mode of action as negative regulators might be a characteristic feature of Raf-like MAP3Ks in *Arabidopsis*.

Our results with physiological and molecular analyses suggest that MAP3Kδ4 may act downstream of auxin signaling. Besides MKK7, several MAPK cascade components are reported to be associated with auxin action. MPK2 was phosphorylated rapidly after 2,4-D treatment in auxin-starved BY-2 cells (Mizoguchi et al. 1994). Constitutively active ANP1, ANP2, and ANP3 suppress *GH3* promoter induction by auxin in *Arabidopsis* protoplasts, suggesting that ANPs block auxin action (Kovtun et al. 2000). To elucidate the function of MAP3Kδ4, auxin-related MAPK cascade components that interact to MAP3Kδ4 should be investigated. MAP3Kδ4 contains a PAS domain in the N-terminal region. Further research to elucidate the function of the PAS domain should determine the function of MAP3Kδ4. In addition, more detailed biochemical investigations are needed to clarify the target molecules of MAP3Kδ4 kinase.

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