



# Genetic analysis of the *Arabidopsis* protein kinases *MAP3Kε1* and *MAP3Kε2* indicates roles in cell expansion and embryo development

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*MAP3Kε1* and *MAP3Kε2* are a pair of *Arabidopsis thaliana* genes that encode protein kinases related to *cdc7p* from *Saccharomyces cerevisiae*. We have previously shown that the *map3kε1;map3kε2* double-mutant combination causes pollen lethality. In this study, we have used an ethanol-inducible promoter construct to rescue this lethal phenotype and create *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* double-mutant plants in order to examine the function of these genes in the sporophyte. These rescued double-mutant plants carry a yellow fluorescent protein (YFP)-*MAP3Kε1* transgene under the control of the alcohol-inducible *AlcA* promoter from *Aspergillus nidulans*. The double-mutant plants were significantly smaller and had shorter roots than wild-type when grown in the absence of ethanol treatment. Microscopic analysis indicated that cell elongation was reduced in the roots of the double-mutant plants and cell expansion was reduced in rosette leaves. Treatment with ethanol to induce expression of YFP-*MAP3Kε1* largely rescued the leaf phenotypes. The double-mutant combination also caused embryos to arrest in the early stages of development. Through the use of YFP reporter constructs we determined that *MAP3Kε1* and *MAP3Kε2* are expressed during embryo development, and also in root tissue. Our results indicate that *MAP3Kε1* and *MAP3Kε2* have roles outside of pollen development and that these genes affect several aspects of sporophyte development.

**Keywords:** MAP kinase, *MAP3Kε1*, *MAP3Kε2*, cell expansion, embryo development, *Arabidopsis*

## INTRODUCTION

The *Arabidopsis* genes *MAP3Kε1* and *MAP3Kε2* encode protein kinases that were originally named as members of the MAP kinase kinase (MAP3K) gene family (MAPK Group, 2002). More recent analysis has indicated that *MAP3Kε1* and *MAP3Kε2* are more closely related to *cdc7p* from *Schizosaccharomyces pombe* and *Cdc15p* from *Saccharomyces cerevisiae*, which are not MAP3Ks (Jouannic et al., 2001; Champion et al., 2004a,b). *Cdc7p* is one of the components of the septation initiation network (SIN). The SIN has been shown to regulate the formation of the septum in fission yeast after chromosome segregation has been completed (Simanis, 2003). The functionally equivalent pathway in the budding yeast *S. cerevisiae* is called the mitotic exit network (MEN), and it regulates cytokinesis and mitotic exit (Simanis, 2003). SIN-like elements such as *sid1p*-, *cdc16*-, and *mob1p*-related proteins have been identified in both the *Arabidopsis* and rice (*Oryza sativa*) genomes (Bedhomme et al., 2008), leading to the speculation that a SIN-like pathway may exist in plants.

The *Arabidopsis* genome encodes the following signaling proteins closely related to the core elements of the SIN pathway: *AtMAP3Kε1*, *AtMAP3Kε2*, *AtSGP1*, *AtSGP2*, *AtMAP4Kα1*, and *AtMAP4Kα2* (Champion et al., 2004b; Bedhomme et al., 2008). These *Arabidopsis* genes partially rescue homologous fission yeast mutants and encode proteins that interact with their fission yeast counterparts. There is currently, however, no direct

evidence in support of the idea that plants use these SIN-related components to regulate cytokinesis (Champion et al., 2004b; Bedhomme et al., 2008).

We have previously reported that single-mutant plants carrying T-DNA null alleles for either *map3kε1* or *map3kε2* displayed no obvious abnormal phenotypes, while the double-mutant combination caused pollen lethality (Chaiwongsar et al., 2006). These results suggested that functional compensation could be occurring between *MAP3Kε1* and *MAP3Kε2* such that the loss of one gene is compensated for by the second gene (Liljegren et al., 2000). This appears to be the case for pollen development, but remained an untested hypothesis for the sporophyte since the pollen-lethality of the double-mutant combination prevents the formation of a homozygous double-mutant sporophyte. It has been reported that *MAP3Kε1* is expressed in all organs of the plant, and that *MAP3Kε1* and *MAP3Kε2* are expressed strongly in regions of the sporophyte that contain actively dividing cells (Jouannic et al., 2001; Chaiwongsar et al., 2006; Bedhomme et al., 2009). These reports indicate that *MAP3Kε1* and *MAP3Kε2* are likely to have functions outside of pollen development.

In order to generate *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* double-mutant plants we made use of an alcohol-inducible transcription system to rescue the pollen lethal phenotype of the *map3kε1;map3kε2* double-mutant combination. This system is derived from the filamentous fungus *Aspergillus nidulans* and has been successfully

adopted for use in plants (Caddick et al., 1998; Roslan et al., 2001; Laufs et al., 2003). It is composed of the *alcR*-encoded transcriptional activator (AlcR) and a promoter derived from the *alcA* promoter (Kulmburg et al., 1992). In *A. nidulans*, these elements allow for the controlled activation of a series of structural genes important for alcohol catabolism (Pickett et al., 1987). In our study we used the *alcR/alcA* system to express yellow fluorescent protein (YFP)-*MAP3Kε1* in *map3kε1;map3kε2* double-mutant pollen, thereby allowing for the generation of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* plants. We report here on our phenotypic analysis of these rescued *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* double-mutant plants. Taken together our genetic analyses indicated that *MAP3Kε1* and *MAP3Kε2* are involved in multiple aspects of plant development, including root cell elongation, rosette leaf expansion, and embryo development.

## RESULTS

### USING AN ALCOHOL-INDUCIBLE PROMOTER CONSTRUCT TO PRODUCE RESCUED *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* DOUBLE-MUTANT PLANTS

We have previously described the T-DNA null alleles *map3kε1* and *map3kε2* (Chaiwongsar et al., 2006). In that previous work we observed that homozygous single-mutant lines for either *map3kε1* or *map3kε2* did not display any obvious abnormal phenotypes when grown under standard laboratory conditions. We also found that the *map3kε1;map3kε2* double-mutant combination caused pollen lethality (Chaiwongsar et al., 2006). These results suggested that there is functional redundancy between *MAP3Kε1* and *MAP3Kε2* during pollen development. Because *map3kε1;map3kε2* pollen is non-viable, however, it is not straightforward to generate a plant that is homozygous double-mutant for these T-DNA null alleles. For this reason one cannot directly explore via genetic analysis the function of this pair of potentially redundant genes in the sporophyte.

In order to overcome this challenge we needed an experimental strategy that would allow *map3kε1;map3kε2* double-mutant pollen to be viable, which would in turn allow a homozygous double-mutant sporophyte to be formed. The strategy that we chose was to place the wild-type *MAP3Kε1* coding region under the transcriptional control of an alcohol-inducible promoter system and induce expression of *MAP3Kε1* during pollen and embryo development so that these processes could proceed normally. After seed had been produced by these plants, the progeny could be grown under conditions that do not induce expression of the *MAP3Kε1* transgenic construct, thereby allowing us to observe any phenotypic consequences of the reduced levels of *MAP3Kε1* and *MAP3Kε2*.

We began by constructing a plasmid in which a YFP tag is translationally fused to the wild-type *MAP3Kε1* coding sequence (CDS) under the transcriptional control of the ethanol-inducible *alcA* promoter (Figure 1A; Caddick et al., 1998; Roslan et al., 2001; Deveaux et al., 2003). We included the YFP tag on this construct so that we could monitor YFP-*MAP3Kε1* expression at the protein level via fluorescence microscopy. This construct was then introduced via *Agrobacterium*-mediated transformation into plants that were homozygous for *map3kε2* and heterozygous for *map3kε1*. PCR-based genotyping was used to identify *map3kε1<sup>-/+</sup>;map3kε2<sup>-/-</sup>* primary transformants that were also

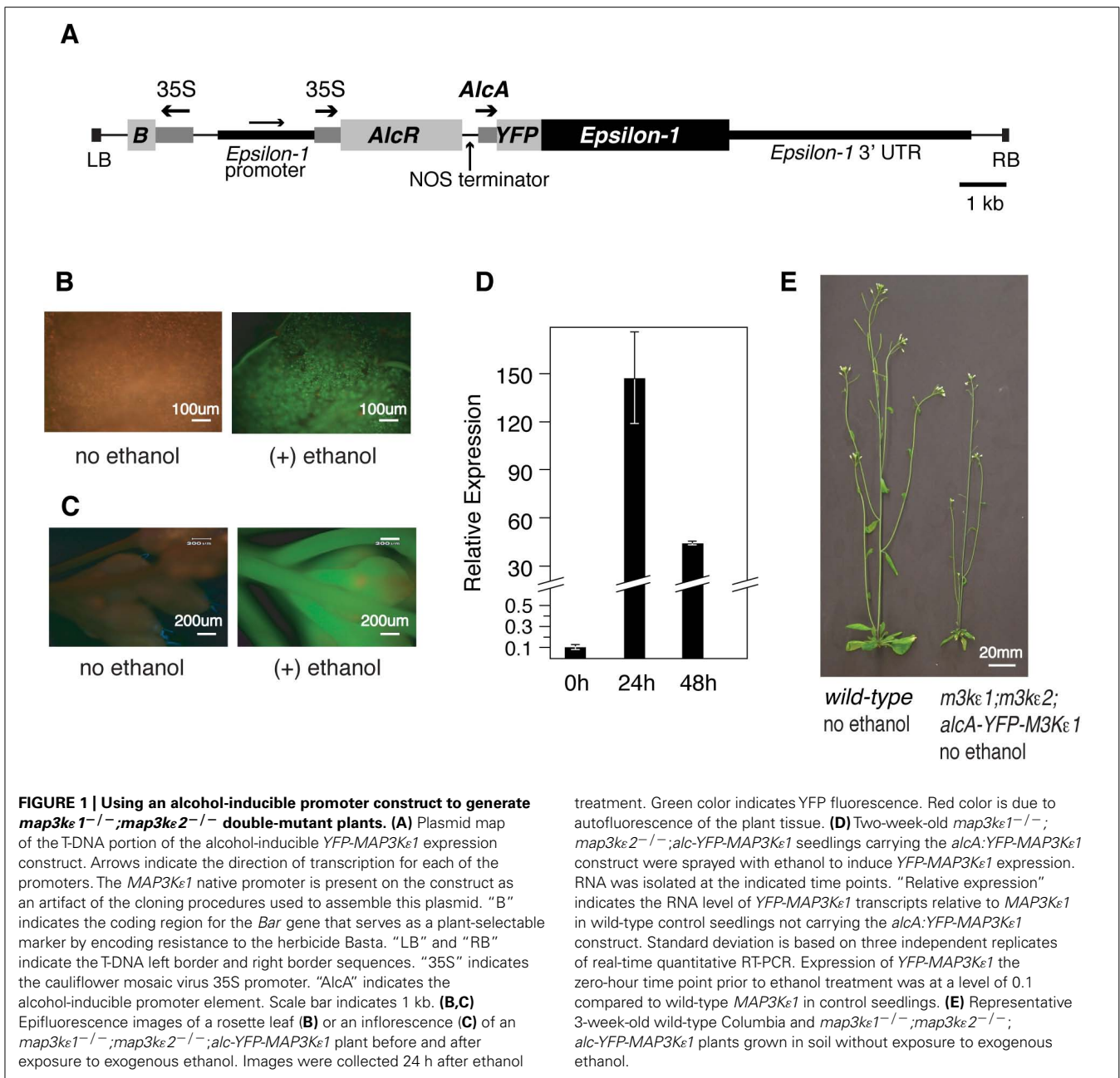
carrying the *alcA:YFP-MAP3Kε1* construct. In order to determine if these plants carried a functional copy of the *alcA:YFP-MAP3Kε1* construct, we monitored YFP-*MAP3Kε1* protein expression using fluorescence microscopy. Twenty-four hours after treatment with a 1% ethanol spray, strong induction of YFP fluorescence was observed throughout the aerial tissue of the plants, including leaves and flowers (Figures 1B,C), indicating that the *alcA:YFP-MAP3Kε1* construct present in these lines was performing as expected.

In order to rescue the lethal phenotype displayed by *map3kε1;map3kε2* double-mutant pollen, developing flowers of *map3kε1<sup>-/+</sup>;map3kε2<sup>-/-</sup>;alcA:YFP-MAP3Kε1* plants were sprayed with 1% ethanol on a daily basis throughout their life-cycle in order to activate expression of *alcA:YFP-MAP3Kε1* and allow double-mutant pollen grains to survive. Progeny from these ethanol-treated plants were genotyped and several *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alcA:YFP-MAP3Kε1* individuals were identified. These results indicated that the *alcA:YFP-MAP3Kε1* construct was able to rescue the *map3kε1;map3kε2* pollen lethal phenotype, and that the YFP tag did not interfere with *MAP3Kε1* function during pollen development.

Progeny derived from eight independently transformed *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* lines were screened using quantitative reverse-transcriptase PCR to find the line with the lowest background level of YFP-*MAP3Kε1* expression. In the absence of ethanol treatment, the double-mutant line chosen for phenotypic analysis in this study expressed YFP-*MAP3Kε1* at a level that was 10-fold lower than that of native *MAP3Kε1* expressed in wild-type plants (Figure 1D). When treated with exogenous ethanol, seedlings carrying this construct displayed strong induction of the YFP-*MAP3Kε1* gene up to a level ca. 150 times higher than that of native *MAP3Kε1* expressed in wild-type plants (Figure 1D). When grown in soil in the absence of exogenous ethanol treatment, progeny of this *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* line had an overall reduction in the size of their rosette leaves and bolts (Figure 1E).

### THE REDUCED LEAF SIZE OF *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* PLANTS IS PARTIALLY RESCUED BY ETHANOL TREATMENT

In order to determine if the reduced size of rosette leaves seen in *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants grown in the absence of ethanol was due to the low level of *MAP3Kε1* expression in these plants relative to wild-type, we performed the following experiment. Two pots of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants were grown side-by-side, along with wild-type controls. One pot of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants was sprayed with 1% ethanol every 2 days throughout development, while the second was sprayed with water only. We also subjected wild-type Columbia plants to the same regimen of water and ethanol treatments to determine if ethanol treatment had any effect on the growth of wild-type plants. As seen in Figure 2A, treatment with ethanol did not affect the growth of wild-type plants. Spraying *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants with ethanol to induce YFP-*MAP3Kε1* expression, however, resulted the partial rescue of the reduced-leaf-size phenotype (Figures 2B,C).

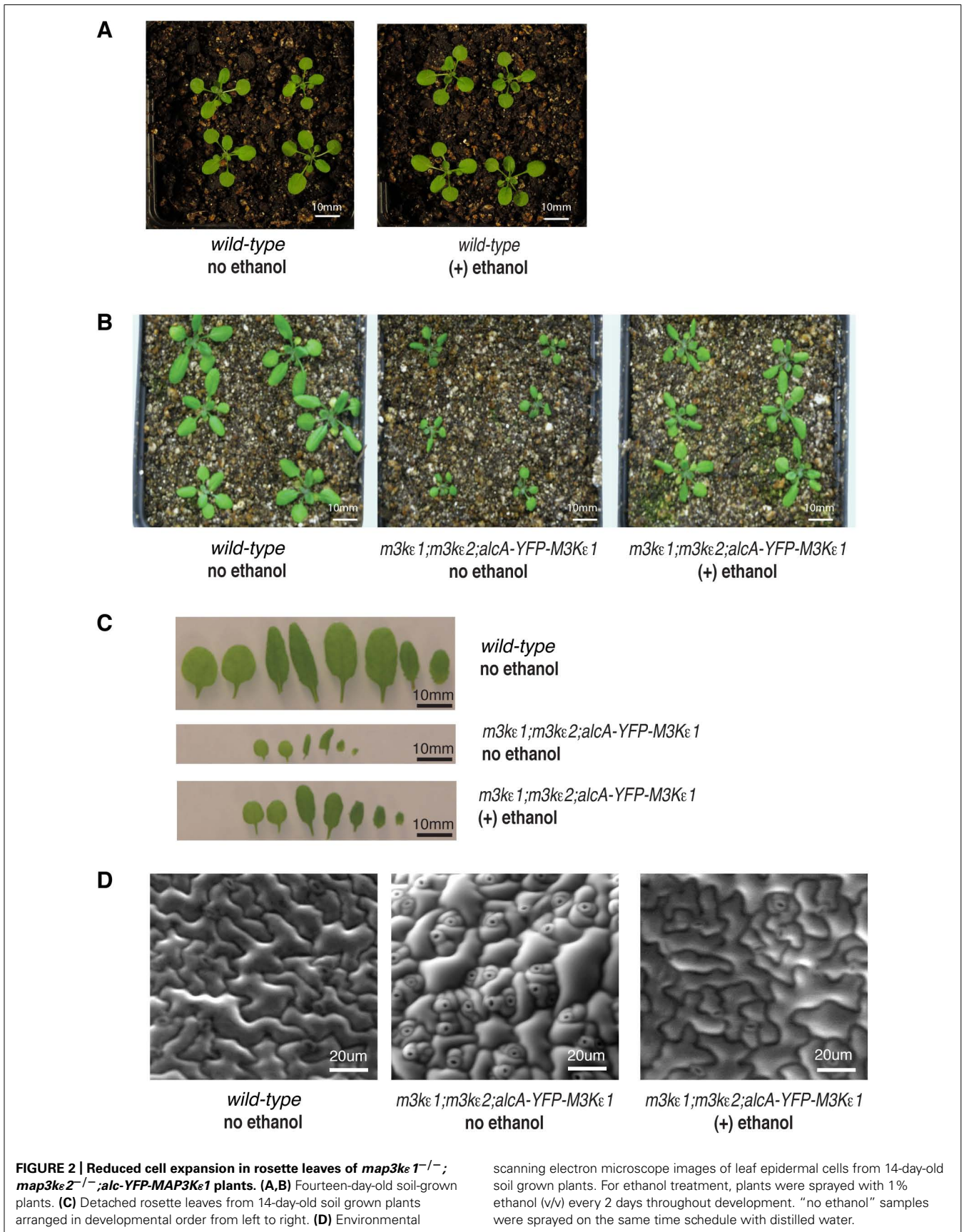


We also performed scanning electron microscopy of the leaf epidermis of *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* plants to observe the size and shape of the epidermal cells. This analysis revealed that the epidermal cells of untreated *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* plants were heterogeneous in size, with a majority of the cells being much smaller than those of wild-type (Figure 2D). Some relatively large cells were also observed in the untreated mutant lines, but these cells did not have the typical jig-saw shape of wild-type epidermal cells. These results indicate that *MAP3Kε1/2* may be important for the normal expansion of leaf epidermal cells. The cellular phenotypes displayed by the untreated *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* plants were partially rescued when the plants were

sprayed with ethanol every 2 days throughout development to induce expression of *YFP-MAP3Kε1* (Figure 2C).

#### ROOT LENGTH IS REDUCED IN *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* PLANTS

To investigate the role of *MAP3Kε1/2* in root growth, seeds of the *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* mutant line as well as a wild-type control were sown on agar plates containing growth media. Root length was then measured after 5 days of growth on vertically oriented plates. We observed that the primary roots of the *map3kε1*<sup>-/-</sup>;*map3kε2*<sup>-/-</sup>;*alc-YFP-MAP3Kε1* plants were ca. 40% shorter than wild-type (Figure 3A). In order to further investigate the contribution of *map3kε1*



and *map3kε2* to this phenotype, we performed a time-course experiment to measure root length over the course of 9 days of growth on vertical agar plates. As additional controls, this experiment included *map3kε1* and *map3kε2* homozygous single-mutant lines as well as the *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* line.

As shown in **Figure 3B**, the primary roots of *map3kε1* homozygous single-mutant lines were significantly shorter than those of wild-type. This phenotype is fully rescued by the presence of an ectopic copy of the wild-type *MAP3Kε1* genomic locus (**Figure 3B**). By comparison, the roots of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants were found to be even shorter than those of the *map3kε1* single-mutant.

We next attempted to use ethanol-induction to rescue the short-root phenotype of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants. We tested a variety of ethanol-treatment procedures but were not able to generate substantial *YFP-MAP3Kε1* expression in root tissue as determined by fluorescent microscopy, and no phenotypic rescue was observed.

#### REDUCED CELL EXPANSION IN *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* ROOTS

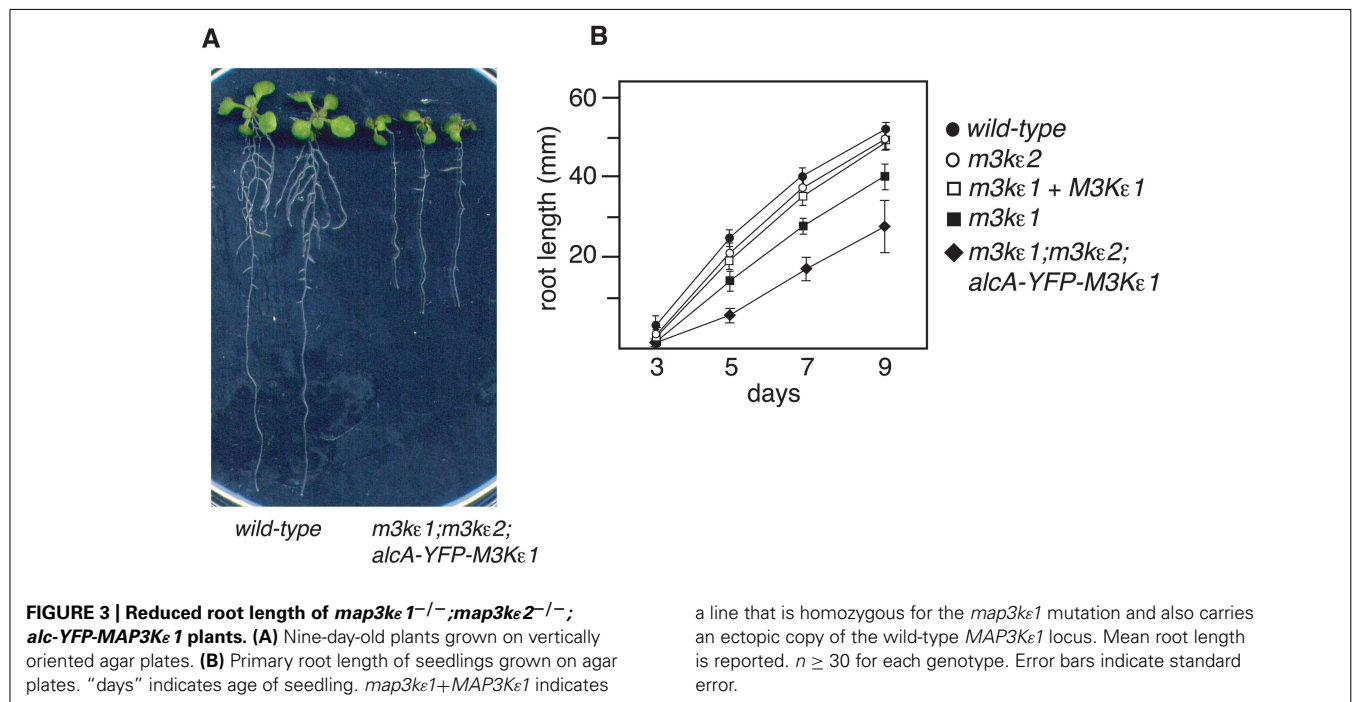
To further explore the cause of the short-root phenotype displayed by *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants, epidermal cell length was measured in three different zones of 7-day-old roots. These zones correspond to the root meristem, the elongation zone, and the mature zone where cell elongation has ceased (Cruz-Ramirez et al., 2004). Root epidermal cells of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* seedlings were shorter than wild-type in all of these zones. On average the root epidermal cells were ca. 20% shorter in the *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants when compared to wild-type, indicating that cell

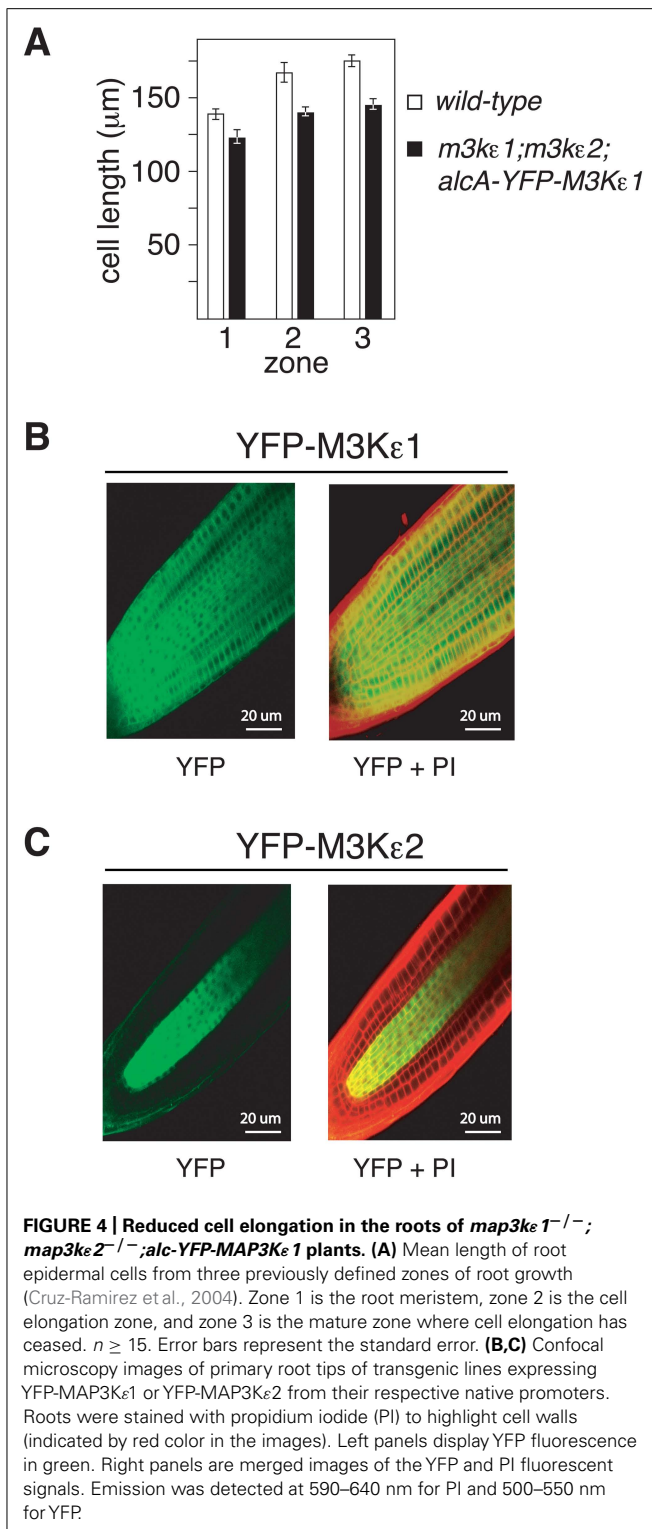
elongation is reduced (**Figure 4A**). Because the overall length of the primary root in *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants is ca. 40% less than wild-type (**Figure 3**), reduced cell elongation alone cannot fully account for the total reduction in root length. The mutant lines must also have fewer total cells in the primary root, suggesting a reduction in the rate of new cell production in the roots of *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants.

We also documented the expression pattern of *MAP3Kε1* and *MAP3Kε2* in *Arabidopsis* primary roots using YFP fusion constructs. *YFP-MAP3Kε1* and *YFP-MAP3Kε2* translational fusions under the transcriptional control of their respective native promoters were introduced into wild-type *Arabidopsis* plants via *Agrobacterium*-mediated transformation. Transgenic lines were generated and fluorescence microscopy was used to detect YFP-*MAP3Kε1* or YFP-*MAP3Kε2* expression. After seed germination, YFP-*MAP3Kε1* was strongly expressed throughout the root apical meristem (**Figure 4B**). By contrast, YFP-*MAP3Kε2* expression in the root apical meristem was largely restricted to vascular tissues (**Figure 4C**). These results indicate that *MAP3Kε1* and *MAP3Kε2* are both expressed in the root apical meristem region with overlapping, but distinct expression patterns.

#### THE *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* DOUBLE-MUTANT COMBINATION CAUSES EMBRYO LETHALITY

*map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants produce fewer seeds than wild-type when grown in the absence of exogenous ethanol (**Figure 5B**). The mutant plants produce an average of 15 seeds per silique, compared to 55 seeds per silique for wild-type (**Figure 5B**). Although *map3kε1;map3kε2* pollen is not viable (Chaiwongsar et al., 2006), Alexander's staining showed that pollen from *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1*





plants was viable in the absence of exogenous ethanol treatment, suggesting that the *alcA:YFP-MAP3Kε1* construct is expressed in the absence of ethanol treatment in developing pollen (data not shown). This phenomenon might be due to the fact that the ethanolic fermentation pathway is normally

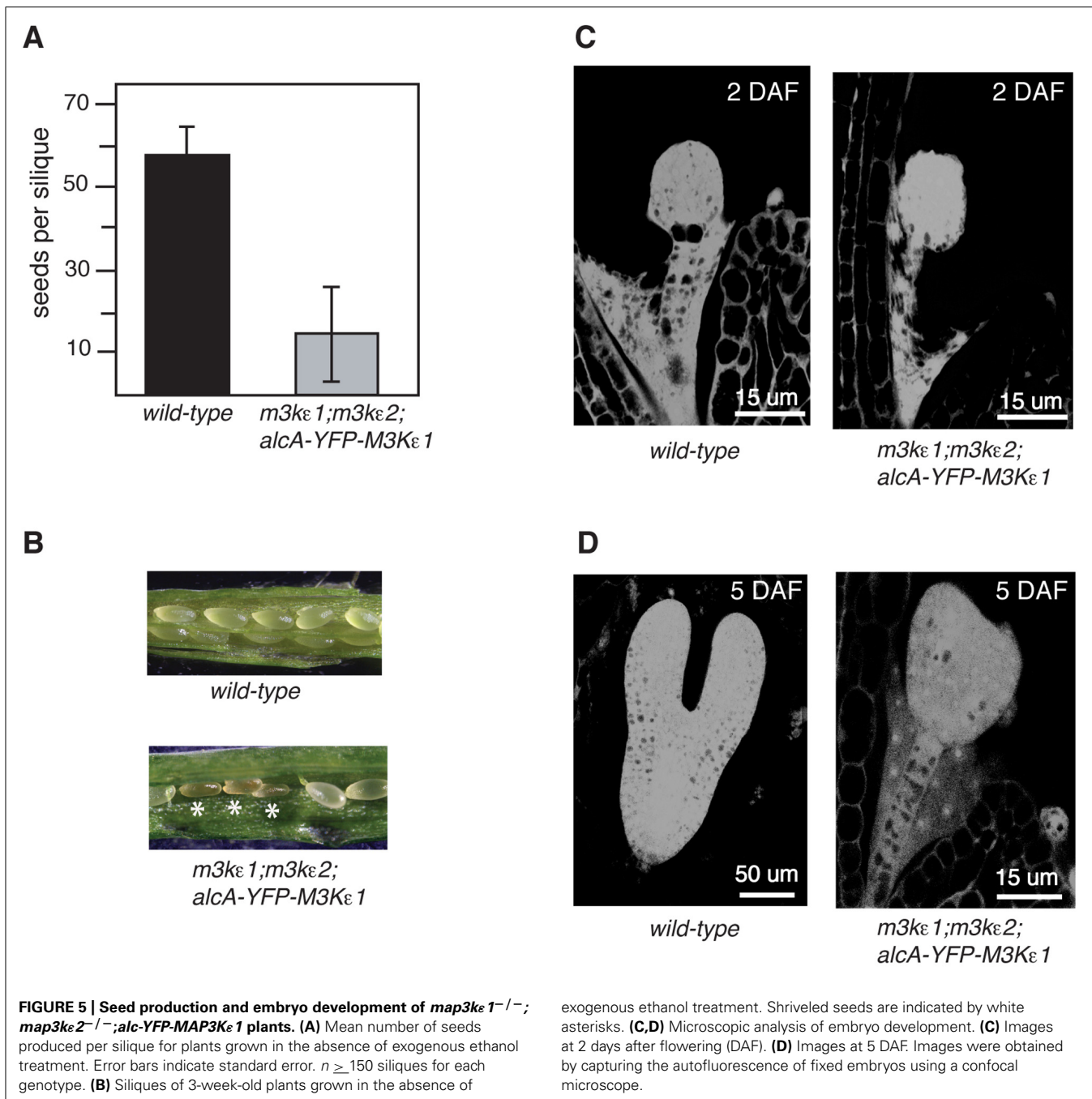
activated during pollen development (Mellema et al., 2002), resulting in the production of acetaldehyde and ethanol, which can both act as inducers of the *alcA* promoter (Tadege and Kühlemeier, 1997).

We have previously shown that *map3kε1;map3kε2* double-mutant female gametophytes develop normally (Chaiwongsar et al., 2006). Therefore, the decrease in seed production of the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants may be due to a disruption of seed development, rather than reduced pollen fitness. To test this possibility, *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants were grown and allowed to self-pollinate in the absence of exogenous ethanol treatment. Developing seeds from the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants were then analyzed microscopically. Siliques from wild-type and *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants were removed from the main shoot and compared. During normal *Arabidopsis* seed development, the external appearance of immature seeds changes from white to green, and then to brown at ca. 15–17 days after flowering (DAF). At 7 DAF, we observed that a portion of the seeds from the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants were smaller than wild-type and brown (Figure 5A). By 16 DAF, a majority of the seeds from *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* siliques appeared collapsed and shriveled (data not shown).

Immature seeds within a single wild-type silique normally develop at approximately the same rate (Sparkes et al., 2003). In wild-type plants grown in our laboratory conditions, the embryos reached the globular stage by 2 DAF and the torpedo stage by 5–6 DAF (Figures 5C,D). However, many of the embryos from *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants grown under the same conditions displayed delayed development. All of the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* embryos reached the globular stage at the normal time of 2 DAF, but at 6 DAF development was stalled at the globular or transition stage in many of the embryos (Figure 5D). At 7 DAF, most of the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* seeds had collapsed and the embryos were arrested at either the globular or heart stage (data not shown).

#### MAP3Kε1 AND MAP3Kε2 ARE EXPRESSED DURING EMBRYOGENESIS

The arrest of embryo development observed with *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* plants suggested that *MAP3Kε1*, *MAP3Kε2* play an important role in normal embryo development. If these proteins are directly involved in embryo development, then one would expect the proteins to be expressed in developing embryos. To directly explore this question we used the *YFP-MAP3Kε1* and *YFP-MAP3Kε2* translational fusion constructs described above to determine if these proteins are expressed in developing embryos. These constructs express the fusion proteins using the gene's respective native promoters. YFP fluorescence was detected via fluorescence microscopy in the developing embryos of plants expressing *YFP-MAP3Kε1* and *YFP-MAP3Kε2* (Figure 6). *YFP-MAP3Kε1* expression was observed throughout all the tissues of the embryo. By contrast, the expression pattern of *YFP-MAP3Kε2* was noticeably concentrated in the developing vascular cells (Figure 6).

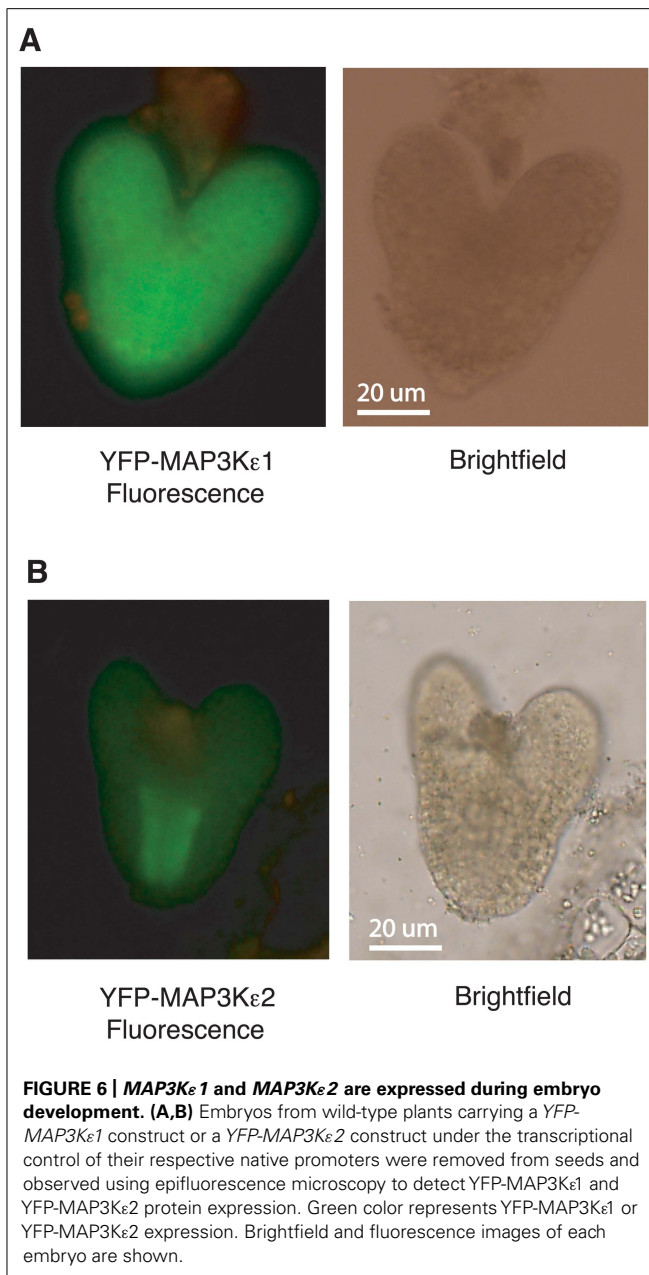


## DISCUSSION

We have previously shown that the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup> double-mutant combination causes pollen lethality (Chaiwongsar et al., 2006). In order to study the function of MAP3Kε1/2 in the sporophyte, we used an ethanol-inducible system to provide conditional expression of MAP3Kε1 during pollen development, thereby rescuing the *map3kε1*; *map3kε2* pollen lethality and allowing us to generate *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup> double-mutant plants. Using this system, YFP-MAP3Kε1 expression could be strongly induced by spraying plants with a solution of 1% ethanol. It should be noted that the basal level of expression from the

*alc-YFP-MAP3Kε1* construct in the absence of exogenous ethanol treatment was not zero. The *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* line that we chose for detailed study expressed YFP-MAP3Kε1 at a level that was 10% that of the wild-type MAP3Kε1 gene in wild-type plants. The presence of detectable transcription from the *alcA* promoter in transgenic plants in the absence of exogenous inducer is commonly observed and is possibly caused by the production of an endogenous inducer in tissue where the oxygen level is low (Roberts et al., 2005).

The residual expression of YFP-MAP3Kε1 by the *alc* promoter that we observed in our experiments highlights some of the



advantages and disadvantages of using the *alc* promoter to rescue pollen lethal mutations. Residual expression can be seen as a disadvantage of this system because it prevents one from obtaining a true null allele for the gene of interest, so one cannot observe the effect of the complete loss of a protein on the growth and development of the plant. Residual expression could also, however, be seen as an advantage of the system. In cases where the complete absence of a gene product causes embryo lethality, residual expression from the *alc* promoter may be able to provide enough gene product to allow the embryo to develop to maturity so that the effect of the partial loss of the gene product of interest can be evaluated in developing seedlings and plants. In the case of our study, the residual expression of *MAP3Kε1* driven by the *alc* promoter in

the absence of ethanol was sufficient to allow seedling germination and plant growth, but cell expansion in roots and leaves was compromised. The leaky expression of the *alc* promoter therefore allows one to produce what is effectively a “weak” allele of a locus for which a null allele would otherwise be lethal. If a true null allele is desired, then it may be possible to use other inducible expression systems such as the dexamethasone-inducible system, the beta-estradiol-inducible system, or a heat shock-inducible promoter in order to obtain a lower background level of gene expression (Borghini, 2010). Alternatively, a pollen-specific promoter (Twell et al., 1990) could be used to rescue the pollen-lethal mutation during pollen development, thus allowing for the formation of a homozygous-mutant embryo. If the basal expression level of the pollen-specific promoter in other stages of plant development was sufficiently low, then one would be able to observe the effect of loss of that gene product during embryo development and beyond.

*map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants were able to germinate and grow in the absence of ethanol treatment. We observed that these *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants had reduced primary root cell elongation and reduced epidermal leaf cell expansion. This reduced cell expansion may explain the overall small size of the mutant plants. The induction of YFP-*MAP3Kε1* expression in these plants via exogenous ethanol treatment could partially rescue the leaf phenotypes, indicating that the *map3kε1* mutation in the *map3kε2* background is responsible for these phenotypes.

The short-root phenotype of *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants could not be rescued by exogenous ethanol treatment. Despite the application of various concentrations of exogenous ethanol and various methods of ethanol application, including direct application or exposure of plants to ethanol vapor, we could not detect substantial expression of YFP-*MAP3Kε1* in root tissue, even though YFP-*MAP3Kε1* expression was strongly induced in the leaves of these same plants (data not shown). These results suggest that the ethanol-inducible construct used in these experiments may not be suitable for providing inducible gene expression in root tissue.

We have previously shown that *MAP3Kε1* is localized to the plasma membrane (Chaiwongsar et al., 2006), suggesting that *MAP3Kε1* may be involved in the process of cell expansion by performing an as of yet unknown function at the plasma membrane. This view is supported by the abnormality of the plasma membrane in *map3kε1*; *map3kε2* double-mutant pollen (Chaiwongsar et al., 2006). In addition, the thicker intine layer observed with *map3kε1*; *map3kε2* double-mutant pollen suggests that *MAP3Kε1* may also affect cell wall synthesis. Future studies will be needed to pinpoint the specific aspect of cell expansion that is influenced by *MAP3Kε1* and *MAP3Kε2*.

Using fluorescence microscopy and YFP-tagged fusion proteins, we observed that *MAP3Kε1* and *MAP3Kε2* were expressed in the embryos. A majority of the embryos that develop on *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants arrest their development at the globular or heart stages. Because *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants have a low basal level of transcription from the *alcA*:YFP-*MAP3Kε1* construct, it seems likely that viable seeds are able to form on *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc*-YFP-*MAP3Kε1* plants as a result



of slightly higher *alc-YFP-MAP3Kε1* activity in a given developing embryo. We frequently observed that the first siliques that develop on *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants yield more viable seeds than later siliques, but it is not clear what the mechanism responsible for this differential seed yield is.

In roots the expression of *MAP3Kε2* seems to be focused in the area of vascular tissue, while *MAP3Kε1* is more uniformly distributed across all tissue types. In addition, we observed that *map3kε1<sup>-/-</sup>* single-mutant plants have shorter roots than wild-type, whereas *map3kε2<sup>-/-</sup>* single-mutants are indistinguishable from wild-type. These results suggest that *MAP3Kε1* can compensate for the absence of *MAP3Kε2*, while the converse is not true. The embryo-lethality observed in *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>;alc-YFP-MAP3Kε1* plants indicates that absence of both gene products is lethal, suggesting substantial functional redundancy within this pair of genes. One interesting question raised by these results is how the expression of *MAP3Kε2* in the vascular tissue of roots is able to compensate for the absence of *MAP3Kε1* in the non-vascular tissues of *map3kε1<sup>-/-</sup>* single-mutant plants. It is possible that a low level of *MAP3Kε2* expression occurs outside of vascular tissue that is below the level of detection afforded by the visualization of YFP fluorescence. Alternatively, *MAP3Kε2* expression could be induced in non-vascular tissues in the *map3kε1<sup>-/-</sup>* mutant background. Further study will be required to determine the extent to which *MAP3Kε1* and *MAP3Kε2* have unique versus redundant roles in plant growth and development.

In this study we have demonstrated how an ethanol-inducible promoter system can be used to rescue a pollen-lethal mutation, thereby allowing one to study mutations in the sporophyte that would otherwise be lethal. Our initial analysis of the resulting *map3kε1<sup>-/-</sup>;map3kε2<sup>-/-</sup>* double-mutant plants indicated a role for these kinases in the processes of cell elongation and embryo development. Further studies will be needed to pinpoint the specific pathways in which *MAP3Kε1* and *MAP3Kε2* function. The work reported here provides a starting point for more detailed functional analysis of this pair of genes and also provides an example of the utility of the alcohol-inducible system for studying pollen-lethal mutations.

## MATERIALS AND METHODS

### PLANT GROWTH CONDITIONS AND MUTANT ALLELES

*Arabidopsis thaliana* (Columbia ecotype) were grown under continuous light at 22–25°C in soil. To activate expression of the alcohol-inducible *alcA-YFP-MAP3Kε1* construct in plants growing in soil, the plants were sprayed with 1% (v/v) ethanol every 2 days throughout development.

### PLASMID CONSTRUCTS

In order to create the *YFP-MAP3Kε1* fusion construct, a wild-type *MAP3Kε1* genomic clone previously described (Chaiwongsar et al., 2006) was modified using site-directed mutagenesis to add recognition sites for the restriction enzymes *AvrII* and *AgeI* immediately after the start codon. The YFP CDS was then PCR amplified from a plasmid vector using PCR primers that added an *NheI* site to the 5' end of the CDS and an *AgeI* site to the

3' end. This PCR-amplified fragment containing the YFP CDS was then ligated to the modified *MAP3Kε1* clone using sticky ends generated by *AvrII*, *AgeI*, and *NheI* cleavage. *NheI* sticky ends are identical to those produced by *AvrII*. The resulting construct contains the YFP CDS fused in frame to the 5' end of the *MAP3Kε1* coding region and retains the *MAP3Kε1* native promoter. The same strategy was used to construct the *YFP-MAP3Kε2* fusion construct. The resulting plasmids were introduced into plants using *Agrobacterium*-mediated transformation (Clough and Bent, 1998).

In order to construct the ethanol-inducible version of the *YFP-MAP3Kε1* fusion protein, recognition sites for the restriction enzyme *RsrII* were introduced immediately downstream of the ATG start codon of the *YFP-MAP3Kε1* coding region described above. A 3.7 kb fragment from the plasmid pBinSRNACATN (Caddick et al., 1998) was then PCR amplified using primers that added *RsrII* sites to the PCR product and cloned into the *RsrII* sites in *YFP-MAP3Kε1*. This 3.7 kb fragment contained an *AlcR* expression cassette as well as the *AlcA* promoter. The resulting plasmid contains the *YFP-MAP3Kε1* gene under the transcriptional control of the *AlcA* promoter (Figure 1A). This vector was introduced into *Arabidopsis* plants via *Agrobacterium*-mediated transformation (Clough and Bent, 1998).

### AGAR PLATE SEEDLING GROWTH ASSAYS

Seeds were surface sterilized with 95% (v/v) ethanol for 5 min, air dried, and sown on 0.5× Murashige and Skoog basal salt mixture media (pH 5.8; Sigma) with 0.7% agar (w/v). After sowing, seeds were allowed to imbibe in the dark at 4°C for 2 days and were then grown on vertically oriented plates under constant light at 22–24°C. To measure the root lengths, images were captured using a flat-bed scanner and analyzed with Adobe Illustrator software.

### MICROSCOPY

Confocal microscopy was used to collect images of root epidermal cells to measure cell length. For these experiments, cell walls were stained using propidium iodide (PI) and optical sections were obtained using a Zeiss LSM 510 Meta Confocal microscope (Zeiss, Thornwood, NY, USA). For PI staining, fresh plant tissue was transferred to a solution of 10 μg/mL of PI (Sigma) for 15–30 min. Imaging was performed using a 568-nm excitation line and an emission window of 585–610-nm. Epifluorescence images were captured digitally using an Olympus DP70 camera (Center Valley, PA, USA) attached to an Olympus BX60 epifluorescence microscope (Center Valley, PA, USA).

To detect YFP fluorescence in embryos, embryos were removed from the seed coats at various stages and viewed with an epifluorescence microscope. To study embryo development, embryos were fixed with 4% glutaraldehyde (in 12.5 mM cacodylate, pH 6.9) overnight. After fixation, the embryos were dehydrated through a 20, 40, 60, 80, and 100% ethanol series for 20 min per step. After dehydration, embryos were cleared in 1:1 (v/v) benzyl benzoate:benzyl alcohol for minimum of 2 h. Embryos were then mounted with glycerol and observed with a Zeiss LSM 510 Meta Confocal microscope with a 488-nm argon laser and an LP539 filter to detect autofluorescence.

## QUANTITATIVE RT-PCR

In order to measure the expression level of *YFP-MAP3Kε1* expression in the *map3kε1*<sup>-/-</sup>; *map3kε2*<sup>-/-</sup>; *alc-YFP-MAP3Kε1* lines, total RNA was isolated from rosette leaf tissue using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was prepared using 100 μg of total RNA with the Super Script First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). The resulting first-strand cDNA was diluted 1:100 with water and used as a template for real-time, quantitative RT-PCR amplification using an iCYCLER PCR system (BioRad, Hercules, CA, USA). SYBR Green (Molecular Probes, Eugene, OR, USA) was used to detect RT-PCR product accumulation. Primers for the actin gene

*ACT2* were used as a control (Genbank Accession: U41998). To detect *MAP3Kε1* expression, the predicted cDNA sequence of *MAP3Kε1* was used to design the following pair of PCR primers: ε1-RT-A1, 5'-AAAAACATTGTGAAGTATCTTGGGTCGTC-3'; ε1-RT-A2, 5'-GCTTCTTTACGAATTCGCGAGAACGATC-3' (Chaiwongsar et al., 2006).

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