

Arabidopsis MLK3, a Plant-specific Casein Kinase 1, Negatively Regulated Flowering and Phosphorylated Histone H3 in vivo

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Research article

Keywords: Arabidopsis, flowering, MLK3, phosphorylation, plant-specific casein kinase 1

Posted Date: October 17th, 2019

DOI: <https://doi.org/10.21203/rs.2.16145/v1>

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Abstract

Background: Casein kinase 1 (CK1) family members are highly conserved serine/threonine kinase present in most eukaryotes with multiple biological functions. Arabidopsis MUT9-like kinases (MLKs) belong to a clade CK1 specific to the plant kingdom and have been implicated collectively in modulating flowering related processes. Three of the four MLKs (MLK1/2/4) have been characterized, however, little is known about MLK3 , the most divergent MLKs.

Results: We demonstrated that compared with wild type, *mlk3* , a truncated MLK3 , flowered slightly early under long day conditions and ectopic expression of MLK3 rescued the morphological defects of *mlk3* , indicating that MLK3 negatively regulates flowering. GA 3 application accelerated flowering of both wild type and *mlk3* , suggesting that *mlk3* had normal GA response. The recombinant MLK3-GFP was localized in the nucleus exclusively. In vitro kinase assay revealed that the nuclear protein MLK3 phosphorylated histone 3 at threonine 3 (H3T3ph). Mutation of a conserved catalytic residue (Lysine 175) abolished the kinase activity and resulted in failure to complement the early flowering phenotype of *mlk3* . Interestingly, the global level of H3T3 phosphorylation in *mlk3* did not differ significantly from wild type, suggesting the redundant roles of MLKs in flowering regulation. The transcriptomic analysis demonstrated that 425 genes significantly altered expression level in *mlk3* relative to wild type. The *mlk3 mlk4* double mutant generated by crossing *mlk3* with *mlk4* , a loss-of-function mutant of MLK4 showing late flowering, flowered between the two parental lines, suggesting that MLK3 played an antagonistic role to MLK4 in plant transition to flowering.

Conclusions: A serine/threonine kinase encoding gene MLK3 is a casein kinase 1 specific to the plant species and represses flowering slightly. MLK3 located in nucleus catalyzes the phosphorylation of histone H3 at threonine 3 in vitro and an intact lysine residue (K175) is indispensable for the kinase activity. This study sheds new light on the delicate control of flowering by the plant-specific CK1 in Arabidopsis.

Background

Casein kinase 1 (CK1) family members were first characterized in the 1970s for their catalytic activity to phosphorylate casein *in vitro* [1]. These highly conserved serine/threonine-selective enzymes are ubiquitously expressed and play diverse cellular roles in eukaryotic organisms ranging from yeast to humans [2, 3]. During evolution, the *CK1* genes evolved an ancient duplication event, resulting in the split of the family into two subgroups, of which one contains members from plant species alone [2, 4]. *Mut9* from green algae *Chlamydomonas* was the founding member of the plant-specific *CK1* and the kinase predominantly phosphorylated histone H3 at threonine 3 [4].

In the last decade, lots of progress has been made on researches of *CK1s* from plants, especially Arabidopsis. The four *MUT9-LIKE KINASEs* (*MLK1-4*) have revealed to collectively impact Arabidopsis growth and development by involving in multiple physiological processes [5-9]. Previously, we found that

the *mlk1 mlk2* double mutant with pleiotropic defects including dwarfism, altered leaf shape and low fertility, was hypersensitive to osmotic stresses [10], suggesting the essentiality for plant growth and stress response. MLKs, also designated Photoregulatory Protein Kinases (PPKs), have been reported to implicate in the fine-tuning of plant response to solar radiation by coordinating the co-action of photoreceptors: phytochrome and cryptochrome [11]. For MLKs' involvement in phytochrome, the hint was from the observation that individual MLKs was co-purified with the circadian clock component ELF3 by affinity purification at the presence of phytochrome B (phyB) [12]. Ni and coauthors' finding that MLKs interacted and phosphorylated both phyB and phytochrom-Interacting Factor3 (PIF3) provided direct molecular evidence [13]. For the blue-light signaling, it was proved that phosphorylation of the blue-light receptor cryptochrome 2 (CRY2) was catalyzed by MLKs and the kinases functioned as interacting partners of CRY2 [11]. The physical structure analysis of MLKs revealed that both the conserved N-terminal domain and the non-kinase domain at C-terminal facilitated the interaction with PIF3 and CRY2 in response to red and blue light, respectively [11, 13].

Recently, MLKs, also described as Arabidopsis EL1-like (AEL) proteins, were documented to phosphorylate ABA signal receptors PYRABACTIN RESISTANCE/PYR-LIKE (PYR/PYL) proteins to promote their degradation by ubiquitination, and triple mutants of *mlks* were hypersensitive to ABA treatment due to eliminated ABA responses [8]. Interestingly, one of the triple mutants, *mlk1,3,4*, showed elevated level of salicylic acid (SA)-induced defense marker genes, and MLKs were identified as interacting proteins with RADICAL-INDUCED CELL DEATH1 (RCD1), a positive regulator of SA signaling [7]. Taken together, Arabidopsis *MLKs* collectively play critical roles in diverse biological processes including stress response, light signaling and immunity.

Besides the common roles shared by *CK1s*, several *CK1s* have been shown to involve in flowering regulation in both monocot and dicot. In rice, for example, mutation of *Early Flowering 1 (EL1)/Heading Date 16 (Hd16)*, a plant-specific *CK1*, caused plants flowered 5-6 days earlier than wild type (Dai and Xue 2010). *EL1/Hd16* has been proven to act as a multifunctional kinase to phosphorylate hierarchical flowering-regulating proteins, such as DELLA protein SLENDER RICE1 (SLR1), circadian clock component Pseudo-Response Regulator 37 (OsPRR37) and floral repressor GRAIN NUMBER, PLANT HEIGHT, AND HEADING DATE7 (Ghd7) [14-16]. On the other hand, Arabidopsis *mlk4* flowered late relative to wild type [12]. Recent study showed that MLK4 interplaying with circadian clock component CCA1 to accelerate flowering [17]. The two cases suggested the opposite roles of the plant-specific *CK1s* in regulating flowering time. This study investigated the function of *MLK3*, the most divergent *MLKs*, in flowering by focusing on a truncated *MLK3* mutant allele with slight early flowering phenotype. Our molecular, histochemical and genetic findings revealed *MLK3* function in flowering repression and the *in vitro* activity of the nuclear protein in catalyzing phosphorylation of H3 at threonine 3

Results

MLK3 with conserved kinase domains belongs to a separate subgroup of CK1s specific to plant kingdom

Previously, we have identified four plant-specific casein kinase 1 encoding genes, namely *MLK1-4* from Arabidopsis [10]. The four MLKs shared sequence identity of 67.9% - 91.1%. Homology analysis of MLKs and the homologs from several crops demonstrated that the plant-specific CK1s were classified into two main branches (I and II). MLK3, distinct from its paralogs, was grouped into a separate branch (Additional file 1, Figure S1). Consistently, the phylogenetic analysis showed that MLK3 was relatively distant from the other three MLKs (Fig. 1a). Sequence alignment showed that MLK3 shared the conserved CK1 functional domains including substrate recognition region, kinase catalytic loop, ATP binding site and a predicted nuclear localization signal [4] (Additional file 2: Figure S2). The predicted isoelectric point of MLKs ranges from 9.09 to 9.66 (Additional file 3: Table S1), suggesting the preference to acidic substrates, such as serine and threonine residues. Thus, albeit divergent from other MLKs in Arabidopsis, MLK3 possessed the common features of CK1, implying its enzymatic activity as a kinase in phosphorylating target protein(s).

***MLK3* was ubiquitously expressed and the *MLK3*-GFP recombinant protein was localized in the nucleus**

Three *MLKs* (*MLK1*, 2 and 4) have been functionally identified [17-19]. To investigate the biological role of *MLK3*, the most divergent *MLK*, we first examined its spatial and temporal expression patterns using semi-quantitative RT-PCR. As shown in Figure 1b, *MLK3* was expressed in roots, stems, leaves and flowers, which is in agreement with the results from eFP Browser (<http://bbc.botany.utoronto.ca/efp>) [20]. Comparison of the absolute transcription level demonstrated that *MLK3* transcript is the lowest, while *MLK4* is the highest, which is about 2.8-fold of *MLK3* (Additional file 4: Figure S3).

Considering that *MLK3* has a predicted nuclear localization signal (Additional file 2: Figure S2) [4, 10], the subcellular localization of *MLK3*-GFP recombinant protein was examined. As expected, when transiently expressed in tobacco leaves by infiltration, the green signal of the *MLK3*-GFP fusion protein was observed exclusively in the nucleus of the leaf epidermal cells as indicated by DAPI staining, while the signal of 35S::GFP control displayed a universal distribution in the epidermal cells of the tobacco leaves (Fig. 1c). Hence, consistent with the previous findings in tobacco and Arabidopsis protoplasts [8, 11, 12], *MLK3*, like its paralogs, is a nuclear protein, implying its potential role in histone modification.

MLK3* phosphorylated histone H3 at threonine 3 *in vitro

Given that nuclear protein *MLK3* shares the canonical features of CK1, we tested whether *MLK3* functions as protein kinase. First, *MLK3* was fused with maltose binding protein (MBP) and expressed in *E. coli* BL21 (DE3) strain. The purified *MLK3*-MBP recombinant protein was then incubated with the phosphoryl donor ATP and substrate before being dotted on membrane. Finally, immuno-blotting was performed using an antibody specifically against phosphorylated H3T3 [4]. Our results showed that strong immune-signal (bottom row) was detected with substrate H3T3ph peptide (a phosphor-histone H3 (Thr3) peptide) (Fig. 2a), which was used as positive control, confirming the specificity of the antibody. For substrate of unmodified histone H3 peptide (H3), anti-H3T3ph signal was detected when both ATP and the recombinant protein *MLK3*-MBP were present (middle row), while anti-H3T3ph signal was undetectable in the absence of *MLK3*-MBP fusion protein (upper row) (Fig. 2a). The detection of the phosphorylated

histone H3 at threonine 3 indicated that with ATP as phosphoryl donor, the recombinant protein MLK3-MBP catalyzed *in vitro* phosphorylation of unmodified histone H3 peptide substrate. Hence, MLK3 phosphorylated histone H3 at threonine 3 (H3T3ph), a predominant target of the plant-specific kinase Mut9 and MLK1 in *Chlamydomonas* and Arabidopsis, respectively [4, 10].

Conserved lysine (K) 146 of MLK3 is essential for MLK3-modulated *in vitro* phosphorylation of histone H3T3

It has been reported that the conserved lysine residue (K174 for Mut9p, K175 for MLK4/PPK1) was essential for the catalytic activity of the kinases [4, 11, 21]. To test whether the counterpart lysine (K146) of MLK3 is critical for phosphorylation of histone H3T3, the conserved K146 was point mutated to arginine (R). The MBP-fused MLK3 (K146R) was purified from *E. coli*, then the kinase activity was examined by dot blotting as mentioned above and the reaction without enzyme was served as negative control. Expectedly, H3T3ph peptide, the positive control, showed anti-H3T3ph signal in the immuno-analysis (bottom row) (Fig. 2b). No signal was detected for unmodified histone H3 peptide substrate with ATP served as phosphoryl donor, no matter the mutated recombinant protein MLK3 (K146R)-MBP was supplied or not (Fig. 2b). These results indicated that unlike MLK3, the point-mutated MLK3 (K146R) was catalytically inactive. Therefore, an intact lysine at the conserved position is crucial for the substrate phosphorylation mediated by the plant-specific CK1.

MLK3 affected leaf growth and flowering time

To address the biological function of *MLK3*, a T-DNA insertion line (SALK_017102) was obtained [10]. PCR analysis revealed that the T-DNA was integrated into the 12th exon of *MLK3*, resulting in a truncated peptide of 498 amino acid residues (Fig. 3a, b). In homozygous *mlk3* mutant, *MLK3* transcript flanking the insertion site was undetectable by RT-PCR, while a transcript upstream of the insertion site was detected (Fig. 3c), suggesting the partial expression of *MLK3*. Hereafter, the primers flanking the T-DNA insertion site were used to analyze the expression of *MLK3*. Morphologically, during vegetative stage *mlk3* was slightly smaller than wild type under LD (Fig. 3d). Measurement of the rosette leaf numbers showed that *mlk3* had 1.8, 2.4 and 3.2 fewer leaves on average than wild type in Week-2, 3 and 4, respectively ($P < 0.05$) (Fig. 3e), suggesting the progressive retardance of leaf growth in *mlk3*. Our calculation of leaf area (the 5th leaf) demonstrated that the fifth leaf of *mlk3* was about 2.4-5.0 mm² smaller than that of wild type during the three weeks ($P < 0.05$) (Fig. 3f).

For flowering time, Huang *et al.*, [12] revealed that in terms of the days to inflorescence at one centimeter, statistically *mlk3* had a minor fewer number of days than wild type, indicating *mlk3* flowered slightly earlier. Consistently, we observed that under LD, *mlk3* flowered at 19.2 days after germination (DAG), while wild type flowered at 22.3 DAG ($P < 0.05$) (Fig. 4a, b). Consequently, at five-week old *mlk3* displayed more siliques (16/plant vs 8/plant, $P < 0.01$) than wild type at the same stage (Fig. 4c). No abnormal flowering time was observed under short day (SD) conditions (Additional file 5 Table S2). Therefore, truncation of *MLK3* altered leaf growth and flowering time simultaneously under LD.

The negative role of *MLK3* in flowering regulation required the intact lysine (K) 146

To confirm the role of *MLK3* in flowering regulation, *MLK3* CDS driven by the 35S promoter was introduced into *mlk3* mutant. The transcriptional analysis of *MLK3* using semi-quantitative RT-PCR showed that distinct from *mlk3* mutant, *MLK3* transcript was detected in the transgenic *mlk3* plants expressing *35S::MLK3* (e.g. Line 8) (Fig. 5a), indicating the expression of *MLK3* in the transgenic plants. For flowering time, different from *mlk3*, the two independent transgenic lines (Lines 4 and 8) flowered at a similar time to wild type (Fig. 5b). In addition, the transgenic plants possessed a similar number of rosette leaves to that of wild type (Additional file 6: Figure S4). These results indicated that constitutive expression of *MLK3* rescued the morphologic abnormalities of *mlk3* in both leaf growth and flowering time.

To determine whether an intact K146 is critical for *MLK3*-mediated flowering, *35S::MLK3 (K146R)* was introduced into *mlk3* plants. RT-PCR showed that a similar intensity of *MLK3* transcript was detected in the transgenic plant and wild type (e.g. Line 6) (Fig. 5a). In contrast, the flowering time analysis of the two independent lines (Lines 3 and 6) demonstrated that similar to *mlk3*, DAG of the both lines was significantly fewer than wild type ($P < 0.05$) (Fig. 5b), suggesting that the transgenic lines flowered earlier. Consistently, the leaf number of the transgenic *mlk3* ectopically expressing *MLK3 (K146R)* did not significantly differ from that of *mlk3* (Additional file 6: Figure S4). These results indicated that unlike *MLK3*, which successfully restored the early-flowering phenotype of *mlk3*, the catalytically inactive *MLK3 (K146R)* expressed constitutively did not alter either flowering time or leaf growth. Therefore, the conserved lysine K146, which is essential for phosphorylation of H3T3, is indispensable for *MLK3*-mediated flowering repression.

Truncation of *MLK3* did not significantly affect the transcriptional level of the major flowering regulators nor the global intensity of phosphorylated H3T3

To profile the transcriptome of *mlk3*, RNA-sequencing was carried out. Consistent with the truncation of *MLK3*, the unique reads of *mlk3* matching the exons downstream of the T-DNA insertion site was clearly depleted compared with wild type, while no significant difference was monitored upstream of the T-DNA interruption (Additional file 7: Figure S5). By the criteria of $|\text{Log}_2\text{FC}| \geq 1$ and $P < 0.01$, a total of 425 genes were differentially expressed with 133 up-regulated and 292 down-regulated in *mlk3* relative to wild type (Additional file 8: Figure S6a). None of the transcript of *MLK3* paralogs was significantly changed in *mlk3*, implying no clear compensation of other *MLKs*. Based on gene annotation, no flowering regulator was differentially expressed in *mlk3* (Additional file 9: Table S3), suggesting that truncation of *MLK3* may not significantly affect the known flowering signaling components at the transcriptional level. Functional categorization of the DEGs based on gene ontology (GO) annotations revealed that the genes categorized to “negative regulation process”, such as “developmental process” and “multicellular organismal process”, were up-regulated, while the genes categorized to “positive regulation process” of the above two biological processes were down-regulated (Additional file 8: Figure S6b).

To compare the global level of H3T3ph between *mlk3* and wild type, western blot was performed with anti-H3T3ph antibody. The intensity of H3T3ph in *mlk3* was not notably different from that of wild type (Additional file 10: Figure S7), suggesting the functional redundancy of other *MLKs*, especially *MLK1* and *MLK2* [10]. Therefore, truncation of *MLK3* caused no significant alteration on either the transcriptional level of the main components of flowering pathway or the intensity of H3T3ph globally.

***MLK3* acts antagonistically to *MLK4* in regulating flowering time**

It has been documented that the loss-of-function mutant of *MLK4*, the closest paralog of *MLK3*, flowered late [12, 17]. To investigate the genetic relationship between *MLK3* and *MLK4* in flowering regulation, we generated the *mlk3 mlk4* double mutant by crossing the two single mutants (Fig. 6a). In homozygous *mlk3 mlk4*, the expression level of both *MLK3* and *MLK4* was eliminated to that of in the corresponding single mutants (Fig. 6b). Given that *FT* transcript was reduced significantly in *mlk4* mutant [17], the expression level of *FT* in *mlk3 mlk4* was tested by RT-qPCR. As shown in Fig. 6b, *FT* in *mlk3 mlk4* was about 60% of wild type, while in *mlk3* and *mlk4*, it was about 135% and 52% of wild type, respectively (Fig. 6b), indicating a compromised level of *FT* in *mlk3 mlk4* relative to its parental lines. Statistical analysis of the average DAG showed that *mlk3 mlk4* flowered at 30.2 DAG, while the two parental lines flowered at 19.7 DAG for *mlk3* and 32.8 DAG for *mlk4*, respectively (Fig. 6c), indicating that *mlk3 mlk4* flowered later than *mlk3* but earlier than *mlk4*. These results suggested that *MLK3* acted antagonistically to *MLK4* in regulating flowering time.

Discussion

Casein kinase 1 (CK1) is a universal and conserved ser/thr protein kinase family in eukaryotes [3]. In mammals, six CK1 isoforms (alpha, beta, gamma, delta and epsilon) have been reported to involve in a variety of cellular processes such as circadian rhythm, chromosome segregation and cellular differentiation by phosphorylating a wide range of key regulatory proteins [3]. Plant kingdom possesses an independent clade kinase evolutionarily related but phylogenetically distinct from mammalian CK1. The plant-specific CK1 family in higher plants expanded due to the lineage duplication event [4, 22]. Based on sequence analysis, green algae *Chlamydomonas reinhardtii* has one, namely *Mut9*, and Arabidopsis genome encodes four, rice six and soybean nine of the plant-specific *CK1* members, respectively [4, 22].

Several plant-specific *CK1s* have been documented to implicate in the transition from the vegetative to the reproductive stage through flowering pathways, such as phytohormone GA signaling, circadian clock and light signaling [5, 16, 23]. Consequently, flowering time was affected by mutation of the CK1 encoding genes in model plant rice and Arabidopsis. On one hand, the rice mutant *early flowering1 (el1)*, which is deficient in a plant-specific casein kinase I, exhibited early flowering with slow leaf emergence rate [15, 16]. On the other hand, mutation of Arabidopsis *MUT9-LIKE KINASE4 (MLK4)* flowered late [12, 17]. These findings indicate the two plant-specific *CK1s* from rice and Arabidopsis oppositely regulate flowering time. Consistent with the former case, we found that truncation of *MLK3* slightly accelerated

flowering and the *mlk3 mlk4* double mutant generated by crossing *mlk3* with the late flowering mutant *mlk4* compromised the flowering time of the two parent lines. Therefore, we propose that *MLK3* is a negative flowering regulator and functions antagonistically to *MLK4*. The distinct functions of *MLK3* and *MLK4* in flowering probably attribute to the opposite effect on flowering integrator *FT*. Additionally, *MLK3*, compared with *MLK4*, had lower affinity with the blue-light receptor *CRY2* (1.9% vs 5.9% of relative abundance) [11]. Unsurprisingly, the latter one was co-purified with two of the evening complex components, *ELF3* and *ELF4*, while the former one interplayed with *ELF3* alone [11, 12]. Hence, the plant-specific *CK1s* act non-redundantly in regulating flowering time.

It appears that the intact kinase activity of *MLK3* is indispensable for Arabidopsis flowering regulation. Albeit relatively divergent from the other three *MLKs*, *MLK3* shared the common functional domains with a sequence identity ranging from 67.9% to 72.7% [4, 10]. As expected, the nuclear protein *MLK3*, like its homolog *MUT9* and *MLK1*, *in vitro* phosphorylated histone H3 at threonine 3 (H3T3), a predominant phosphosite of *Mut9* in *Chlamydomonas* [4, 10]. In contrast, the enzymatic activity of *MLK3*(K146R) was abolished by mutating a conserved lysine residue (K146). This is consistent with the elimination of the catalytic activity of *Mut9* or *MLK4/PPK1* caused by mutating the counterpart lysine, i.e. K174 for *Mut9* in *Chlamydomonas* and K175 for Arabidopsis *MLK4/PPK1*, respectively [4, 11]. These findings suggested that the plant-specific *CK1s* shared the similar catalytic structure and mechanism in phosphorylating target proteins. In agreement with the *in vitro* result, constitutive expression of *MLK3* but not the point-mutated *MLK3* (K146R) complemented the defects of *mlk3* in flowering. Thus, the intact enzymatic activity of *MLK3* was required for proper flowering repression.

Diverse proteins including histones and flowering signaling components have been documented to be phosphorylated by the plant-specific *CK1s*. For example, *MLK4* *in vitro* phosphorylated histone H2A on serine 95 although no notable difference of phosphorylated H2A S95 was detected between the late flowering *mlk4* mutant and wild type [17]. Our results that *MLK3* phosphorylated histone H3 at T3 *in vivo* and mutation of the kinase activity on H3T3 could not rescue the early flowering phenotype of *mlk3* suggest that *MLK3*-mediated phosphorylation of H3T3ph plays a critical role in flowering. In addition to histone proteins, recent studies on *mlk3/ppk4-combined MLKs/PPKs* triple mutants proved that *MLK3/PPK4*, together with its paralogs, targeted light signaling receptors or coordinators. For instance, the level of phosphorylated *PIF3*, a negatively regulator of flowering, was reduced in red-light hypersensitive triple mutant *ppk124* and *ppk134* [13, 24], suggesting that normal light-induced *PIF3* phosphorylation required the *MLKs/PPKs* collectively. For rice *CK1* protein *EL1*, *DELLA* protein *SLR1* was found to be phosphorylated specifically and *EL1* was required for the negative effect of *SLR1* on GA signaling including flowering regulation [16]. These findings support the notion that *MLK3*, together with its homologs, are involved in flowering regulation by phosphorylating a diverse spectrum of proteins. It would be interesting to identify *MLK3* target protein(s), especially the components of flowering pathways. Future study on loss-of-function mutant would provide further evidence of *MLK3* in Arabidopsis flowering regulation.

Conclusions

Arabidopsis MUT9-like (MLK) family of Ser/Thr kinase enzymes, specific to the plant lineage, has been implicated in multiple processes regulating flowering. Unlike *MLK4*, which promoted flowering, *MLK3* played a negative role in the shift to the reproductive phase. Albeit divergent from its paralogs, *MLK3* shared the conserved kinase activity in phosphorylating histone H3 at threonine 3, and *MLK3*-mediated phosphorylation was essential for flowering repression. Therefore, besides the collective involvement of the four *MLKs* in modulating the flowering pathway components, *MLK3* acted antagonistically to *MLK4* in flowering regulation.

Methods

Plant materials and growth conditions

Arabidopsis seeds of ecotype Col-0, the T-DNA insertion lines of GK-756G08 and SALK_017102 were obtained from Dr. Cerutti's lab at University of Nebraska-Lincoln [10]. Imbibed seeds were treated at 4 °C for three days before germination in soil. Plants were grown under normal conditions (21 °C) with long-day (LD) (16 h light/8 h dark) with light supplied at 100 $\mu\text{mol}/\text{m}^2/\text{s}$.

Plasmid constructs and plant transformation

For *pMLK3::GUS*, a fragment of 1.5 kb upstream of the transcription start site of *MLK3* was amplified from genomic DNA, and was cloned into pCAMBIA1301 vector using Pst I and Bgl II sites after confirmed by sequencing. For *35S::MLK3* construct, *MLK3* CDS was amplified from reverse transcribed cDNA. The confirmed sequence was ligated into pROK2 vector after digestion by BamH I and Sac I. For point mutation, the oligonucleotide primers containing the desired mutation of *MLK3* CDS were used according to the instruction of QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies). Primers were shown in Additional file 11: Table S4.

In vitro protein kinase activity assay

The open reading frame (ORF) of *MLK3* without the stop code (TGA) was re-amplified. The sequence confirmed amplicon was cloned into Not I and BamH I sites of vector pMAL-c5X (NEB). For generation of MLK3 (K146R)-MBP, the point-mutated *MLK3* plasmid was used as template with the same primers for MLK3-MBP. Sequence verified PCR product was digested by Not I and BamH I, then ligated into pMAL-c5X. The MBP fused protein was expressed in *E. coli* BL21 (DE3) strain. The recombinant protein was purified with Amylose Resin (NEB) after induction for 16 hours by IPTG (0.1 mM). Protein kinase activity was assayed by dot blotting with antibody against H3T3 phosphorylation (Upstate, 07-424) as described [10]. An unmodified histone H3 peptide (residues 1-21) biotin conjugate (Upstate 12-403) was used as substrate.

mRNA sequencing and data analysis

For mRNA-sequencing, rosette leaves from 11-day-old plants grown under LD were used to isolate total RNA with TRIzol reagent (Invitrogen). Three independent replicates were used for RNA extraction and library preparation according to the Illumina's protocol. The libraries were sequenced using a Genome Analyzer (Illumina) and the transcription analysis was processed with a regular RNA-sequencing workflow on bioconductor. Briefly, the reference genome of Arabidopsis (ftp://ftp.ensemblgenomes.org/pub/plants/release-42/fasta/arabidopsis_thaliana/dna/) was used to map the clean reads after trimming the raw read data by TopHat 2.1.1 [25]. Gene expression values were calculated and differentially expressed genes were determined by DESeq2 package [26]. Gene Ontology plotting was performed using Bioconductor packages ggplot2, clusterProfiler [27] and org.At.tair.db.

Immunoblot analysis

For dot blotting, reactions containing substrate, ATP and phosphatase inhibitor (Roche Applied Science) were carried out in liquid medium in the presence or absence of MLK3-MBP/MLK3 (K146R)-MBP at 30 °C for 60 min. One ul of the reactions was spotted on a nitrocellulose filter and air dried. Ponceau S stained membrane was pictured to indicate protein loading and was applied to regular immune blotting. For western blot, total protein isolated from two-week-old plants was separated on 15% SDS-PAGE. Antibody against H3T3 phosphorylation (Upstate, 07–424) was used with a modification-insensitive anti-H3 antibody (Abcam, ab1791) as an internal loading control.

Subcellular localization and nuclear staining

To generate MLK3-GFP fusion protein, the CDS of *MLK3* was cloned into pENTR/D-TOPO (Life Technologies) and recombined into destination vector pK7FWG2.0 using Gateway LR Clonase II Enzyme Mix (Life Technologies). The agrobacteria expressing *35S::GFP* or *35S::MLK3-GFP* were separately infiltrated into tobacco leaf with needle-free syringe. Subcellular localization was examined using a confocal laser scanning microscope (Olympus) 36 h after infiltration. VECTASHIELD with DAPI was used to stain the nucleus before capturing the image.

Accession gene IDs

MLK3/PPK4 (At2g25760), *MLK1/PPK2* (At5g18190), *MLK2/PPK3* (At3g03940) and *MLK4/PPK1* (At3g13670).

Abbreviations

CK1: casein kinase 1

MLKs: MUT9-like kinases

PPKs: photoregulatory protein kinases

AEL1: Arabidopsis *early-flowering 1*-like

H3T3ph: phosphorylation of histone H3 at threonine 3

DAG: days after germination

Declarations

Acknowledgements

We thank Dr. Heriberto Cerutti (University of Nebraska-Lincoln) for kindly providing the Arabidopsis seeds and Dr. Changbin Chen (University of Minnesota) for critically reading the manuscript.

Authors' contributions

ZW and QY designed the experiments; ZW, JK, TZ and RY performed experiments; SJ and Z-HW analyzed the data; ZW and JK wrote the paper. All authors read and approved the final manuscript.

Funding

This work was financed by the National Natural Science Foundation of China (31772663), the Agricultural Science and Technology Innovation Program (ASTIP-IAS-TS-14) and the Fundamental Research Funds for Central Non-profit Scientific Institution (2017ywf-2d-3). The funding bodies did not play a role in the design of the study, collection, analysis and interpretation of data, or in writing the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

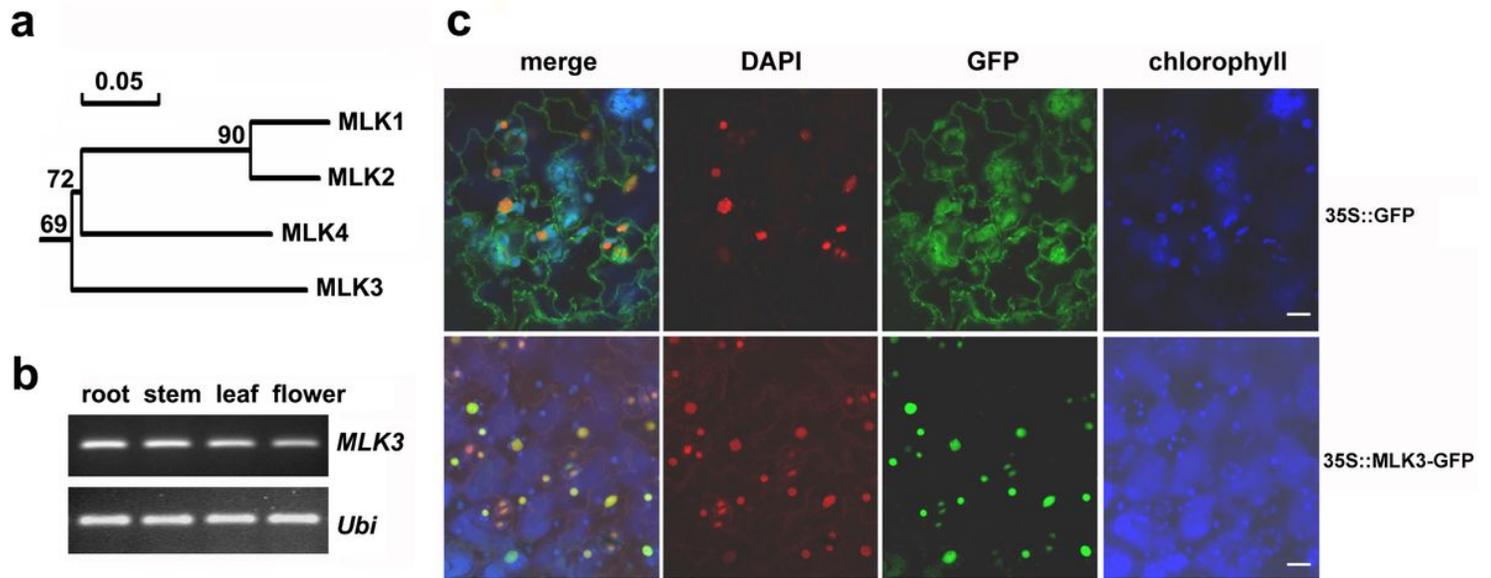


Figure 1

Phylogenetic analysis and expression pattern of MLK3 (a) Phylogenetic analysis of the four MLKs using DNAMAN (version 7.0). (b) Expression analysis of MLK3 transcript by RT-PCR. Total RNA of roots and leaves was isolated from 2-week-old plants, and total RNA of stems and flowers was from mature plants. POLYUBIQUITIN 10 (At4g05320) was used as an internal standard. (c) Subcellular localization of MLK3-GFP fusion protein in tobacco leaf. Agrobacterium expressing 35S::MLK3-GFP and 35S::GFP was infiltrated into tobacco leaves separately. Transiently expressed GFP was imaged by confocal fluorescence microscopy (Olympus) 36 hours after the infiltration. Nucleus was indicated by DAPI staining. Bars= 10 μ m.

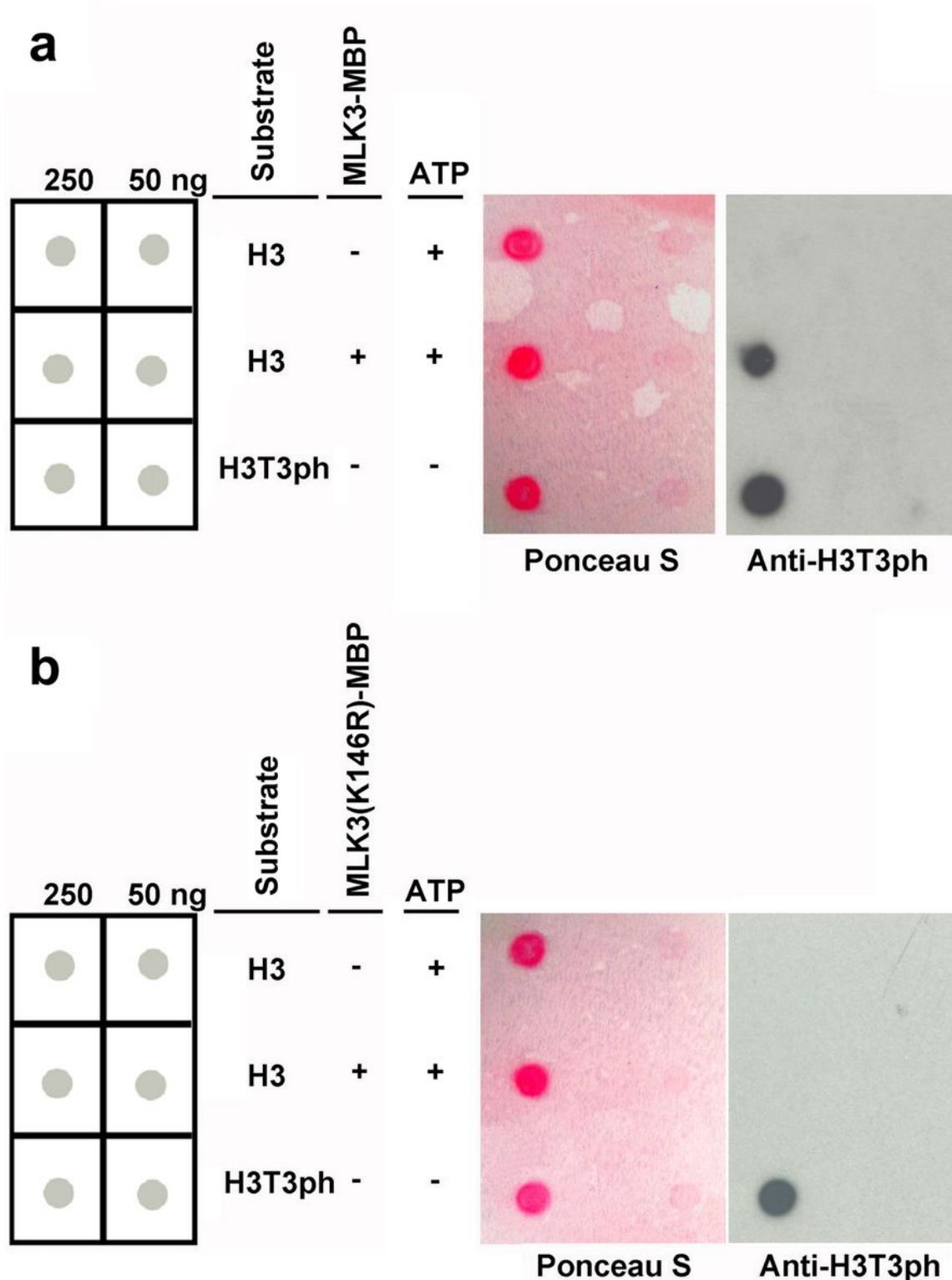


Figure 2

In vitro kinase activity assay of MLK3 The kinase activity assay of the recombinant protein MLK3-MBP (a) and MLK3 (K146R)-MBP (b) on H3 at threonine 3 by dot blotting. MLK3 and MLK3 (K146R) were fused with maltose binding protein (MBP) separately. H3, an unmodified histone H3 peptide (residues 1-21), biotin conjugate (Upstate 12-403) was used as substrate. The reaction mixture was spotted on a nitrocellulose membrane and phosphorylation was examined by immune-blotting with an antibody

against H3T3ph (Upstate, 07–424). Peptide H3T3ph, phospho-histone H3 (Thr3) peptide (residues 1-21), biotin conjugate, synthesized by BIOMATIK Corporation, was used as positive control. Two dosages of peptide (250 ng and 50 ng) were dotted on the membrane in parallel as indicated.

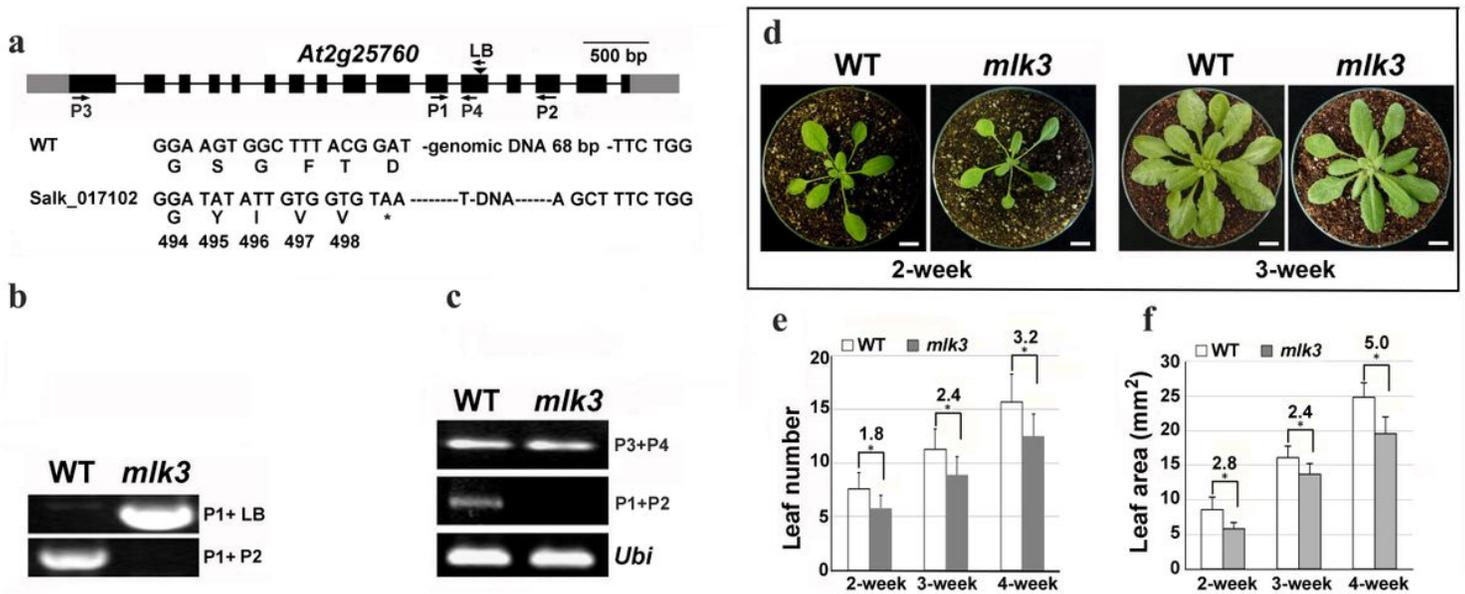


Figure 3

Leaf growth was retarded in *mlk3* by truncation of MLK3 (a) Schematic representation of MLK3 (*At2g25760*) locus with a T-DNA insertion. Introns, exons and un-translated regions were represented as lines, filled boxes and gray boxes, respectively. The triangle indicated the T-DNA insertion site of *mlk3* (SALK_017102). Arrows indicated the locations of primers (LB, P1 & P2, P3 & P4) used for genotyping and the transcript detection. Translation of MLK3 was listed in wild type and *mlk3* mutant with star representing the stop codon. (b) Genotyping of *mlk3* by PCR with indicated primer combinations. (c) Transcriptional analysis of MLK3 by RT-PCR using primers of P1 & P2 and P3 & P4 as indicated in (a). (d) Images of wild type and *mlk3* plants of 2-week-old and 3-week-old. Bar = 1 cm. (e) Comparison of the leaf numbers between wild type and *mlk3* in the indicated time points. * indicated the significant difference from wild type (Student's t-test, $P < 0.05$). Leaf number difference between the two genotypes was indicated. Leaf with petiole was counted from 15 plants of each genotype and three replicates were conducted independently. (f) Comparison of leaf area between wild type and *mlk3* in the indicated time points. The 5th leaf from 15 plants of each genotype was measured and leaf area was calculated using ImageJ (<https://imagej.nih.gov/ij/download.html>). Three batches of plants were analyzed independently. The leaf area with significant difference between the two genotypes was indicated as * (Student's t-test, $P < 0.05$). The difference of leaf area between the two genotypes was indicated (mm²).

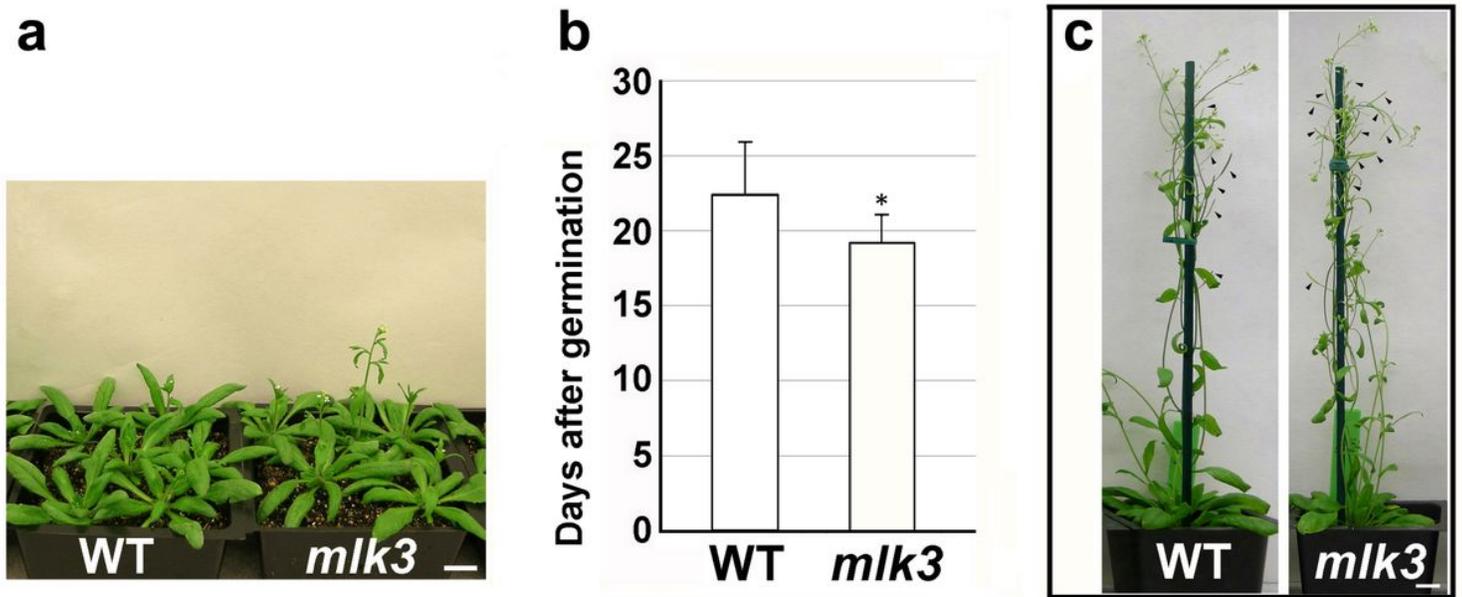


Figure 4

Truncation of MLK3 caused slight early-flowering (a) Representative image of 24-day-old plants grown under LD. Bar = 2 cm. (b) Flowering time analysis. Days after germination (DAG) was measured as the number of days from germination to the emergence of the first flower under LD. Plants ($n > 30$) were counted from three independent replicates. * indicated the significant difference from wild type (Student's t-test, $P < 0.05$). (c) Representative image of mature plants (five-week-old) under LD. Arrowheads indicated young siliques. Bar = 1 cm.

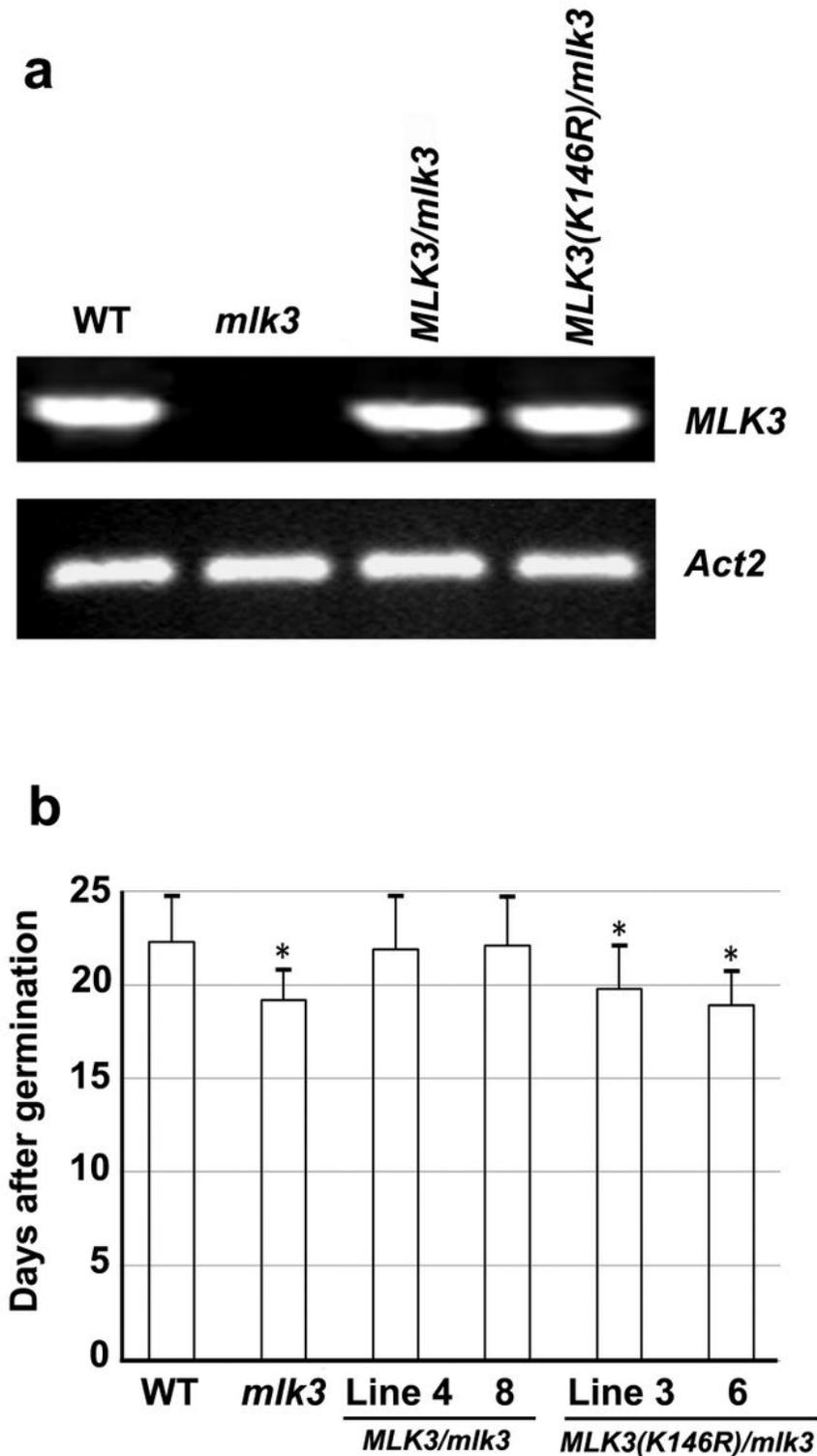


Figure 5

Analysis of MLK3 transcript and flowering time analysis of *mlk3* plants expressing 35S::MLK3 or 35S::MLK3(K146R) (a) Transcriptional analysis of MLK3 by semi-quantitative RT-PCR. Total RNA was isolated from 10-day-old plants of the indicated genotypes. Line 8 and Line 6 were analyzed for the transgenic plants of 35S::MLK3/*mlk3* or 35S::MLK3(K146R)/*mlk3*, respectively, and Actin 2 (At3g18780) was used as internal control. (b) Flowering time analysis of the indicated transgenic plants under LD.

Two independent transgenic lines were analyzed for the transgenic plants. * indicated significant difference from wild type (Student's t-test, $P < 0.05$). Three biological replicates were performed with at least 15 plants in total and the Fig. represented mean \pm SD.

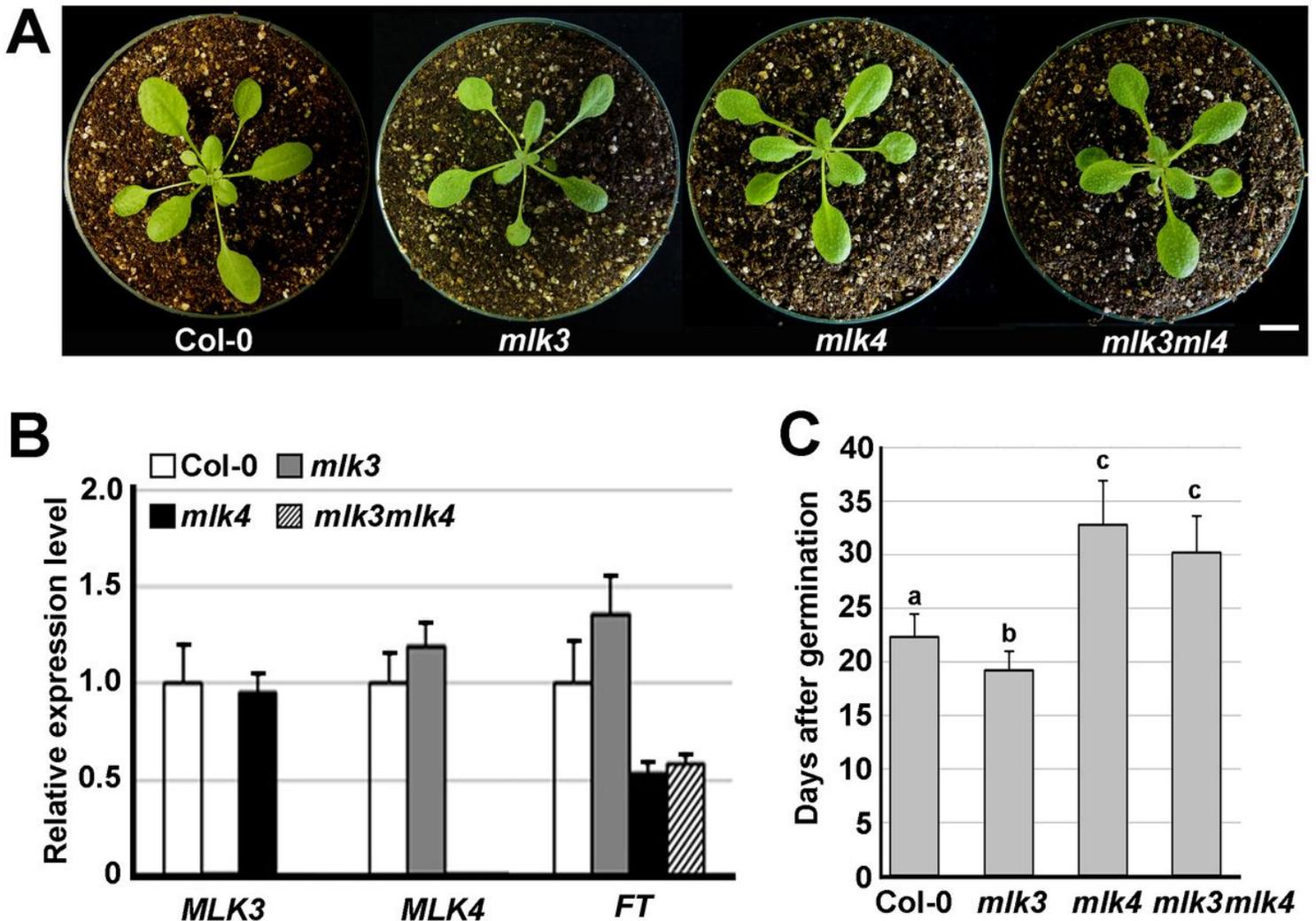


Figure 6

Analysis of flowering time and the gene expression level in *mlk3 mlk4* double mutant (a) Two-week-old plants of the indicated genotypes, scale bar = 1 cm. (b) Relative gene expression level in the four indicated genotypes with Actin 2 used as internal control. (c) The statistical analysis of DAG of the indicated plants under LD. DAG was counted from three independent batches of plants with about 15 plants for the individual line. Data represented mean \pm SD. Different letters indicated significant difference between the genotypes ($P < 0.05$).

Supplementary Files

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