

Full Length Research Paper

Casein kinase 1-Like 3 is required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis

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The homozygous T-DNA mutant of a casein kinase 1-Like 3 (*ckl3*) was identified. The quantitative real-time polymerase chain reaction (QRT-PCR) results showed that *CKL3* was highly expressed in flowers and roots, but less in stems, leaves and leafstalks. It was found that *CKL3* gene was induced by abscisic (ABA). When grown in the presence of increasing concentration of exogenous ABA, the *ckl3* mutant showed were more sensitive than wild type to the inhibition of seed germination and seedling root growth by applied ABA. In presence of all ABA, NaCl and mannitol concentrations tested, the germination percentage of *ckl3* mutant seeds was lower than that of wild type. In the presence of exogenous ABA, NaCl and mannitol, wild-type seeds showed higher germination percentages than the *ckl3* mutants at different stages of development. Wild type seedlings showed a reduced inhibition of root growth compared with *ckl3* plants under different ABA concentration treatment. Also, compared with wild-type plants, the expressions of the ABA and abiotic stress-responsive genes including *ABI1*, *ABI4*, *ABI5*, *ABF3*, *KIN1*, *RAB18*, *SOS3*, and *DREB1A* decreased, but *RD22* and *RD29B* increased in *ckl3* mutants. Taken together, these results suggested *CKL3* is required for abscisic acid regulation of seed germination, root growth and gene expression, and was involved in salt and osmotic stress response in the early development stage. This study provides important clues to casein kinase I activities in ABA signaling and plant development.

Key words: *Arabidopsis*, casein kinase 1-like 3 (*CKL3*) gene, phenotype, abscisic acid (ABA) signal transduction.

INTRODUCTION

The casein kinase 1 (CK1) family of monomeric serine-threonine protein kinases is found in eukaryotic organisms from yeast to humans. Mammals have seven family members including alpha, beta 1, gamma 1, gamma 2, gamma 3, delta and epsilon. CK1 is present in different cell types and in several subcellular compartments, including the membranes, nucleus and cytoplasm of eukaryotes and additionally in the mitotic spindle in mammalian cells (Fish et al., 1995). The family members have the highest homology in their kinase domains and

differ from most other protein kinases by the presence of the sequence S-I-N instead of A-P-E in kinase domain VIII (Hanks and Hunter, 1995). Outside the kinase domain, CKI family members fall into subfamilies that have little homology to each other and differ in the length and amino acid sequence of their N- and C-terminal extensions. CK1 members appear to have a similar catalytic activity and substrate specificity as predicted from their highly conserved catalytic domain. The S/Tp-X-X-S/T is one consensus phosphorylation site, where S/Tp refers to a phospho-serine or phospho-threonine, X refers to any amino acid and the underlined residues refer to the target site. Thus, this CKI consensus site requires priming by another kinase. CKI also phosphorylates a related unprimed site, which optimally contains a cluster

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of acidic amino acids N-terminal to the target S/T including an acidic residue at n-3 and a hydrophobic region C-terminal to the target S/T (Price et al., 2006). Furthermore, CK1 has no an activation mechanism controlled by second messengers, which is different from many other protein kinase families. Therefore, the distinct function of each isoform within the cell is achieved partially due to their subcellular targeting to specific compartments or tight association with the target molecules. Domain-swapping experiments about yeast CK1 isoforms show that the interaction between CK1 and its substrates is controlled by the ability of the kinase to associate stably with different subcellular compartments. There are four CK1 homologues in budding yeast, which are essential to yeast vitality. The YCK1/2, and 3 localize to the plasma membrane via palmitoylation and HRR25 localizes to the nucleus (Vancura et al., 1994; Wang et al., 1996). However, the non-redundant HRR25 and YCKs are shown to functionally complement each other when localization signals are swapped, supporting that the subcellular targeting of each isoform is an important determinant for its specific function.

CK1 isoforms function as regulators of multiple signal transduction pathways in most eukaryotic cell types. They are involved in Wnt signaling (Davidson et al., 2005; Price, 2006), circadian rhythms (Lee et al., 2009; Etchegaray et al., 2009), cell morphogenesis (Robinson et al., 1993), cell cycle progression (Behrend et al., 2000; Petronczki et al., 2006) and DNA repair (Dhillon and Hoekstra, 1994). The CK1 has been shown to regulate BR signaling in rice (Liu et al., 2003) and rice flowering time (Cheng and Xue, 2010). CK1 also regulates cell-to-cell communication (Lee et al., 2005) and modifies cell shape by phosphorylating tubulins in Arabidopsis (Lee et al., 2009; Ben-Nissan et al., 2008, 2010). There has been however, no any study on how CK1 is involved in the regulation of abscisic acid (ABA) signaling pathway.

The Arabidopsis genome encodes 14 casein kinase 1-like (CKL) isoforms from 13 genes that are distinctively localized to the cytoplasm, nucleus, endoplasmic reticulum, or uncharacterized punctuate structures (Lee et al., 2005). It is also different in overall expression patterns of each isoform at the cellular and tissue level (<https://www.geneinvestigator.ethz.ch>), indicating that CKL members likely perform non-redundant biological functions. The biological functions of most members of the CK1 family in higher plants remain unknown. Hence, in the present study, to understand the function of *CKL3* gene in Arabidopsis we isolated a *ckl3* mutant and investigated its possible phenotypes in ABA signaling pathways.

MATERIALS AND METHODS

Plant material and growth conditions

The T-DNA insertion mutant of *CKL3* gene in Arabidopsis was

identified in the Salk collection corresponding to donor stock numbers SALK_016571c, and it was named *ckl3* in the present study. Columbia-0 ecotype *Arabidopsis* was used as wild type (WT) and *ckl3* mutant for germination and gene expression analyses. The plants were routinely grown in a growth chamber under 40% humidity, a temperature of 22°C, and with a 16-h light/8-h dark photoperiod at 100 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of light. For *in vitro* culture, seeds were surface-sterilized in 70% ethanol for 10 min, soaked for 10 min in 7% NaClO and finally washed four times in sterile distilled water. Stratification of the seeds was conducted during day 3 at 4°C. Afterward, seeds were sowed on Murashige and Skoog (1962) plates containing solid medium composed of Murashige and Skoog basal salts and 1% (w/v) Suc, solidified with 1% (w/v) agar and the pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber.

Mutant identification by polymerase chain reaction (PCR) screening

The SALK_016571c line containing a single T-DNA insertion in *ckl3* was obtained from the Salk Institute Genomic Analysis Laboratory. To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from seedlings and submitted for PCR genotyping using the following *ckl3* primers: LP: 5'-CTTTGGT GAGAATTTCTCC-3'; RP: 5'-ACAATCGCGGTAC AGTAAACG-3'. The T-DNA left border primer of the pROK2 vector used was LBpROK2 (5'-TGGTTCACGTAGTGGGCCATC-3').

Abiotic stress

Plants were grown on Murashige and Skoog plates supplemented with 1.5% Suc. After two weeks, Arabidopsis seedlings were treated with different concentration of exogenous ABA, NaCl and mannitol, respectively. Plant materials were collected and frozen in liquid nitrogen for RNA isolation.

RNA extraction and quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was isolated using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd). First strand cDNA was reverse transcribed from DNase I-treated RNA with oligo (dT) as the primer. QRT-PCR analyses were performed using Sybr Green PCR Master mix (Applied Biosystems) in Mx3000P thermal cycler (Stratagene) and data were analyzed with MxPro software (Stratagene). The primers used are indicated in Table1. The QRT-PCR was carried out in a total volume of 25 μL containing 2 μL of the reverse-transcribed product above, 0.2 μM of each primer and 1x SYBR green PCR master mix (TaKaRa Co. Ltd.). The PCR protocol starts with a denaturing step for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and a primer extension reaction at 60°C for 1 min. All PCR reactions and the negative controls were run in duplicates with three biological replicates each.

Germination assays and root growth

To score seed germination, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% Suc and different concentrations of ABA (0.05, 0.15 or 0.3 μM). To determine sensitivity to inhibition of germination by high osmoticum, the medium was supplemented with different concentrations of salt (50, 100 or 150 mM) and 150 mM mannitol. Each value represents the average germination percentage of about 300 seeds at least three replicates.

Table 1. RT-PCR primer sequences.

Gene	Primers sequence (5' - 3')
<i>Actin-2</i>	F:CACTGTGCCAATCTACGAGGGT R:CACAAACGAGGGCTGGAACAAG
<i>CKL3</i>	F: AGCATCCTCAACTTCTCTATG R: AGCATTCTCTGTTCCATCAA
<i>RD22</i>	F:TTCGGAAAAGCGGAGAT R:CTTTGAAGGCCAAGTGGT
<i>RD29B</i>	F:AAGGAGACGCAACAAGGG R:ACGGTGGTGCCAAGTGAT
<i>KIN1</i>	F:CGCTGGCAAAGCTGAGGA R:TTCGGATCGACTTATGTATCGT
<i>ABI1</i>	F:TGGCTGGAGAAGTGAAGAAA R:CGGCGCAACTGACTCAATCT
<i>ABI4</i>	F:CGGTGGGTTGAGTCTATCAA R:CGGATCCAGACCCATAGAACA
<i>ABI5</i>	F: GGAGGTGGCGTTGGGTTT R:GGGCTTAACGGTCCAACCA
<i>SOS3</i>	F:GCCGGTCCATGAAAAAGTCA R:TCTTCTCGCTCGATGAATCCA
<i>DREB1A</i>	F:GGCGGGTCGTAAGAAGTT R:GATCCGTCGTCGCATCAC
<i>DREB2A</i>	F:CTGTTGAGACTCCTGGTT R:GAGGTATTCCGTAGTTGA
<i>ABF3</i>	F: AATGGCGGATTCTATGGATT R:GCATCTGTAGTGGCTGAG

The root growth assay for scoring ABA sensitivity was done by measuring root growth six days after the transfer of 6-day old seedlings onto vertical Murashige and Skoog plates containing 0 or 10 μ M ABA.

RESULTS

Analyses of *ckl3* mutant

Homozygous individuals were identified by PCR analyses. Sequencing of the T-DNA flanking region in *ckl3* showed that the insertion was localized 57 nucleotides downstream from the ATG start codon (Figure 1A). The numbers (1 to 10 lines) were identified

to be homozygote by PCR analysis (Figure 1B and C). In this study, the homozygous plants, namely, *ckl3* mutant, were selected for further studies.

Expression of *CKL3* gene in the different tissues

The expression profile showed that *CKL3* was highly expressed in flowers and roots, but less in stems, leaves and leafstalks (Figure 2A).

Expression of *CKL3* in response to ABA

The expression patterns for the *CKL3* gene were

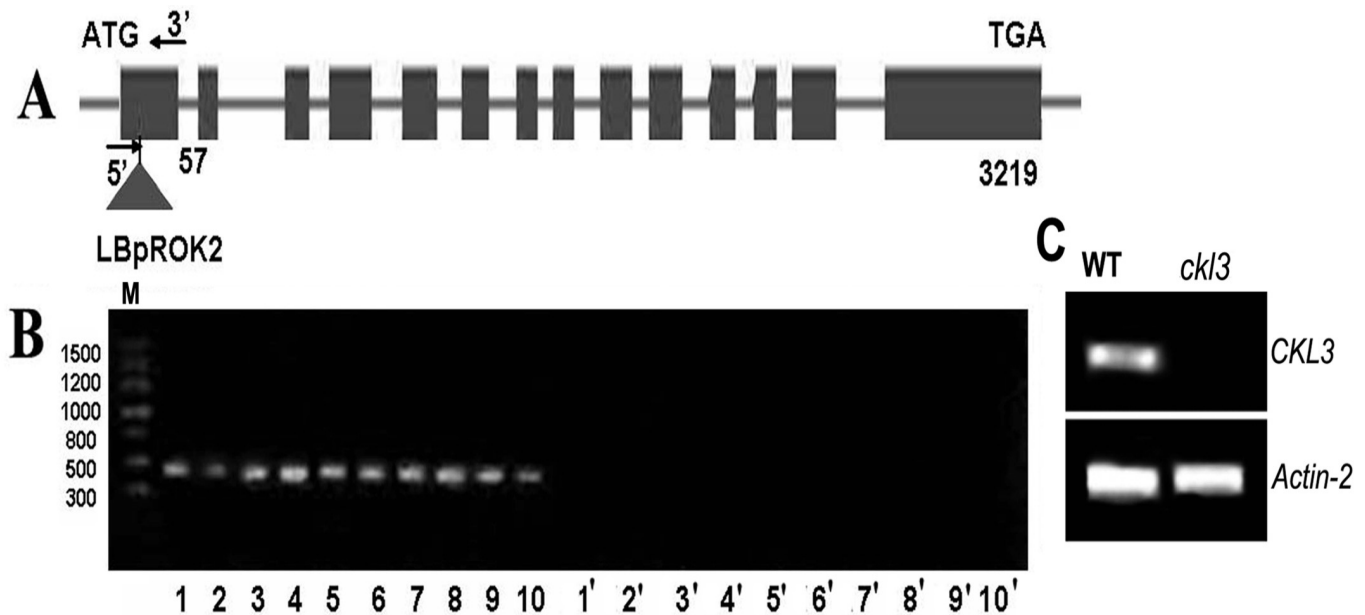


Figure 1. Identification of the *ckl3* with T-DNA insertion by PCR. (A), Scheme of the *CKL3* gene and localization of the T-DNA insertions in *ckl3* mutant. The numbering begins at the ATG translation start codon and arrows denote the direction and position of the PCR primers. LBpROK2, the T-DNA left border primer. (B), Genomic DNA PCR products generated from *CKL3* with various primer combinations are shown. Lanes 1 to 10 correspond to PCR products using the primers 5' + LBpROK2; lanes 1'-10' correspond to PCR products using the primers 5' + 3'; Lane M, DNA maker. (C), RT-PCR result shows absence of *CKL3* transcript in mutant genotype. *Actin-2* is used as an internal control.

observed under ABA stress (Figure 2B). It was noted that the 10 μ M ABA treatment induced an increase in mRNA level of the *CKL3* gene. The expression level increase of the *CKL3* gene could be observed with the ABA treatments after 1 h (Figure 2B) and it was highest after 2 h. These results show that the *CKL3* gene was likely to be a regulation factor in ABA signaling pathway.

Phenotype analysis

Progeny of *ckl3* homozygous individuals was harvested and different analyses to test their sensitivity to ABA, NaCl and mannitol were performed. First, the sensitivity of the mutant to inhibition of seed germination was analyzed (Figures 3 to 5). In the presence of 0.05 μ M ABA, the average seed germination percentage of wild type was higher than that of *ckl3* mutants on the second and third day after 3 days of cold treatment, and after 4 days, *ckl3* mutant seeds showed a germination ratio similar to wild type (Figure 3). In the presence of 0.15 or 0.3 μ M ABA, the average seed germination percentage of wild type was higher than that of *ckl3* mutants from the second to seventh day after 3 days of cold treatment (Figure 3). More also, in the presence of 50 or 100 mM NaCl, the average seed germination percentage of wild type was higher than that of *ckl3* mutants from the second to seventh day after 3 days of cold treatment (Figure 4). In addition, in the presence of 150 mM NaCl,

the average seed germination percentage of wild type was higher than that of *ckl3* mutants from the third to seventh day after 3 days of cold treatment (Figure 4). While in the presence of 150 mM mannitol, the average seed germination percentage of wild type was higher than that of *ckl3* mutants from the second to seventh day after 3 days of cold treatment (Figure 5).

Furthermore, the root growth assay was analyzed for scoring ABA sensitivity after 6 days of the transfer of 6-day old seedlings onto vertical Murashige and Skoog plates containing 0 or 10 μ M ABA. The *ckl3* seedlings showed an increased inhibition of root growth compared with wild-type plants (Figure 6).

Expression of ABA and abiotic stress responsive genes

ABA induces the expression of many genes that are important for adaptation to stress. The expression of the *CKL3* gene can be induced by the ABA (Figure 2B). Therefore, the expression of some ABA and abiotic stress-responsive genes was monitored by quantitative real-time PCR in the present study. The results show that compared with wild-type plants, the expression of the ABA and abiotic stress-responsive genes including *ABI1*, *ABI4*, *ABI5*, *ABF3*, *KIN1*, *RAB18*, *SOS3* and *DREB1A* decreased, while *RD22* and *RD29B* increased in *ckl3* mutants (Figure 7).

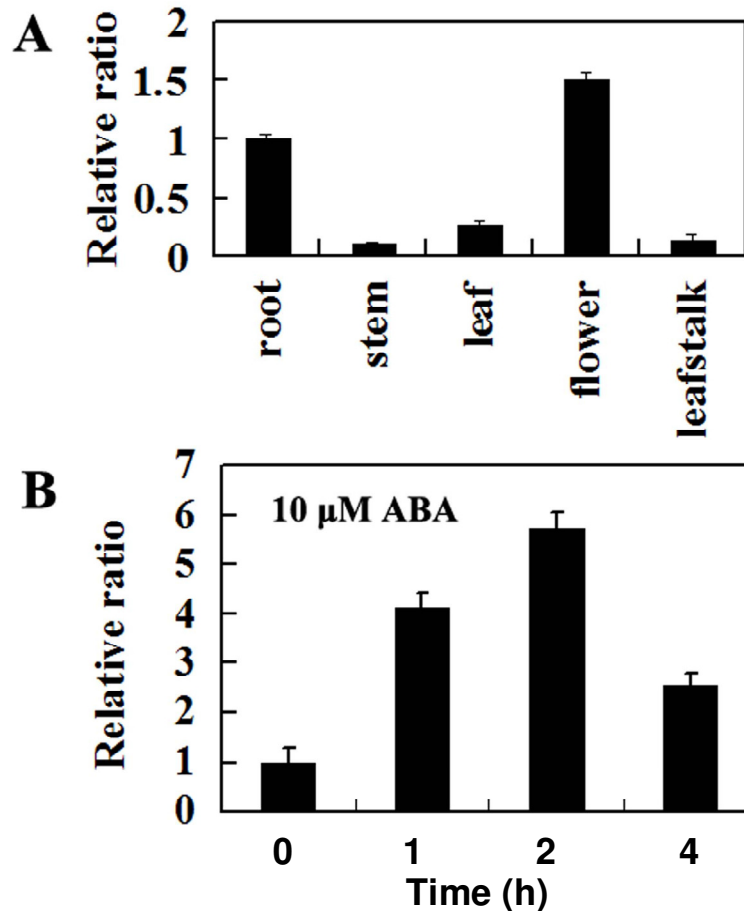


Figure 2. The Real-time RT-PCR analysis of *CKL3* gene. (A) The expression analysis of *CKL3* gene in stem, root, leaf, leafstalk and flower of 4-week old wild type plants. (B) The expression analysis of *CKL3* gene in whole seedlings of 2-week old wild type at various time points (h) post-treatment with 10 μ M ABA.

DISCUSSION

The CK1 members are evolutionarily conserved eukaryotic protein kinases and play fundamental roles in various cellular, physiological and developmental processes. The evolutionary conservation of these genes indicates their essential roles. Although, multiple CK1 isoforms are present in the plant genome, a few biological functions of CK1s have been reported. In rice, OsCK11 regulates root development (Liu et al., 2003). OsEL1, a casein kinase, is a novel regulator of GA signaling and has important functions in controlling rice flowering time by regulating GA responses (Cheng and Xue, 2010). In sesame, SeCK1 transcripts were predominantly expressed in developing seeds and were induced approximately threefold by exogenous application of ABA. SeCK1 acts as a positive regulator of the SeFAD2 promoter via phosphorylation of the SebHLH transcription factor (Kim et al., 2010). In *Arabidopsis* and tobacco, plasmodesmata-associated casein kinase I

(PAPK) specifically phosphorylates the C-terminal residues of tobacco mosaic virus movement protein (TMV MP), which may play a regulatory role in macromolecular trafficking between plant cells (Lee et al., 2005). *Arabidopsis* casein kinase 1-like 6 (CKL6), which contains a microtubule-binding domain, play a role in anisotropic cell growth and shape formation through the regulation of microtubule organization, possibly through the phosphorylation of tubulins (Ben-Nissan et al., 2008).

The ABA plays important regulatory roles in many plant stress and developmental responses throughout the plant life cycle, particularly in the ability to sense and respond to various unfavorable environmental conditions, including drought, salt, and cold stresses during vegetative growth. Although, previous studies indicated that CK1 members are involved in multiple signaling pathways including circadian rhythm, growth and morphogenesis, vesicular trafficking, DNA repair, cytokinesis, and cell cycle progression (Davidson et al., 2005; Price, 2006; Lee et al., 2009; Etchegaray et al., 2009; Behrend et al.,

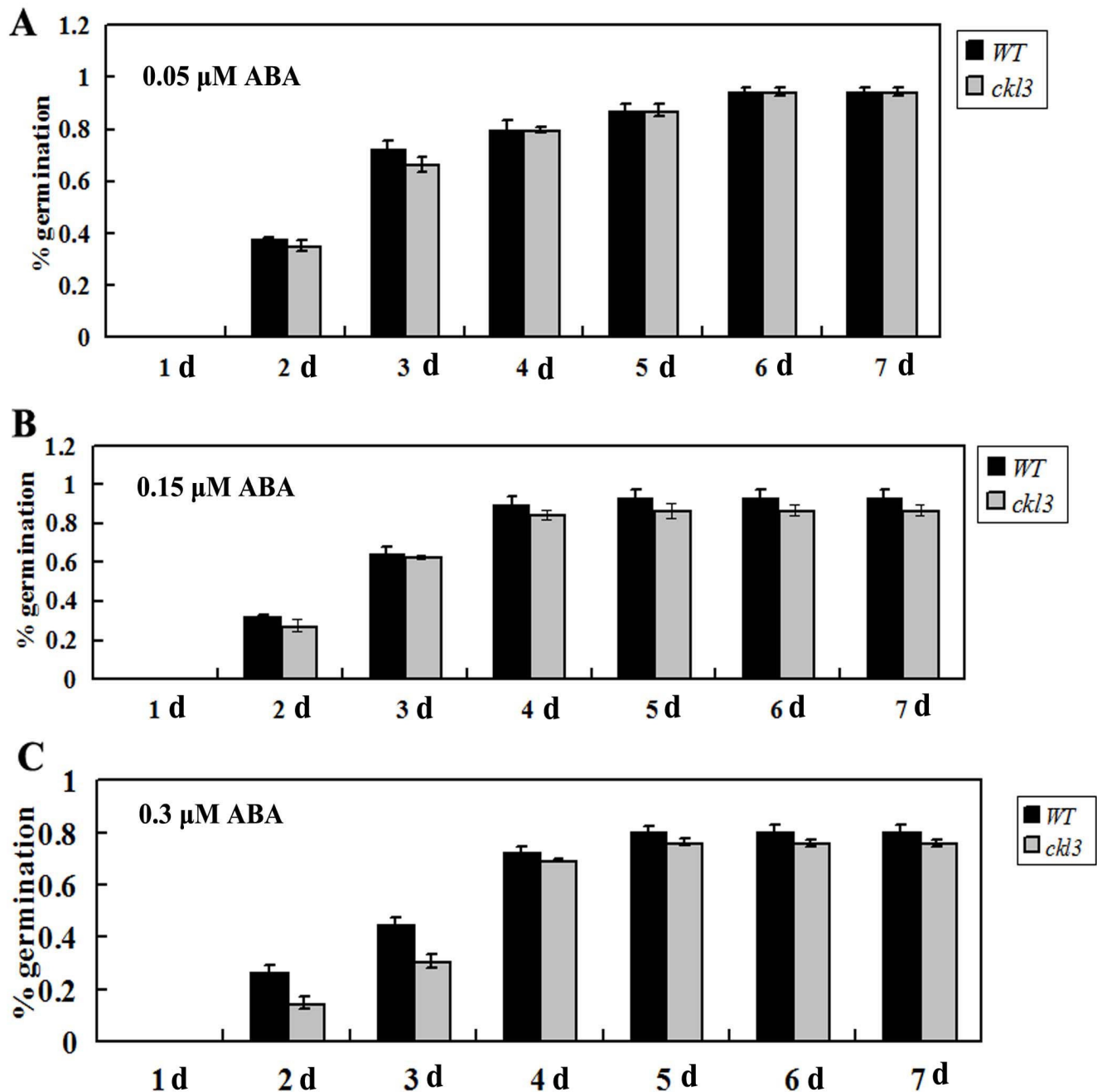


Figure 3. Germination efficiency of wild-type and *ckl3* mutant seeds on days 1 to 7 of treatment with different ABA concentrations. (A) 0.05 μM ABA; (B) 0.15 μM ABA; (C) 0.3 μM ABA. Values are averages \pm SD for three independent experiments. Approximately 300 seeds of each genotype are sowed on each plate and scored for germination.

2000; Petronczki et al., 2006; Robinson et al., 1993; Dhillon and Hoekstra, 1994; Liu et al., 2003; Cheng and Xue 2010; Lee et al., 2009; Ben-Nissan et al., 2008, 2010), there is no report of its role in ABA signaling pathway in plants. In this study, we found that *CKL3* regulates seed germination and involved in ABA, salt and osmotic stress response in *Arabidopsis*. There are at least 21 putative CK1 isoforms in *Arabidopsis* genome

(Klimczak et al., 1995) and 14 CKI-like genes have been isolated (Lee et al., 2005). The results of a search using the GENEVESTIGATOR tool (<http://www.genevestigator.ethz.ch/>) show that 20 *Arabidopsis* CK1 genes are constitutively expressed in all organs or tissues.

Our results also showed *CKL3* transcripts were expressed in various organs such as roots, stems, leaves, flowers and leafstalks, especially highly expressed in

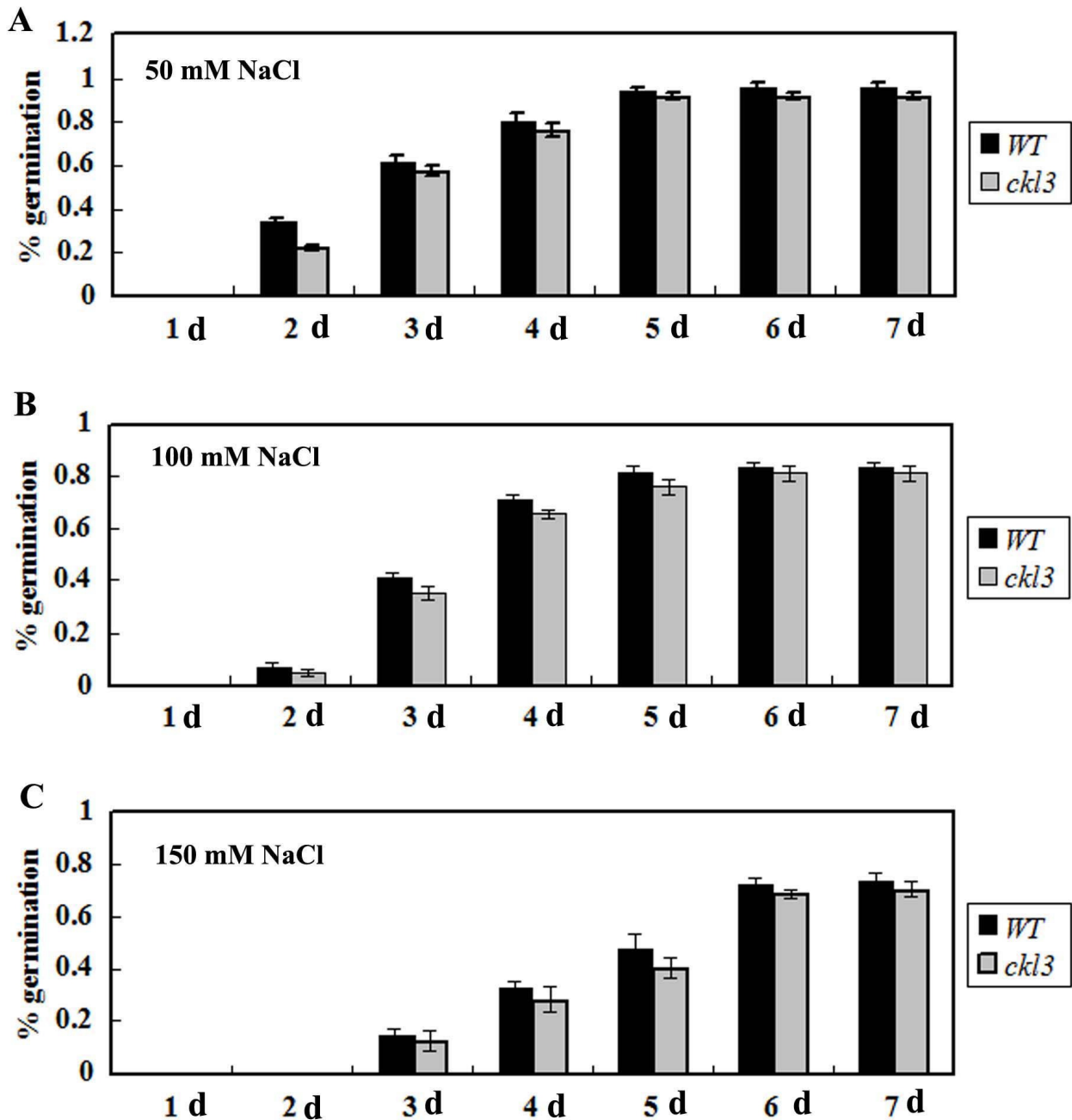


Figure 4. Germination efficiency of wild-type and *ckl3* mutant seeds on day 1 to 7 of treatment with different NaCl concentrations. (A) 50 mM NaCl; (B) 100 mM NaCl; (C) 150 mM NaCl. Values are averages \pm SD for three independent experiments. Approximately 300 seeds of each genotype are sowed on each plate and scored for germination.

flowers and roots (Figure 2). The expression of the *CKL3* transcripts was induced by exogenous application of ABA, suggesting that *CKL3* may play roles in plant responses to various environmental stresses. Therefore, we selected and isolated a homozygous *ckl3* mutant by PCR to further study the physiological function of *CKL3* gene. For a physiological characterization of the *ckl3*

mutant in comparison with wild type seeds, we tested germination behavior under different conditions and at different stages of development. In presence of all ABA, NaCl and mannitol concentrations tested, the germination percentage of *ckl3* mutant seeds was lower than that of wild type (Figures 3 to 5). In the presence of exogenous ABA, wild-type seeds showed higher germination

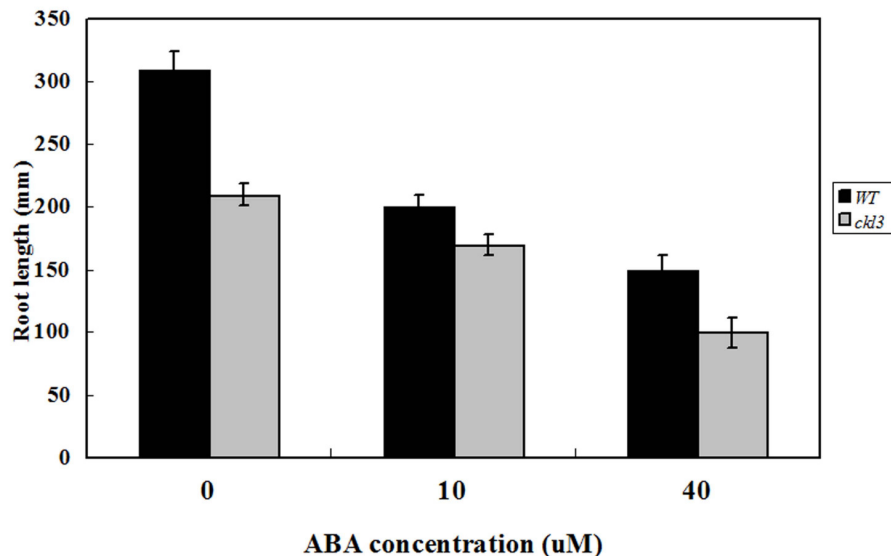


Figure 5. Germination efficiency of wild-type and *ckl3* mutant seeds on day 1 to 7 of 150 mM mannitol treatment. Values are averages \pm SD for three independent experiments. Approximately 300 seeds of each genotype are sowed on each plate and scored for germination.

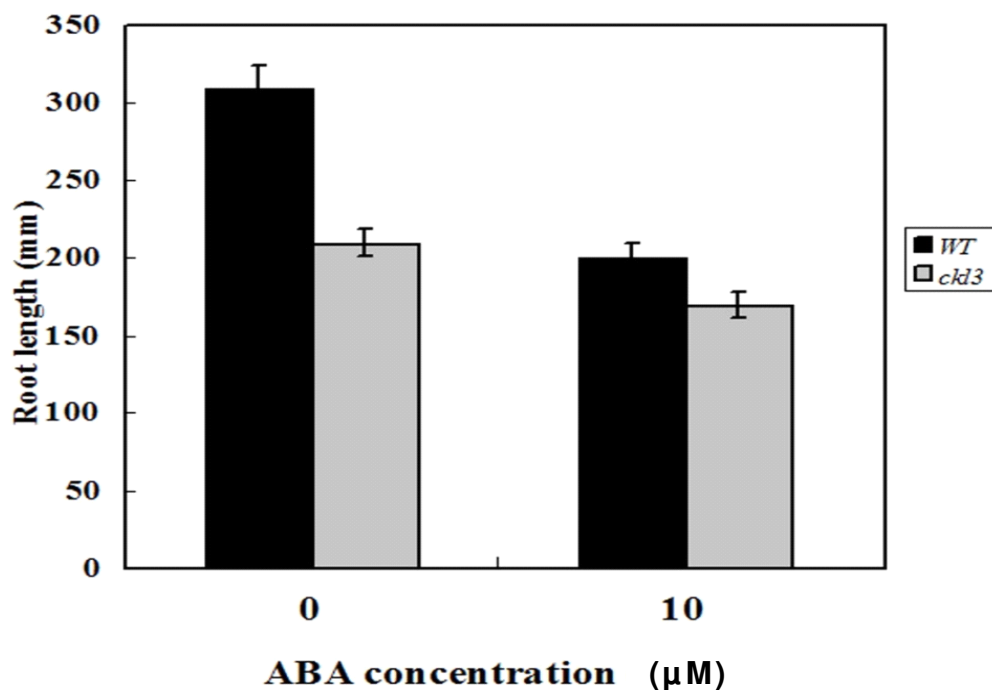


Figure 6. Seedlings of wild-type and *ckl3* mutant are grown for 6 days on ABA-free medium and then incubated vertically for 6 days on an agar plate supplemented with 0 or 10 μM ABA.

percentages than the *ckl3* mutants at different stages of development and wild-type seedlings showed a reduced inhibition of root growth compared with *ckl3* plants (Figures 3 to 5). Therefore, the *ckl3* mutant showed the significant ABA sensitive phenotypes in seed germination

and root growth.

It is clear that identifying *CKL3* as a new regulator of ABA responses would be a crucial step in understanding the *CKL3* physiological function in *Arabidopsis*. Furthermore, compared with wild-type plants, the expression of

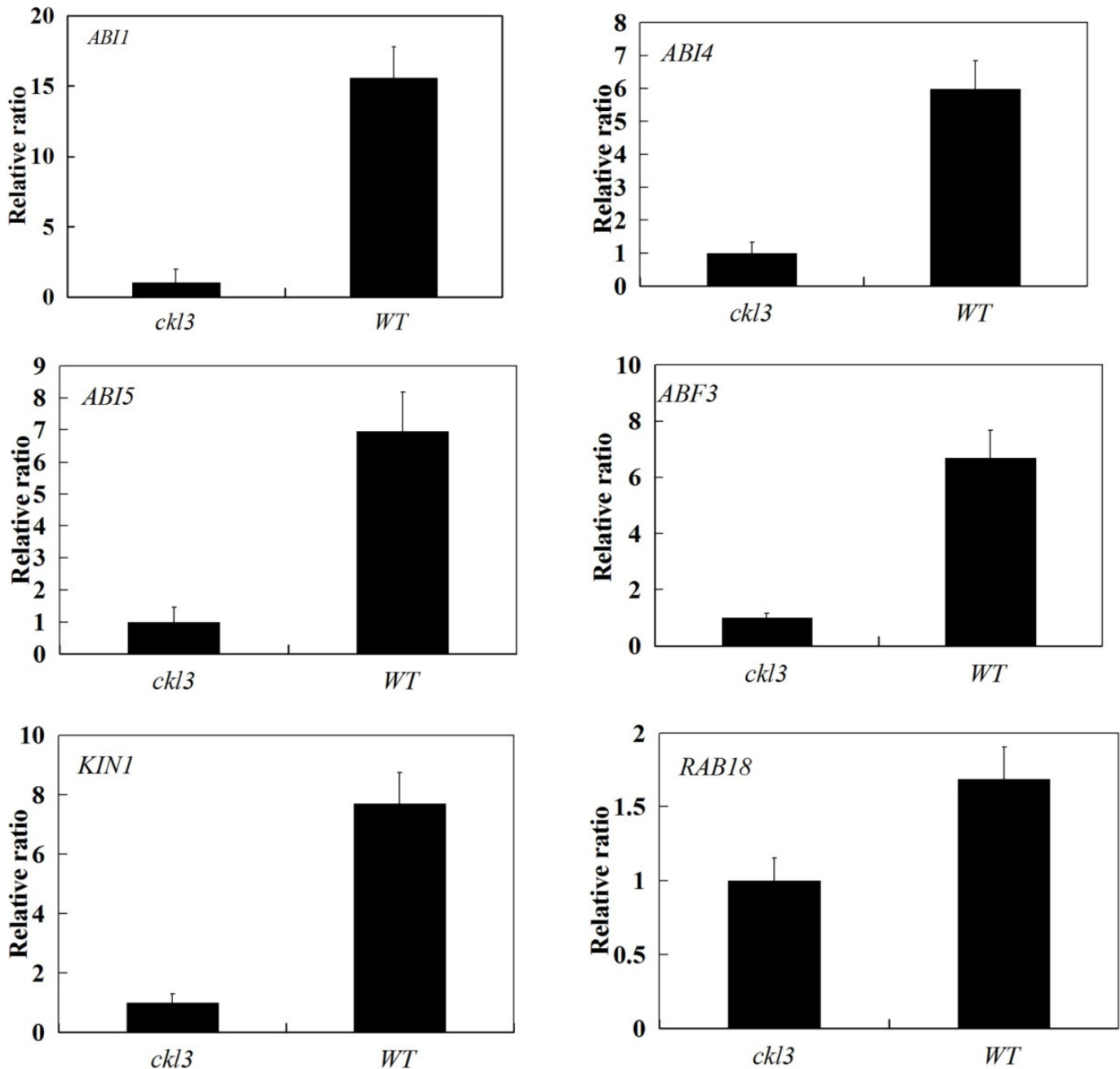


Figure 7. The expression analysis of ABA and abiotic stress-responsive genes including *ABI1*, *ABI4*, *ABI5*, *ABF3*, *KIN1*, *RAB18*, *SOS3*, *DREB1A*, *RD22* and *RD29B* in wild type and *ckl3* mutant. QRT-PCR was performed with total RNA from leaves of 40-day old plants under LD conditions. These experiments were repeated at least three times.

the ABA and abiotic stress-responsive genes including *ABI1*, *ABI4*, *ABI5*, *ABF3*, *KIN1*, *RAB18*, *SOS3* and *DREB1A* decreased, but *RD22* and *RD29B* increased in *ckl3* mutants. Thus, there is a question regarding whether this phenotype of the *ckl3* mutant can be accounted for by an indirect mechanism (that is; *CKL3* regulate the expression of a gene that in turn regulates the expressions of the ABA and abiotic stress-responsive

genes) or whether there is a more direct and yet unknown mechanism by which *CKL3* can regulate gene expression. Taken together, these results suggest *CKL3* was required for abscisic acid regulation of seed germination, root growth and gene expression and was involved in salt and osmotic stress response in the early development stage in *Arabidopsis*. This study therefore provided important clues to casein kinase I activities in

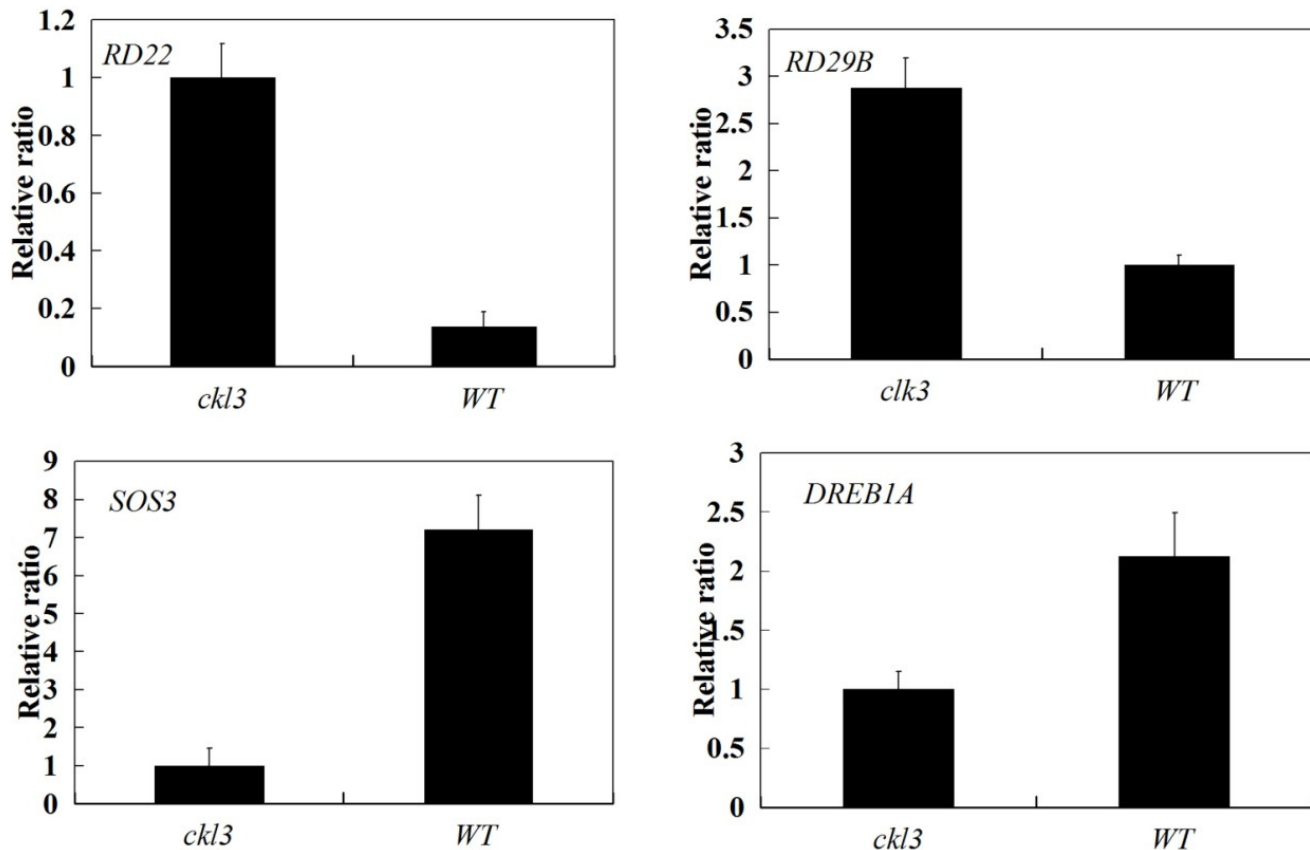


Figure 7. Contd.

ABA signaling and plant development.

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