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Arabidopsis casein kinase 1-like 8 enhances NaCl tolerance, early flowering, and the expression of flowering-related genes

Ji-hong Zhang[a], He-long Sun[a], Xiao-ying Zhao[b] and Xuan-ming Liu[b]

[a]School of Chemical Engineering, Xiangtan University, Xiangtan, People's Republic of China; [b]College of Biology, Hunan University, Changsha, People's Republic of China

ABSTRACT

Members of the casein kinase 1 (CK1) family are evolutionarily conserved eukaryotic protein kinases involved in various cellular, physiological, and developmental processes in yeast. However, the biological roles of CK1 members in plants are poorly understood. Here, we report that an *Arabidopsis* CK1 member named casein kinase 1-like 8 (CKL8) was ubiquitously expressed in all plant organs, mainly in the stems of seedlings according to quantitative real-time PCR. Western blotting showed a remarkable expression of the *AtCKL8* gene in transgenic plants induced by high salinity. A histochemical assay of AtCKL8 promoter::GUS expression revealed that the AtCKL8 promoter is very active in both seedlings and adult plants subjected to the salinity stress, while no GUS activity was detectable in all the transgenic plants grown under normal conditions. In a subcellular distribution analysis, the AtCKL8-GFP fusion protein was localized mainly in the cell membrane. AtCKL8-overexpressing transgenic plants showed an insensitivity to high salinity and an early flowering phenotype. Overall, these findings suggest that AtCKL8 plays a positive role in NaCl signaling and improves salt stress tolerance in transgenic *Arabidopsis*.

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KEYWORDS
Arabidopsis; casein kinase; NaCl stress; histochemical staining; subcellular localization

Introduction

The casein kinase (CK1) family is an evolutionarily conserved eukaryotic Ser/Thr protein kinase family composed of a highly similar catalytic domain and a variable domain mostly located at the C terminus (Gross & Anderson 1998; Vielhaber & Virshup 2001).

Biological function of CK members in plants is not well known although these evolutionarily conserved enzymes are expected to play important roles in various physiological and developmental processes such as light signaling, circadian rhythms, hormone responses, cell cycle control, salicylic acid-mediated defense, abiotic stress responses, and flowering time (Espunya et al. 1999; Hardtke et al. 2000; Hidalgo et al. 2001; Riera et al. 2004; Portolés & Más 2007; Moreno-Romero et al. 2008), which are demonstrated in other eukaryotic organisms.

Salt stress is a major abiotic stress that inhibits crop growth (Munns & Tester 2008). Higher plants have evolved diverse and elaborate mechanisms to protect themselves from salt stress through a series of physiological and morphological changes, such as Salt Overly Sensitive regulatory pathway and the mitogen-activated protein kinase cascade (Zhu 2002; Goyal et al. 2005; Verslues & Juenger 2011). Core sets of transcription factor family genes are differentially expressed in response to elevated external salinity (Golldack et al. 2011), including AP2/ERF (Kasuga et al. 1999), basic helix–loop–helix (Jiang et al. 2009), and NAC (Tran et al. 2004) families.

Arabidopsis encodes 14 CK1-like (CKL) members from 13 genes which differentially localize to the cytoplasm, nucleus, ER, or vesicle-like punctate structures (Lee et al. 2005). The recombinant OsCK11 protein encoding a CK1 member in rice localizes to the nucleus, perhaps suggesting *OsCK11* involvement in the regulation of gene transcription, which was induced upon brassinosteroid treatment (Liu et al. 2003). The role of CKL6, in addition to associating with punctuates structures, partitions to the cortical microtubules and plays a role in polarized cell expansion (Ben-Nissan et al. 2008). The result is shown that CKL6 phosphorylates Ser413/420 of tubulin β, and overexpression of CKL6 can alter the cortical microtubule organization during interphase (Ben-Nissan et al. 2008). CK2 alpha subunits positively regulate response to hormone abscisic acid (ABA), salt (NaCl) stress and lateral root development in an overlapping manner (Mulekar & Huq 2015). CKL3 was involved in abiotic stress, such as hormone, salt, and osmotic stress (Wang et al. 2011). CKL2 participate in ABA signaling in higher plant and play a positive regulatory role (Cui et al. 2014). *Arabidopsis* CKL3 and CKL4 were identified as key regulators in blue light signaling via phosphorylating the receptor cryptochrome 2 (Tan et al. 2013). *Arabidopsis* CKL8 regulated ethylene synthesis by phosphorylating ACS; expression of tomato CKL8 in fruits results in the delayed ripening of tomatoes (Tan & Xue 2014).

Overexpression of one of the CK2 regulatory subunits (CKβ3) in *Arabidopsis* leads to early flowering under short-day conditions (Sugano et al. 1999). The involvement of CK2 in regulating circadian clock and photoperiod pathway is better understood. Both α and β subunits regulate the circadian clock by phosphorylating the central clock components, CCA1 and LHY (Sugano et al. 1999; Daniel et al. 2004). Overexpression of two of the CK2 regulatory subunits (CKB3 and CKB4) in *Arabidopsis* leads to defect in circadian clock and altered flowering under both long- and short-day conditions (Sugano et al. 1999; Perales et al. 2006). The CKB4 level is also regulated post-translationally by the

CONTACT Ji-hong Zhang jihongzh01@xtu.edu.cn; Xuan-ming Liu xml05@hnu.edu.cn

circadian clock, resulting in altered flowering time (Perales et al. 2006). CK2 alpha subunits also redundantly regulate the flowering time under both short- and long-day conditions (Mulekar et al. 2012; Mulekar & Huq 2012).

In this study, we isolated an *Arabidopsis* T-DNA insertion mutant line, Salk_054509 and Salk_025753c. We sought to further investigate the biological roles of AtCKL8 in stress response by overexpressing the gene in *Arabidposis*. We generated transgenic plants ectopically expressing the AtCKL8 gene, and these plants had enhanced salt-tolerance phenotypes. The results obtained suggest that AtCKL8 may be involved in NaCl signal transduction pathways, and be induced to express in response to salt stress in AtCKL8-overexpressing transgenic *Arabidopsis* plants. Our data showed that AtCKL8 promoted *Arabidopsis* flowering under LD conditions, which was probably due to enhanced expression of flowering-related genes.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana T-DNA insertion mutants, SALK_054509 and SALK_025753c, were obtained as heterozygous T3 populations from the *Arabidopsis* Biological Research Center (ABRC, Columbus, OH). All the seeds (including WT (Columbia-0 (Col-0), mutants, or overexpressing transgenic plants) were surface sterilized and vernalized at 4°C for two days before placed on half strength Murashige and Skoog (1/2 MS) medium (Murashige & Skoog 1962) solidified with 0.8% agar. One or two-weeks-old seedlings were transferred from plates into plastic pots filled with compost soil. *Arabidopsis* plants were grown aseptically in a growth chamber (22°C, ~120 µmol photos $m^{-2}s^{-1}$ under a 16-h light/8-h darkness photocycle).

RNA extraction

Total RNAs were separately extracted from different *A. thaliana* tissues using Trizol reagent (Invitrogen, USA) (Sambrook & Russell 2001). Total RNAs of 14-days-old seedlings from different tissues, or total RNAs of leaves detached from 20-days-old plants were subjected to DNase treatment by using DNA-free kit (Ambion, GrandIsland, NY). All the samples were separately harvested frozen in liquid nitrogen and stored at −80°C until use.

Plasmids construction and plant transformation

All plasmid constructs were generated by using GATEWAY™ cloning technology (Invitrogen, GrandIsland, NY). The construction of various DNA cloning vectors and fluorescent fusion proteins under the control of the cauliflower mosaic virus 35S promoter is described elsewhere (Lee et al. 2005; Ben-Nissan et al. 2008).

A transcriptional fusion of the AtCKL8 promoter to a GUS reporter gene was made as follows: We performed PCR on the *Arabidopsis* (Columbia-0) genomic DNA with 35S-Promoter-F and 35S-Promoter-R primers in order to amplify the 5′ flanking DNA sequences between −1008 and +76 bp of the AtCKL8 gene. The 1.1-kb PCR fragment was cloned into the pCAMBIA-1301 binary vector, thereby creating the pCAMBIA-1301-CKL8 plasmid. For creation of the AtCKL8-GFP chimeric construct (35S-GW-GFP-AtCKL8), primers 35S-Gw-GFP-F and 35S-Gw-GFP-R were used to PCR amplify the AtCKL8 coding region. Following GATEWAY™ cloning technology, the PCR product was cloned into a 35S-GW-GFP binary vector that contained a GFP reporter gene. Primers used for plasmids' construction were listed in Table 1. All the plasmids constructed above were verified by DNA sequencing. All the plasmids constructed were then transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into *Arabidopsis* plants (Col-0) by the floral dip method, respectively, to generate transgenic plants (Clough & Bent 1998). Transgenic plants were selected on MS medium supplemented with 30 mg/L kanamycin. In order to better characterize their phenotype, we selected three T2 homozygous lines carrying a single copy transgene in *Arabidopsis*.

AtCKL8 gene expression analysis

For the qRT-PCR analysis of the gene expression of AtCKL8 in Arabidopsis, cDNA was synthesized from 10 µg total RNA using the PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Dalian of China) with oligo dT primer. All the samples were prepared to a final volume of 10 µl. qPCR-PCR was performed on an MxPro-Mx3000P Real-Time Thermal Cycling System using 2×SYBR Fast qPCR Mix (Takara) as the fluorescence probe reaction system, according to the manufacturer's instructions. All the primers of the related genes were designed based on the sequences downloaded from the TAIR database (http://www.arabidopsis.org/). Gene-specific primer pairs used here were listed in Table 1. PCR amplification was carried out in 96-well optical reaction plates heated for 10 min 95°C to activate the hot start Taq DNA polymerase, followed by 40 cycles as follows: 95°C for 45 s, 56°C for 45 s, and 72°C for 45 s, followed by 72°C for 10 min (Park et al. 2008). Expression levels were normalized to ACTIN2 expression levels. All RT-PCR experiments were carried out at least in triplicates.

Stress treatments, phenotypic, and morphometric analysis of transgenic Arabidopsis plants

To examine the osmotic tolerance of AtCKL8-overexpressing transgenic plants, salt stress and mannitol treatment were used to assess the tolerance and investigate the role of AtCKL8 during osmotic stress. For NaCl tolerance stress, six-days-old seedlings were transferred to 1/2 MS medium supplemented with various NaCl concentrations (0, 30, 50, 70, and 100 mM) for 14 days. The root lengths of wild-type and transgenic lines were analyzed. For morphometric analysis, seven-days-old seedlings were transferred to soil, and three-weeks-old plants were treated wtih 300 mM NaCl or 400 mM mannitol for six days (Park et al. 2008). The effects of different adversity stresses on seedling growth were determined by comparing the morphological variations in photographs taken using a digital camera.

Western-blot analysis

Total plant proteins for western-blot analysis were extracted from two-weeks-old plants of 35S:CKL8 transgenic plants. Protein concentration was determined by the method of Bradford (1976). Fifteen micrograms of total protein was

Table 1. Primers used for PCR and qPCR analysis.

Genes	Forward (5′-3′)	Reverse (5′-3′)
Actin2	CACTGTGCCAATCTACGAGGGT	CACAAACGAGGGCTGGAAGAGC
FT	CAACCCTCACCTCCGAGAATAT	TGCCAAAGGTTGTTCCAGTTGT
ZTL	ATGAAGAGGGAGGTCTTTTTCC	GAGTCAACCGCAATCTATTCATC
GI	GGATAGTCAAAGTGTTGGTGGAAC	TCATTCCGTTCTTCTCTGTTGTT
FKF	TTCCGTTAGAGGTTGGGATG	TTATGTTTGAACACAGGATACGAGA
35S-Gw-GFP	GGGGACAAGTTTGTACAAAAAAGCA GGCTTCATGGATCGTGTGGTGGTAAGT	GGGGACCACTTTGTACAAGAAAGCTGG GTCTCACTTCCTCTTTCCATTCCCTATG
35S-Promoter	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCCTTATGTTCTCGCGAATATCC	GGGGACCACTTTGTACAAGAAAGCTGG GTCCTAGAAAAAGTTCACCAAATG

loaded per well for SDS-PAGE. The proteins were electrophoretically transferred to Hybond-C membranes (Amersham) using the Trans-Blot Cell (Bio-Rad). Anti-GFP antibodies (Invitrogen) were used for western-blot analyses. For analyses of AtCKL8 protein being induced by western blots, protein was extracted from two-weeks-old rosettes of AtCKL8-overexpressing transgenic plants which were treated with 100 mM NaCl or by soaking roots for different times (0, 48, 72, 96, and 120 h).

Transient expression of AtCKL8-GFP in onion epidermal cells

To determine the subcellular localization of the AtCKL8-GFP fusion protein, the constructed vector 35S-GW-AtCKL8-GFP was then co-introduced into onion epidermal cells via gene gun transformation (Wang et al. 2011; Alinsug et al. 2012). The green fluorescence, which demonstrated the location of the expressed gene, was observed by confocal laser scanning microscopy (Olympus).

Analysis of AtCKL8 promoter-GUS expression

The AtCKL8 promoter-GUS construct (pCAMBIA-1301) was transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into *Arabidopsis* plants by the floral dip method (Clough & Bent 1998). Transformants were selected as described previously (Kim et al. 2003). Histochemical GUS assays of the transgenic plants were performed according to the protocol described by Jefferson et al. (1987).

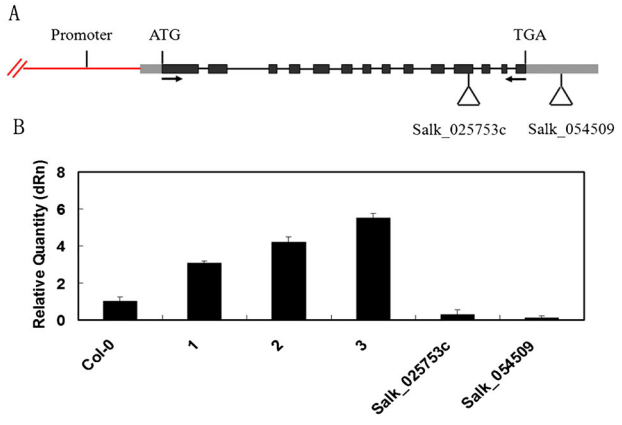


Figure 1. Molecular characterization of *ckl-8*. (a) The genome structure of *ckl-8* alleles. The gray and black boxes, black horizontal thick and red horizontal thick lines indicate the 5′- or 3′-untranslated region, exon, intron and promoter region, respectively. (b) Expression of AtCKL8 transcript was determined in wild-type, T-DNA insertion mutant and AtCKL8-overexpressing transgenic plants by quantitative PCR. Lines 1 to 3 are AtCKL8-overexpressing transgenic lines.

Results

Isolation and characterization of AtCKL8 mutants in Arabidopsis

To investigate the biological roles of AtCKL8, we isolated mutants with a T-DNA insertion in the AtCKL8 gene from the SALK collection. Two homozygous T-DNA insertion lines (Salk_025753c and Salk_054509), named *ckl*8-1 and *ckl8*-2, respectively, were screened and identified. The positions of the T-DNA insertions in the *AtCKL8* gene are indicated in Figure 1(a).

Our quantitative PCR results revealed that three of the five plants expressed much higher levels of AtCKL8 than those of the wild-type and the other T-DNA insertion mutant plants (Figure 1(b)). Of the three lines of AtCKL8-overexpressing transgenic plants, we selected line 3 for subsequent studies. The *AtCKL8* gene expression profiles in two T-DNA insertion mutant plants were much lower than those in wild-type plants, which confirms that they were T-DNA mutants.

The Arabidopsis *CKL8* gene promotes flowering time under long-day (LD) conditions

The possible role of AtCKL8 in plant development was investigated using an overexpression approach. Early flowering was observed in all of the overexpressing transgenic plants obtained under LD conditions (Figure 2(a)). To determine the flowering phenotype of each of the plants, we measured the rosette leaf number (LN) at the time of bolting. The average LN values were significantly lower in overexpressing transgenic plants (6.0) than in controls (9.2) (Figure 2(b)).

Overexpression of AtCKL8 upregulates the expression of FT and GI genes, but does not markedly affect the expression of endogenous ZTL or FKF genes

To gain a deeper understanding of AtCKL8 function in the early flowering phenotype, the expression of the flowering-related genes, namely FLOWER LOCUS T (*FT*) , ZEITLUPE (*ZTL*), FLAVIN-BINDING KELCH REPEAT F-BOX (*FKF*) and GIGAS (*GI*), was examined in the T-DNA mutant plants, AtCKL8-overexpressing lines, and wild-type plants under LD conditions (Figure 3). Under LD growth conditions, we observed higher levels of *GI* and *FT* expression in the transgenic lines compared with the wild-type and T-DNA insertion mutant plants. There were no significant differences in the transcription of endogenous *ZTL* and *FKF* expression in the transgenic lines compared with the wild-type and T-DNA insertion mutant plants (Figure 3). These results indicated that overexpression of AtCKL8 led to upregulated expression of flowering-related genes under LD conditions.

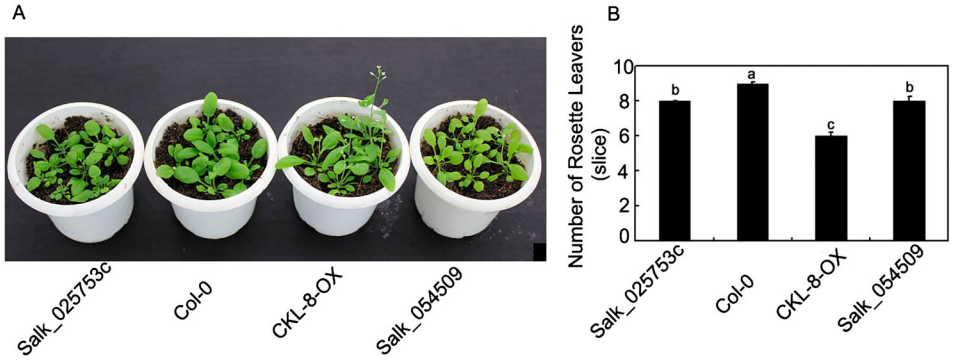


Figure 2. Phenotypical analysis of all transgenic plants. (a) Flowering time phenotype of control, T-DNA insertion mutant, and AtCKL8-overexpressing transgenic plants are as described above. (b) Bar graph showing the average number of rosette leaves under LD (16-h light/8-h dark) conditions. Mean values ± SD are given ($n \geq 30$). The different letters represent significant differences according to Duncan's multiple range test at $P < .05$.

Overexpression of AtCKL8 enhances tolerance to salt stress

To clarify the role of AtCKL8 under salt stress, wild-type, T-DNA insertion mutant plants, and AtCKL8-overexpressing plants were subjected to salinity treatments. The germination efficiencies and root growth of all seeds were investigated. As shown in Figure 4(a), wild-type seeds germinated poorly on the third day. However, the germination rate of all seeds was approximately 100% on the seventh day. In 30 mM NaCl, the germination of all seeds was promoted slightly (Figure 4(b)). Increasing the salt concentration to 100 mM had little effect on the germination of seeds from AtCKL8-overexpressing transgenic lines, whereas it had a greater inhibitory effect on the germination of seeds from the WT and T-DNA insertion mutant plants (Figure 4(c)). After culturing in normal 1/2 MS medium for six days, the seedlings were transferred to MS medium with 0, 30, 50, 70, and 100 mM NaCl for 14 days. Root length did not differ significantly between WT plants and transgenic lines in normal 1/2 MS medium (Figure 4(d)). In 30 mM NaCl, the root growth of all seedlings was accelerated slightly. In 100 mM NaCl, the root growth of all of seedlings was significantly retarded. With the 50, 70, and 100 mM NaCl treatments, the roots of the AtCKL8-overexpressing transgenic plants were slightly longer than those of the WT and T-DNA insertion mutant plants (Figure 4(b)). This indicated that the AtCKL8-overexpressing transgenic plants were more tolerant of salt stress than WT *Arabidopsis* and T-DNA insertion mutant plants.

Overexpression of AtCKL8 increased protein expression under salinity treatment

To reveal the expression patterns of AtCKL8, we initially determined the relative levels of *AtCKL8* gene transcripts using RT-PCR of total RNA extracted from various organs of eight-days-old *Arabidopsis* wild-type plants (Col-0), including the leaf, petiole, stem, flower, and root. AtCKL8 was expressed in all organs at relatively low levels but at

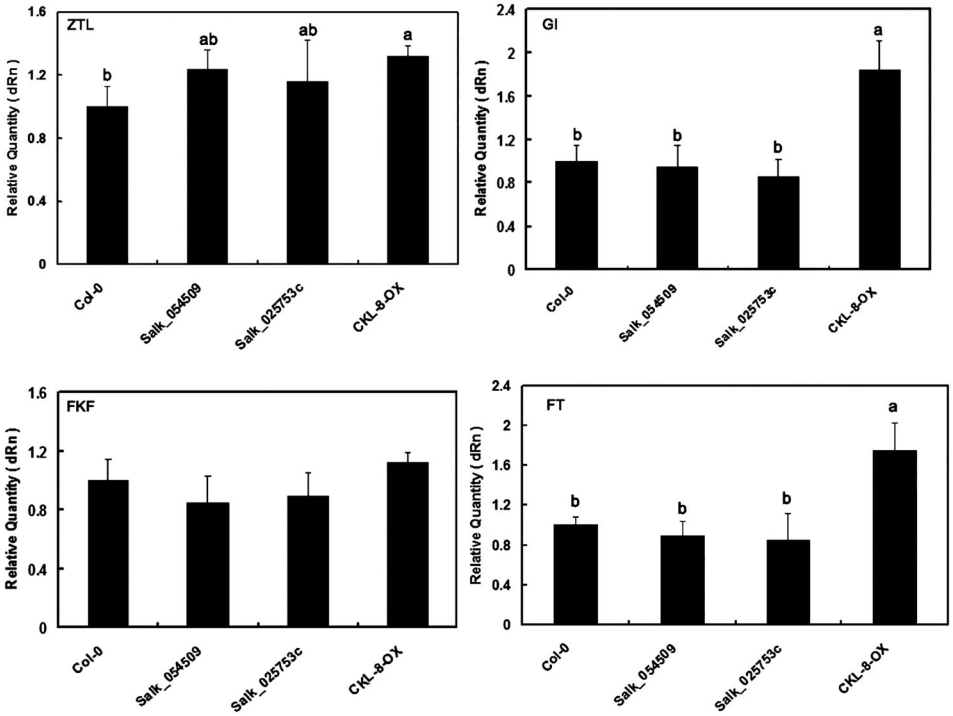


Figure 3. Comparison of various flowering-related genes' transcription levels in wild-type, T-DNA insertion mutant, and At-CKL8 overexpressing transgenic plants by quantitative RT-PCR. Each sample was tested in triplicate, and data represent the means (± SD). The different letters represent significant differences according to Duncan's multiple range test at $P < .05$.

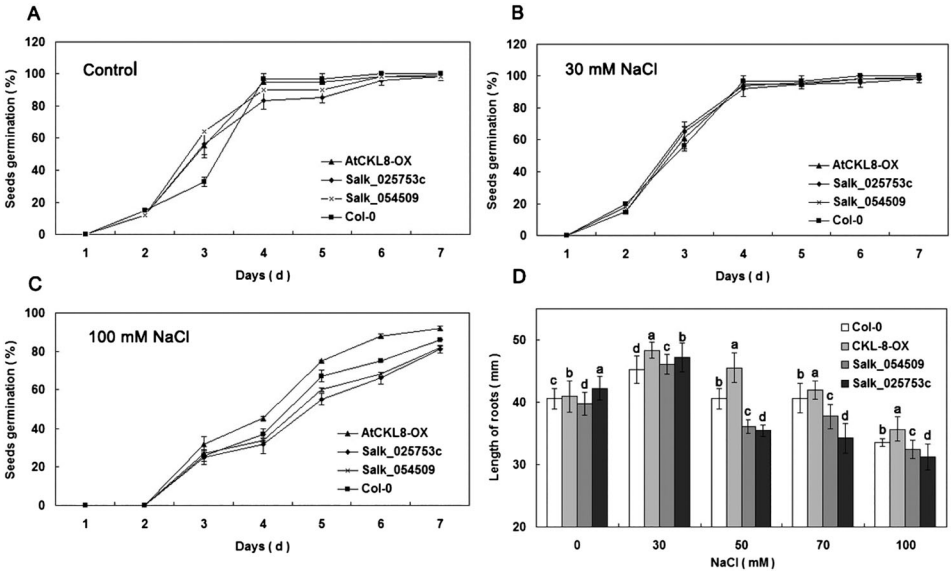


Figure 4. Seed germination of wild-type (Col-0), T-DNA insertion mutant, and AtCKL8-overexpressing transgenic plants in response to NaCl: germination rates in half-strength MS medium containing 0 (a), 30 (b) or 100 mM NaCl (c) over time. (d) Effects of salt on the root length of WT, T-DNA insertion mutant, and AtCKL8-overexpressing transgenic plants. After all of the seeds had germinated for 6 days in 1/2 MS medium, the seedlings were transferred to new 1/2 MS solid agar plates supplemented with 0, 30, 50, 70, or 100 mM NaCl for 14 days. Mean values ± SD are given ($n \geq 30$). The different letters represent significant differences according to Duncan's multiple range test at $P < .05$.

relatively higher levels in the stem, which was consistent with the Genevestigator database (Figure 5(a)). Western-blot analysis indicated that the expression of the AtCKL8 protein was induced by 100 mM NaCl in AtCKL8-overexpressing transgenic plants (Figure 5(b)). In response to NaCl stress, expression of the AtCKL8 protein increased gradually, peaking at 96 h and then decreased at 120 h. In comparison, there was only a very weak signal at 0 h. Therefore, we concluded that AtCKL8 protein expression was induced under salt stress conditions.

AtCKL8-overexpressing transgenic plants show tolerance to NaCl stress

To gain insight into the function of AtCKL8, we investigated the mechanism of increased tolerance to NaCl and mannitol in AtCKL8-overexpressing transgenic plants. We used two types of osmotic stress to determine whether AtCKL8 overexpression provided protection in *Arabidopsis*. First, three-weeks-old plants grown in soil were watered at three-day intervals with 300 mM NaCl and 400 mM mannitol for six days. After the NaCl treatment, the AtCKL8-overexpressing transgenic plants grew robustly, while the wild-type and T-DNA insertion mutant plants showed clear signs of wilting, with chlorotic leaves (Figure 6(c)). Compared with the untreated controls, none of the plants subjected to mannitol treatment showed obvious phenotypic changes (Figure 6(b)). Therefore, phenotypic differences observed under salt stress were apparent, suggesting that AtCKL8 plays a relevant role in the salt stress response.

Expression patterns analysis of AtCKL8 during Arabidopsis *growth and development*

To examine the spatial and developmental expression patterns of *AtCKL8* in more detail, 1.1 kb of the genomic sequence upstream of the AtCKL8 open reading frame (ORF; −1008 to +76 bp from the first ATG) was fused to the GUS reporter gene, and the resulting construct was transfected into wild-type *Arabidopsis* plants.

Histochemical staining for GUS suggested that AtCKL8 is expressed in all tissues after salt stress treatment (Figure 7). During seedling development in 100 mM NaCl MS medium, GUS expression was detected in the leaf veins, leaf primordia, and roots of 10-days-old seedlings (Figure 7(a)). In the reproductive organs of mature plants exposed to 300 mM NaCl for two days, strong GUS expression was observed in stigmas and anthers of flowers (Figure 7(b)). AtCKL8 promoter activity was also detected in the receptacle of the silique during petal abscission and in the veins of petals (Figure 7(c)). In immature seeds, high GUS expression was observed in the placentae of silique (Figure 7(d)). Moreover, histochemical GUS assays in transgenic plants indicated little AtCKL8 promoter activity in all organs under normal growth conditions (data not shown).

The AtCKL8-GFP fusion protein localizes to the cell membrane

To identify subcellular localization of the AtCKL8 protein, its ORF was introduced into the pCAMBIA 1304-GFP vector upstream of GFP gene to create an AtCKL8-GFP translational fusion construct under control of the cauliflower mosaic virus 35S promoter. The AtCKL8-GFP fusion plasmid was co-expressed in onion epidermal cells using a particle bombardment assay. Gene expression was assessed by fluorescence from the GFP marker and observed under a confocal microscope. These results indicated that the AtCKL8-GFP fusion protein was targeted mainly to cell membranes, and partially to the cell nucleus in onion epidermal cells (Figure 8).

Discussion

Salt stress results in both ionic and osmotic stresses, which lead to secondary stresses, such as oxidative stress and

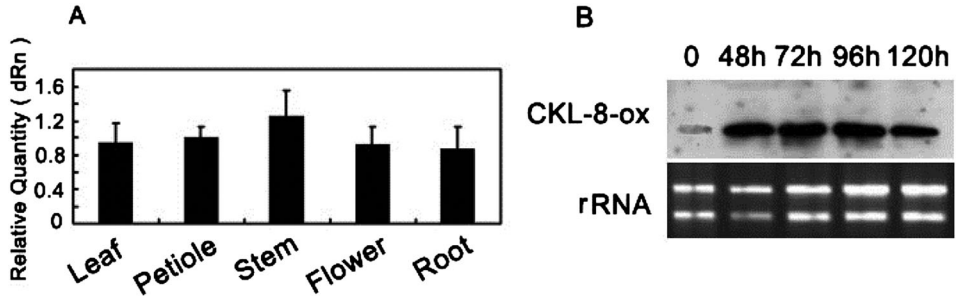


Figure 5. Expression analysis of the AtCKL-8 gene. (a) mRNA expression of the *AtCKL8* gene in different tissues and organs from wild-type *Arabidopsis* plants by RT-PCR. The values are shown as means ± standard error of three independent experiments, each with at least three replicates. Total RNA was extracted from the leaves of WT plants. (b) Western-blot analysis. Total leaf protein was extracted from AtCKL8 transgenic lines after treatment with 100 mM NaCl for 0–120 h, separated by SDS-PAGE, and then subjected to immuno-blotting using an anti-GFP antibody.

nutrient-deficiency stress. It negatively affects photosynthesis, energy production, lipid metabolism, membranes integrity, and enzyme activity (Chinnusamy et al. 2006; Sahi et al. 2006).

The expression of floral integrators (e.g. *FT* and *SOC1*) and their upstream regulators (e.g. *CO* and *FLC*) is a critical molecular marker for the flowering time phenotype under both LD and short-day conditions (Imaizumi & Kay 2006; Kim et al. 2009). Although the role of the *Arabidopsis* CK2 alpha subunits in regulating flowering time has been established, only the CK2 alpha triple mutant, and not the alpha single mutants, displayed the late flowering phenotype under both LD and short-day conditions (Mulekar et al. 2012, Mulekar & Huq 2015). Involvement of the *Arabidopsis* CKL8 gene in controlling flowering time has not yet been reported. The data showed that AtCKL8-overexpressing transgenic plants flowered earlier than did the wild-type and single-mutant plants under LD conditions, suggesting that AtCKL8 is involved in the photoperiod pathway (Figure 2).The level of *FT* mRNA largely determines the flowering time (Li et al. 2015). *FKF* and *GI* are also circadian clock components (Sawa et al. 2007; Baudry et al. 2010). We observed increased *FT* and *GI* expression in AtCKL8-overexpressing transgenic plants compared with wild-type and T-DNA insertion mutant plants under LD conditions (Figure 3), supporting the hypothesis that the *AtCKL8* gene modulates flowering time via the photoperiod pathway. However, no visible phenotype was observed in our T-DNA insertion mutants, which might indicate functional redundancy because *Arabidopsis* encoded 14 CKL gene members.

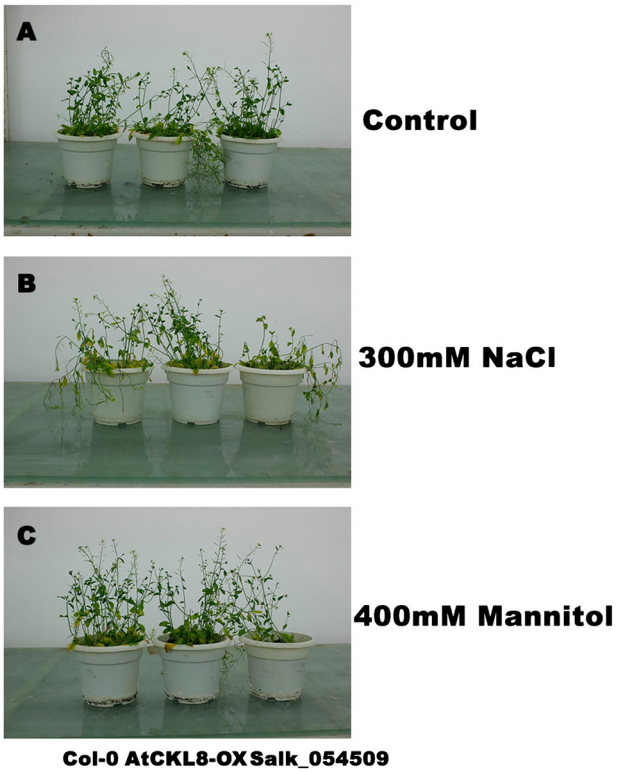
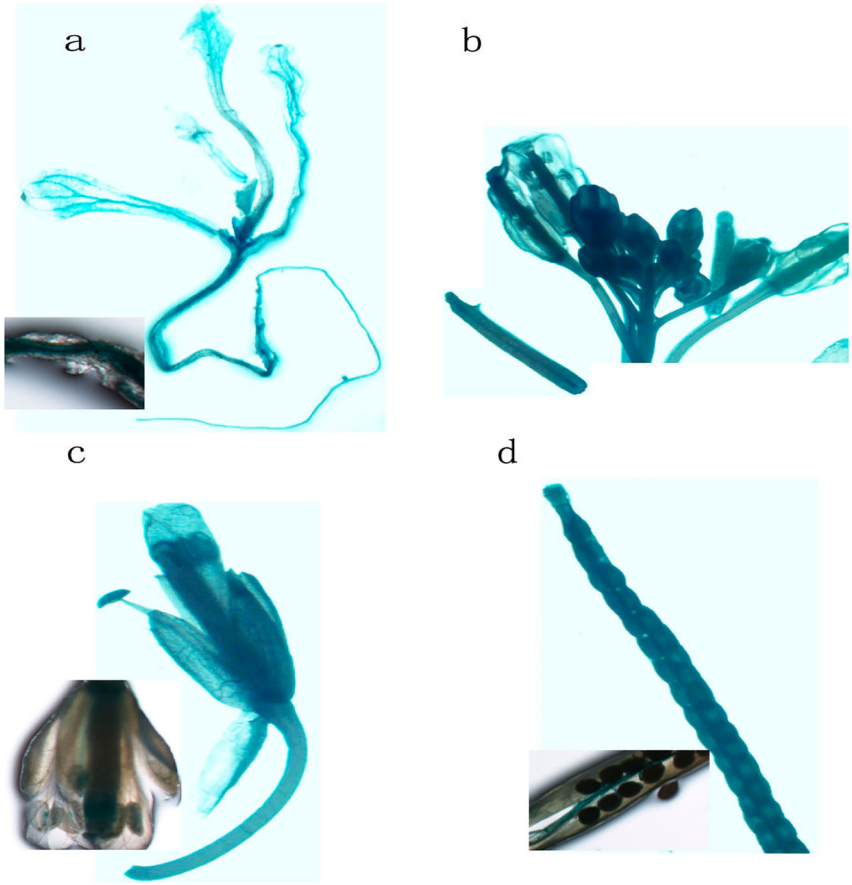


Figure 6. Adversity stress response of wild-type (WT), T-DNA insertion mutant, and AtCKL8-overexpressing transgenic plants. All plants were grown in compost soil under greenhouse conditions for 3 weeks. (a) control, (b) 300 mM NaCl, and (c) 400 mM mannitol. Photographs were taken after six days of treatment.

In this study, we found that AtCKL8 is a member of the *Arabidopsis* CK1 kinase family, that is constitutively expressed in various organs and expressed at very low levels in all tissues (Figure 5). The AtCKL8-overexpressing transgenic plants expressed significantly more protein when subjected to high-salt stress (Figure 5). Consistent with these notions, members of the CK1 family are constitutively active and can be isolated as active enzymes from many different organisms, tissues, and cell lines (Tuazon & Traugh 1991). Each isoform also appears to exhibit different expression patterns at the cellular and tissue levels, indicating that CKL members likely perform non-redundant biological functions (Lee 2009). CKL6 is ubiquitously expressed in various tissues at a relatively low level not detectable by conventional Northern analysis (Ben-Nissan et al. 2010). OsCKL1 expressed constitutively and ubiquitously in various tissues but was also inducible by brassinolide or ABA treatment (Ben-Nissan et al. 2010). We found that AtCKL8-overexpressing transgenic plants showed greater tolerance for salt stress in terms of these physiological and morphological parameters (Figures 4 and 6). In the AtCKL8 promoter::GUS expression analysis, high-salt stress also induced *AtCKL8* promoter activity (Figure 7). The consistent relationship between the transcript accumulation and GUS expression for AtCKL8 strongly suggests that NaCl plays a predominant role in the regulation of AtCKL8 gene expression. From these results, we inferred that AtCKL8 participates in salt stress signaling pathways. However, more studies are required to determine the exact mechanism by evaluating the different AtCKL8 protein functions.

In *Arabidopsis*, at least five CKL members, including CKL6, were localized in punctuate particles, the identities of which are not yet known. Lee et al. (2005) found that the CKL1, CKL6, CKL8, CKL10, and CKL13 isoforms exhibited a predominantly punctuate fluorescence pattern at the cell periphery. However, CKL2 is localized in the cytoplasm and



Figuer 7. Histochemical localization of GUS expression in the vegetative and reproductive organs of transgenic *Arabidopsis* plants carrying AtCKL8 promoter::GUS constructs: (a) 10-days-old seedling, (b) Floral stage-flower buds and anther, (c) A flower with petals and style, and (d) Immature legume with ovule.

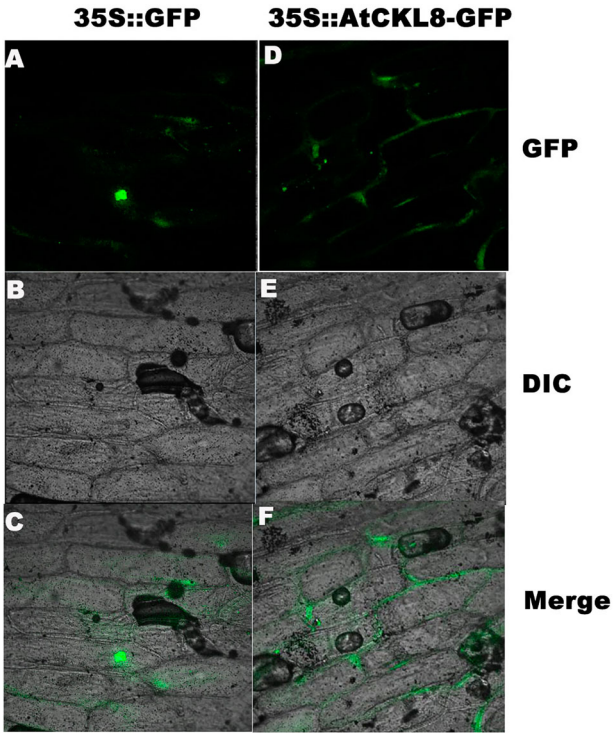


Figure 8. Subcellular location of the AtCKL8-GFP protein. (a–c) The control GFP protein. (d–f) The CKL8-GFP fusion protein. Onion epidermal cells were bombarded with plasmids harboring the GFP coding region 35::GFP (a–c) or with a 35S::CKL8-GFP fusion construct (d–f). GFP accumulated in cell membrane, partially in the nucleus. At-CKL8-GFP fusion protein accumulated in the cell membranes and partially in the nucleus. Fluorescence was examined 18 h after transfection under a confocal laser scanning microscope.

nucleus (Lee et al. 2005). In yeast and humans, CKL isoforms have variable domains at either the N or C terminus (Gross & Anderson 1998). In *Arabidopsis*, all CKL isoforms appeared to contain C-terminal extensions with variable lengths of ~20–180 amino acid residues. The multiple and dynamic subcellular locations of CKL6 are likely a key mechanism enabling this kinase to serve various biological functions in plants (Lee 2009). The subcellular localization of CKL8 showed that AtCKL8 was localized mostly at the cell membranes and partially in the nucleus (Figure 8), confirming the previous research results.

Disclosure statement

No potential conflict of interest was reported by the authors.

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ORCiD

He-long Sun http://orcid.org/0000-0001-8011-1955

References

Alinsug MV, Chen FF, Luo M, Tai R, Jiang L, Wu K. 2012. Subcellular localization of class II HDAs in *Arabidopsis thaliana*: nucleocytoplasmic shuttling of HDA15 is driven by light. PLoS One. 7:e30846.

Baudry A, Ito S, Song YH, Strait AA, Kiba T, Lu S, Henriques R, Pruneda-Paz JL, Chua NH, Tobin EM, et al. 2010. F-box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control *Arabidopsis* clock progression. Plant Cell. 22:606–622.

Ben-Nissan G, Cui W, Kim DJ, Yang Y, Yoo BC, Lee JY. 2008. *Arabidopsis* casein kinase 1-like 6 contains a microtubule-binding domain and affects the organization of cortical microtubules. Plant Physiol. 148:1897–1907.

Ben-Nissan G, Yang YD, Lee JY. 2010. Partitioning of casein kinase 1-like 6 to late endosome-like vesicles. Protoplasma. 240:45–56.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.

Chinnusamy V, Zhu J, Zhu JK. 2006. Salt stress signaling and mechanisms of plant salt tolerance. Genet Eng. 27:141–177.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16:735–743.

Cui Y, Ye JZ, Guo XH, Chang HP, Yuan CY, Wang Y, Hu S, Liu XM, Li XS. 2014. *Arabidopsis* casein kinase 1-like 2 involved in abscisic acid signal transduction pathways. J Plant Interact. 9:19–25.

Daniel X, Sugano S, Tobin EM. 2004. CK2 phosphorylation of CCA1 is necessary for its circadian oscillator function in *Arabidopsis*. Proc Natl Acad Sci. 101:3292–3297.

Espunya MC, Combettes B, Dot J, Chaubet-Gigot N, Martinez MC. 1999. Cell-cycle modulation of CK2 activity in tobacco BY-2 cells+. Plant J. 19:655–666.

Golldack D, Lüking I, Yang O. 2011. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep. 30:1383–1391.

Goyal K, Walton LJ, Tunnacliffe A. 2005. LEA proteins prevent protein aggregation due to water stress. Biochem J. 388:151–157.

Gross SD, Anderson RA. 1998. Casein kinase I: spatial organization and positioning of a multifunctional protein kinase family. Cell Signal. 10:699–711.

Hardtke CS, Gohda K, Osterlund MT, Oyama T, Okada K, Deng XW. 2000. HY5 stability and activity in *Arabidopsis* is regulated by phosphorylation in its COP1 binding domain. EMBO J. 19:4997–5006.

Hidalgo P, Garretón V, Berríos CG, Ojeda H, Jordana X, Holuigue L. 2001. A nuclear casein kinase 2 activity is involved in early events of transcriptional activation induced by salicylic acid in tobacco. Plant Physiol. 125:396–405.

Imaizumi T, Kay SA. 2006. Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci. 11:550–558.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907.

Jiang Y, Yang B, Deyholos MK. 2009. Functional characterization of the *Arabidopsis* bHLH92 transcription factor in abiotic stress. Mol Genet Genomics. 282:503–516.

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol. 17:287–291.

Kim DH, Doyle MR, Sung S, Amasino RM. 2009. Vernalization: winter and the timing of flowering in plants. Annu Rev Cell Dev Biol. 25:277–299.

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S. 2003. CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. Plant Cell. 15:411–423.

Lee JY. 2009. Versatile casein kinase 1: multiple locations and functions. Plant Signal Behav. 4:652–654.

Lee JY, Taoka KI, Yoo BC, Ben-Nissan G, Kim DJ, Lucasa WJ. 2005. Plasmodesmal-associated protein kinase in tobacco and *Arabidopsis* recognizes a subset of non-cell-autonomous proteins. Plant Cell. 17:2817–2831.

Li L, Li X, Liu YW, Liu HT. 2015. Flowering responses to light and temperature. Sci China Life Sci. 12:1–6.

Liu W, Xu ZH, Luo D, Xue H-W. 2003. Roles of *OsCKI*1, a rice casein kinase I, in root development and plant hormone sensitivity. Plant J. 36:189–202.

Moreno-Romero J, Espunya MC, Platara M, Arinŏ J, Martínez MC. 2008. A role for protein kinase CK2 in plant development: evidence obtained using a dominant-negative mutant. Plant J. 55:118–130.

Mulekar JJ, Bu Q, Chen F, Huq E. 2012. Casein kinase II α subunits affect multiple developmental and stress-responsive pathways in *Arabidopsis*. Plant J. 69:343–354.

Mulekar JJ, Huq E. 2012. Does CK2 affect flowering time by modulating the autonomous pathway in *Arabidopsis*? Plant Signal Behav. 7:292–294.

Mulekar JJ, Huq E. 2015. *Arabidopsis* casein kinase 2 α4 subunit regulates various developmental pathways in a functionally overlapping manner. Plant Sci. 236:295–303.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 59:651–681.

Murashige Y, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 15:473–497.

Park HY, Seok HY, Park BK, Kim SH, Goh CH, Lee BH, Lee CH, Moon YH. 2008. Overexpression of *Arabidopsis* ZEP enhances tolerance to osmotic stress. Biochem Biophys Res Commun. 375:80–85.

Perales M, Portolés S, Más P. 2006. The proteasome-dependent degradation of CKB4 is regulated by the *Arabidopsis* biological clock. Plant J. 46:849–860.

Portolés S, Más P. 2007. Altered oscillator function affects clock resonance and is responsible for the reduced day-length sensitivity of CKB4 overexpressing plants. Plant J. 51:966–977.

Riera M, Figueras M, López C, Goday A, Pagés M. 2004. Protein kinase CK2 modulates developmental functions of the abscisic acid responsive protein Rab17 from maize. Proc Natl Acad Sci. 101:9879–9884.

Sahi C, Singh A, Kumar K, Blumwald E, Grover A. 2006. Salt stress response in rice: genetics, molecular biology, and comparative genomics. Funct Integr Genomics. 6:263–284.

Sambrook J, Russell D. 2001. Molecular cloning a laboratory manual. 3rd ed. New York, NY: Cold Spring Harbor Laboratory Press.

Sawa M, Nusinow DA, Kay SA, Imaizumi T. 2007. FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. Science. 318:261–265.

Sugano S, Andronis C, Ong MS, Green RM, Tobin EM. 1999. The protein kinase CK2 is involved in regulation of circadian rhythms in *Arabidopsis*. Proc Natl Acad Sci. 96:12362–12366.

Tan ST, Dai C, Liu HT, Xue HW. 2013. *Arabidopsis* casein kinase1 proteins CK1.3 and CK1.4 phosphorylate cryptochrome2 to regulate blue light signaling. Plant Cell. 25:2618–2632.

Tan ST, Xue HW. 2014. Casein kinase 1 regulates ethylene synthesis by phosphorylating and promoting the turnover of ACS5. Cell Rep. 9:1692–1702.

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2004. Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell. 16:2481–2498.

Tuazon PT, Traugh JA. 1991. Casein kinase I and II-multipotential serine protein kinases: structure, function, and regulation. Adv Second Messenger Phosphoprotein Res. 23:123–164.

Verslues PE, Juenger TE. 2011. Drought, metabolites, and *Arabidopsis* natural variation: a promising combination for understanding adaptation to water-limited environments. Curr Opin Plant Biol. 14:240–245.

Vielhaber E, Virshup DM. 2001. Casein kinase I: from obscurity to center stage. IUBMB Life. 51:73–78.

Wang M, Yu DS, Guo X, Cui Y, Li X, Zhang J, Zhao L, Chang H, Hu S, Zhang C, et al. 2011. Casein kinase 1-Like 3 is required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. Afr J Biotechnol. 10:13219–13229.

Zhu JK. 2002. Salt and drought stress signal transduction in plants. Annu Rev Plant Biol. 53:247–273.