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Original Article

Arabidopsis CK2 family gene *CKB3* involved in abscisic acid signaling

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(With 5 figures)

Abstract

CKB3 is a regulatory (beta) subunit of CK2. In this study *Arabidopsis thaliana* homozygous T-DNA mutant *ckb3* was studied to understand the role of *CKB3* in abscisic acid (ABA) signaling. The results shown: *CKB3* was expressed in all organs and the highest expression in the seeds, followed by the root. During seed germination and root growth the *ckb3* mutant showed reduced sensitivity to ABA. The *ckb3* mutant had more stomatal opening and increased proline accumulation and leaf water loss. The expression levels of number of genes in the ABA regulatory network had changed. This study demonstrates that *CKB3* is an ABA signaling-related gene and may play a positive role in ABA signaling.

Keywords: proline, T-DNA mutant, ABA signaling, expression.

Gene da família Arabidopsis CK2 *CKB3* envolvido na sinalização de ácido abscísico

Resumo

CKB3 é uma subunidade reguladora (beta) de CK2. Neste estudo, o mutante homozigoto *ckb3* de *Arabidopsis thaliana* foi estudado para entender o papel da *CKB3* na sinalização de ácido abscísico (ABA). Os resultados apresentados: *CKB3* foi expresso em todos os órgãos e a maior expressão nas sementes, seguida pela raiz. Durante a germinação das sementes e o crescimento radicular, o mutante ckb3 mostrou sensibilidade reduzida ao ABA. O mutante ckb3 teve mais abertura estomática e aumento do acúmulo de prolina e perda de água nas folhas. Os níveis de expressão do número de genes na rede reguladora da ABA haviam mudado. Este estudo demonstra que *CKB3* é um gene relacionado à sinalização ABA e pode desempenhar um papel positivo na sinalização ABA.

Palavras-chave: prolina, mutante do T-DNA, sinalização ABA, expressão.

1. Introduction

Plants are protected by the expression of various stress-related genes to synthesize hormones and regulatory plant growth and development as well as mediators of environmental stress responses (Sreenivasulu et al., 2012; Gnutt et al., 2017). Among various phytohormones abscisic acid (ABA) is the major hormone which is the central regulator of abiotic stress resistance in plants and coordinates an array of functions (Finkelstein, 2013; Wani and Kumar, 2015), enabling plants to cope with different stresses. A previous study shown that ABA acts as a stress signal in plants and plays an important role in modulating plant response to various biotic and abiotic stresses including cold drought salinity stress and so on (Ma et al., 2019; Wang et al., 2019). Genetic and chemical studies exemplifying ABA in regulating seed maturation and dormancy are important as they have showed that a reduction this hormone level is associated to decreasing seed dormancy (Li et al., 2012; Lee et al., 2015).

Casein kinase 2 (CK2) is an essential and evolutionary conserved Ser/Thr protein kinase and is a heterotetramer composed of two catalytic (CK2α) and two regulatory subunits (CK2β) (Litchfield, 2003). Specifically, CK2 contains four α-subunits and four β-subunits, αA/CKA1, αB/CKA2, αC/CKA3and αcp and β1/CKB1, β2/CKB2, β3/CKB3 and β4/CKB4 in the Arabidopsis genome (Salinas et al., 2006). Molecular genetics studies have further showed that CK2 is a critical component of the circadian clock systems of various organisms including the long-day plant *Arabidopsis* (*Arabidopsis thaliana*) (Allada and Meissner, 2005; Portolés and Más, 2007; Ogiso et al., 2010). According to reports CK2 is involved in various stress responses including heat, UV, drought, hormone

responses, and so on (Wang et al., 2014; Olesen et al., 2015; Zhang et al., 2020; Nagatoshi et al., 2018).

CKB3 is a regulatory (beta) subunit of CK2 involved in regulation of the circadian clock in Arabidopsis (Sugano et al., 1999). Plant hormone and stress-response elements were found through gene chip analysis, including ABRE (ABA-responsive element), AuxRE (auxin-responsive element), CGTCA-motif (MeJA-responsive element) and HSE (Heat-responsive element). Thus the expression of *CKB3* gene was also regulated by hormones and stresses and that it might play an important role in hormone and stress-response pathways.

In this study to understand the role of *CKB3* in abiotic stress signaling *Arabidopsis thaliana* homozygous T-DNA mutant *ckb3* was used. The physiological and biochemical indicators were measured, such as the germination, root growth, hypocotyl elongation, stomatal apertures, water-loss rate and so on, then combined the expression of *CKB3* gene in response to various stresses of T-DNA mutant *ckb3* and Col-0to analysis the role of CKB3 in abiotic stress. The results provide a basis for further study of *CKB3* involved in various stress responses.

2. Material and Methods

2.1. Identification of homozygous T-DNA insertion mutants

The *Arabidopsis thaliana* Columbia wild-type (Col-0) was used as an Arabidopsis wild-type. From the *Arabidopsis* Biological Resources Center (ABRC) purchased the T-DNA insertion mutants *ckb3* (Salk_093548 with Col-0 as background). Using tri-primer-PCR method to identify homozygous T-DNA insertion *mutants*, and the primers information were listed in Table 1. The primers were provided by ATIDB (the *Arabidopsis thaliana* Integrated Database).

2.2. Germination assays and root growth

To surface-sterilize the seeds for germination assay, using 75% ethanol to wash the seeds for 30 s followed by 20% NaClO for 10 min, then washed the seeds six to ten times with sterile distilled water and then placed in 4 °C for vernalization. The seeds were planted on Murashige and Skoog (MS) medium that contained 3% sucrose and 8% agar (PH = 5.8-6.0) with different concentrations of ABA (0 μM, 0.3 μM, 0.6 μM, 1 μM) after 4 days and then transferred to a growth chamber at 22 °C with about 80 μmol photons m^{-2} s^{-1} under 16 h of light/ 8 h of dark and 60% relative humidity. About root elongation analysis, the seeds were surface-sterilized using the above methods. The seedings were grown in MS plates for 3 days and then transferred to ABA-containing (10 μM, 40 μM) or ABA-free MS medium and continued to grow for 6 days before measurement.

Table 1. Primer sequences used for identifying *Arabidopsis thaliana* homozygous T-DNA insertion.

Primer name	Primer sequence 5'-3'
ckb3-F	AACGTGGATCACAAACTTTACTTG
ckb3-R	TCCACTCACATCAGACCCTTC
R0	ATTTTGCCGATTTCGGAAC
CKB3F	CTCCTCCGTCTCACTTCACC
CKB1R	TTCCAACTCCTCTTGCTCCT

2.3. Stomatal aperture measurement

Using rosette leaves of 4-week-old plants to measure the stomatal aperture. To incubate the detached leaves in solution containing 10 mM MES, 50 mM KCl and 10 mM $CaCl_2$ (pH 6.15) for 2 h under light. Then add ABA to the solution to the final concentration was 1 M. After the detached leaves were treated for 2 h, the stomatal apertures were measured as described previously (Sun et al., 2012).

2.4. Determination of the water-loss rate

To detect plant water-loss rate used the method was described by Shan et al. (2012). Getting the rosette leaves from an approximately 3-week-old T-DNA insertion mutants and Col-0 plants to detect the water-loss rate. Put the detached rosette leaves in clean filter paper, and then placed into a growth chamber with 25 °C and humidity of 60%. To recorded the fresh weight every 30 min. Each experiment was repeated three times.

2.5. Proline content measurement

To incubate the 14-day-old seedlings of Col-0 and T-DNA insertion mutants in MS solution containing 3% sucrose and 8% agar (PH = 5.8-6.0) with different concentrations of ABA (0 or 100 μmol). The 14-day-old seedling plants were collected and using the sulfosalicylic acid method to extract proline (Qin et al., 2014). The experiment was performed in triplicates.

2.6. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the 14-day-old Col-0 and mutant with TriZol (Takara) which was incubated in an ABA solution. To synthesize the first strand cDNA using the Maxima® First cDNA Synthesis Kit (Fermentas). Using a SYBR® Green I kit (TOYOBO, Japan) to perform quantitative PCR (qRT-PCR) in an Mx3000P thermal cycler (Stratagene USA). The procedure of the PCR reactions was started with a denaturing step for 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C and a primer extension reaction at 55 °C for 1 min. The ACTIN2 gene was used as an internal control. All qRT-PCR tests were run in duplicates each with three biological replicates. The primers information was listed in Table 2. Analyze the data using MxPro (Stratagene) software.

3. Results

3.1. CKB3 T-DNA insertion mutants homozygous identification

Through PCR and qRT-PCR technology to identify the CKB3 T-DNA insertion mutants homozygous plants. The flanking region sequences of the T-DNA insertion mutant *ckb3* showed that the insertion was located 80 bp

downstream of the ATG start codon and inverted insertion. In the end we got three individuals homozygous of T-DNA insertion mutant *ckb3* (Figure 1B). Through qRT-PCR technology to analyze the expression of homozygous of T-DNA insertion mutant *ckb3*. The result showed that the expression of CKB3 gene in T-DNA insertion mutant plant was zero indicating that the T-DNA insertion severely impaired the *CKB3* gene expression.

3.2. Expression of the CKB3 gene in different organs and in response to various stresses

The expressions of *CKB3* gene were analyzed in roots, stems, rosette, cautine leaves, flowers, silique and seeds of *Arabidopsis* (Figure 1D). *CKB3* was expressed in all organs and the highest expression in the seeds, followed by the root. To analyze the expression of *CKB3* gene under different stresses, the seedlings of 14 days old were

Table 2. Primer sequences used in quantitative RT-PCR.

Primer	Primer sequence 5'-3'
ABI3 (AT3G24650) F	ATGAAAAGCTTGCATGTGGC
ABI3 (AT3G24650) R	TCATTTAACAGTTTGAGAAGT TGG
OST1 (AT4G33950) F	GGATCAACCGGGCCAAAG
OST1 (AT4G33950) R	TGAGTGCCTGCAGGAGGAA
ABF2 (AT1G45249) F	TTACAGGCAAGGATCATGGA
ABF2(AT1G45249) R	CACGGAAACAAACAACCAAG
RAB18 (AT5G66400) F	AGCTCTAGCTCGGAGGATGA
RAB18 (AT5G66400) R	CATGATGACCTGGCAACTTC
ABI5 (AT2G36270) F	TGGAGGCGAGGGTGGTGTTG
ABI5 (AT2G36270) R	CGGGAATGAAGGATCACCGG
EM1 (AT3G51810) F	CTTGATGAGAAGGCGAAGCA
EM1 (AT3G51810) R	CTCAATCCCTTCTTCCTCCG
ACTIN2 (AT3G18780) F	TGGCGTCAAAGCAACTGAGC
ACTIN2 (AT3G18780) R	CACAAACGAGGGCTGGAACAAG

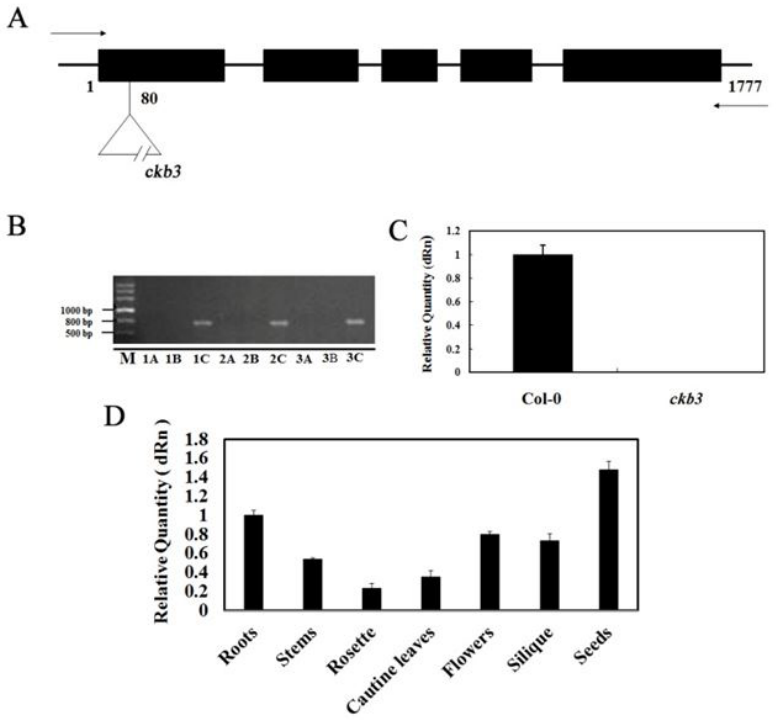

Figure 1. The identifications of the T-DNA insertion homozygous mutants and *CKB3* transgenic plants of *Arabidopsis thaliana*. (A) A schematic structure of the *ckb3* gene and the T-DNA locations in the *ckb3* mutants; (B) Homozygotes for the T-DNA insertions identified by three primers. M is the marker, and 1, 2, 3 are homozygous mutants; (C) real-time fluorescence quantitative PCR analysis of the *CKB3* gene expression in the T-DNA insertion mutants with actin-2 used as control; (D) Q-PCR analysis of the CKB3 gene expression in different organs of Arabidopsis with actin-2 used as control.

treated with ABA, GA, IAA and NaCl for different lengths of time (Figure 2). The expression of *CKB3* peaked when treated by ABA for 6 h, IAA stress for 1 h. The expression decreased at first and then increased with time when treated by GA and NaCl. The results indicated that *CKB3* may participate in the ABA stress signaling pathway.

3.3. CKB3 is involved in the ABA-mediated inhibition of seed germination and root elongation

CKB3 gene has the higher expression in seeds and roots than the other organ, to certain the effect *CKB3* on seed germination and root growth. The seeds of T-DNA insertion mutant *ckb3* and Col-0 were sown in a MS medium added with different concentrations of ABA after surface disinfection. The seed germination data was checked from 1 day to 7 day. The T-DNA mutant *ckb3* displayed a higher germination rate than the wild-type Col-0 (Figure 3) in the presence of 0.3 μmol/L, 0.6 μmol/L and 1μmol/L ABA. The *ckb3* was less sensitive to ABA than the wild type. When treated with 10 μM ABA or 40 mM NaCl, the *ckb3* had apparently higher root growth than Col-0 (Figure 4A). These results were match to the seed germination assay.

3.4. CKB3 gene affects stoma aperture, water loss and proline contents under ABA-mediated

Based on previous research results, in order to survive harsh conditions the plants can produce ABA to change stomatal openness, water loss and proline content (Schroeder et al., 2001; Verslues and Bray, 2006; Seiler et al., 2014; Eisenach et al., 2017). The results showed that the stomatal apertures of *ckb3* were larger than those of the wild type with ABA treatment (10 μmol/L ABA) (Figure 4B). These results indicated that *CKB3* might play a negative role under the influence of ABA in ABA-regulated stomatal closure. In this study shown the leaves of T-DNA insertion mutant *ckb3* lost water at a slower rate than the Col-0 leaves (Figure 4C). To certain whether *CKB3* affects proline accumulation in plants in response to ABA, the proline contents of the wild type *ckb3* plants in response to ABA was determined. As shown in Figure 4D, the T-DNA insertion mutant *ckb3* had significantly higher accumulated proline than Col-0. Thus, under the influence of ABA, *CKB3* aslo plays a negative role.

3.5. CKB3 regulates the expression of ABA and stress responsive genes

In order to confirm whether *CKB3* is involved in the ABA signaling pathway, expression levels of ABA signaling pathway related genes *ABI3*, *ABI5*, *ABF2*, *OST1*, *RAB18* and *EM1* (Yoshida et al., 2015; Skubacz et al., 2016; Gao et al., 2016; Wang et al., 2018). As shown in Figure 5, the expression level of ABI3 in T-DNA mutant plant was much lower than this in Col-0, but the expression levels were equal in ckb3 T-DNA mutant and Col-0 with ABA treatment displayed. The expression levels of *ABI5*, *OST1*, *ABF2* and *EM1* in the *ckb3* plants were lower. These results showed that (Figure 5) *CKB3* gene can regulate the expression level of ABA and stress-related genes, indicating that *CKB3* may positively affect the ABA signaling.

4. Discussion

A previous have shown that several CK family genes are involved in the ABA signaling pathway, such as that three nuclear-located CK2 α-subunits (CKA1-3) in Arabidopsis have a synergistic role in ABA-induced blockage (Mulekar et al., 2012). CKA4 gene is an enhancing factor in abiotic stress signalling through modulating the expression of some molecular players in retrograde signaling (Wang et al., 2014). CKB1 is involved in abscisic

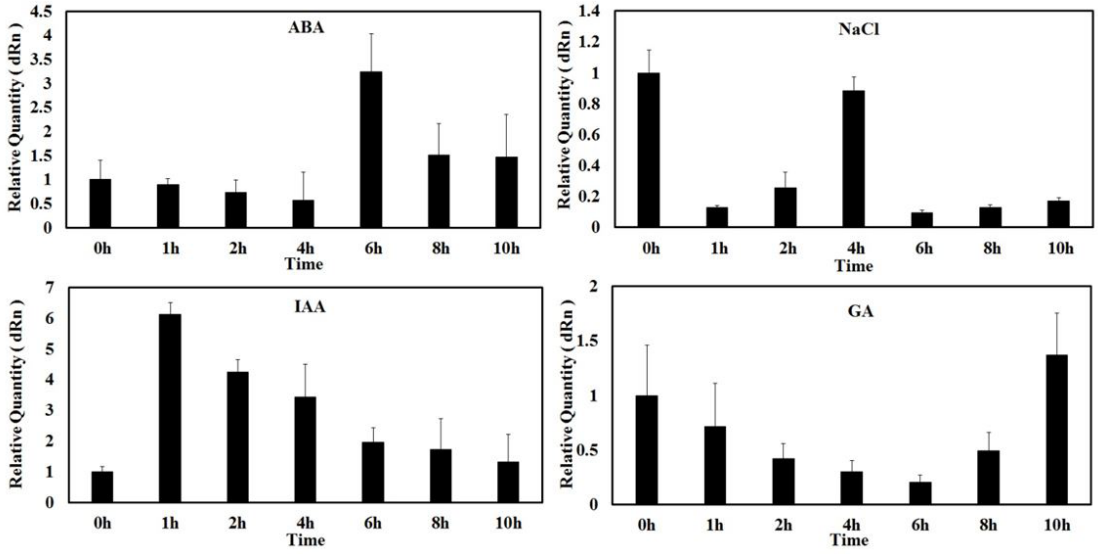

Figure 2. Real-time fluorescence quantitative PCR analysis of the expression of *Arabidopsis thaliana CKB3* gene in response to exogenous ABA, NaCl, IAA and GA. *Actin-2* was used as control.

acid to regulate stress responses in Arabidopsis thaliana (Yuan et al., 2017). Previous studies have shown that *CKB3* gene is a key component of the plant circadian clock system, including the long-day plant Arabidopsis, there is no reports on the involvement of this gene in stress response. Through gene chip analysis we found certain plant hormone and stress-response elements, such as ABRE (ABA-responsive element), AuxRE (auxin-responsive element), CGTCA-motif (MeJA-responsive element) and HSE (Heat-responsive element). In this study to understand the role of *CKB3* in abiotic stress signaling Arabidopsis thaliana homozygous T-DNA mutant *ckb3* was used. The germination, root growth, hypocotyl elongation, stomatal apertures, water-loss rate, proline content and the expression of *CKB3* in response to ABA of T-DNA mutant *ckb3* and Col-0 were measured. These results can determine whether *CKB3* gene is involved in the ABA signaling pathway.Although expression of *CKB3* in all organs, its expression increased in roots and seeds and this result agrees with the expression of *CKB1* gene (Yuan et al., 2017). The *ckb3* mutant showed reduced sensitivity to ABA during seed germination and seedling growth more stomatal opening and increased proline accumulation, these results indicate *CKB3* may be play a negative role in regulating seed germination, seedling growth, stomatal opening and proline accumulation under the influence of ABA. *ABI3* (ABA Insensitive 3) plays a negative feedback regulatory role in seed germination (Reyes and Chua, 2007). *ABI5* (ABA Insensitive 5) is a basic leucine zipper transcription factor that plays a pivotal role in the regulation of early seedling growth and seed germination in the abiotic stresses and ABA (Skubacz et al., 2016). Without ABA treatment the expression analysis of stress-responsive genes showed that the expressions of *ABI3* and *ABI5* were lower in *CKB3* T-DNA mutants plants than in Col-0 plants, the expression *ABI3* was equal in T-DNA mutants *ckb3* and Col-0 and *ABI5* was lower in *CKB3* T-DNA mutants plants than in Col-0 plants when treatment with ABA.

These results shown *CKB3* plays a role in regulating seed germination under the influence of ABA. *OST1* is well characterized at molecular and physiological levels

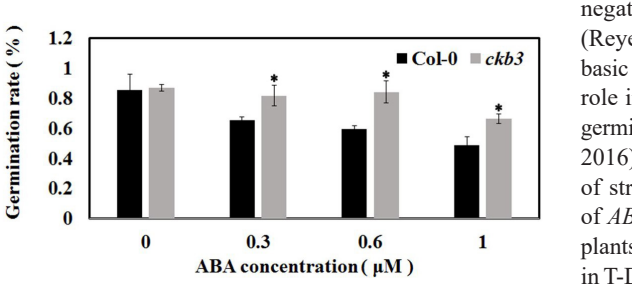

Figure 3. Effects of exogenous ABA on germination inhibition of seeds from Col-0, *Arabidopsis thaliana* homozygous T-DNA mutant ckb3. *indicate that the value of Two-way ANOVA was P< 0.05, this value indicate significant differences between the Col-0 and mutant.

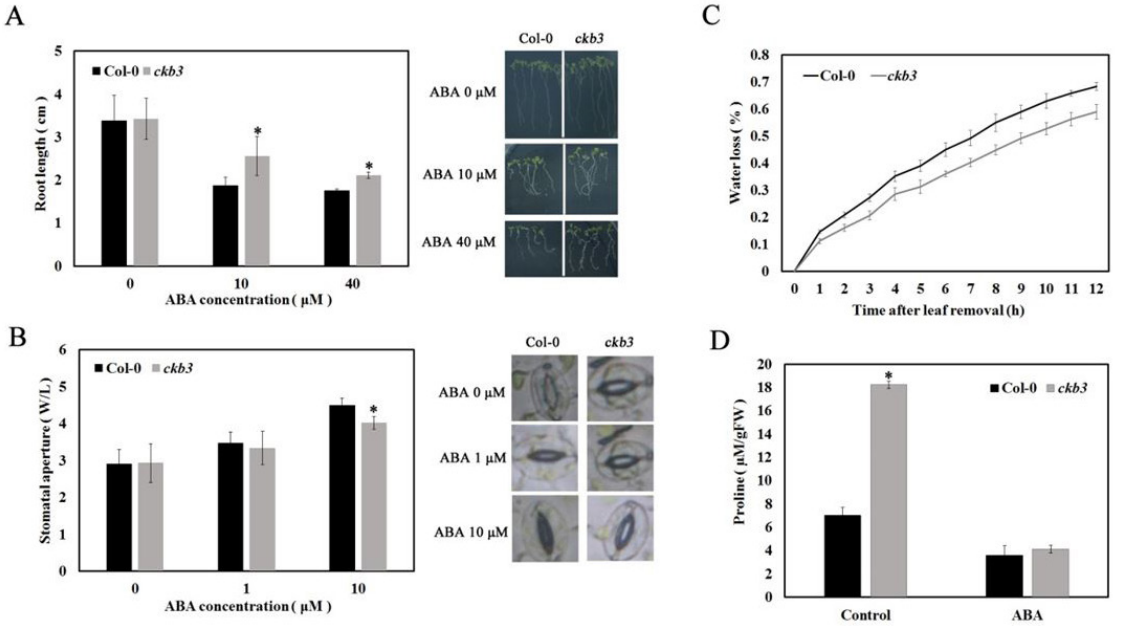

Figure 4. The analysis of root length, water-loss rate, stomatal aperture and proline accumulation in wild type and mutants of *Arabidopsis thaliana*. (A) The analysis of root length with ABA treatments; (B) Stomatal aperture of the wild type, and *ckb3* in response to ABA treatments; (C), Assessment of the water-loss rate. (D) Proline accumulation of Col-0, and *ckb3* in response to ABA. Data are expressed as the ratio of the traverse to the longitudinal diameter diameter (T/L) of stomata. *indicate that the value of Two-way ANOVA was P< 0.05, this value indicates significant differences between the Col-0 and mutant.

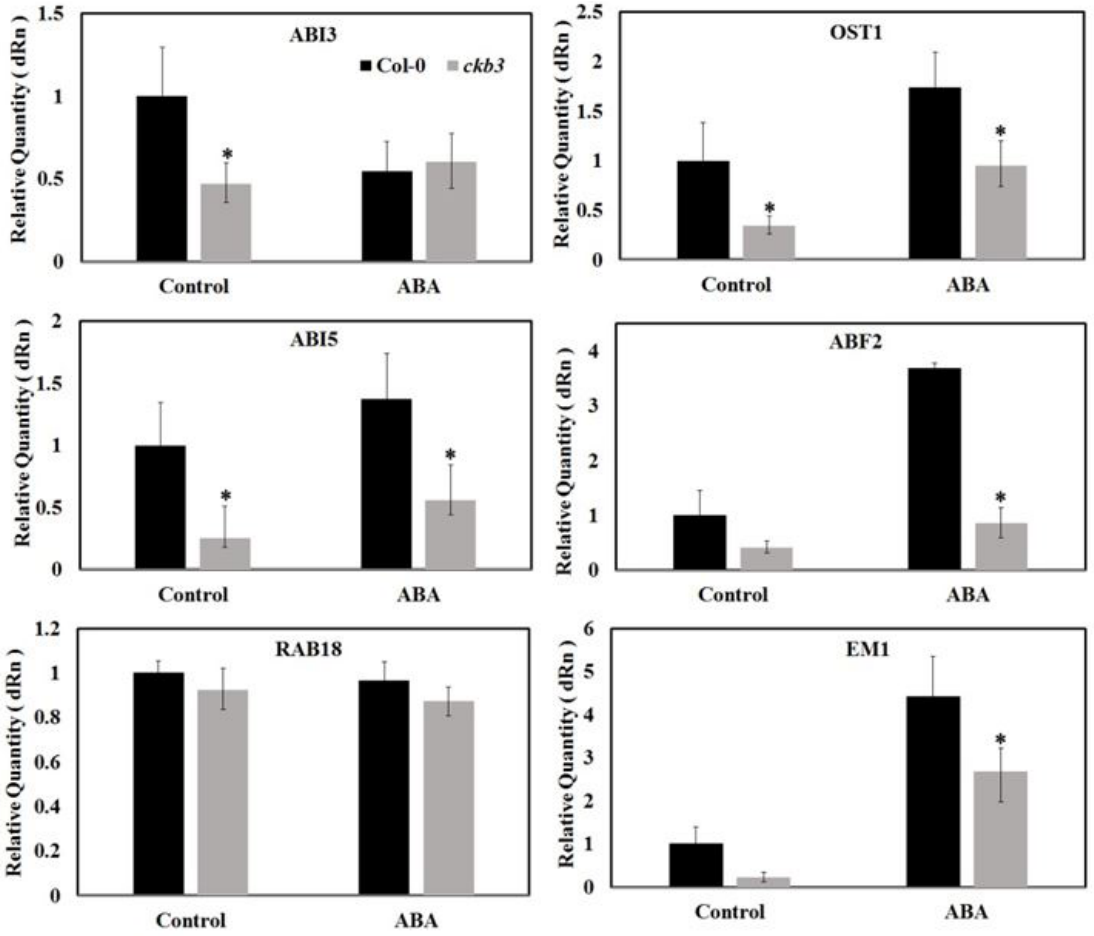

Figure 5. Expression of ABA and stress-responsive genes in wild-type Col-0 and *Arabidopsis thaliana* homozygous T-DNA mutant *ckb3* in response to ABA with actin-2 used as control. *indicate that the value of Two-way ANOVA P< 0.05.

to control stomata closure in response to water-deficit stress (Xuanyuan et al., 2017). After ABA processing the *OST1* expression was lower in the T-DNA mutant *ckb3* than in the Col-0 plants. The expression of *ABF2* (ABRE binding factor 2) and *EM1* was similar to the level of *OST1*. The expression of *ABF2* has been reported to be strongly induced by salt, drought and ABA (Zandkarimi et al., 2015; Zhou et al., 2016). *EM1* (Early Methionine-Labeled) was ABA-related gene the expression can be induced by *ABI5* (Skubacz et al., 2016). These results indicate that *CKB3* is an ABA signaling related gene and future is to explore whether this gene is involved in the plant stress response.

Acknowledgements

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