Plastid casein kinase 2 knockout reduces abscisic acid (ABA) sensitivity, thermotolerance, and expression of ABA- and heat-stress-responsive nuclear genes

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Abstract

Plastid casein kinase 2 (CK2) is a major Ser/Thr-specific enzyme for protein phosphorylation in the chloroplast stroma and its kinase activity is regulated by redox signals. To understand the role of CK2 phosphorylation of plastid proteins in abiotic stress signalling, an Arabidopsis plastid CK2 (CKA4) knockout mutant was investigated in terms of the plant response to abscisic acid (ABA) and heat stress. CKA4 expression was upregulated by ABA and heat treatment. The cka4 mutant showed reduced sensitivity to ABA during seed germination and seedling growth, and increased stomatal aperture and leaf water loss with a slightly reduced leaf ABA level. The cka4 mutant was more sensitive to heat stress than the wild-type Columbia-0. The expression levels of a number of genes in the ABA regulatory network were reduced in the cka4 mutant. Many heat-upregulated genes (heat-shock factors and heat-shock proteins) were also reduced in the cka4 mutant. The cka4 mutant showed reduced expression levels of plastid-encoded RNA polymerase target genes (atpB and psbA). CKA4 knockout mutation also resulted in a reduction in expression of some critical genes (PTM, ABI4, and PRS1) involved in retrograde signalling from the chloroplast to the nucleus. Similar results were observed in mutant plants with the knockout mutation in both CKA4 and CKA3, which encodes a nuclear CK2 α3 subunit. CKA3 expression was not responsive to ABA and heat stress. These results suggest that CKA4 is an enhancing factor in abiotic stress signalling through modulating the expression of some molecular players in retrograde signalling.

Key words: ABA insensitivity, gene expression, knockout mutation, plastid casein kinase 2, retrograde signalling, thermotolerance.

Introduction

Casein kinase 2 (CK2) is an evolutionary conserved Ser/Thr protein kinase and is generally composed of two different elements (catalytic α-subunit and regulatory β-subunit), which can combine to form a tetramer αβ2 (Litchfield, 2003; Mulekar and Huq, 2013). The genome of Arabidopsis thaliana contains eight genes coding for four α-subunits (αA/
CKA1, αB/CKA2, αC/CKA3, and αcp) and four β-subunits (β1/CKB1, β2/CKB2, β3/CKB3, and β4/CKB4) (Salinas et al., 2006). In Arabidopsis, three α-subunits (αA/CKA1, αB/CKA2, and αC/CKA3) are located in the nucleus, while αcp contains a chloroplast-targeting sequence motif and is localized exclusively in the chloroplast (Salinas et al., 2006). No β-subunits are present in the chloroplast (Salinas et al., 2006).

CK2 phosphorylates a wide range of proteins (Meiggs and Pinna 2003; Mulekar and Huq, 2013). Nuclear CK2 is known to play a role in several important processes in plants, such as cell division and expansion (Moreno-Romero et al., 2008), photomorphogenesis (Hardtke et al., 2000), circadian rhythms (Sugaro et al., 1998, 1999; Lu et al., 2011), flowering time (Mulekar and Huq, 2012), auxin response (Marquès-Bueno et al., 2011), and abscisic acid (ABA) response (Mulekar et al., 2012).

Involvement of nuclear CK2 in ABA signalling attracts the attention of abiotic stress biologists, as ABA plays a key role in gene regulation in plants under drought and salt stresses (Nakashima and Yamaguchi-Shinozaki, 2013). ABA also regulates various aspects of physiological and developmental processes in plants, such as seed maturation, dormancy and germination, and plant growth (Finkelstein et al., 2002; Nakashima and Yamaguchi-Shinozaki, 2013). Upon perception of drought and salt stresses, ABA levels in plant cells increase, which leads to stomatal closure to reduce leaf water loss and modulates expression of stress-related genes (Fujita et al., 2011). Knockout mutants of CK2 α-subunits in Arabidopsis show insensitivity to ABA-induced blockage of seed germination and cotyledon greening (Mulekar et al., 2012), indicating its role in modulating ABA signalling, although the molecular connection of CK2 in ABA signalling is still unknown. To date, the major molecular players in the core ABA signalling network have been identified (Umezawa et al., 2010; Raghavendra et al., 2010; Nakashima and Yamaguchi-Shinozaki, 2013).

Nuclear CK2 is also involved in the heat response in the mammalian system. Heat induces the relocation of CK2 and activation of its activity (Gerber et al., 2000; Soncin et al., 2003). Mammalian heat-shock factor (HSF) I is phosphorylated by CK2. HSF1 activation by heat is correlated with the thermal activation of nuclear CK2, and overexpression of CK2 activates HSF1 activity (Soncin et al., 2003). However, the role of CK2 in modulating the plant heat response is currently unknown. HSF plays a central role in the regulation of heat-shock proteins (HSPs) in all eukaryotic organisms, including plants (Scharf et al., 2012). HSPs are molecular chaperones and are responsible for stabilizing proteins and membranes and assisting in protein refolding under heat stress (Wang et al., 2004). Plants possess basal thermotolerance to cope with temperatures above the optimal for growth and acquired thermotolerance to enhance resistance to otherwise lethally high temperatures. Acquired thermotolerance relies on acclimation to permissive high temperatures, during which time a large number of heat-responsive genes (e.g. HSFs and HSPs) are rapidly induced (Kotak et al., 2007; Wang et al., 2004; Xue et al., 2014).

Studies of chloroplast CK2 have to date focused exclusively on its role in phosphorylation of chloroplast proteins. The chloroplast-targeting CK2 α-subunit is known to be present in other plant species in addition to Arabidopsis, namely plastid transcription kinase (PTK) or cpCK2 (Baginsky et al., 1999; Ogrzewalla et al., 2002; Jeong et al., 2004). PTK/cpCK2 is located in the stroma of chloroplasts and is the major kinase for phosphorylation activity in the stroma (Bayer et al., 2012). However, only a few PTK/cpCK2 substrates have been verified experimentally to date, which include sigma factors (SIG1 and SIG6), components of the plastid-encoded RNA polymerase complex (Ogrzewalla et al., 2002; Schweert et al., 2010b; Türkeri et al., 2012), RNA-binding proteins (Liere and Link, 1997; Reiland et al., 2009), chloroplast nucleoid-associated protein (MF1) (Jeong et al., 2004) and the β-subunit of the chloroplast ATPase complex (Kanekatsu et al., 1998). Large-scale phosphoproteome profiling in Arabidopsis has shown that potential CK2 phosphorylation sites are over-represented among 174 phosphoproteins in the chloroplast (Reiland et al., 2009). The potential target substrates include carbonic anhydrase, polynucleotide phosphorylase (PNPase), transcriptionally active chromosome (TAC) subunits (TAC10 and TAC16), and a thylakoid-associated kinase STATE TRANSITION 7 (STN7) (Reiland et al., 2009). STN7 is known to be involved in retrograde signalling from the chloroplast to the nucleus for regulation of nuclear-encoded photosynthetic genes (Pesaresi et al., 2009). The kinase activities of both PTK/cpCK2 and STN7 are regulated by redox signals in the chloroplast (Pfannschmidt and Liere, 2005; Schönberg and Baginsky 2012). Interestingly, the currently known pathway of STN7 retrograde signalling involves ABA INSENSITIVE4 (ABI4; León et al., 2013), one of its functions is connected to ABA signalling. Recently, retrograde signalling has also been identified to play an important role in heat-mediated induction of HSF and HSP genes, and downregulation of chloroplast ribosomal protein S1 inhibits the activation of HSFA2 and HSP gene expression in Arabidopsis during heat stress (Yu et al., 2012). The potential roles of PTK/cpCK2 in phosphorylating STN7 and modulating the expression of plastid genes, which could have a profound effect on nuclear gene expression, indicate that PTK/cpCK2 might have a role in retrograde signalling. Furthermore, redox signalling in the chloroplast, which regulates PTK/cpCK2 kinase activity, is also known to be connected to abiotic stress signalling such as under drought and heat-stress conditions (Sierla et al., 2013; Suzuki et al., 2012).

In this work, we investigated the potential role of Arabidopsis plastid CK2 αcp (hereafter named CKA4) in modulating plant responses to ABA and heat stress and regulating nuclear genes during these processes using knockout mutants to provide experimental evidence for its involvement in retrograde signalling. In addition to a cka4 mutant, two cka3 cka4 double-knockout mutants (a single T-DNA insertion knocks out both genes) were also used to consolidate observations of the cka4 mutant phenotypes and to investigate the potential additive role of CKA3, a nuclear CK2 α-subunit, in plant responses to ABA and heat stress. The cka4 and cka3 cka4 mutants showed reduced ABA insensitivity phenotypes during seed germination and seedling growth. These ABA-insensitivity phenotypes were supported...
by attenuation of ABA-mediated upregulation of ABA-responsive genes in the mutants. The knockout mutation of cka4 and cka3 cka4 also resulted in reduced thermotolerance and a reduction in expression of heat-responsive HSF and HSP genes during heat stress. No additive role of CKA3 to CKA4 in the Arabidopsis response to ABA and heat stress was seen. The knockout mutation of CKA4 also reduced the expression levels of some target genes of the plastid-encoded RNA polymerase and critical genes involved in known retrograde signalling pathways. These results provide experimental evidence that plastid CK2 is involved in retrograde signalling in plant responses to ABA and heat stress.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as a wild-type control. The cka4 (CS311135), cka3-cka4-1 (SALK_022432c), and cka3-cka4-2 (CS355016) T-DNA insertion mutant lines were obtained from the Arabidopsis Biological Resource Center (http://www.arabidopsis.org). For ABA treatment in a liquid medium, the seedlings were grown initially on Murashige and Skoog (MS) agar plates and were then transferred to a MS liquid medium with or without ABA supplement. MS agar plates were MS medium containing 1% (w/v) sucrose and 0.8% (w/v) agar. Details of ABA and without ABA supplement. MS agar plates were MS medium containing 1% (w/v) sucrose and 0.8% (w/v) agar. Details of ABA and heat treatments and the age of the plants are given in the figure legend.

RNA isolation and expression analysis using quantitative reverse transcription (RT)-PCR

Total RNA was isolated from plants using a Total RNA Purification System kit (Invitrogen). The purified RNA samples were treated with gDNA Eraser (Takara) to remove genomic DNA according to the manufacturer’s instruction. cDNA was synthesized using a PrimeScript™ RT reagent kit (Takara). For quantitative real-time PCR analysis, triplicate PCRs were carried out for each sample in a Mx3000P Real-Time Thermal Cycler (Stratagene). The gene-specific primers used for quantification of mRNA are listed in Supplementary Table S1 at JXB online. The Actin2 gene was used as an internal control. The following amplification procedure was used: 95 °C for 15 min, followed by 45 amplification cycles of 95 °C for 15 s, 56 °C for 40 s, and 72°C for 10 s. The specificity of real-time PCR amplification was confirmed by a single peak in melting temperature curve analysis of real-time PCR-amplified products. The quantification of relative mRNA levels was determined as described by Xue et al. (2011).

Verification of T-DNA insertion mutation

First, PCR analysis was used to verify the cka3 cka4 and cka4 mutants and to obtain homozygotes of cka3-cka4-1, cka3-cka4-2, and cka4. The positions of primers used for PCR amplification are indicated in Fig. 1A and primers sequences are shown in Supplementary Table S1. Secondly, the expression levels of CKA3 and CKA4 were determined in the leaves of 30-d-old plants of the mutants using quantitative real-time PCR analysis and primers designed downstream of T-DNA insertion sites in CKA4 and CKA3. CS31135 obtained from the Arabidopsis Biological Resource Center had a second T-DNA insertion at the At1g66110 gene besides the CKA4 knockout mutation. To verify that the At1g66110 mutation was absent in the progeny of CS311135, specific primers for At1g66110 downstream of the T-DNA-insertion site (Supplementary Table S1) were used to determine the expression level of the At1g66110 gene. Progeny of CS311135 with the presence of the cka4 mutation and absence of the At1g66110 knockout mutation were used for this study.

Fig. 1. CK2 α4 (CKA4) and α3 (CKA3) transcript levels in CS311135 (cka4), SALK_022432c (cka3-cka4-1), and CS355016 (cka3-cka4-2) mutants with a T-DNA insertion. (A) Schematic illustration of the CKA4 and CKA3 genes and localization of the T-DNA insertion in cka4 and cka3 cka4 mutants. Exons and introns are represented by black rectangles and thick lines, respectively. The 5' and 3' untranslated regions (UTRs) are depicted as white rectangles. The locations of the T-DNA insertions are indicated by triangles. The positions of PCR primers are indicated by arrows. (B) The CKA3 and CKA4 transcript levels in the rosette leaves of 30-d-old Col-0, cka4, and cka3 cka4 mutants and were determined using real-time PCR. Relative expression level was normalized to Col-0, and values are means±standard deviation (SD) of three biological replicates. Cka3a4-1, cka3-cka4-1, cka3a4-2, cka3-cka4-2.
Measurements of proline and malondialdehyde (MDA)

After treatment, the samples were frozen in liquid nitrogen and kept at −80 °C until proline and MDA assays were carried out. Free proline concentrations in seedlings were determined using the method described by Bates et al. (1973). MDA extraction and measurement were according to Zhang et al. (2009).

Leaf water loss and stomatal aperture measurement

Leaf water-loss analysis was carried out using 30-d-old plants grown in soil. Rosette leaves (±0.1 g) were cut from the wild-type and mutant plants and placed in the growth cabinet at 22 °C with the lights on. Leaf water loss was monitored over a 2 h period. Water loss at each time point was presented as the percentage of initial fresh weight.

Stomatal aperture was measured using whole-leaf imaging as described by Pei et al. (1998). Rosette leaves of 30-d-old plants were used for preparation of epidermal peels and treated with or without ABA (1 or 10 μM) in a stomatal opening solution (20 mM KCl, 1 mM CaCl₂, and 5 mM MES/KOH; pH 6.15) for 4 h in a growth cabinet. A Nikon TE 2000 inverted fluorescence microscope with ACT-2U software was used to measure stomatal apertures of rosette epidermal peels. The aperture ratios (ratio of stomatal pore width to length) of 40–50 stomata from Col-0 or mutants were determined in each experiment. Three independent experiments were conducted to obtain the mean values of stomatal aperture ratios for each genotype.

Measurement of ABA levels

The rosette leaves were detached from 30-d-old plants grown in soil and then incubated in liquid MS medium with or without 10 μM ABA for 4 h. Tissue ABA levels were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS 8030 plus; Shimadzu, Japan). A total of 200 mg (fresh weight) of ground leaf sample was well mixed with 1 ml of 80% methanol in an ultrasonic bath and then kept at 4 °C overnight. After being centrifuged at 15 200g for 10 min, the supernatant was collected and dried under vacuum. Dried extract was dissolved in 200 μl of 0.1 M sodium phosphate solution (pH 7.8). The solution was passed through a Sep-Pak C₁₈ cartridge (Waters, USA), followed by elution with 1500 μl of 80% methanol. The eluate was dried under vacuum and then redissolved in 50 μl of 10% methanol; 5 μl of the solution was injected into the LC-MS/MS 8030 system in which an Acquity UPLC BEH column (2.1 mm inner diameter × 100 mm, particle size 1.7 μm; Waters, USA) was used. The column temperature was set at 40 °C and the eluant flow rate was set at 0.25 μl min⁻¹. The mobile phase comprised solvent A (0.02% acetic acid) and solvent B (acetonitrile) in a gradient mode [time (min)/solvent A (%)/solvent B (%) was 0/90/10, 4/30/70, 5/50/100, and 6/90/10]. The mass spectrometer was set to a multiple reaction monitoring mode using electrospray ionization in a negative ion mode, with a nebulizing gas flow at 3 l min⁻₁, a drying gas flow rate was set at 0.25 ml min⁻¹, a desolvation temperature at 250 °C, and a heat block temperature at 480 °C. 2H₆-ABA (Olchemim, Czech Republic) was used as an internal standard. For ABA, the ionization conditions were employed, while for 2H₆-ABA, the ionization conditions (pre-bias voltages of 19 V for quadrupole 1 and 28 V for quadrupole 3, collision energy of 10 eV, mass-to-charge ratio of 263:153.2) were employed. The seedlings were placed on MS agar plates supplemented with various concentrations of ABA (0, 10, or 40 μM) and grown for 7 d in a growth chamber at 22 °C with a 16 h light/8 h dark cycle. For heat treatment, the seedlings were subjected to a heat-treatment regime (37 °C for 2 h, 22 °C for 2 h, and 45 °C for 2.5 h) and then grown at 22 °C for 5 d. For dark-growth experiments, 2.5-d-dark-grown seedlings were exposed to the above heat-treatment regime and then grown at 22 °C for 5 d in the dark. Root and hypocotyl lengths were measured at the end of the experimental growth periods.

Results

CK2 α₄ (CKA4) knockout mutant and α₃ α₄ (CKA3 CKA4) double-knockout mutants

To elucidate the role of CKA4 (At2g23070) in the ABA and heat-stress signalling and the potential additive role of CKA3, we investigated three T-DNA insertion mutants: a cka₄ mutant (CS311135) and two cka₃ cka₄ double mutants (SALK_022432c and CS355016). The presence and position of the T-DNA insertion in the progeny of the three mutants were confirmed by PCR using primers flanking the T-DNA insertion sites (data not shown). The T-DNA insertion sites in these three mutants are illustrated in Fig. 1A. CKA4 is located adjacent to CKA3 (At2g23080). The two genes run in opposite orientation and part of the coding sequence of one gene is the promoter of the other. Therefore, a T-DNA insertion in SALK_022432c and CS355016 was expected to affect the expression of both CKA4 and CKA3. However, the T-DNA insertion mutation of CKA4 in CS311135 is unlikely to interfere with the expression of CKA3. To determine whether CKA4 and CKA3 expression was affected by T-DNA insertion in these mutants, quantitative RT-PCR was performed using primers targeting CKA4 and CKA3 transcript sequences downstream of its T-DNA insertion site. As shown in Fig. 1B, neither CKA4 nor CKA3 transcripts were detectable in the leaves of SALK_022432c and CS355016. Therefore, these two mutant lines represented CKA3 CKA4 double-knockout mutants [cka₃ cka₄-1 (SALK_022432c) and cka₃ cka₄-2 (CS355016)]. In CS31135, only the CKA4 transcript level was not detectable and the expression level of CKA3 was similar to Col-0 wild-type plants. Therefore, CS31135 is a cka₄ mutant. CS31135 obtained from the Arabidopsis Biological Resource Center also has a mutation in At1g66110 encoding a protein belonging to the DUF577 family of unknown function. The At1g66110 mutation was eliminated in the progeny of CS31135, as evidenced by the presence of a similar transcript level of the At1g66110 gene between Col-0 and the CS31135 progeny containing cka₄ (Supplementary Fig. S1 at JXB online). The absence of the CKA4 and CKA3 CKA4 transcript in the progeny of these T-DNA insertion mutants indicated that they were homozygous.

A loss-of-function mutation of CKA3 CKA4 or CKA4 leads to reduced ABA sensitivity

ABA is known to regulate seed germination. The efficiency of seed germination in MS agar plates supplemented with various concentrations of ABA was examined by scoring
radicle emergence rates 5 d after vernalization. No significant difference in germination between mutants and wild-type Col-0 with 0 and 0.3 µM ABA was observed (Fig. 2A). With ABA concentrations of 0.6 and 1 µM, two cka3 cka4 double mutants had a higher germination rate than Col-0. This high germination rate was also observed in CS311135, indicating that CKA4 knockout alone can account for the observed ABA-insensitive phenotype.

The effect of CKA4 and CKA3 CKA4 mutation on the germination rate of seeds under salt or osmotic (mannitol) stress was also investigated. Seed germination was carried out in MS agar plates supplemented with NaCl (0, 50, 100, or 150 mM) or mannitol (0, 50, 150, or 300 mM). It is well established that ABA accumulates in plants under these stresses (Zhu, 2002). Significantly higher germination rates were observed in the three mutants than in Col-0 plants at NaCl concentrations above 100 mM or mannitol concentrations above 150 mM (Fig. 2B, C).

This study also investigated whether the loss of function of CKA4 and CKA3 CKA4 affected the ABA-mediated root growth inhibition. High concentrations of ABA are known to inhibit root growth (Sharp and LeNoble, 2002). Germinated Col-0 and mutant seedlings were grown on MS agar plates containing different concentrations of ABA (0, 10, or 40 µM). The lengths of the primary roots of these plants were measured after 7 d of growth. As shown in Fig. 2D, the cka4 and cka3 cka4 mutants grew significantly longer roots than Col-0 seedlings on medium containing 10 µM ABA, indicating that the sensitivity of seedling root growth to ABA was reduced in the mutants.

Disruption of CKA4 increases stomatal aperture and leaf water loss rate

Each stoma is surrounded by two guard cells, which control the opening and closure of the stomatal aperture by their relative turgor pressure. ABA is known to play an important role in controlling stomatal aperture. To elucidate the potential role of CKA4 in stomatal regulation and the additive role of CKA3, the stomatal aperture of rosette leaves was examined in cka4 and cka3 cka4 mutants. As shown in Fig. 3A and B, the cka4 mutant had a significantly wider stomatal aperture size than Col-0 plants with 0 or 1 µM ABA treatment. However, the stomata of the mutant were still able to close effectively at the high ABA concentration (10 µM). Similar results were observed in the cka3 cka4 double mutants.

The large stomatal aperture in the cka4 and cka3 cka4 mutants without ABA treatment might be attributed to either a deficiency in endogenous ABA or to ABA insensitivity. Therefore, the ABA levels were measured in the leaves of Col-0 plants and the cka4 mutant with or without 10 µM ABA treatment. As shown in Fig. 3C, there was a slight but statistically significant difference in the leaf ABA levels between Col-0 and the cka4 mutant without ABA treatment. However, no difference was observed between Col-0 and the cka4 mutant with 10 µM ABA treatment for 4 h.

Stomatal closure is known to be controlled by OPEN STOMATA 1 (OST1) and the expression level of OST1 is regulated by ABA (Mustilli et al., 2002). Quantitative RT-PCR analysis showed that the constitutive expression level of OST1 in three mutants was markedly lower than that of Col-0 (Fig. 3D). The reduction in the OST1 expression level in these mutants was still observed at a low ABA concentration (1 µM). At the high ABA concentration, no difference was observed between the mutants and wild-type plants. These expression results are in line with the increased stomatal aperture sizes in the mutants without or with a low ABA concentration treatment.

The stomatal aperture size was expected to be positively associated with the water-loss rate of plant leaves. To provide further supporting evidence for the effect of CKA4 mutation on stomatal aperture, leaf water loss was measured in the mutants and wild-type plants over a 2 h period. The leaf water loss in the three mutants was markedly quicker than in Col-0 plants (Fig. 3E). Furthermore, water loss at the whole-plant level was also examined. In the well-watered conditions, no obvious difference was observed in growth between the mutants and Col-0 (Fig. 3F). With withdrawal of water supply, the plants of the three mutants in pots utilized water more quickly than Col-0 plants, which caused the mutant plants to wilt and wither earlier than Col-0 plants (Fig. 3F).

CKA4 is upregulated by ABA, and CKA4 or CKA3 CKA4 mutation attenuates upregulation of ABA-responsive genes by ABA

To investigate whether CKA4 is involved in ABA-mediated gene regulation, the ABA responsiveness of CKA4 and the effect of CKA4 and CKA3 CKA4 mutation on the expression of ABA-responsive genes were examined. CKA4 expression was upregulated by ABA in Col-0 plants (Fig. 4A, B). A maximum upregulation of CKA4 expression by ABA was seen at 10 µM and 6 h after ABA treatment in 2-week-old Col-0 plants. In contrast, CKA3 was not regulated by ABA in Col-0 (Fig. 4C). For the investigation of CKA4 involvement in regulation of genes in ABA signalling pathway, the expression levels of six ABA signalling downstream genes [RESPONSIVE TO DEHYDRATION (RD) 29A, RD29B, RD22, RESPONSE TO ABA 18 (RAB18), KIN1, and KIN2] were compared between the Col-0 and mutant plants after 10 µM ABA treatment for 6 h. RD29A, RD29B, RD22, and RAB18 are frequently used as ABA signalling downstream marker genes (Lång and Palva, 1992; Lee et al., 2010). Arabidopsis KIN1 and KIN2 are cold- and ABA-inducible downstream genes and encode small polypeptides (Kurkela and Borg-Franck, 1992). Expression analysis revealed that induction of the six ABA-responsive marker genes by ABA was attenuated in the cka4 and cka3 cka4 mutants (Fig. 4D). The expression level of an ABRE BINDING FACTOR3 (ABF3) was also lower in the mutant lines than in Col-0 (Fig. 4D), which is one of the important transcriptional activators of many ABA-upregulated downstream genes in vegetative organs and has been shown to upregulate RD29 and RAB18 (Kang et al., 2002). These results indicated that CKA4 is a positive regulator in ABA signalling.
Fig. 2. Reduced ABA sensitivity phenotypes of cka4 and cka3 cka4 mutants. (A–C) The effects of ABA (A), NaCl (B), and mannitol (C) on seed germination. Col-0 and mutant seeds were germinated on MS agar plates containing 1% sucrose and different concentrations of ABA, NaCl, or mannitol. Radicle emergency rates were determined 5 d after vernalization. Values are means±SD of 300 seeds. (D) Sensitivity of root growth to ABA in Col-0, cka4, and cka3 cka4 mutants. Seedlings of Col-0 and the mutants were grown for 6 d on ABA-free medium and then incubated vertically for 7 d on an MS agar plate supplemented with or without ABA. Values are means±SD of 200 seedlings. Asterisks indicate significant differences between mutant and Col-0 (*P<0.05, **P<0.01, using Student’s t-test). (This figure is available in colour at JXB online.)
cka3 and cka3 cka4 mutants show reduced thermotolerance

The involvement of CKA4 and the possible additive role of CKA3 in plant adaptation to heat stress were investigated in the cka4 and cka3 cka4 mutants. Three experiments were performed to evaluate the thermosensitivity of the mutants relative to Col-0 wild-type plants. In the first experiment, 4-week-old seedlings were exposed to a heat-treatment...
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regime with heat acclimation (37 °C for 2 h, 22 °C for 2 h, and 45 °C for 2.5 h). As shown in Fig. 5A, the cka4 and cka3 cka4 mutants were more sensitive to heat stress, showing severer damage by heat stress than Col-0. This indicates that the acquired thermotolerance is impaired in these mutants. In the second experiment 2-d-old germinated seeds were directly exposed to high temperature (45 °C for 90 min), which is commonly used to assess basal thermotolerance. This treatment resulted in much severer growth retardation and mortality in the mutants compared with Col-0 (Fig. 5B), demonstrating the reduced basal thermotolerance in the mutants. The third experiment was to provide statistical data on the survival rates after heat stress between the mutants and Col-0 plants by exposing 2-d-old seedlings to the heat-treatment regime with heat acclimation, as described for the first experiment. The cka4 and

Fig. 4. ABA-mediated expression changes of CKA4, CKA3, and potential downstream genes in the shoots of 2-week-old plants. (A) Relative CKA4 expression level in Col-0 plants treated with various concentrations of ABA for 6 h. (B) Relative CKA4 expression level in Col-0 plants treated with 10 µM ABA for up to 10 h. (C) Relative CKA3 expression level in Col-0 plants treated with 10 µM ABA for up to 8 h. (D) A comparative expression analysis of ABA-responsive genes in the seedlings of Col-0 and mutants treated with 10 µM ABA for 6 h. Values are means±SD of three biological replicates. In (B) and (C), the relative expression level in ABA-treated samples was normalized to the sample without treatment (control) at each time point. In (D), the relative expression level was normalized to Col-0 under control conditions. Asterisks indicate significant differences between mutant and Col-0 or between ABA treatment and control (*P<0.05, **P<0.01).
cka3 cka4 mutants showed significantly lower survival rates than Col-0 (Fig. 5C). It appeared that the cka3 cka4 double mutants had a similar survival rate to the cka4 mutant, indicating that there was no additive effect of CKA3 to the reduced thermotolerance caused by the CKA4 loss-of-function mutation.

To investigate further the role of CKA4 in acquired thermotolerance, root and hypocotyl elongation assays were performed. No significant differences in root and hypocotyl elongation were observed between Col-0 plants and the three mutants under control conditions. Exposure of 5-d-old seedlings to the heat-treatment regime with heat acclimation
as described above reduced root elongation in both Col-0 and the three mutants (Supplementary Fig. S2A, B at JXB online). The root-length reduction in the cka4 and cka3 cka4 mutants was more pronounced than that in Col-0 plants. The reduction of the hypocotyl length was also observed in the three mutants, but not in the wild-type seedlings in this heat-stress regime (Supplementary Fig. S2C, D). These results demonstrated that CKA4 or CKA3 CKA4 mutation causes significant impairment of root and hypocotyl elongation after heat stress.

Cka4 and cka3 cka4 mutants show reduced proline accumulation and increased MDA content after heat stress

Increased proline accumulation is known to occur in plants under abiotic stress conditions, mainly for stress protection (Szabados and Savouré, 2010). High temperature can lead to increased lipid peroxidation, which is quantified by measuring the amount of MDA (Hulbert et al., 2007). Therefore, proline and MDA contents were measured in Col-0 and three mutant plants after heat stress. No differences in the proline and MDA contents were observed in the leaves of 1-month-old plants between Col-0 and the mutants under non-stress conditions (Fig. 6). Both proline and MDA contents increased in Col-0 and the three mutants after heat stress. However, the cka4 and cka3 cka4 mutants accumulated significantly less proline in the leaves than Col-0 plants (Fig. 6A), which may indicate a reduced stress-protective capacity in the mutants. Significantly higher lipid peroxidation occurred in the mutants after heat stress, as indicated by the higher MDA content in the mutants than in Col-0 (Fig. 6B). These data demonstrated the relative severity of heat-stress damage in the cell membrane between the mutants and Col-0.

CKA4 expression is upregulated during heat stress and recovery phase, and disruption of CKA4 reduces expression of heat-induced genes

To gain insights into the molecular mechanisms of reduced thermotolerance caused by CKA4 mutation, the expression response of CKA4 to heat stress and a comparative expression analysis of representative HSF and HSP genes were performed. Quantitative RT-PCR analysis showed that CKA4 expression in the leaves of Col-0 plants was significantly upregulated during heat acclimation (Fig. 7A, B). The CKA4 mRNA level remained elevated after a 2 h recovery at 22 °C in the heat-treatment regime adopted in this study (Fig. 7B). Most interestingly, a very high level of CKA4 transcript accumulation was observed during the recovery phase, 24 h after the heat-treatment regime (Fig. 7C). No significant change in the expression of CKA3 during heat stress was observed in Col-0 plants (Fig. 7D).

During heat acclimation, the expression levels of HSFs (HSFA1d, HSFA2, and HSFA3) and HSPs (HSP18.2, HSP21, HSP25.3, HSP26.5, HSP90.1, and HSP101) were markedly upregulated (Fig. 7E). This heat-induced upregulation was apparently less in cka4 and cka3 cka4 mutants than in Col-0 (Fig. 7E). The attenuation in the heat-induced upregulation of HSP25.3 and HSP101 transcripts in the mutants was particularly pronounced. A decrease in the accumulation of these gene transcripts in the mutants indicated a reduced thermotolerance capacity in these plants. Again, no significant differences in the heat-induced transcript accumulation of these HSF and HSP genes were observed between cka4 and cka3 cka4 double mutants, suggesting no additive role of CKA3 to CKA4 in the heat-mediated regulation of these heat-stress genes.

Fig. 6. Accumulation of proline and MDA in Col-0 and cka4 and cka3 cka4 mutants after heat stress. (A) Proline content in the leaves of 30-d-old plants of Col-0 and mutants with or without a heat-treatment regime (37 °C for 2 h, 22 ºC for 2 h, and 45 ºC for 2.5 h). (B) MDA content in the leaves of 30-d-old plants of Col-0 and mutants with or without the heat-treatment regime. Samples were harvested immediately after the heat-treatment regime. Values are means±SD of three biological replicates. Asterisks indicate significant differences between the mutants and the Col-0 (*P<0.05).

CKA4 knockout reduces the expression of critical genes involved in retrograde signalling pathways and some plastid-encoded RNA polymerase target genes

Alterations in the expression levels of nuclear genes in the cka4 mutant suggested that CKA4 is involved in retrograde signalling from the chloroplast to the nucleus. One known retrograde
Fig. 7. Expression levels of CKA4, CKA3, HSF, and HSP genes in the leaves of 4-week-old plants (A) Expression levels of CKA4 in Col-0 plants during heat treatment (37 °C). (B) Expression levels of CKA4 in Col-0 plants during a heat-treatment regime (37 °C for 2 h, 22 °C for 2 h and 45 °C for 2.5 h). Pre-heat samples were harvested before heat treatment. (C) Expression levels of CKA4 in Col-0 plants during the recovery phase. Samples were harvested during the 22 °C recovery phase after the heat-treatment regime. (D) Expression levels of CKA3 in Col-0 plants during heat treatment (37 °C). (E) Expression levels of HSF and HSP genes in Col-0 plants and mutants treated at 37 °C for 3 h. Control samples were without heat treatment. In (A)–(D), relative expression levels in treated samples was normalized to samples without treatment (control) at each time point. In (E), the relative expression level was normalized to Col-0 plants under control conditions. Values are means±SD of three biological replicates. Asterisks indicate significant differences between treated and control or between mutant and Col-0 plants (*P<0.05, **P<0.01).
signalling pathway involves GEMONES UNCOUPLED 1 (GUN1, a plastid RNA-binding protein), a PHD-type transcription factor with a transmembrane domain (PTM) and ABI4; ABI4 is a central regulator for this retrograde signalling and is also involved in the ABA signalling pathway (León et al., 2013). To investigate whether genes involved in this retrograde signalling pathway are affected by the CKA4 mutation, the expression levels of these three genes in the mutant and Col-0 plants under normal and ABA-treated conditions were determined. As shown in Fig. 8, the expression level of ABI4 was significantly reduced in the cka4 and cka3 cka4 mutants under both normal conditions and ABA treatment. A significant reduction in PTM expression was also observed in the mutants with ABA treatment but not in the normal conditions. In contrast, the expression level of GUN1 was higher in the mutants than in Col-0 in both control and ABA-treated plants.

For retrograde signalling during heat stress, a heat-responsive nucleus-encoded plastid ribosomal protein S1 (PRS1) has been shown to be involved in this process (Yu et al., 2012). This study also showed that the expression level of PRS1 was elevated in heat-treated plants (Fig. 8), but its heat-induced upregulation was attenuated in the cka4 and cka3 cka4 mutants. No significant differences in the expression levels of PRS1 under control conditions and ABA treatment were observed between Col-0 and the mutants.

Plastid CK2 is known to phosphorylate some components of plastid-encoded RNA polymerase (PEP) complex and has

![Fig. 8. Expression levels of genes involved in retrograde signalling pathways and plastid-encoded RNA polymerase target genes in Col-0, cka4, and cka3 cka4 mutants. The leaves of 4-week-old plants were used for analysis. Plants were treated with either 10 µM ABA for 6 h or at 37 °C for 3h in comparison with no treatment (control). Relative expression level was normalized to Col-0 under control conditions and values are means±SD of three biological replicates. Asterisks indicate significant differences between mutant and Col-0 plants (*P<0.05).](image)
be proposed to have a role in regulating expression of some plastid-encoded genes (Schweer et al., 2010b). The availability of the cka4 mutant provided us an opportunity to test this hypothesis. This study analysed the expression levels of three known PEP target genes [psbA coding for photosystem II D1 protein, atpB coding for the β-subunit of chloroplast ATPase complex, and trnK (tRNA-Lys); Schweer et al., 2010b]. Expression levels of psbA and atpB were significantly reduced in the cka4 and cka3 cka4 mutants under normal conditions as well as with ABA and heat treatment in comparison with Col-0 plants, while trnK expression was not affected (Fig. 8).

cka4 and cka3 cka4 mutants show normal growth phenotypes under non-stress conditions

In order to see whether the reduced ABA sensitivity and thermotolerance was due to alteration in growth as a result of CKA4 mutation, a comparative growth analysis was performed. As shown in Supplementary Fig. S3 at JXB online, no growth defects were observed in the cka4 and cka3 cka4 mutants. The morphologic appearance, rosette size and shoot and root dry matter production in 4-week-old plants were similar between Col-0 and three mutants. These data indicated that CKA4 knockout mutation does not affect plant morphology and growth under normal growth conditions.

Discussion

This study showed that a knockout mutation in the plastid CK2 gene (CKA4) resulted in reduced ABA sensitivity and thermotolerance in Arabidopsis, which was further supported by the same phenotypes observed in two cka3 cka4 double-knockout mutants. Alterations in these phenotypes in the cka4 and cka3 cka4 mutants were accompanied by a reduction in signal transduction for inducing expression of ABA- or heat-stress-responsive downstream genes in the nucleus. This indicates that the phosphorylation process by plastid CK2 enhances retrograde signalling from the plastid to the nucleus. Thus, this study demonstrated that plastid CK2 is a positive regulator in retrograde signalling in the plant response to ABA and heat stress.

A previous knockout mutation study has shown that three nuclear-located CK2 α-subunits (CKA1–3) in Arabidopsis have a synergistic role in ABA-induced blockage of seed germination and cotyledon greening (Mulekar et al., 2012), but the molecular basis of this ABA insensitivity in these mutants was not investigated. Although the upstream pathway of nuclear CK2 involvement leading to this ABA insensitivity is expected to differ from the plastid CK2, we thought that cka3 cka4 double mutants might have severer ABA-insensitivity phenotypes during seed germination and seedling growth. However, no significant differences in the phenotypes and expression levels of downstream genes were observed between the cka4 mutant and cka3 cka4 mutants in the plant response to ABA. Similarly, the reduced thermodulation and expression of heat-responsive genes in the cka4 mutant did not differ significantly from those in the cka3 cka4 double mutants during heat stress. This indicates that plastid CK2, through retrograde signalling, may play a dominant role over the nuclear CKA3 in regulating gene expression during the processes investigated. However, whether nuclear CK2 α-subunits in plants are involved in signal transduction during heat stress is currently unknown, although mammalian nuclear CK2 phosphorylates HSF1 (Soncin et al., 2003), a key regulator in the mammalian cell heat response. CKA4 has been shown to have the highest expression level among four α-subunit genes, with CKA3 being the lowest in the leaf, root, stem, and flower organs of Arabidopsis (Salinas et al., 2006). In particular, the function of the three nuclear CK2 α-subunit genes is likely to be redundant. This could be one factor for contributing to the lack of observable additive effect of CKA3 seen in the cka3 cka4 double mutant lines. However, the two cka3 cka4 double mutants used in this study can serve as two independent lines for confirming the phenotypic changes resulting from CKA4 mutation, as these double mutants have a CKA4 knockout mutation by T-DNA insertion at two different locations from the cka4 mutant.

ABA regulates many important plant processes such as seed germination, dormancy, seedling growth, and stomatal closure (Finkelstein et al., 2002; Mustilli et al., 2002; Fujii et al., 2007). The molecular mechanisms underlying ABA responses in plants have been intensively studied by biochemical and genetic approaches (Pandey et al., 2005). The major molecular players in the core ABA signalling network have been identified by molecular and genetic analyses (Fujii et al., 2009; Umezawa et al., 2010; Raghavendra et al., 2010; Nakashima and Yamaguchi-Shinozaki, 2013). The reduced sensitivity phenotypes of the cka4 mutant in response to exogenous ABA during seed germination and seedling growth indicate that CKA4 is involved in modulating ABA signalling. CKA4 expression was upregulated by ABA, which may serve as enhancement for its action in this process. The reduced ABA sensitivity in the cka4 mutant is particularly evident in the following two datasets: (i) significantly reduced inhibition of root growth in the mutants in the presence of 10 μM ABA (Fig. 2D), and (ii) a significant reduction in the ABA-mediated upregulation of six nuclear-encoded ABA-responsive downstream genes (RD22, RD29A, RD29B, RAB18, KIN1, and KIN2) in an experiment where 10 μM ABA was used for induction (Fig. 4D). A significant reduction in the expression level of ABF3 was also observed in the cka4 mutant. ABF3 is an important transcriptional activator in vegetative organs and binds to ABA-responsive elements (Yoshida et al., 2010), which are present in many downstream genes (e.g. RD29B and RAB18) of the ABA regulatory network (Kang et al., 2002).

Interestingly, the stomatal apertures of the cka4 mutant were significantly wider than those of Col-0 plants under normal conditions and following treatment with a low concentration of ABA. Expression analysis revealed that the expression level of OST1 was significantly lower in the cka4 mutant than in Col-0 under these conditions. OST1, expressed in stomatal guard cells and vascular tissue, positively regulates ABA- and reactive oxygen species-mediated stomatal closure (Mustilli et al., 2002). OST1 controls the activity of guard cell anion
channel SLAC1 (Geiger et al., 2009). Because of the wider opening of the stomata in the mutant, the leaf water loss in the mutant was more rapid than in Col-0 and the mutant became more susceptible to drought stress. The wider opening of the stomatal aperture in the cka4 mutant than in Col-0 under normal conditions appears to be related at least partly to its lower leaf ABA level. As part of the ABA synthetic pathways is located in the chloroplast (Seo and Koshiba, 2002), the CKA4 knockout may also affect the activities of some enzymes involved in the chloroplast part of the ABA synthetic pathways. However, the CKA4 knockout did not affect plant growth under normal growth conditions.

In addition to its role in ABA signalling, this study showed that CKA4 also participated in heat-stress signalling. Knockout mutation of CKA4 resulted in increased plant susceptibility to heat stress and reduced the survival rate of plants after exposure to a lethally high temperature. Both basal and acquired thermotolerance capacities were affected by CKA4 mutation. The root and hypocotyl elongation rates after heat treatment were significantly reduced in the cka4 mutant, indicating that more severe heat injury occurred in the mutant than in Col-0. The cka4 mutant plants accumulated more MDA after heat stress. MDA is a secondary end product of the oxidation of polyunsaturated fatty acids and is considered an indicator of general lipid peroxidation (Hodges et al., 1999). Thus, the increased MDA level indicates increased lipid peroxidation in the mutant line after heat treatment. The cka4 mutant plants accumulated less proline during heat stress than Col-0. Proline has long been recognized as one of the general abiotic stress protectants. It functions as a molecular chaperone, is able to protect protein integrity, and enhances the activities of many enzymes (Szabados and Savouré, 2010), which can be beneficial for plant recovery from heat stress. The reduced level of proline in the cka4 mutant may indicate that the general protection system is impaired due to the absence of the plastid CK2.

The reduced thermotolerance in the cka4 mutant may be partly attributed to impairment in ABA signalling, as it is known that some ABA-deficient (aba1, aba2, and aba3) and -insensitive (abi1, abi2, and abi3) mutants are more sensitive to heat stress (Larkindale et al., 2005; Penfield, 2008). However, the HSP levels in these ABA-deficient and -insensitive mutants are unaffected (Larkindale et al., 2005). In contrast, cka4 mutation resulted in reduced expression levels of HSPs, indicating that the impact of CKA4 on thermotolerance also involves a unique pathway. It is known that HSPs have an important protective role during heat stress (Wang et al., 2004; Zhang et al., 2009). HSPs accumulate during the heat-acclimation stage for plants to acquire some degree of thermotolerance to lethally high temperatures (Wang et al., 2004). Increased levels of HSP accumulation in plants can enhance thermotolerance (Bita and Gerats, 2013; Grover et al., 2013). HSFs are central regulators of HSP genes (Pirkkala et al., 2001; Scharf et al., 2012; Xue et al., 2014). In this study, the heat-induced expression levels of six representative HSP genes were found to be lower in the cka4 mutant than in Col-0, which explains the low thermotolerance in the mutant. The low expression levels of HSP genes were accompanied by reduced expression levels of three HSFs analysed (HSFA1d, HSFA2, and HSFA3), suggesting that CKA4 is a positive regulator of the heat-acclimation process, likely to be upstream of the HSF genes.

The CKA4 transcript was also upregulated during heat stress. Interestingly, the CKA4 transcript level was markedly elevated during the recovery phase and peaked at 24 h after the completion of heat treatment. Deregulation of some genes days after heat exposure has been observed in the previous studies (Pecinka and Schield, 2012; Popova et al., 2013). This study also showed that the transcript level of HSP101 remained elevated 24 h after heat treatment and that the post-heat transcript level of HSP101 was lower in the cka4 and cka3 cka4 double mutants than in Col-0 (Supplementary Fig. S4 at JXB online). HSP101 is considered to be one of the HSPs playing a key role in the acquisition of thermotolerance in plants (Gurley, 2000; Quetsch et al., 2000). It is possible that the higher levels of HSPs during the post-heat-stress period can also help plant recovery from heat injury.

Another possible role of CKA4 in protecting plants from heat injury is that CKA4 may be involved in phosphorylation of HSPs in the chloroplast. Phosphorylation of some HSPs by CK2 has been observed in the mammalian system (Lees-Miller and Anderson, 1989; Ishihara et al., 2000). In plants, a number of HSPs are present in the chloroplast (Chen and Vierling 1991; Shi and Theg, 2010) and are known to play important roles in protecting photosynthetic apparatus (Chauhan et al., 2012). Examination of the high-confidence list of identified chloroplast phosphoproteins reveals that no chloroplast HSPs are phosphorylated (Reiland et al., 2009), indicating that this is an unlikely route for the observed impact of CKA4 on thermotolerance.

Our findings on the loss-of-function mutation of a plastid CK2 resulting in a reduction in ABA- or heat-induced expression of many nuclear genes suggest that CKA4 has a role in retrograde signalling. It is known that the expression of many chloroplast proteins involved in photosynthesis is coordinately regulated in plants (Chu et al., 2013). Such coordination is essential to achieve the balance of photosynthetic components, such as ribulose-1,5-bisphosphate carboxylase/oxygenase, which contains a plastid-encoded large subunit and a nuclear-encoded small subunit. Plastid CK2 has been shown to phosphorylate the components (e.g. SIG1 and SIG6) of the PEP complex (Ogrzewalla et al., 2002; Schweer et al., 2010b; Türkeri et al., 2012), implicating its role in regulation of plastid gene expression. The phosphorylated Arabidopsis SIG6 (AtSIG6) has a higher binding affinity to the promoter of Arabidopsis atpB than the unphosphorylated protein (Türkeri et al., 2012). Plastid CK2 phosphorylation of AtSIG6 occurs at multiple sites (Schweer et al., 2010a). A complementation study of an AtSIG6 knockout mutant with AtSIG6 phosphorylation site mutants has shown that a mutation in many critical phosphorylation sites reduces the expression level of atpB in Arabidopsis but has no obvious effect on the expression of psbA based on non-quantitative northern blot hybridization analysis (Schweer et al., 2010a).
This study provides direct experimental evidence that the expression levels of two PEP target genes (atpB and psbA) were reduced by the knockout mutation of a plastid CK2. Although no obvious effect of AtSIG6 phosphorylation site mutation on psbA expression was observed in the study of Schweer et al. (2010a), this might be due to the use of single-phosphorylation-site mutants in their study. The effect of mutations in multiple phosphorylation sites of ATSIG6 on psbA expression has not been tested to date. The alterations in the levels of some critical plastid proteins are likely to have some effect on the expression of nuclear genes. For example, the heat-stress-mediated upregulation of HSF2 and some HSP genes is inhibited by downregulation of a plastid ribosomal protein S1 (PRS1) (Yu et al., 2012), which is involved in plastid protein synthesis. In fact CKA4 knockout also reduced the expression of PRS1, indicating that CKA4 is likely to be an upstream regulator of PRS1, which subsequently influences the expression levels of some HSF and HSP genes. However, the pathway of this heat-stress retrograde signalling is currently unknown.

A well-known retrograde signalling pathway from the chloroplast to the nucleus involves GUN1, PTM, and ABI4. PTM and ABI4 are transcription factors. PTM is associated with the chloroplast envelope membrane and can move to the nucleus to activate the expression of ABI4 (Chi et al., 2013). In this retrograde signalling, ABI4 is a regulator for modulating expression of nuclear genes and is also involved in ABA and sugar signalling pathways (León et al., 2013). The expression levels of PTM and ABI4 were reduced in the cka4 mutant with ABA treatment, suggesting that this retrograde signalling is modulated by CKA4. STN7 kinase is involved in the GUN1–PTM–ABI4 retrograde signalling pathway (Chi et al., 2013) and is regulated by the redox state of chloroplast (Pesaresi et al., 2009). STN7 is suggested to be a potential substrate of plastid CK2 (Reiland et al., 2009). Whether CKA4 modulating the GUN1–PTM–ABI4 retrograde signalling pathway is through phosphorylation of STN7 awaits further investigation. Furthermore, plastid CK2 is also regulated by the redox state chloroplast (Pfannschmidt and Liere, 2005). Thus, CKA4 may have a role in linking the redox signal in the chloroplast to ABA and heat-stress signalling in the nucleus.

In summary, the present study provides a substantial body of work that supports a role for the plastid CK2, CKA4, in positive regulation in the ABA and heat-stress signalling pathways. Knockout mutation of CKA4 led to reduced sensitivity in the ABA-mediated blockage of seed germination and seedling growth and reduced thermotolerance. These phenotypes observed in the cka4 mutants were attributed to its function in modulating the expression levels of ABA- and heat-responsive genes in the nucleus via retrograde signalling. CKA4 mutation negatively influences retrograde signalling via the GUN1–PTM–ABI4 and PRS1 pathways. These findings provide an exciting opportunity for identification of the additional molecular players in ABA and heat-stress signalling pathways and exploitation of CKA4 as a potential modulator for improvement of plant tolerance to various abiotic stresses in the future.

**Supplementary data**

Supplementary data are available at JXB online.

**Supplemental Table S1.** Sequences of oligonucleotide primers used in this study.

**Supplementary Fig. S1.** The expression level of At1g66110 gene in Col-0 and cka4 mutant (the progeny of CS311135).

**Supplementary Fig. S2.** Root and hypocotyl elongation in Col-0, cka4, and cka3 cka4 mutants after heat treatment.

**Supplementary Fig. S3.** Comparative analysis of growth parameters of wild-type Col-0, cka4, and cka3 cka4 mutant plants.

**Supplementary Fig. S4.** The expression level of HSP101 at the 24 h recovery phase after a heat-treatment regime.

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**References**


Plastid CK2 regulates nuclear ABA- and heat-responsive genes


