Arabidopsis Casein Kinase 2 α 4 subunit regulates various developmental pathways in a functionally overlapping manner

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Running title: CK2 α 4 subunit plays functionally overlapping roles in *Arabidopsis thaliana*

Abstract

Casein kinase 2 (CK2) is an essential and well-conserved Ser/Thr kinase that regulates proteins in a posttranslational manner. CK2 has been shown to affect a large number of developmental processes across eukaryotes. It is a tetrameric protein composed of a dimer of alpha (catalytic) and beta (regulatory) subunit each. In our previous study we showed that three of the four CK2 α subunits in Arabidopsis act in a functionally redundant manner to regulate various developmental pathways. In this study we constructed two independent CK2 $\alpha 4$ RNAi lines in the CK2 alpha triple mutant background. Through functional characterization of these RNAi lines we show that the fourth α subunit in Arabidopsis also functions redundantly in regulating ABA response, lateral root formation and flowering time. CK2 $\alpha 4$ -GFP localizes to the chloroplast in transgenic Arabidopsis seedlings, consistent with the presence of a chloroplast localization signal at the amino-terminus of CK2 $\alpha 4$ subunit. Taken together, our results suggest a functionally overlapping role for the CK2 $\alpha 4$ subunit in regulating various developmental processes in plants.

Keywords: Kinase, CK2 $\alpha 4$, flowering time, ABA response, lateral roots

1. Introduction

The group of enzymes termed protein kinases comprises an essential set of enzymes for all living organisms. Kinases add one/more phosphate groups to their substrate protein which often regulates its cellular function and/or abundance. The phosphate group can be removed by an oppositely acting enzyme called phosphatase. The reversibility of this posttranslational modification allows cells to rapidly respond to a stimulus. Casein kinase 2 (CK2) is an essential and ubiquitous Ser/Thr kinase present in all eukaryotes. The enzyme is a tetramer composed of one dimer of regulatory (beta) and catalytic (alpha) subunits each [1]. Although, CK2 is primarily a Ser/Thr kinase, it has been shown to phosphorylate Tyrosine residues in a few cases [2,3]. CK2 recognizes S/TXXD/E/Yp/Sp as a consensus sequence and is one of the few kinases that can utilize both ATP and GTP as phosphate source [4]. In mammals, CK2 has been extensively studied as a potential target for anticancer drugs due to its role in the cell cycle regulation controlling both cell division as well as apoptosis [5].

CK2 has been shown to regulate various developmental and stress response pathways through functional studies from various species in plants (Arabidopsis, maize, rice, tobacco, wheat and mustard) [6–11]. Compared to over 300 targets in animal system, there is less number of known CK2 substrates in plants; however, the list is steadily growing [1,12]. In Arabidopsis, both alpha and beta subunits are redundantly encoded by four genes each and are ubiquitously expressed spatially and developmentally. All the CK2 alpha subunits except $\alpha 4$ are nuclear localized. CK2 $\alpha 4$ subunit has an N-terminal chloroplast localization signal; however, it also contains the internal conserved Nuclear Localization Signal (NLS) [10]. This subunit also has the highest transcript level spatially and developmentally amongst the four alpha subunits [13]. The regulatory beta subunits are found both in the nucleus and cytoplasm. Both the subunits exist in a complex and as well as monomers in the cell [10].

This genetic and functional redundancy in both the catalytic and regulatory subunits has long hindered identification of the roles of CK2 in plant development. In the past few years, two approaches, creating an inducible dominant negative catalytic subunit mutant and creating higher order catalytic subunit knock out mutants have given important insights into involvement of CK2 in various pathways in Arabidopsis. The study carried out using an inducible dominant negative alpha subunit mutant showed that CK2 positively regulated the development of lateral roots in

Arabidopsis. Also, the dominant negative mutant was defective in cell cycle and cell expansion and root and shoot growth. However, prolonged induction of defective alpha subunit was lethal supporting essential nature of the enzyme [14]. Two independent studies using different CK2 alpha subunit T-DNA insertion lines to create higher order mutants have shown involvement of nuclear CK2 alpha subunits in various pathways in Arabidopsis [13,15]. CK2 alpha subunits positively regulate response to hormone ABA, Salt (NaCl) stress and lateral root development in an overlapping manner. They also redundantly regulate the flowering time under both short and long day conditions. [13,16].

A recent study reported the characterization of T- DNA insertion knock out lines of the chloroplast localized CK2 $\alpha 4$ subunit. The CK2 $\alpha 4$ single knock out mutant was found to be hyposensitive to ABA and salt stress similar to CK2 alpha triple mutant. The CK2 $\alpha 4$ knockout mutant was also hypersensitive to heat stress and showed reduced expression of genes involved in retrograde signaling [17]. Identification and characterization of a chloroplastic CK2 in rice has shown that the phosphorylation sites for target proteins in monocots and dicotyledonous plants have evolved to be different suggesting plastid CK2 regulation has changed over time in monocots and dicotyledonous plants [18].

In this study we characterized the function of the $\alpha 4$ subunit of CK2 in Arabidopsis thaliana using RNAi approach. CK2 is an essential enzyme and a quadruple alpha subunit mutant is likely to be lethal. Previous studies have also shown that the CK2 alpha subunits are functionally redundant and the single mutants did not exhibit any noticeable phenotype. Hence, we developed constitutive RNAi lines to down regulate CK2 $\alpha 4$ gene in both the wild type (Col-0) and CK2 alpha triple ($\alpha 123$) mutant background to better understand the function of $\alpha 4$ subunit.

2. Materials and methods

2.1. Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, <http://www.sungro.com/>) under constant light at $24 \pm 0.5^\circ\text{C}$. Seeds were surface sterilized and plated on Murashige–Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described [19]. After stratification at 4°C in the dark, seeds were transferred to

constant white light chamber at $22 \pm 0.5^{\circ}\text{C}$ for respective number of days. For lateral root number, seedlings were grown under white light at 22°C for 9 days on a vertical plate. The number of lateral roots visible to the naked eye was counted. The experiments were repeated at least three times.

2.2. Construction of CK2 $\alpha 4$ RNAi and CK2 $\alpha 4$ -GFP over expression transgenic lines

A 200 bp region starting from the stop codon in the 3' UTR of Arabidopsis CK2 $\alpha 4$ gene (TAIR: AT2G23070) was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector pB7GWIWG2(II),0 [20]. The recombinant construct was introduced into Wild type (Col-0) and CK2 alpha triple mutant plants via Agrobacterium mediated transformation [21]. The reduction in the transcript level of CK2 $\alpha 4$ in the homozygous RNAi lines was confirmed through quantitative real time PCR. The list of primers used is in Table 1. Full length open reading frame encoding CK2 $\alpha 4$ gene was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector called pB7FWG2 [20]. The recombinant vector was introduced into wild type Col-0 plants via Agrobacterium mediated transformation as described [21].

2.3. Protein extraction and CK2 kinase assays

Protein extraction and CK2 kinase assays were performed as described [22–24]. Briefly, crude extracts were prepared by homogenizing 2-week-old green seedlings in buffer [40 mM HEPES, pH 7.4, 15 mM MgCl_2 , 1mM EDTA, 10 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM sodium orthovanadate, 40 mM β -glycerophosphate and 10% glycerol]. The homogenate was centrifuged at 16 000 g for 15 min at 4°C . Protein concentration was determined using the Bradford protein assay method (Bio-Rad Laboratories, <http://www.bio-rad.com/>). For the CK2 phosphorylation assays, 25 μl kinase assay mixtures contained 50 mM HEPES–KOH, pH 7.6, 5 mM MgCl_2 , 2.4 mM DTT, 0.2 mM γ -[32P]ATP (~250 c.p.m. per pmol), 100 mM KCl, ~1 pmol CK2 and ~10–20 pmol rWheIF4B. For the extracts from wild-type, triple mutant plants and homozygous RNAi lines in both wild type and triple mutant background, 0.5 μg of total protein was added for each reaction. The reaction was incubated at 30°C for 10 min and terminated by the addition of 6X SDS loading buffer. Samples were boiled for 3 min and separated on an 8% SDS-PAGE gel. The gels were dried and exposed to a PhosphorImager.

2.4. Quantitative RT-PCR assays for floral integrator genes

Quantitative RT-PCR was performed as previously described [25]. Briefly, samples were collected every 4 h starting at ZT0 from 12-day-old wild-type, CK2 α triple mutant and one of the two RNAi lines in the triple mutant background seedlings grown under SD and LD conditions. Total RNA was extracted using a Spectrum plant total RNA kit (Sigma-Aldrich, <http://www.sigmaaldrich.com/>) and reverse transcribed using MMLV (Invitrogen, <http://www.invitrogen.com/>) as per the manufacturer's protocol. The qRT-PCR assays used the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems, <http://www.appliedbiosystems.com/>). Real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems). PP2A (TAIR: At1g13320) was used as a control for normalization of the expression data. The resulting cycle threshold (Ct) values were used for calculation of the levels of expression of different genes relative to PP2A as follows: $2^{\Delta Ct}$ where $\Delta Ct = Ct(PP2A) - Ct(\text{specific gene})$. Primer sequences used for qRT-PCR are listed in Table 1.

2.5. Abscissic acid response assays

Seeds were surface sterilized and plated on MS GM-Suc supplemented with different concentrations of hormone (ABA). After stratification at 4°C in the dark, seeds were placed under continuous light at 22°C. The numbers of germinated seeds and open, green cotyledons were recorded by observation under a microscope. At least 50 seeds for each genotype were plated for each experiment and the experiment was repeated at least three times.

2.6. Light fluorescence microscopy

Arabidopsis transgenic seedlings expressing CK2 $\alpha 4$ -GFP were grown on MS plates for 2 weeks under white light. Leaf and root sections from these seedlings were observed under 100X magnification using Zeiss Axiovert fluorescent light microscope.

3. Results

3.1. Development of CK2 $\alpha 4$ RNAi lines in wild type and alpha triple mutant background

In our previous study, we showed that the three nuclear subunits are functionally redundant and hence the single subunit mutants do not show any phenotype [13]. Thus, to get an insight into the function of the fourth, chloroplast localized alpha subunit of CK2, we requested

the CK2 $\alpha 4$ T-DNA insertion knock out lines (CS311135) from ABRC stock center. However, we were unable to obtain any homozygous or heterozygous *cka4* mutant plants from the segregating population. Hence, we developed RNAi lines that down regulated the CK2 $\alpha 4$ gene expression in both the wild type and CK2 alpha triple mutant background.

All four CK2 alpha subunit genes in Arabidopsis show high level of conservation in the nucleotide sequence. Hence, we chose a 200 bp nucleotide sequence in the 3' UTR of the CK2 $\alpha 4$ gene (AT2G23070) downstream of the stop codon. This nucleotide sequence is specific to the CK2 $\alpha 4$ gene and is not found in other three alpha subunit genes. Plasmid vector containing this sequence was introduced into both wild type (Col-0) and CK2 alpha triple mutant plants. We obtained several independent lines in both the backgrounds. However, based on the reduction in transcript as well as enzyme activity we chose two lines in each background for further characterization. All the four selected lines show significant reduction in the CKA4 transcript (Figure1 A, B). We also checked the expression of the rest of the alpha subunit genes in the RNAi lines in wild type background (Figure1A). We found no significant difference in their expression confirming that the down regulation in the transcript was specific to CK2 $\alpha 4$.

CK2 alpha subunit is the catalytic subunit of the CK2 holoenzyme. Hence, we examined the relative enzyme activity in the total plant extracts from 2 week old seedlings of the RNAi lines using recombinant Wheat eIF4B as a substrate (Figure 1D). Wheat eIF4B is a verified CK2 substrate which is robustly phosphorylated by wild type Arabidopsis CK2 α subunit and this phosphorylation is not affected by presence of CK2 β subunit [24]. Results show that the RNAi lines in the wild type background show 70-80% relative kinase activity while the relative kinase activity in the RNAi lines in CK2 alpha triple mutant background is reduced to 40 to 50% (Figure 1C). Although the RNAi lines show up to 70-75% reduction in the transcript level it does not correlate with the reduction seen in the relative kinase activity of the plant extracts. CK2 is an essential enzyme and CK2 knock out in yeast is not viable [26]. Hence, it is likely that the RNAi lines with severe reduction in kinase activity may not be viable and hence were not obtained from the screen. However, looking at the functional redundancy of CK2 alpha subunits, it is still interesting to investigate the phenotypes of these RNAi lines as it would provide insights into the overlapping functions of the alpha subunits that may not be uncovered through characterization of single, double and triple knockouts. We found that both the RNAi lines in the

wild type background did not show statistically significant and consistent results for all the phenotypes tested. Hence, in this paper we have focused on characterizing the RNAi lines in the CK2 alpha triple mutant background.

3.2. CK2 α 4 promotes flowering under both short day and long day conditions

Previous studies from rice showed involvement of CK2 in controlling flowering time in plants. In rice, a well-known QTL, heading date 6 (Hd6) encodes the alpha subunit of CK2 and is responsible for delaying flowering under long days [27]. In Arabidopsis, we found that the three nuclear CK2 alpha subunits redundantly promoted flowering under both short and long day conditions [13]. Only the CK2 alpha triple mutant plants flower later than the wild type while the alpha single and double mutant combinations flowered similar to wild type under these conditions. Vernalization and treatment with Gibberellic Acid rescued the flowering phenotype of the CK2 alpha triple back to wild type levels suggesting that the CK2 alpha subunits control flowering through the autonomous pathway [13,16]. For the CK2 α 4 RNAi lines, we found that the RNAi lines in the wild type background flowered similar to wild type under both SD and LD conditions (data not shown). However, the RNAi lines in the alpha triple mutant background (α 123- α 4i) showed delayed flowering under both SD and LD with respect to the alpha triple mutant (Figure 2 A, B, and 3 A, B). Under long day conditions, the two independent RNAi lines were late flowering in terms of number of rosette leaves but not with respect to the number of days to flower (Figure 2 A, B). Similar results were obtained under short day conditions, the two independent RNAi lines (α 123- α 4i) flowered significantly later than the both wild type (Col-0) and alpha triple mutant in terms of number of rosette leaves but not in terms of number of days (Figure 3 A, B). Overall these data suggest that CK2 α 4 regulates flowering time in a functionally overlapping manner with other alpha subunits.

3.3. CK2 α 4 subunit redundantly regulates flowering time genes under both short and long day conditions

Delayed flowering under short day and/or long day is a result of changes in the expression levels of *CO* and/or *FLC* the two genes that control flowering time in Arabidopsis and/or their respective floral integrator target genes *FT* and/or *SOC1* [28,29]. In the CK2 alpha

triple mutant reduced expression of *FT* and *SOC1* and significantly higher *FLC* levels under both LD and SD conditions was reported [13]. To investigate the molecular bases of delayed flowering in the RNAi lines we performed a time course quantitative real time PCR assay for these four key flowering time genes. Under LD conditions, there is no significant difference in the *CO* level while *FLC* levels are slightly higher in $\alpha 123\text{-}\alpha 4i$ line than triple mutant (Figure 2 C, E). Both *SOC1* and *FT* have slightly reduced levels than the triple mutant (Figure 2 D, F) Under SD conditions, the RNAi line shows similar trend. The RNAi line shows slightly increase in the *FLC* expression and small reduction in the levels of *FT* and *SOC1* with respect to the triple mutant. The expression of *CO* was not altered under these conditions (Figure 3 E, D, F, and C). Thus, it appears that all four alpha subunits regulate the expression of flowering time genes in Arabidopsis in a functionally overlapping manner.

3.4. CK2 $\alpha 4$ promotes lateral root development

In mammals, the role of CK2 in regulating cell cycle and cell division is well documented [5]. In Arabidopsis, the inducible dominant negative mutant of CK2 shows defects in cell cycle and cell expansion. As a result the dominant negative mutant shows severe reduction in the number of lateral roots and also reduction in the level of expression of genes involved in cell cycle. These studies suggest that CK2 may play a role in auxin signaling pathway [14,30]. The CK2 alpha triple mutant also shows significantly less number of lateral roots than the wild type [13]. To examine whether CK2 $\alpha 4$ has any role in lateral root development, we counted the number of lateral roots in 9 day-old Arabidopsis seedlings. None of the RNAi lines in the wild type background shows any significant difference in the number of lateral roots (data not shown). However, the two lines in the alpha triple mutant background show significantly less number of lateral roots than the triple mutant (Figure 4 A, B). This suggests that the CK2 $\alpha 4$ subunit also contributes to the lateral root development in functionally overlapping manner.

3.5. CK2 $\alpha 4$ positively regulates block of seed germination in the presence of ABA

CK2 has been shown to modulate the function of Rab17, an ABA responsive protein in Maize [31]. In Arabidopsis, the nuclear CK2 alpha subunits contribute synergistically to the block of seed germination in response to the hormone ABA and Salt (NaCl) in a concentration dependent manner [13]. Also, a recently published study shows that both CK2 $\alpha 4$ single knock-

out and CK2 $\alpha 3\alpha 4$ double knock-out mutants are hyposensitive to ABA induced block of seed germination [17]. We analyzed the seed germination response to ABA in the CK2 $\alpha 4$ RNAi lines. We find that only one of the two RNAi lines in the wild type background was hyposensitive to ABA with respect to seed germination (data not shown). However, both the RNAi lines in the triple mutant background showed enhanced hyposensitivity with respect to the CK2 triple mutant in a concentration dependent manner (Figure 5). We find that there is however no difference between the CK2 alpha triple mutant and the RNAi lines with respect to cotyledon greening in the presence of ABA (data not shown).

3.6. CK2 $\alpha 4$ localizes primarily to chloroplasts in leaf tissue

Previous research indicates that CK2 $\alpha 4$ subunit contains a conserved N-Terminus chloroplast targeting signal and has been shown to localize to chloroplasts in transient assays in Tobacco leaves [10]. To examine subcellular localization in transgenic plants *in vivo*, we constructed GFP tagged CK2 $\alpha 4$ over expression lines in Col-0 background. We observed leaves and root samples from 2 week old seedlings under fluorescent microscope. Results show that that most of the CK2 $\alpha 4$ -GFP is localized to the chloroplasts of the guard cells (Figure 6). We examined extensively in root and leaf cells for nuclear staining; however, no consistent GFP fluorescence was observed in nucleus of any cell types.

4. Discussion

Protein kinase CK2 is a well-studied kinase in the mammalian system. With over 300 known target proteins CK2 is known to be an essential kinase and affects important developmental processes [1,4]. Similar to mammals, in plants, CK2 has been implicated in various developmental pathways supported by a small yet steadily growing list of substrates [12].

In the model plant *Arabidopsis thaliana*, high redundancy in genes encoding both alpha and beta subunits has prevented dissection of the physiological role of CK2 in plant development for a long time. Of the four alpha subunit genes in *Arabidopsis*, three are nuclear localized. The gene encoding for the fourth alpha subunit contains an N-terminus chloroplast localization sequence and this subunit is shown to be chloroplast localized in transient assays in tobacco leaves [10]. In our previous study we characterized CK2 alpha triple mutant for various phenotypes. CK2 alpha triple mutant was a complete knock-out for the three nuclear-localized

alpha (catalytic) subunits in Arabidopsis. Using the triple knock-out mutant we were able to identify new functions of CK2 in Arabidopsis. Surprisingly, in spite of complete absence of any nuclear alpha subunits, the CK2 alpha triple plants did not show any extreme developmental abnormalities under normal growth conditions [13]. Among all the CK2 alpha subunits, $\alpha 4$ is expressed at the highest level throughout Arabidopsis development [32]. Thus, in the absence of a null mutant of CK2 $\alpha 4$ subunit, we used an RNAi approach to investigating the role of CK2 $\alpha 4$ in the development of Arabidopsis. Analyses of transcript levels of all 4 subunits indicated that CK2 $\alpha 4$ subunit was specifically down regulated in the RNAi lines tested (Figs. 1A, B). Enzymatic assays also showed significant down regulation of CK2 activity in multiple independent transgenic lines (Fig. 1C, D), establishing that these lines can be used to analyze the function of CK2 $\alpha 4$ subunit. Using these lines, the phenotypic characterization and molecular evidence presented here suggest that CK2 $\alpha 4$ subunit contributes to the roles of nuclear alpha subunits redundantly in regulating multiple developmental pathways including flowering time under both long- and short- days, lateral root development and ABA responses.

A role for the CK2 alpha subunits in regulating the flowering time has previously been established, although, only the CK2 alpha triple mutant and not the alpha single mutants displayed the late flowering phenotype under both long- and short-day conditions [13]. This is consistent with the lack of any phenotype in the CK2 $\alpha 4$ RNAi lines in the wild type background (data not shown). However, in the CK2 $\alpha 4$ RNAi lines in the triple mutant background, flowering time was modestly but significantly delayed under both long and short day conditions (Figs. 2A, B, 3A, B). The gene expression analyses also supports this conclusion where a modest difference was observed for the *FLC* and *SOC1* expression in the RNAi lines in the triple background compared to the triple mutant (Figs. 2E, F, 3E, F). Thus, it appears that like the other three alpha subunits, CK2 $\alpha 4$ subunit also controls flowering in Arabidopsis in an overlapping manner possibly through the autonomous pathway as previously hypothesized [13,16].

It is well known that CK2 controls cell cycle and cell expansion in mammals [5]. CK2 dominant negative mutant shows a strong defect in cell cycle reflected in significantly less number of lateral roots [14]. CK2 is also speculated to affect auxin signaling which may also result in defect in the lateral root formation [33,34]. Consistent with these data, our results also

show that CK2 $\alpha 4$ subunit plays a functionally overlapping role in the pathway controlling development of lateral roots in Arabidopsis (Figure 4).

The two RNAi lines in the CK2 alpha triple mutant background show enhanced hyposensitivity towards the block of seed germination under various concentrations of ABA with respect to the wild type as well as CK2 alpha triple mutant (Figure 5). This is consistent with the results published in a recent paper where they found that CK2 $\alpha 4$ single knock-out and *a3a4* knock out mutants are also hyposensitive to ABA treatment [17]. In our previous study we found that the nuclear CK2 α subunits control seed germination in the presence of ABA in a synergistic manner. All three double knock out combinations showed enhanced hyposensitivity than any individual alpha subunit mutant [13]. The exact mechanism by which CK2 modulates this response is yet unknown. However, it has been shown that the CK2 $\alpha 4$ and *a3a4* double knock out plants contain significantly reduced endogenous ABA levels than the wild type (Col-0) plants [17]. Also, ABA treatment induces CK2 $\alpha 4$ expression and the $\alpha 4$ and *a3a4* double knock outs show reduction in ABA responsive genes with respect to the wild type [17]. A recent paper showed that CK2 phosphorylates Maize OST1 which increases its affinity towards PP2C and triggers its degradation that modulates ABA responses such as stomata opening [35]. It is likely that the CK2 alpha triple mutant contains reduced ABA levels and all CK2 alpha subunit genes function redundantly in this pathway.

Although the phenotypic and molecular characterization of CK2 $\alpha 4$ RNAi lines in CK2 alpha triple mutant background provides us important insights into the function of CK2 $\alpha 4$ in the development of Arabidopsis, it also presents a quandary about how a chloroplast localized enzyme regulates such a diverse biological processes possibly controlled by nuclear genes. Although CK2 $\alpha 4$ has been shown to be localized to the chloroplast, this subunit also contains the internal NLS which is conserved in all CK2 alpha subunits in Arabidopsis [10]. In addition, there are other examples of proteins that are localized and functioning in both nucleus as well as chloroplast in Arabidopsis e.g. HEMERA [36]. To test this possibility, we made transgenic plants overexpressing CK2 $\alpha 4$ fused to GFP and expressed constitutively using a 35S promoter in wild type background. However, despite extensive investigation, we only observed chloroplast localization of this subunit confirming previous transient results in a stable transgenic plant background (Figure 6). Thus, in one hand, these data supports a hypothesis where chloroplast

localized CK2 $\alpha 4$ subunit regulates these diverse biological process by controlling retrograde signaling pathways as this pathway have been shown to regulate plant growth and development extensively [37–40]. Alternative, it is still possible that a small undetectable amount of CK2 $\alpha 4$ is localized in the nucleus and regulates these diverse pathways. Further studies are necessary to draw a definitive conclusion about the function of the CK2 $\alpha 4$ subunit.

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Figure Legends

Figure 1: CK2 α 4 RNAi lines display reduced kinase activity compared to wild type plant extracts *in vitro*. A) Quantitative RT-PCR showing the transcript level of all four CK2 α subunit genes in Col-0, α 123 and two CK2 α 4 RNAi lines in the wild type background. *PP2A* was used as the reference gene. B) Quantitative RT-PCR showing the transcript level of all four CK2 α subunit genes in Col-0, α 123 and two CK2 α 4 RNAi lines in the α 123 background. *PP2A* was used as the reference gene. C) Bar graph showing the level of reduction in kinase activity of the CK2 α 4 RNAi lines compared to wild type and α 123 triple mutant plants. The experiment was repeated three times and the data are expressed as mean $\pm$ SE. * and **, indicates statistically significant difference with a p value <0.05 and <0.01, respectively when compared between wild type and alpha triple and between alpha triple and α 4 RNAi lines. D) Gel photograph displaying reduced kinase activity for CK2 α 4 RNAi lines compared to wild type and α 123 triple mutant plant extracts *in vitro*.

Figure 2: CK2 α 4 RNAi lines display late flowering phenotype under long day (LD) conditions. Bar-graph showing average number of rosette leaves (A) and days to flower at bolting (B) under LD conditions. Plants were grown under LD (16h light/8h dark) conditions. The experiment was repeated three times ($n \geq 15$) and the data are expressed as mean $\pm$ SE. * and **, indicates statistically significant difference with a p value <0.05 and <0.01, respectively when compared between wild type and alpha triple and between alpha triple and α 4 RNAi lines. Quantitative RT-PCR analyses for flowering time genes *CO* (C), *FT* (D), *FLC* (E) and *SOC1* (F) in CK2 α 4 RNAi lines in α 123 mutant background compared to α 123 and Col-0. Samples were collected every 4 hours starting at ZT0 under both LD and SD conditions. Expression of the reference gene *PP2A* in the wild type background is set to 1 and the relative expressions in the wild type and triple mutant are calculated in relation to *PP2A*. Data are presented for three biological replicates and three technical replicates for each biological replicate, and are expressed as mean $\pm$ SE.

Figure 3: CK2 α 4 RNAi lines display late flowering phenotype under short day (SD) conditions. Bar-graph showing average number of rosette leaves (A) and days to flower at bolting (B) under SD conditions. Plants were grown under SD (8h light/16h dark) conditions. The data are expressed as mean $\pm$ SE. * and **, indicates statistically significant difference with a p value <0.05 and <0.01, respectively when compared between wild type and alpha triple and between

alpha triple and $\alpha 4$ RNAi lines. Quantitative RT-PCR analyses for flowering time genes *CO* (C), *FT* (D), *FLC* (E) and *SOC1* (F) in CK2 $\alpha 4$ RNAi lines in $\alpha 123$ mutant background compared to $\alpha 123$ and Col-0. Samples were collected every 4 hours starting at ZT0 under both LD and SD conditions. Expression of the reference gene *PP2A* in the wild type background is set to 1 and the relative expressions in the wild type and triple mutant are calculated in relation to *PP2A*. Data are presented for three biological replicates and three technical replicates for each biological replicate, and are expressed as mean $\pm$ SE.

Figure 4: CK2 $\alpha 4$ subunit promotes lateral root development. A) Bar-graph shows the average number of lateral roots for Col-0, $\alpha 123$ and CK2 $\alpha 4$ RNAi lines in $\alpha 123$ mutant background. Seedlings were grown on MS-agar plates for nine days at 22⁰C under white light. The experiment was repeated three times ($n \geq 12$ for each genotype) and the data are expressed as mean $\pm$ SE. * and **, indicates statistically significant difference with a p value < 0.05 and < 0.01 , respectively when compared between wild type and alpha triple and between alpha triple and $\alpha 4$ RNAi lines. B) Photographs of seedlings showing reduced number of lateral roots for the Col-0, $\alpha 123$ and CK2 $\alpha 4$ RNAi lines in $\alpha 123$ mutant background.

Figure 5: CK2 $\alpha 4$ subunit positively regulates ABA-induced block of seed germination. Line graph showing reduced seed germination of Col-0, $\alpha 123$ and CK2 $\alpha 4$ RNAi lines in $\alpha 123$ mutant background in response to increasing concentrations of ABA. The experiment was repeated three times ($n \geq 50$ for each genotype) and the data are expressed as mean $\pm$ SE.

Figure 6: CK2 $\alpha 4$ subunit is localized to chloroplast in transgenic Arabidopsis seedlings. Photographs showing the guard cells expressing CK2 $\alpha 4$ -GFP at 100X magnification under light fluorescence microscope. Guard cells under bright field (Panel A), guard cells expressing CK2 $\alpha 4$ -GFP (Panel B) and merged Bright field and GFP image (Panel C). Bar = 5 μ M.

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