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Role of Casein Kinase 2 (CK2) alpha subunits in the growth and development of *Arabidopsis thaliana*

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Dedication

Dedicated to Aai (Shubhada Mulekar), baba (Jayant Mulekar), Mangal Atya (Mangal Joag) and Sangutai (Dr. Sangeeta Kohli). I love you and thank you for being there for me, always!

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Role of Casein Kinase 2 (CK2) alpha subunits in the growth and development of *Arabidopsis thaliana*

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Casein kinase 2 (CK2) is a conserved Ser/Thr kinase present in all organisms. It is a tetrameric protein composed of two alpha and two beta subunits. In Arabidopsis, there are 4 genes encoding for each alpha (catalytic) and beta (regulatory) subunit of CK2. The role of CK2 alpha subunits in the growth and development of Arabidopsis was studied through characterization of T-DNA insertion mutants (for α1, α2, α3) and RNAi approach (for α4). Results show that all 4 CK2 alpha subunits positively regulate flowering under both short day and long day conditions, possibly in a functionally redundant manner. Elevated levels of *FLC* and reduced levels of *SOC1* in the CK2 alpha triple mutant compared to wild type (Col-0) suggest that the alpha subunits possibly act through the autonomous pathway to regulate flowering. The alpha subunits also synergistically promote inhibition of seed germination and cotyledon greening in the presence of ABA and salt (NaCl). I also found that the CK2 alpha subunits redundantly control lateral root formation in Arabidopsis.

CK2 has been shown to be involved in light signaling and phosphorylates the regulatory proteins HY5, HFR1 and PIF1 in the pathway. PIF1 belongs to a family of bHLH transcription factors (Phytochrome Interacting Factors) which function as repressors of photomorphogenesis. In the presence of light, activated phytochromes

(red/far red light photoreceptors) translocate to nucleus and interact with PIFs, which leads to rapid phosphorylation of PIFs followed by their degradation through the 26S proteasome. An *in vitro* kinase assay was performed to test whether CK2 phosphorylates all the PIF family proteins. Results show that CK2 phosphorylates PIF1, PIF3 and PIF5 but does not phosphorylate PIF4, PIF6 and PIF7. Up to 10 putative CK2 sites in PIF3 protein were mapped and mutated however the mutant protein was still significantly phosphorylated by CK2. The CK2 phospho-deficient mutant of PIF1 (PIF1-7M) showed reduced homo-dimerization capacity than the wild type in yeast two hybrid assays. However, further studies are required to access the functional significance of it.

Overall, this research uncovers novel functions for CK2 alpha subunits in the growth and development of *Arabidopsis thaliana*.

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Chapter 1: Protein kinase CK2 regulates various growth and developmental processes in plants[1]

Post translational modification of proteins is a common mechanism used by all eukaryotes to regulate protein function and stability. Phosphorylation/dephosphorylation of proteins is one of the most common post-translational modifications. This mechanism is advantageous to the cells as it is reversible. Proteins can be phosphorylated by protein kinases and dephosphorylated by protein phosphatases. Protein kinase CK2 (formerly known as casein kinase II) is a dual specificity Ser/Thr kinase present in all eukaryotes with a high degree of structural and functional conservation. The holoenzyme is a tetramer composed of two alpha (catalytic) and two beta (regulatory) subunits (Litchfield, 2003). However, many lines of evidence suggest that both the regulatory and catalytic subunits can exist independently as monomers both in the nucleus and cytoplasm and exhibit independent functions (Filhol *et al.*, 2004). The first X-ray crystallographic structure of CK2 has been elucidated using the maize CK2 α subunits (Niefind *et al.*, 1998). Although a minimum consensus sequence (Ser-Xaa-Xaa-Acidic) for the CK2 phosphorylation site has been defined, a great amount of deviation from the consensus sequence was observed with the known CK2 substrates (Pinna, 2003).

In mammals, CK2 has been extensively studied and shown to phosphorylate more than 300 known substrate proteins involved in multiple developmental pathways including control of the cell cycle and the circadian clock (Litchfield, 2003; Tamaru *et al.*, 2009). The functional characterization of CK2 from many plant species (Arabidopsis, maize, rice, tobacco, wheat and mustard) has shown similar involvement of CK2 in diverse pathways (Salinas *et al.*, 2001; Riera *et al.*, 2001*a*; Kato *et al.*, 2002; Ogrzewalla

[1] Parts of this chapter have been previously published (doi: 10.1093/jxb/ert401). I am the first and single author while my supervisor Dr. Enamul Huq is the corresponding author.

et al., 2002; Ogiso *et al.*, 2010). In plants, unlike animals, both alpha and beta subunits of CK2 are often encoded by multiple genes (Salinas *et al.*, 2006). For example, in *A. thaliana* there are four genes encoding alpha and beta subunits. One of the alpha subunits (alpha 4) has been shown to be targeted to the chloroplast (Salinas *et al.*, 2006) and reported to phosphorylate a plastid Sigma factor (AtSIG6) in Arabidopsis (Schweer *et al.*, 2010). All the genes encoding CK2 subunits are ubiquitously expressed both spatially and developmentally (Salinas *et al.*, 2006). However, according to a recent study, there is an increase in both the CK2 enzyme activity as well as the transcript levels in pollen (Moreno-Romero *et al.*, 2011). Recent research in maize shows the presence of additional alpha and beta subunit genes by characterizing one new beta subunit (CK2β4) and two new isoforms of alpha subunits (CK2α2 and CK2α4), both containing putative chloroplast transit peptides (Vélez-Bermúdez *et al.*, 2015). Protein kinase CK2 has a steadily growing list of substrates in plants (Table 1.1). The list includes proteins that function as transcription factors as well as other cellular proteins involved in basic physiological processes such as translation and the cell cycle. The high degree of redundancy in the genes encoding the catalytic as well as regulatory subunits in plants compared to animal systems, adds another level of complexity in analyzing CK2 functions.

A complete knock-out of CK2 in the yeast system (*S. cerevisiae*) is not viable suggesting a vital role in cell survival (Padmanabha, R., *et al.* , 1990). In plants, both CK2 subunits are encoded by multigene families; hence, a single gene knock-out mutant is not likely to show any abnormal phenotype. Two different strategies have been used to study the role of CK2 alpha subunits in Arabidopsis: creating higher order knock-out mutants and generation of a dominant negative transgenic plant. Two independent studies using the CK2 alpha triple mutant (*α1α2α3*) have shown that CK2 alpha subunits

positively regulate flowering time, ABA and salt stress responses, and in general plant growth and development (indicated by smaller cotyledon size, shorter root and hypocotyl) (Lu *et al.*, 2011; Mulekar *et al.*, 2012). The inducible dominant negative mutant was generated by expressing an inactive catalytic subunit cloned downstream of an inducible promoter. The inducible dominant negative mutant seedlings were defective in cell cycle and cell expansion, and in root and shoot growth. Prolonged induction of the transgene was lethal suggesting the vital nature of CK2 function in plants (Moreno-Romero *et al.*, 2008). A recent study characterized T-DNA knock out mutants of the chloroplastic CK2 alpha 4 subunit in Arabidopsis. The CK2 alpha 4 single knock out mutant was found to be hyposensitive to ABA and salt stress similar to the 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 (Wang *et al.*, 2014). 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 (Lu *et al.*, 2014). The studies using T-DNA insertion mutants and dominant negative mutants have been instrumental in uncovering new biological roles for this enzyme. In this review we will discuss various pathways that are regulated by CK2 in plants with a focus on light signaling and circadian clock.

LIGHT SIGNALING

Plants being sessile use light as a major developmental cue. To perceive light of different wavelengths, plants have multiple families of photoreceptors with different absorption spectra that confer differential sensitivity to different colors of light. When grown in the dark, the seedlings are etiolated and are marked by an elongated hypocotyl,

closed apical hook and closed cotyledons (called skotomorphogenesis). The de-etiolation begins after the seedlings are exposed to light and is marked by a shortened hypocotyl, straight apical hook, and open, green and expanded cotyledons (called photomorphogenesis). This stark change in the phenotype is achieved by rapid changes in gene expression. The skotomorphogenic development of a plant under dark conditions is characterized by high levels of repressor proteins (such as Phytochrome Interacting bHLH transcription Factors, PIFs, required mainly in the dark) and constant degradation of the positive transcription factors required for seedling deetiolation (such as HY5, HFR1, LAF1 and others). The photomorphogenic development results in the opposite scenario leading to the stabilization of the positive factors and the degradation of the negative regulators. One of the critical steps in the light signal transduction pathway is the differential regulation of the stability of these positively and negatively acting transcription factors, which results in large-scale changes in gene expression. A critical balance of both positive factors and negative factors is essential to achieve optimal growth of the plant under both light and dark conditions (Huq, 2006).

The involvement of protein kinase CK2 in light signaling in Arabidopsis was first shown using transgenic lines in which CK2 expression was knocked down by antisense RNA (Lee *et al.*, 1999). The adult antisense transgenic plants displayed smaller leaves. At the seedling stage, the antisense lines had elevated transcript levels of *CHALCONE SYNTHASE* (*CHS)* in the dark, and chlorophyll a/b-binding protein (*CAB*) mRNA and small subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase (*RBCS*) under light conditions. This differential gene expression of the light regulated genes can be attributed to a compound effect of reduced CK2 activity on its multiple targets.

HY5, a positive regulator of photomorphogenesis, was a substrate for phosphorylation by CK2, provided further evidence for CK2 involvement in light

signaling pathways (Hardtke *et al.*, 2000). HY5, a bZIP transcription factor, is one of the most important positively acting factors that promote photomorphogenesis in light (Lau and Deng, 2012). In darkness, HY5 is constantly degraded through the 26S proteasome pathway aided by the E3 ubiquitin ligase activity of COP1. HY5 contains a putative CK2 phosphorylation site in the N-terminal region, which overlaps with the COP1 interaction domain. Phosphorylation by CK2 reduced the affinity of HY5 for COP1, resulting in its increased stability. CK2 mediated phosphorylation of HY5 also results in decreased DNA binding capacity *in vitro*. Both phosphorylated and non-phosphorylated forms of HY5 are found *in vivo* (Hardtke *et al.*, 2000). Thus, CK2 adds a level of finer regulation by stabilizing HY5 protein from degradation yet decreasing its capacity to act as a transcription factor. Another positive regulator of photomorphogenesis HFR1, a bHLH transcription factor, is found to be under a similar regulation by CK2. HFR1 is also a substrate for COP1 and is degraded in the dark through the 26S proteasome pathway (Duek *et al.*, 2004; Yang *et al.*, 2005). CK2 phosphorylates HFR1 at multiple serine residues in the N-terminal region of the protein. Similar to HY5, the CK2 mediated phosphorylation results in the increased stability of HFR1. Light signals have been shown to enhance the phosphorylation and subsequent stabilization of HFR1 (Park *et al.*, 2008). Thus, CK2-mediated phosphorylation reduces interaction between HY5/HFR1 with COP1 resulting in stabilization of these factors to promote photomorphogenesis.

During photomorphogenesis, in contrast to the positively acting factors, the negatively acting factors (e.g., Phytochrome Interacting Factors, PIFs) are rapidly degraded in response to light signals through the 26S proteasome-mediated pathway (Castillon *et al.*, 2007; Leivar and Quail, 2011*a*). Interestingly, PIF1, a negative factor, is also a substrate for CK2 (Bu *et al.*, 2011*a*). CK2 phosphorylates seven Ser/Thr residues scattered throughout PIF1. Three of these serine residues at the C-terminus of PIF1

(Ser464-466) are crucial for the stability of PIF1. Phosphorylation of PIF1 by CK2 aids its degradation under red light which is reflected in the slower degradation kinetics of the mutant phospho-deficient PIF1 protein *in vivo* (Bu *et al.*, 2011*a*). Thus, in spite of not being a light regulated kinase, CK2 promotes plant development under light not only by stabilizing the positive regulators but also by promoting degradation of the negative regulators such as PIF1 (Fig.1.1) (Bu *et al.*, 2011*b*).

Although CK2 phosphorylates various positive and negative regulators in light signaling, its role in this pathway is still poorly understood. Does CK2 phosphorylate other PIFs? Recently, the phosphorylation status of PIF3 under both dark and light has been shown (Ni *et al.*, 2013). Some of the phosphorylation sites identified in PIF3 appear to be similar to CK2 phosphorylation sites. Other PIFs also have predicted CK2 sites. Further examination of whether other PIFs are substrates for CK2 and the functional significance of this phosphorylation needs to be investigated *in vivo*. This may be achieved by expressing both phospho deficient and phospho mimic forms of CK2 substrates in the corresponding mutant backgrounds. These functional complementation assays with the homozygous lines expressing mutant forms of PIFs will be helpful in understanding the contribution of CK2 mediated phosphorylation in light signaling pathway.

CIRCADIAN CLOCK

All living organisms maintain an endogenous biological clock that regulates their behavior, physiology, development and overall fitness in natural environment. In mammals these processes include the sleep cycle, core body temperature, and hormone production to name a few; while in plants, the clock is essential for the regulation of growth and development including flowering time, leaf movements, photosynthesis and

pathogen resistance. All circadian clocks consist of input pathways, a central oscillator and output pathways. Light and temperature act as inputs to entrain the central oscillator composed of positive and negative factors in an autoregulatory negative feedback loop. The entrainment of the endogenous clock helps synchronize robust rhythms against daily fluctuations of the natural environment (Mohawk *et al.*, 2012; Nagel and Kay, 2012).

The molecular players that are part of the central oscillator are different in different organisms, yet they all form a negative feedback loop. For example, in mammals PER1-3 and CRY1 and 2 are the negative factors, and CLOCK and BMAL-1 act as positive factors; while in Drosophila the same roles are performed by PERIOD and TIMELESS, and CLOCK and CYCLE proteins, respectively. In the fungus *Neurospora crasa*, Frequency (FRQ) is the negative regulator in the feedback loop and inhibits the activity of its transcriptional activator proteins White Collar-1 (WC1) and White Collar-2 (WC2) (Brunner and Schafmeier, 2006). However, in the model plant *Arabidopsis thaliana*, based on the recent findings, the core clock is proposed to work as a repressilator circuit composed of a three inhibitor ring oscillator instead of the common autoregulatory negative feedback loop. The three components include the two MYB transcription factors CCA1 and LHY, which repress the transcription of genes encoding proteins that form the evening complex (EC). The EC in turn represses the PRR (Pseudo Response Regulator) family proteins. The PRRs (including TOC1) complete the circuit by repressing the CCA1/LHY gene expression. The activation of PRRs by CCA1/LHY is identified as the morning loop while the autoinhibition of EC is the evening loop (Pokhilko *et al.*, 2012; Nagel and Kay, 2012).

In animals, mutations in the CK2 subunits lead to defects in the normal functioning of the clock. For example, in Drosophila *Timekeeper*, *tik* is a result of a dominant negative mutation in the catalytic (alpha) subunit of CK2 (Lin *et al.*, 2002).

Similarly, in plants, mutations in the CK2 subunits lead to defects in flowering time, an output trait of the circadian clock. In Arabidopsis, the CK2 alpha triple mutant (*α1α2α3*) shows significantly delayed flowering under short days which can be rescued by both Gibberelic Acid (GA) and vernalization treatment. None of the single or double CK2 alpha mutants shows the delayed flowering phenotype, suggesting a redundant role for the alpha subunits in the pathway (Lu *et al.*, 2011; Mulekar *et al.*, 2012). Another independent study using Arabidopsis CK2 alpha triple mutant (*α1α2α3*) showed period lengthening for the majority of the central oscillator genes without affecting their amplitude and robustness. The triple mutant plants showed increased CCA1 abundance compared to wild type, which might partly explain the lengthening of period in the mutants (Lu *et al.*, 2011). Heading date 6 (Hd6), a well-studied Quantitative Trait Locus (QTL) in rice, encodes the alpha subunit of CK2 (Takahashi *et al.*, 2001). A functional Hd6 allele is known to delay flowering in rice under long-day conditions by modulating Heading date 1 (Hd1, the Arabidopsis CONSTANS ortholog) activity. The rice ortholog of CCA, OsLHY, is phosphorylated by Hd6; although, a critical serine to glutamate mutation at a conserved CK2 site in OsLHY disconnects the rice CK2 (Hd6) from the rice circadian clock (Ogiso *et al.*, 2010). Thus, the catalytic alpha subunits of CK2 play key roles in optimizing the output pathways in plants.

The CK2 regulatory beta subunits have also been shown to function independently in the circadian clock in Arabidopsis. Over expression of both *CKB3* and *CKB4* in Arabidopsis results in increased CK2 activity and period shortening for a few clock controlled genes with different phases (Sugano *et al.*, 1999; Perales *et al.*, 2006). In the case of *CKB3*, the over expression lines showed early flowering under both short-day and long-day conditions. The CK2 beta subunits are also known to undergo autophosphorylation (Sugano *et al.*, 1999). In the case of CKB4, the autophosphorylation

induces its degradation through the 26S proteasome. Also, *CKB4* over expression lines exhibit period shortening and phase shift for floral induction genes resulting in early flowering (Perales *et al.*, 2006; Portolés and Más, 2007). A detailed review covering the role of CK2 beta subunits in plants has recently been reported (Velez-Bermudez *et al.*, 2011).

Involvement of protein kinase CK2 in regulating the stability or subcellular localization of important clock proteins is well conserved across species. In the fungus Neurospora, CK2 phosphorylates both the positive (WCC) and negative (FRQ) factors in the negative feedback loop and affects their activity and stability respectively (Brunner and Schafmeier, 2006). In Drosophila, CK2 regulates the subcellular localization and stability of the clock components PERIOD (PER) and TIMELESS (TIM) (Meissner *et al.*, 2008). CK2 also phosphorylates the mammalian clock proteins BMAL1 and PER2 to control their nuclear localization and protein stability respectively (Meissner *et al.*, 2008; Tamaru *et al.*, 2009).

Similar to the animal systems, CK2 has been found to regulate central clock components in plants resulting in altered period lengths and flowering time (Fig. 1.2). CK2 phosphorylates key clock regulators, CCA1 and LHY in Arabidopsis (Sugano *et al.*, 1998, 1999; Daniel *et al.*, 2004). Although mutating multiple CK2 sites in CCA1 does not abolish the circadian rhythms in the expression of central oscillator as well as output genes, it affects the amplitude of CCR2 expression in constant light. It also affects the dimerization of CCA1 as well as its interaction with DNA *in vitro* (Sugano *et al.*, 1998, 1999; Daniel *et al.*, 2004). This alteration of DNA binding affinity of CCA1 by CK2 is shown to be required for the temperature compensation of the circadian clock in Arabidopsis (Portolés and Más, 2010).

Thus, the protein kinase CK2 plays a critical role in regulating circadian rhythms in plants as it does in animal systems. CK2 phosphorylates central clock regulators CCA1 and LHY and this phosphorylation is essential for the normal clock function. In addition, both CK2 alpha and beta subunits independently influence the circadian responses in Arabidopsis and in rice. However, there are still some gaps in our understanding of the role played by CK2 in regulating clock function. The molecular mechanisms underlying the observed phenotypes, such as late flowering in the CK2 alpha triple mutant or early flowering in CKB3 over expression lines are not completely known. Although the involvement of CK2 in circadian clock is conserved across species, its functional significance appears to evolve in a species specific manner. Further studies need to be focused on elucidating these specific new functions of CK2 in circadian clock in various plant species.

BEYOND LIGHT AND CLOCK

Protein kinase CK2 is a ubiquitous kinase present in all tissues and is localized in both the cytoplasm and the nucleus. Hence, it is not surprising that CK2 phosphorylates a number of nuclear as well as cytoplasmic proteins that are involved in multiple pathways and modulates their function. Some of the important pathways in plants that involve CK2 include house keeping processes like translation, DNA structure, cell cycle as well as responses to various hormones and stresses. A recent study in rice shows that CK2 controls the phosphate levels by phosphorylating Phosphate Transporter 8 (PT8) under phosphate-sufficient conditions to inhibit its function (Chen *et al.*, 2015). CK2 also phosphorylates proteins involved in various basic cellular functions. Table 1 presents an exhaustive list of CK2 substrates in plants. In this section we will review the work

describing the involvement of CK2 in some of the important physiological processes in Arabidopsis as well as in other plants.

Translational regulation

Translation initiation is a multistep process in eukaryotes and involves a critical step, the formation of a 43S preinitiation complex that contains the 40S ribosomal subunit and various initiation factors (Mathews *et al.*, 2007; Muench *et al.*, 2012). A multifactor complex (MFC) consisting of several initiation factors along with GTP-Met-t-RNA may exist independently or facilitate formation of the pre-initiation complex (Muench *et al.*, 2012). A number of these initiation factors are CK2 substrates. Mammalian eIF5 is phosphorylated by CK2 and this phosphorylation enhances its interaction with eIF2 *in vivo* (Homma *et al.*, 2005*b*). Plant CK2 phosphorylates recombinant initiation factors eIF2α, eIF2β, eIF3c, eIF4B, eIF5 and histone deacetylase 2B from Arabidopsis and wheat (Dennis and Browning, 2009; Dennis *et al.*, 2009). Different combinations of Arabidopsis CK2 alpha and beta subunits show differential activity towards each of these substrates indicating an additional layer of regulation in plants. CK2 phosphorylation regulates the stability of Arabidopsis eIF2β *in vivo* (Dennis and Browning, 2009). CK2 phosphorylates eIF3c heavily and this phosphorylation promotes its interaction with eIF1, eIF2 and eIF5 *in vitro* (Dennis *et al.*, 2009). Based on these data, CK2 appears to regulate the formation of the MFC complex in plants. The phosphorylation of maize eIF5A promotes its nuclear localization as observed by the localization of the transiently expressed phospho-mimic form of ZmeIF5 in maize protoplasts (Łebska *et al.*, 2010*a*). Apart from its function as an elongation factor, eIF5A also displays RNA binding activity (Ma *et al.*, 2010), CK2 mediated phosphorylation of eIF5A might modulate translation by regulating nuclear export of mRNAs associated with eIF5A. Although, the

phosphorylation of the initiation factors has been speculated to play a role in cell cycle progression in the mammalian system (Homma *et al.*, 2005*a*); the *in vivo* implications of this kind of regulation remain unknown in plants.

Cell cycle

It is well known that CK2 positively regulates the process of cell proliferation as well as the suppression of apoptosis in normal mammalian cells (Trembley *et al.*, 2010). Several studies have reported elevated levels of CK2 protein in diverse types of cancers and hence CK2 is emerging as a key target for drug development to treat cancers. Evidence of involvement of CK2 in the regulation of cell cycle in plants has been reported. In synchronized tobacco BY2 cells, CK2 activity oscillates peaking at the G1/S and M phases (Espunya *et al.*, 1999). Also, tissues with high mitotic activity such as pollen and meristem show a higher level of CK2 transcripts indicating a role for CK2 in cell proliferation in these tissues (Moreno-Romero *et al.*, 2011). The effect of CK2 on the expression of the core cell cycle regulators was investigated using microarray analysis of a dominant negative mutant of CK2 in Arabidopsis. As expected, most of the genes known to be up-regulated at the G2/M and S phases were down-regulated in the dominant negative mutant. Moreover, ploidy analysis revealed the absence of the S phase nuclei and the presence of nuclei arrested at both the G1 and G2 phases in the induced cells. A more detailed study showing a direct involvement of CK2 in the cell cycle regulation is required to expand our understanding of the roles of CK2 in regulating the cell cycle in plants.

DNA damage

Maintaining the integrity of genomic DNA is an important house-keeping function in eukaryotic cells. In eukaryotes, High Mobility Group (HMG) proteins form a

heterogeneous group of small proteins associated with chromatin. The HMG proteins bind the DNA in a sequence independent manner through the HMG domain. Maize HMG proteins, HMGB1 and HMGB2/3, are CK2 substrates. CK2 phosphorylation enhances thermal stability of these proteins but reduces their ability to bind linear DNA. However, no change in their ability to recognize DNA minicircles was observed (Stemmer *et al.*, 2002). Another structure specific recognition protein from maize, SSRP1, is also shown to be phosphorylated by CK2 at several residues including two residues present in the HMG domain. CK2 mediated phosphorylation of SSRP1 induces the recognition of UV damaged DNA as indicated by the CD spectrum of the phosphorylated protein (Krohn *et al.*, 2003). Another kind of DNA damage is dsDNA breaks due to ionizing radiation. Some of the mammalian CK2 substrate proteins involved in chromatin remodeling are found to play a role in the dsDNA break repair pathways (Loizou *et al.*, 2004; Ayoub *et al.*, 2008; Parsons *et al.*, 2010). The dominant negative CK2 alpha subunit mutant in Arabidopsis is hypersensitive to different genotoxic agents indicating overall lower genomic stability in the mutant (Moreno-Romero *et al.*, 2012). Thus, CK2 seems to be involved in a variety of DNA damage responses in maize and Arabidopsis.

Phytohormones

Plant growth and development is tightly regulated by various phytohormones that act through a complex network of signal transduction pathways. The hormone signal transduction alters the stability/subcellular localization/function of the downstream proteins and changes the expression of target genes. CK2 has been implicated to play a role in executing signal transduction of various hormones in plants, as described below.

Salicylic Acid

The hormone Salicylic Acid (SA) is involved in activating Systemic Acquired Resistance (SAR) in plants upon infection by pathogens (Vlot *et al.*, 2009). Phosphorylation of TGA2, a member of the TGA group of bZIP transcription factor family by CK2 is shown to be enhanced by SA treatment both *in vitro* and *in vivo* (Kang and Klessig, 2005). Mutation of the CK2 sites in TGA2 to alanine results in abolition of this phosphorylation. The phosphorylated wild type TGA2 shows significantly reduced DNA binding as compared to the mutant TGA2-A protein *in vitro*. However, *in vivo* expression of both the proteins resulted in the induction of the downstream target gene PR-1 to identical levels. This might be attributed to over expression of both the proteins *in vivo*. Thus, CK2 phosphorylation of TGA2 is induced by SA treatment and changes the DNA binding capacity of TGA2 *in vitro;* however, its functional significance *in vivo* has yet to be identified.

Abscisic Acid

Abscisic acid (ABA) is an important plant hormone that regulates seed dormancy and germination, and response to various stresses in plants (Sean *et al.*, 2010). The transcription factors involved in ABA signaling bind to the ABA response elements (ABRE) in the target gene promoters. EmBP-2 and EmBZ-1 are two b-ZIP transcription factors that bind to the ABREs and control expression of the *RAB28* gene during embryo development in maize. CK2 phosphorylates both of these proteins and alters their DNA binding capacity. The expression of EmBZ-1 is induced by ABA and both EmBZ-1 and EmBP-2 activate the expression of *RAB28*, an ABA inducible gene. Thus, CK2 and ABA both modulate the activity of these proteins during embryo development (Nieva *et al.*, 2005). Another CK2 substrate, Responsive to ABA 17 (Rab17) is known to arrest seed germination under stress conditions in maize. The phosphorylation of Rab17 is necessary

for its subcellular localization as well as function. The phospho-deficient mutant Rab17 accumulates in the nucleoli and is unable to arrest germination under high salt stress when over expressed in Arabidopsis suggesting a role for CK2 (Riera *et al.*, 2004).

In Arabidopsis, the CK2 alpha subunits play a key role in response to both ABA and salt stress. The CK2 alpha triple (*α1α2α3*) as well as all the double mutant combinations (*α1α2*, *α2α3* and *α1α3*) are hyposensitive with respect to both germination and cotyledon greening at high concentrations of both ABA and salt (NaCl) (Mulekar *et al.*, 2012). Thus, CK2 positively regulates responses to ABA hormone in both maize and Arabidopsis (Riera *et al.*, 2001, Mulekar *et al.*, 2012). Recently, a SnRK2 from maize Open Stomata 1 (OST1) has been shown to be phosphorylated by CK2. Snf1-Related Kinases 2 (SnRK2) comprise a highly conserved group of kinases in maize and Arabidopsis that are strongly activated by ABA. These SnRK2s are inactivated by ABA-insensitive (ABI)- clade protein phosphatases (PP) 2Cs in the absence of ABA. The CK2 mediated phosphorylation aids OST1 degradation by increasing its binding to PP2C (Vilela *et al.*, 2015). Further studies are necessary to identify more such CK2 substrate(s) that regulate these responses.

Auxin

Auxin controls a variety of developmental processes in plants (Chapman and Estelle, 2009). Auxin is predominantly synthesized in young and developing tissues and is differentially distributed within plant tissues. The polar auxin transport is mediated by AUX1/LAX (influx) and PIN (efflux) transporter protein families (Chapman and Estelle, 2009). Studies performed with the CK2 alpha subunit dominant negative mutant indicate a role for CK2 in auxin transport. The dominant negative mutant shows reduced auxin transport to roots. Microarray analysis shows that some of the *PINs* are misexpressed in

the dominant negative mutant (Moreno-Romero and Martínez, 2008; Moreno-Romero *et al.*, 2008). The inducible dominant negative mutant and the CK2 alpha triple mutant (T-DNA insertion lines) both show a reduced number of lateral roots compared to wild type (Moreno-Romero *et al.*, 2008; Mulekar *et al.*, 2012). Formation of lateral roots is auxin dependent and requires a functional cell division cycle. Thus, the reduced number of lateral roots in both the mutants might be a combined effect of the defect in cell cycle as well as defective auxin transport in these plants. Identification and functional characterization of CK2 substrates will enhance our understanding of how CK2 regulates auxin signaling.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our understanding of the role of CK2 in plants is slowly but steadily growing with the discovery of additional CK2 substrates (Table 1.1). CK2 mRNAs do not appear to be under major transcriptional regulation. However, the enzyme activity of CK2 is partly regulated post-translationally. Although the alpha subunit has the catalytic activity, the regulatory beta subunit modulates the catalytic activity of the alpha subunit for some of the substrates. The CK2 holoenzyme is not regulated as an ON/OFF switch like the MAP kinases that are activated only upon phosphorylation (Goldsmith, 1995). Hence, it appears that CK2 acts as a housekeeping kinase that modifies protein functions in a dynamic way.

Most of the CK2 substrates that have been identified are primarily transcription factors or regulatory proteins functioning in diverse pathways. The phosphorylation of these factors often results in changes in the DNA binding affinity, dimerization, stability, protein-protein interaction and subcellular localization. For protein stability, CK2 appears to play a dual role. For example, CK2-mediated phosphorylation of some substrates (e.g.,

HY5, HFR1 and others) results in stabilization of these substrates, while the phosphorylation of other substrates (e.g., PIF1 and others) results in promotion of their degradation (Hardtke *et al.*, 2000; Park *et al.*, 2008; Bu *et al.*, 2011*a*). In addition, phosphorylation of some substrates is induced by the beta subunits (e.g., PIF1, eIF2β, eIF4B), while the beta subunits also reduce phosphorylation of other substrates (e.g., eIF3c) (Dennis and Browning, 2009; Dennis *et al.*, 2009; Bu *et al.*, 2011*a*). At present, it is not clear whether any sequence-specific phosphorylation site determines either the stabilization or the degradation of these substrates. In addition, there is no correlation between the stability of any substrates and the regulation of their phosphorylation by the beta subunits. Although, CK2 usually phosphorylates at a consensus sequence (Ser-Xaa-Xaa-Acidic), the enzyme allows flexibility within the consensus sequence specificity for the substrates. Therefore, the *in silico* prediction software analyses can yield false positive/negative results. Identification and characterization of a large number of CK2 substrates by proteomics will help design better software to predict these features.

CK2 has been implicated to play a role in multiple developmental and stress responsive pathways in plants. However, the exact molecular mechanisms by which CK2 regulates these pathways still remain elusive. Our knowledge on the effect of CK2-mediated phosphorylation on the limited numbers of plant substrate proteins identified so far is rudimentary. Identification and further characterization of the CK2 substrates in each of these pathways are necessary to understand how CK2 regulates these pathways. Further biochemical and functional analysis of the effect of the CK2 phosphorylation using phospho-deficient and phospho-mimic mutants of the substrate proteins will provide a better mechanistic view on how these substrates play roles in these pathways.

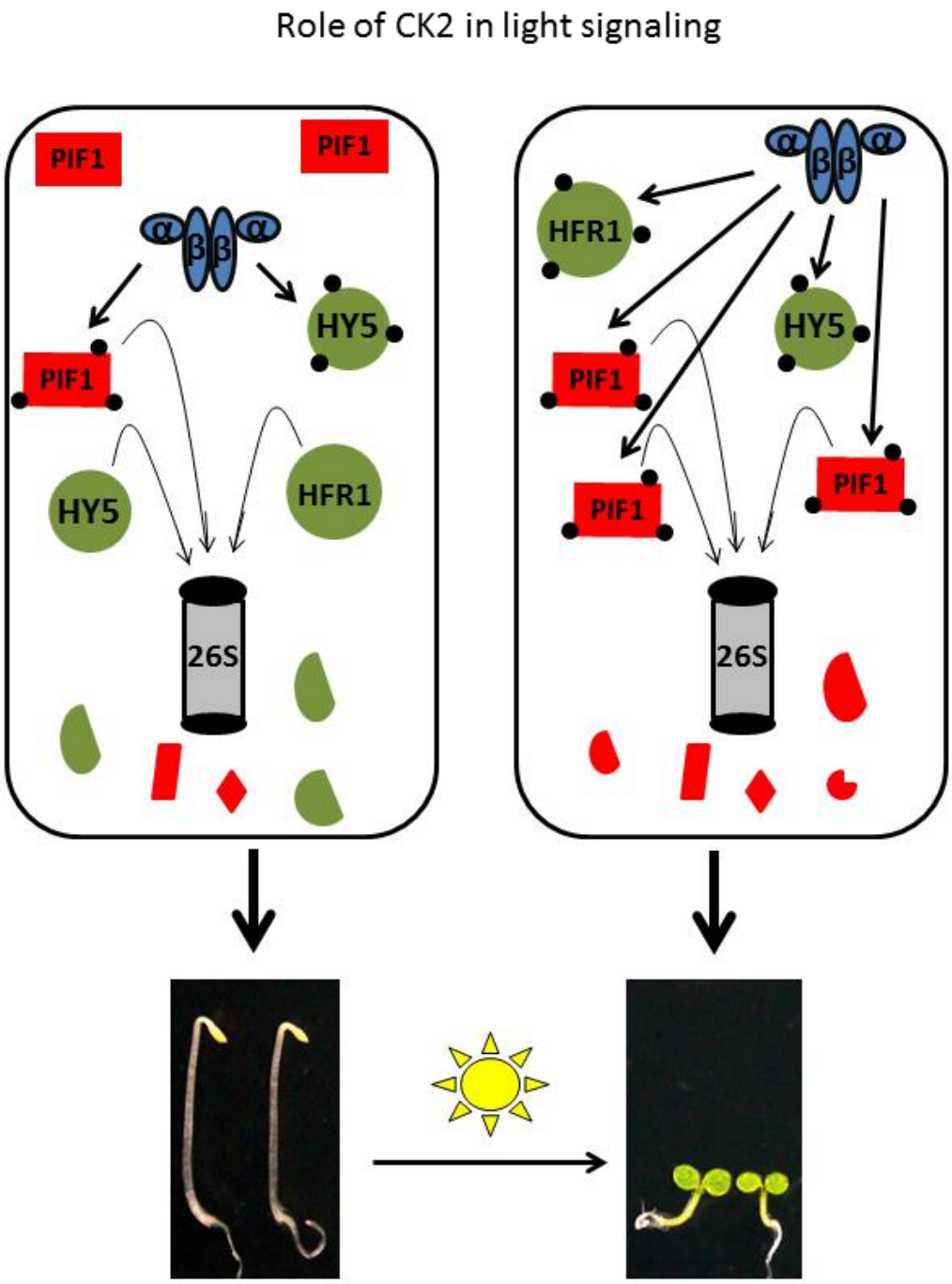

Figure 1.1: CK2 regulates photoperiodic flowering time and circadian clock in plants. (A) CK2 regulates flowering time under both short and long days. (B) CK2 beta subunits regulate period length when overexpressed. (C) CK2 phosphorylates CCA1 and the phosphorylation reduces dimerization of CCA1 resulting in reduced DNA binding.

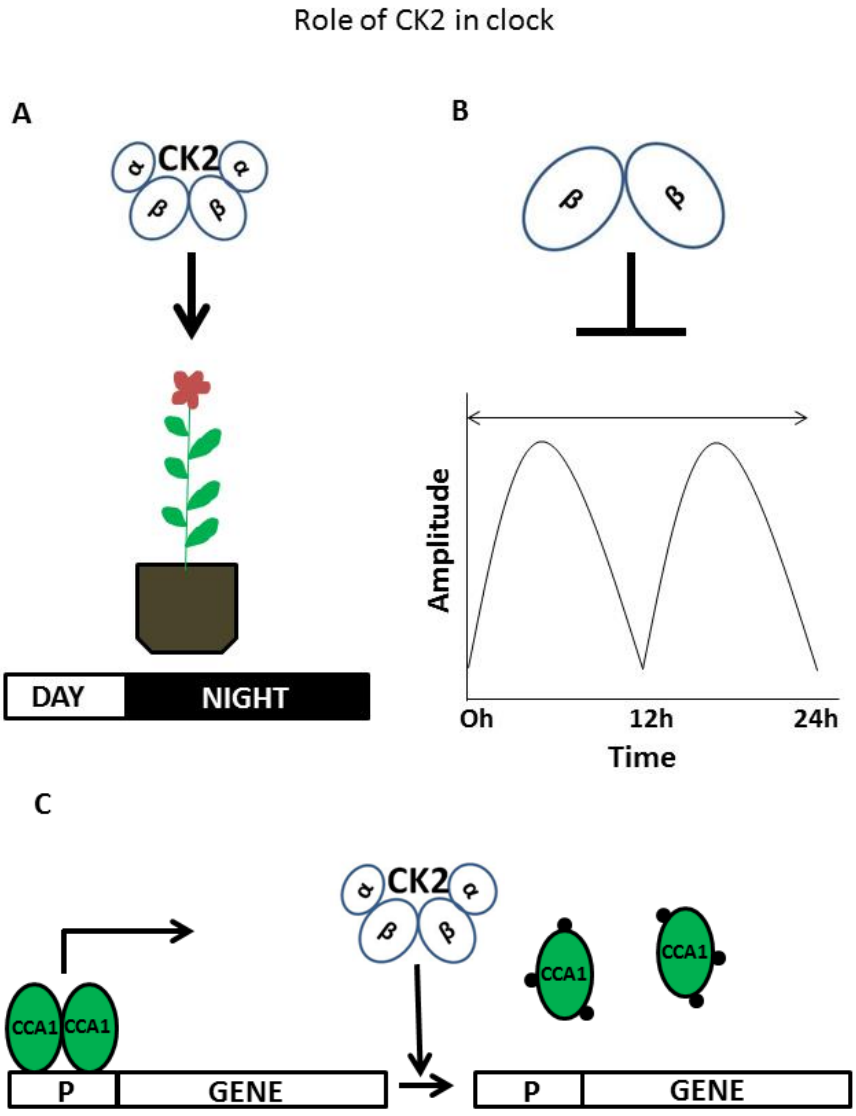

Figure 1.2: CK2 regulates photomorphogenesis by phosphorylating both positive and negatively acting components. In the dark (left panels), the positively acting transcription factors (HY5 and HFR1) are degraded through the 26S proteasome system, and PIF1, a negatively acting transcription factor, is highly abundant in the dark repressing photomorphogenesis resulting in etiolated seedling growth. In response to light (right panels), HY5 and HFR1 are stabilized, and light promotes rapid degradation of PIF1 and other PIFs to promote photomorphogenesis resulting in de-etiolated seedlings. CK2 phosphorylates both the positively and negatively acting components presumably under both dark and light conditions. However, the significance of CK2 phosphorylation is different for these two groups of transcription factors. CK2- mediated phosphorylation stabilizes HY5 and HFR1, while it promotes rapid light-induced degradation of PIF1. A balance between the level of the positive and negative factors optimizes photomorphogenesis.

Table 1.1: Summary of the CK2 substrates identified from plants

No.	Name	Type of protein	Source	Role	Pathway	Reference
1	AT-1	Transacting factor	Pea	Binds to AT-rich promoter	Light signaling	(Datta and Cashmore, 1989)
2	ATBP-1	Transacting factor	Pea	Binds to AT-rich promoter	Light signaling	(Tjaden and Coruzzi, 1994)
3	GBF1	bZIP transcription factor	Arabidopsis	Binds to G-box promoter	Light signaling	(Klimczak *et al.*, 1992)
4	HFR1	bHLH transcription factor	Arabidopsis	Promotes photomorphogenesis	Light signaling	(Park *et al.*, 2008)
5	HY5	bZIP transcription factor	Arabidopsis	Promotes photomorphogenesis	Light signaling	(Hardtke *et al.*, 2000)
6	PIF1	bHLH transcription factor	Arabidopsis	Represses photomorphogenesis	Light signaling	(Bu *et al.*, 2011*a*)
7	CCA-1	Myb-related transcription factor	Arabidopsis	Circadian clock regulator	Circadian clock	(Sugano *et al.*, 1998)
8	LHY	Myb-related transcription factor	Arabidopsis	Circadian clock regulator	Circadian clock	(Sugano *et al.*, 1998)
9	eIF2α	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis *et al.*, 2009)
10	eIF2β	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis *et al.*, 2009)
11	eIF3c	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis *et al.*, 2009)
12	eIF4B	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis *et al.*, 2009)
13	eIF5	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis *et al.*, 2009)
14	eIF5A	Translation elongation factor	Maize	Nucleocytoplasmic shuttling regulation	Translation	(Łebska *et al.*, 2010*a*)
15	HD2B	Translation initiation factor	Wheat, Arabidopsis	Enhance multifactor complex formation	Translation	(Dennis and Browning, 2009)

Table 1.1 (continued)

16	p34	Ribonucleoprotein	Spinach chloroplast	mRNA 3' end processing	Translation	(Kanekatsu *et al.*, 1993)
17	p36	eIF-2 subunit	Wheat germ	Guanine nucleotide Exchange	Translation	(Janaki *et al.*, 1995)
18	PDH65	DNA helicase	Pea	Opens the duplex DNA during nucleic acid transactions	Transcription	(Tuteja *et al.*, 2008)
19	P-proteins	Ribosomal proteins	Maize	Complex with 60S ribosomal subunits	Translation	(Hasler *et al.*, 1991)
20	SIG6	Plastid sigma factor	Arabidopsis	Directs plastid RNA polymerase to promoter	Transcription	(Schweer *et al.*, 2010)
21	HMGB1	High Mobility Group (HMG) proteins	Maize	Chromatin-associated proteins	Transcription, Recombination	(Stemmer *et al.*, 2002)
22	HMGB2/3	High Mobility Group (HMG) proteins	Maize	Chromatin-associated proteins	Transcription, Recombination	(Stemmer *et al.*, 2002)
23	SSRP1	Structure-Specific Recognition Protein	Maize	Chromatin-associated proteins	Transcription and replication	(Krohn *et al.*, 2003)
24	EmBP-2	bZIP transcription factor	Maize	Bind to ABRE	ABA/stress-induced pathway	(Nieva *et al.*, 2005)
25	p23	Co-chaperone protein	Arabidopsis	Chaperone associates with Hsp90	Hormone signaling?	(Tosoni *et al.*, 2011)
26	Rab 17	LEA protein	Maize	Stress-induced protein	ABA/stress-induced pathway	(Riera *et al.*, 2004)
27	TAS-14	LEA protein	Tomato	Stress-induced protein	ABA/stress-induced pathway	(Godoy *et al.*, 1994)
28	TGA-2	bZIP transcription factor	Arabidopsis	Binds to promoters	Salicylic Acid response	(Kang and Klessig, 2005)
29	ZmBZ-1	bZIP transcription factors	Maize	Bind to ABRE	ABA/stress-induced pathway	(Nieva *et al.*, 2005)
30	Apyrase	ATP hydrolysing enzyme	Pea	ATP hydrolysis	ATP synthesis	(Hsieh *et al.*, 2000)

Table 1.1 (continued)

31	Beta-conglycinin	Storage protein	Soybean	Storage protein	Seed storage	(Ralet MC, Fouques D, Leonil J, Molle D, 1999)
32	C2	Proteasome protein	Rice	Protein degradation	Proteasome machinery	(Umeda *et al.*, 1997)
33	Calreticulin	Ca2+ binding protein	Spinach	Ca2+ metabolism	Seed storage	(Baldan *et al.*, 1996)
34	CFOCF1-ATPase	ATPase synthase b subunit	Spinach chloroplasts	ATP synthesis	ATP synthesis	(Kanekatsu *et al.*, 1998)
35	gp96	Lipoxygenase	Soybean	Oxygenation of unsaturated fatty acids	Lipid synthesis	(Ohtsuki *et al.*, 1995)
36	Lamina-like	Lamina matrix protein	Pea	Lamina matrix protein	Nuclear matrix proteins	(Li and Roux, 1992)
37	MFP1	MAR-binding filament-like protein 1	Tomato	DNA binding coiled coil protein	Component of thylakoid membrane and nucleoids	(Jeong *et al.*, 2004)
38	OST1	Open Stomata 1	Maize	ABA signaling	SnRK2	(Vilela *et al.*, 2015)
39	PT8	PO_4 transporter	Rice	Component of plasma membrane	Transport PO_4	(Chen *et al.*, 2015)

Chapter 2: Nuclear CK2 alpha subunits affect multiple developmental and stress-responsive pathways in Arabidopsis[2]

ABSTRACT

CK2 (formerly known as Casein Kinase II), a ubiquitous Ser/Thr kinase, plays critical roles in all higher organisms including plants. The CK2 holoenzyme consists of two catalytic alpha, and two regulatory beta subunits. The Arabidopsis genome has four alpha subunit and four beta subunit genes, and members of both alpha and beta subunit families have been shown to be localized in the cytoplasm, nucleus and also in chloroplasts. However, the biological roles of CK2 subunits have not been fully characterized yet. Here we identified T-DNA insertion mutants in three alpha subunit genes (*α1, α2* and *α3*) and made double and triple mutants. The CK2 *α1α2α3* triple mutants displayed reduced CK2 activity compared to wild type seedlings. Phenotypic characterization showed that CK2 *α1α2α3* triple mutants are late flowering under both long- and short-day conditions. Genes encoding floral integrators are differentially regulated in the triple mutant compared to the wild type plants. CK2 *α1α2α3* triple mutants also displayed reduced hypocotyl growth, smaller cotyledon size and reduced number of lateral roots compared to wild type seedlings under light. ABA-induced block of seed germination and cotyledon greening is reduced in CK2 alpha subunit mutants in an additive manner. Moreover, CK2 alpha subunit mutants are also hyposensitive to a NaCl-induced block of seed germination. Taken together, these data suggest that CK2

[2] Parts of this chapter have been previously published (doi: 10.1111/j.1365-313X.2011.04794.x). Dr. Qingyun Bu is the first co-author, Dr. Fulu Chen is the second author and my supervisor Dr. Enamul Huq is the corresponding author. QB developed the T-DNA mutant lines and kinase assay for CO. FC did some qRT-PCRs for flowering time genes.

alpha subunits affect diverse developmental and stress responsive pathways in Arabidopsis.

INTRODUCTION

Protein phosphorylation/dephosphorylation is a key post-translational mechanism often exploited to regulate physiological processes in all living organisms. CK2 kinase is one of the Ser/Thr kinases involved in regulation of protein function and is present in all eukaryotes. The protein kinase CK2 (also known as Casein Kinase II) is a tetrameric protein composed of two regulatory (beta) and two catalytic (alpha) subunits. Except for plants and the yeast *Saccharomyces cerevisiae*, the regulatory subunit is often encoded by a single gene, while all eukaryotes contain at least two genes encoding for the catalytic subunit (Riera *et al.*, 2001*b*; Espunya *et al.*, 2005; Salinas *et al.*, 2006). This Ser/Thr kinase is evolutionarily well-conserved, with a vast number of physiological targets (>300) identified to date, thus emphasizing its role in the regulation of diverse cellular processes (Litchfield, 2003). In *S. cerevisiae,* a double knockout for CK2 alpha subunit fails to survive, indicating a vital role for this kinase (Padmanabha *et al.*, 1990). The deletion of a single regulatory or catalytic subunit in yeast leads to changes in expression profiles of various genes regulating the cell cycle (Barz *et al.*, 2003), indicating a broad role for CK2 in the cell cycle progression. The recent research in tumor biology indicates that CK2 is also involved in regulation of tumor suppressor genes and prevents cells from undergoing apoptosis (Duncan and Litchfield, 2008).

CK2 kinase activity from various plant species (rice, wheat, maize, tobacco, mustard and Arabidopsis) has been studied and characterized to date (Salinas *et al.*, 2001; Riera *et al.*, 2001*a*; Kato *et al.*, 2002; Ogrzewalla *et al.*, 2002; Ogiso *et al.*, 2010). In *Arabidopsis thaliana* alpha and beta subunits of CK2 are each encoded by four genes.

Expression profiling experiments revealed that these CK2 subunits are ubiquitously expressed in almost all the tissues. One of the alpha subunits (α4) containing a putative chloroplast target peptide is found to have higher expression levels compared to the other subunits that are localized in the nucleus and/or cytoplasm (Salinas *et al.*, 2006). These localization data support the fact that majority of the CK2 substrates found to date are either nuclear proteins or proteins involved in signal transduction.

In Arabidopsis, CK2 plays critical role in various physiological processes such as light signaling, circadian rhythm, hormone responses, cell cycle control and flowering time (Sugano *et al.*, 1999; Hardtke *et al.*, 2000; Portolés and Más, 2007; Park *et al.*, 2008; Bu *et al.*, 2011*a*). The circadian clock component protein, CCA1 is one of the substrates of CK2 kinase. Also, over-expression of one of the CK2 regulatory subunits (CKB3) in Arabidopsis leads to early flowering under short-day conditions (Sugano *et al.*, 1999). Another CK2 regulatory subunit CKB4 is found to influence the Arabidopsis circadian clock through the phosphorylation status of the CKB4 subunit itself (Perales *et al.*, 2006). In light signaling pathways, CK2 phosphorylates both positively and negatively acting transcription factors and regulates their stability and activity. For example, HY5 and HFR1, which are positively acting transcription factors are stabilized by CK2-mediated phosphorylation (Hardtke *et al.*, 2000; Park *et al.*, 2008). The CK2-mediated phosphorylation affects the ability of HY5 to bind to target promoters and also to interact with COP1 (Hardtke *et al.*, 2000). Conversely, the light-induced degradation of PIF1 (and possibly other PIFs) is enhanced by CK2-mediated phosphorylation (Bu *et al.*, 2011*a*). Translation initiation factors from Arabidopsis, wheat and maize are phosphorylated by CK2, affecting their stability, altered complex formation, differential nucleo-cytoplasmic localization and ultimately the efficiency of translation (Dennis and Browning, 2009; Dennis *et al.*, 2009; Łebska *et al.*, 2010*b*). CK2 functions with respect to hormone

responses have been studied in maize and Arabidopsis (Riera *et al.*, 2004; Kang and Klessig, 2005). In maize, CK2 kinase is found to regulate bZIP proteins and their target, Rab17, one of the abscisic acid responsive proteins (Riera *et al.*, 2004; Nieva *et al.*, 2005). CK2 also differentially phosphorylates maize HMBG proteins and modulate their stability and DNA binding activity (Stemmer *et al.*, 2002, 2003; Krohn *et al.*, 2002). In Arabidopsis, salicylic acid is found to induce the CK2 activity leading to enhanced phosphorylation of a certain group of TGA proteins (bZIP transcription factors), negatively influencing their DNA binding ability (Kang and Klessig, 2005). Therefore, as in the case of animal systems, CK2 regulates diverse pathways in plants.

Since CK2 is a vital kinase for survival and also because of the pleiotropic nature of CK2 functions, the physiological characterization of the CK2 mutants has been a challenge. Early experiments using antisense technology to knock down CK2 levels have shown that the transformed plants have a smaller leaf size and number than the wild type. Also, the expression levels of some of the light-regulated genes were reduced in the antisense lines compared to wild type seedlings (Lee *et al.*, 1999). Another recent study carried out using a steroid-inducible dominant negative CK2 mutant revealed a role for CK2 in cell division and cell expansion in Arabidopsis (Moreno-Romero *et al.*, 2008). However, the steroid-inducible expression is more suitable for the early seedling-stage on plates, limiting its use for analyzing adult stage phenotypes (e.g., flowering time). Here we identify T-DNA insertion mutants in three alpha subunit genes (α1, α2 and α3) in Arabidopsis and describe double and triple mutants in all combinations. Phenotypic characterization shows that CK2 alpha subunits affect multiple developmental and stress-responsive pathways in Arabidopsis.

RESULTS

Isolation and characterization of CK2 alpha subunit mutants in Arabidopsis

To investigate the biological roles of CK2, we isolated T-DNA insertion mutants in the CK2 alpha subunit genes from the SALK collection (Alonso *et al.*, 2003). We identified two independent homozygous T-DNA insertion lines for the α1 and α2 genes and one independent homozygous T-DNA insertion line for α3 and named them as *α1-1*, *α 1-2*, *α 2-1*, *α 2-2* and *α 3-1* (Fig. 2.1a). RT-PCR analyses showed that *α 1-1*, *α 2-2* and *α 3-1* are homozygous null alleles as no wild type mRNA was detected by RT-PCR assays in these lines (Fig. 2.1b). However, *α 1-2* and *α 2-1* showed a wild type level message present in the mutants, suggesting that these are not null mutants. The T-DNA is inserted in the 1st and 4th introns in *α 1-2* and *α 2-1*, respectively (Fig. 2.1a). The mRNA size for CK2α1 was slightly larger than the wild type mRNA size in *α 1-2* line possibly due to aberrant transcription start site. In the case of *α 2-1,* the T-DNA might have been spliced out during RNA processing, giving rise to a wild type message level. Because *α 1-1*, *α 2-2* and *α 3-1* did not show any transcript, we used these lines to create double (*α1α2*, *α1α3* and *α2α3*) and triple (*α1α2α3*) mutants for phenotypic characterization.

The CK2 α subunit is the catalytic subunit of CK2 holoenzyme. Because we mutated three out of four alpha subunits, we examined the relative CK2 kinase activity of the wild type and triple mutant plants using recombinant wheat eIF4B as a substrate (Dennis and Browning, 2009). Wheat eIF4B is robustly phosphorylated by the wild type Arabidopsis CK2 alpha subunit, and the beta subunit did not show any effect on the phosphorylation level by alpha subunit (Dennis and Browning, 2009), suggesting that this is a good substrate to examine the difference in kinase activity between the wild type and triple mutant plants. We made extracts from two-week old wild type and triple mutant plants and assayed for CK2 kinase activity as described (Dennis and Browning, 2009).

Results show that the CK2 *α1α2α3* triple mutant has ~70% kinase activity compared to wild type plants (Fig. 2.1c, d). This is consistent with the expression level for different alpha subunit genes in Arabidopsis. Digital expression analyses showed that the CK2 α4 has the highest expression level at all developmental stages compared to the other three alpha subunits mutated here (Fig. 2.9) (Zimmermann *et al.*, 2004). Taken together, these data suggest that CK2 *α1α2α3* triple mutant has significantly reduced CK2 activity, and can be used to assess the role(s) of CK2 alpha subunits in plant growth and development.

CK2 alpha subunits promote flowering time under both long-day (LD) and short-day (SD) conditions

Although a rice CK2 alpha subunit has been shown to be involved in modulating flowering time (Takahashi *et al.*, 2001; Ogiso *et al.*, 2010), involvement of Arabidopsis CK2 alpha subunits in controlling flowering time has not yet been reported. We examined the flowering time phenotypes of all the CK2 alpha subunit single, double and triple mutant combinations under both SD and LD conditions. The results show that the single and double mutants flower at the same time as wild type plants under both SD and LD conditions (Fig. 2.10). However, the triple mutant plants flowered significantly later than the wild type controls under both SD and LD conditions. Both the number of days to flower and the rosette leaf numbers for the triple mutant were higher than the wild type plants (Fig. 2.2a, b), suggesting that CK2 alpha subunits affect flowering time possibly in an overlapping and/or genetically redundant manner.

Gibberellic acid (GA) is required for flowering under SD conditions (Wilson *et al.*, 1992). To investigate whether the delayed flowering phenotype of the CK2 *α1α2α3* triple mutant is due to a defect in GA, we sprayed GA_3 on the meristems of wild type and CK2 *α1α2α3* triple mutant plants under both SD and LD conditions. Results show that the flowering time is reduced in response to GA application for both wild type and the

CK2 *α1α2α3* mutant plants (Fig. 2.11). Under LD conditions, CK2 *α1α2α3* triple mutant flowering was similar to the wild type controls in the presence of GA application (Fig. 2.11b). However, the CK2 *α1α2α3* triple mutant still flowered later than the wild type controls under SD conditions (Fig. 2.11a). These data suggest that GA biosynthesis/signaling may not contribute to the delayed flowering in the CK2 *α1α2α3* triple mutants under these conditions.

To assess whether the delayed flowering is due to a defect in the vernalization response, we treated both wild type and the CK2 *α1α2α3* triple mutant plants for 40 days at 4^0C, and then transferred them to LD and SD conditions to evaluate their flowering time. Results show that vernalization had no (for LD conditions) or modest (for SD conditions) effect on the flowering time phenotypes of the wild type controls (Fig. 2.12a, b). However, the CK2 *α1α2α3* triple mutant plants flowered earlier than the non-vernalized triple mutant plants in response to vernalization treatment under both LD and SD conditions (Fig. 2.12a, b), suggesting that vernalization response is not defective in the triple mutant. Under these conditions, the flowering time for the triple mutant and the wild type control plants were similar (Fig. 2.12a, b). Because CK2 *α1α2α3* triple mutant flowered later under both LD and SD conditions, and responded to vernalization treatment, CK2 alpha subunits may function in the autonomous pathway to regulate flowering time.

Flowering time genes are differentially regulated in CK2 alpha subunit triple mutant

Expression of floral integrators (e.g., *FT* and *SOC1*) and their upstream regulators (e.g., *CO* and *FLC*) are critical molecular markers for flowering time phenotype under both LD and SD conditions (Imaizumi and Kay, 2006; Kim *et al.*, 2009). To examine the molecular basis for the delayed flowering phenotype of the CK2

α1α2α3 triple mutant, we performed time course qRT-PCR assays to determine the gene expression levels in CK2 *α1α2α3* triple mutant and wild type plants under both LD and SD conditions. Under LD conditions, the expression of *CO* is slightly down-regulated while the expression of *FLC* is up-regulated in the triple mutant compared to wild type plants (Fig. 2.3a). As expected, the expression of *FT* and *SOC1* is strongly down-regulated in the triple mutant compared to wild type under these conditions. Under SD conditions, expression of *FT* and *SOC1* is modestly reduced in the triple mutant while expression of *FLC* is modestly up-regulated compared to wild type plants (Fig. 2.3b). Expression of *CO* is similar in both the wild type and triple mutant plants under these conditions. Reduced expression of *FT* and *SOC1* and increased expression of *FLC* under both LD and SD conditions might delay flowering of the triple mutant under these conditions.

Because CK2 *α1α2α3* triple mutant plants flowered later under both LD and SD conditions (Fig. 2.2), and *FLC* expression is up-regulated in the triple mutant compared to wild type seedlings (Fig. 2.3), we examined whether *FLC* expression is down-regulated in response to vernalization treatment. Results showed that *FLC* expression is reduced in both wild type and mutant plants in response to vernalization treatment (Fig. 2.12c). Strikingly, the level of *FLC* is similar in both the wild type and the CK2 *α1α2α3* triple mutant plants in response to venalization. Reduction of *FLC* expression in the CK2 *α1α2α3* triple mutant to a wild type level in response to vernalization is consistent with the rescue of the late flowering phenotype of the triple mutant after vernalization treatment.

CO is not phosphorylated by CK2

Because expression of *CO* and *FT* is down-regulated in the CK2 *α1α2α3* triple mutant (Fig. 2.3), and CO is post-transcriptionally regulated (Valverde *et al.*, 2004), we hypothesized that CO might also be post-translationally regulated by CK2. We purified Arabidopsis recombinant CO using a bacterial expression system and examined whether CK2 can phosphorylate CO *in vitro*. However, CO is not phosphorylated by CK2 in the *in vitro* kinase assay (Fig. 2.13). This result is also consistent with a recent report that rice CO is not phosphorylated by rice CK2 (Ogiso *et al.*, 2010). These data suggest that CK2 regulates *FT* expression by a different mechanism other than regulating CO post-translationally.

CK2 alpha subunit triple mutants display reduced hypocotyl lengths and smaller cotyledons under light

CK2 has been shown to influence the stability of positively and negatively acting transcription factors in light signaling pathways (Hardtke *et al.*, 2000; Park *et al.*, 2008; Bu *et al.*, 2011*a*), and also affect light-regulated gene expression (Lee *et al.*, 1999). To assess the roles of CK2 alpha subunits in regulating photomorphogenesis, we investigated the seedling de-etiolation phenotypes under monochromatic light conditions. Results show that CK2 alpha subunit single and double mutant seedlings displayed similar hypocotyl lengths compared to wild type seedlings both in the dark and in red/far-red light conditions (data not shown). The hypocotyl lengths of the wild type and the *α1α2α3* triple mutant seedlings were largely similar in the dark. However, the *α1α2α3* triple mutant seedlings displayed developmental defects when grown in white light. Approximately 20% of the seedlings did not develop normal roots and failed to green properly (Fig. 2.14). The rest of the seedlings (~80%) developed open and expanded cotyledons, normal roots, and developed as adult plants under greenhouse conditions.

These apparent segregating behaviors are not due to genotyping error, as the majority of the phenotypes characterized here were from the later group.

Fluence rate response curves showed that the *α1α2α3* triple mutant seedlings displayed shorter hypocotyls under a broad range of red light intensities, but not under far-red light conditions (Fig. 2.4a, b, d). Measurement of the cotyledon area showed that the *α1α2α3* triple mutant seedlings also displayed smaller cotyledons at multiple red light intensities (Fig. 2.4c, d). The triple mutant also displayed slightly shorter roots compared to wild type seedlings under red light (Fig. 2.4d). Short hypocotyls, short roots and small cotyledons under red light are indicative of a general growth defect as opposed to a hypersensitive response under red light conditions.

CK2 alpha subunits promote lateral root development

CK2 has been implicated in regulating cell division and cell expansion using a steroid-inducible dominant negative mutant (Moreno-Romero and Martínez, 2008; Moreno-Romero *et al.*, 2008). To investigate whether the CK2 alpha subunit mutants display any defect in lateral root number, we grew seedlings on agar plates vertically at 22^0C for two weeks. We then counted the number of lateral roots. Results show that the average number of lateral roots/plant was unaffected in all three single mutants compared to wild type seedlings (Fig. 2.5a). Among the double mutants, only the *α1α2* double mutant displayed a significantly reduced number of lateral roots compared to wild type (Fig. 2.5a). In addition, the *α1α2α3* triple mutant showed the least number of lateral roots compared to wild type (Fig. 2.5a, b), suggesting that all three alpha subunits are affecting lateral root number in an additive manner.

CK2 alpha subunit mutants are hyposensitive to ABA-induced block of seed germination and cotyledon greening in an additive manner

Previously, CK2 has been shown to modulate developmental functions of maize ABA responsive protein RAB17 by phosphorylation (Riera *et al.*, 2004). To examine whether CK2 alpha subunit mutants are defective in ABA responses, we performed dose-response curves for seed germination and cotyledon greening responses, the two hallmark processes that are inhibited by ABA in a concentration-dependent manner. Results show that with respect to the rates of inhibition for seed germination and cotyledon greening, alpha subunit single mutants are slightly hyposensitive compared to that of wild type seeds (Fig. 2.6a). However, the double mutants displayed a much higher rate of seed germination and cotyledon greening compared to wild type seeds, especially at the highest concentration of ABA (Fig. 2.6b). The triple mutant seeds showed strongly increased rate of seed germination compared to wild type seeds (Fig. 2.6c, left). Interestingly, the triple mutant seeds showed only a slightly increased rate of cotyledon greening compared to wild type seeds (Fig. 2.6c, right). The reduced hyposensitive cotyledon greening phenotype of the triple mutant compared to the double mutants might be explained by the fact that the triple mutant seedlings develop poorly (Fig. 2.14), and ~20% of seedlings are excluded from the assay as they appear defective. Taken together, these data suggest that CK2 alpha subunit mutants are hyposensitive to ABA and are functioning in a synergistic manner in response to ABA.

CK2 alpha subunit mutants are hyposensitive to a NaCl-induced block of seed germination in an additive manner

CK2 alpha subunit from sugar beet is induced by NaCl and has been shown to increase NaCl tolerance in yeast (Kanhonou *et al.*, 2001). Moreover, Arabidopsis seed germination and cotyledon greening are strongly inhibited by NaCl in a

concentration-dependent manner (Ruggiero *et al.*, 2004). To investigate whether CK2 alpha subunit mutants are defective in NaCl responses, we performed NaCl dose response curves for seed germination and cotyledon greening responses. Results show that the alpha subunit single mutants and wild type seeds displayed similar rates of inhibition for seed germination and cotyledon greening (Fig. 2.7a). However, the double mutants displayed much higher rates of seed germination and cotyledon greening compared to wild type seeds (Fig. 2.7b). Interestingly, the triple mutant seeds showed a slightly increased rate of seed germination and cotyledon greening compared to the wild type and double mutant seeds (Fig. 2.7b, c). As discussed above, the reduced hyposensitive phenotype of the triple mutant compared to the double mutants might be due to the poorly developed seedlings of the triple mutant compared to wild type seedlings (Fig. 2.14). Taken together, these data suggest that CK2 alpha subunit mutants are hyposensitive to NaCl and are functioning in a synergistic manner to respond to NaCl.

DISCUSSION

CK2 has been shown to modulate diverse pathways in all eukaryotic organisms. Previously, the biological functions of the α subunit genes have been investigated either using an antisense approach (Lee *et al.*, 1999) or by overexpressing a dominant-negative mutant in the wild type Arabidopsis background (Moreno-Romero *et al.*, 2008). Both of these approaches suffer from various limitations including the lack of specificity for individual alpha subunit genes. Here we identified T-DNA insertion mutants in three of the four alpha subunit genes and demonstrate that these three alpha subunits function in an overlapping and/or additive/synergistic manner in affecting diverse developmental and stress response pathways in Arabidopsis. Our approach has uncovered biological roles of these alpha subunit genes in additional pathways that were not previously identified.

One of the previously unidentified phenotypes for the Arabidopsis alpha subunit mutants is their role in affecting flowering time. The data show that the individual alpha subunit genes and the three combinations of double mutants (*α1α2, α2α3* and *α1α3*) flower at the same time as wild type plants under both LD and SD conditions. However, the *α1α2α3* triple mutant flowered later than wild type plants under these conditions, suggesting that these genes are functioning in an overlapping manner. The late-flowering phenotype along with the increased level of expression of *FLC* under both LD and SD conditions suggest that CK2 alpha subunit genes may function in the autonomous pathway. This is consistent with the fact that the flowering time for the triple mutant was reduced to a wild type level in response to vernalization treatment (Fig. 2.12). In addition, expression of *FLC* in the triple mutant is reduced to a wild type level in response to vernalization treatment (Fig 2.12c). These data suggest that CK2 alpha subunit genes are functioning in the autonomous pathway to regulate flowering time.

Delayed flowering under LD conditions also suggests that the alpha subunits may function in the photoperiod pathway. Previously, overexpression of *CK2 β4* has been shown to regulate *CO* expression due to an aberrant pace of the circadian clock (Portolés and Más, 2007). In addition, CK2 also regulates the pace of the clock by regulating the phosphorylation state of the core clock component CCA1 (Sugano *et al.*, 1999; Daniel *et al.*, 2004). Our data demonstrating reduced expression of *CO* in the CK2 *α1α2α3* triple mutant compared to wild type plants under LD conditions (Fig. 2.3a) supports this hypothesis that CK2 alpha subunit genes also modulate flowering time through the photoperiod pathway. The late flowering phenotype of the Arabidopsis *α1α2α3* triple mutant is consistent with a rice mutant in the alpha subunit gene of rice CK2 (*Hd6*) (Takahashi *et al.*, 2001; Ogiso *et al.*, 2010). However, unlike the rice *Hd6* mutant, which flowers late only under LD conditions, the Arabidopsis *α1α2α3* triple mutant flowered

late under both LD and SD conditions. Arabidopsis is a facultative LD plant while rice is a SD plant. Although the photoperiod pathway components are conserved in Arabidopsis and rice, individual gene functions are not identical. For example, CO activates *FT* expression under LD in Arabidopsis, while rice CO activates *FT* expression under SD conditions. The differences in the flowering time for the Arabidopsis and rice CK2 alpha subunit mutants might be due to the intrinsic differences in the flowering time pathways between Arabidopsis and rice.

Although the triple mutant plants flowered late under both LD and SD conditions, the mechanism by which CK2 alpha subunit genes modulate flowering time is not clear. Increased expression of *FLC* and decreased expression of *CO/FT/SOC1* in the triple mutant compared to wild type plants under both LD and SD conditions provide the molecular basis of the delayed flowering phenotype (Fig. 2.3). However, how CK2 modulates expression of these genes is still unknown. Because CO is post-transcriptionally regulated (Valverde *et al.*, 2004), one possibility is that CK2 phosphorylates Arabidopsis CO to regulate *FT* and *SOC1* expression and thereby promote flowering time. However, kinase assays with purified CO and CK2 holoenzymes showed that Arabidopsis CO is not a substrate for Arabidopsis CK2 *in vitro* (Fig. 2.13). This is also consistent with a recent report that Hd1 (rice CO) is not phosphorylated by Hd6 (rice CK2) *in vitro* (Ogiso *et al.*, 2010). It is possible that CK2 affects the function of upstream factor(s) (e.g., CCA1 and possibly others) that regulates the expression of *CO* and *FLC* and the downstream floral integrator genes (*FT/SOC1)*, as has been proposed (Daniel *et al.*, 2004; Portolés and Más, 2007; Ogiso *et al.*, 2010).

Previously, it was shown that overexpression of a dominant-negative mutant in wild type plants induced short hypocotyl in both dark- and light-grown seedlings (Moreno-Romero *et al.*, 2008). Furthermore, they concluded that the partial de-etiolated

phenotypes of the transgenic seedlings are due to a defect in cell elongation as opposed to constitutive photomorphogenesis in the dark. The data presented here show that all the single, double and triple mutants grew similar to wild type in the dark (Fig. 2.4; data not shown). However, the triple mutant seedlings displayed varying degrees of seedling defects, including shorter hypocotyls and smaller cotyledons compared to wild type seedlings (Figs. 2.5, 2.14), suggesting that the triple mutant is defective in cell elongation/expansion. In addition, shorter hypocotyls and smaller cotyledons are indicative of a general defect in cell elongation/expansion as opposed to a hypersensitive response to light which oppositely regulates cell expansion in these two organs (Briggs and Spudich, 2006). This is also consistent with reduced number of lateral roots for the triple mutant compared to wild type plants as previously observed with the dominant-negative transgenic plants (Fig. 2.5) (Moreno-Romero *et al.*, 2008). Further experiments are necessary to examine whether the triple mutant is defective in auxin signaling as shown in the case of transgenic plants overexpressing a dominant negative mutant form of CK2 alpha subunit (Marquès-Bueno *et al.*, 2011).

In addition to the developmental defects described above, the CK2 double and triple mutants also displayed hyposensitive responses to ABA and salt stress in a synergistic manner (Figs. 2.6, 2.7). How CK2 modulates these responses in Arabidopsis is still unknown. In maize, a group of late embryogenesis abundant (LEA) proteins, named Rab (responsive to ABA) proteins, is highly expressed during seed maturation or induced in response to ABA or osmotic stress in vegetative tissues (Mundy and Chua, 1988). The DNA binding activity of the bZIP proteins (EmBP-2 and ZmBZ-1) that regulate expression of *Rab28* is oppositely regulated by CK2-mediated phosphorylation (Nieva *et al.*, 2005). In addition, Rab17 interacts with CK2 subunits and is phosphorylated by maize CK2 (Riera *et al.*, 2004). Overexpression of the wild type

Rab17, but not the mutant Rab17 that is deficient in CK2-mediated phosphorylation, repressed seed germination and cotyledon greening in Arabidopsis. These data suggest that CK2 modulates the functions of Rab17 by phosphorylation. Although the Arabidopsis homolog of maize Rab17 has not been characterized yet, it is possible that the Arabidopsis CK2 affects the function of the Arabidopsis Rab17 homolog to respond to ABA and NaCl stress conditions.

In summary, the data presented here and elsewhere suggest that CK2 alpha subunits are involved in affecting diverse developmental and stress responsive pathways in Arabidopsis (Fig. 2.8). At the molecular level, CK2 phosphorylates transcription factors and translation initiation factors, and regulates their functions (Hardtke *et al.*, 2000; Park *et al.*, 2008; Dennis and Browning, 2009; Dennis *et al.*, 2009; Łebska *et al.*, 2010*a*; Bu *et al.*, 2011*a*). At the morphological level, CK2 modulates diverse pathways to optimize plant growth and development (Fig. 2.8) (Riera *et al.*, 2001*b*). All organisms have a large number of CK2 substrates *in vivo* that may function in multiple pathways (Riera *et al.*, 2001*b*; Meggio and Pinna, 2003). In animal systems, more than 300 CK2 substrates have been identified so far, and more are predicted to be identified (Meggio and Pinna, 2003). Therefore, the phenotypes of the CK2 mutants will reflect the net effect of CK2 on these diverse pathways that regulate plant growth and development. Identification and characterization of these substrates may shed light on how CK2 affects plant growth and development and their response to stress conditions.

MATERIALS AND METHODS

Plant growth conditions and Phenotypic Analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under constant light at 24 °C ± 0.5°C. Monochromatic R and FR light sources and the

spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) used to measure fluence rates are as described (Shen *et al.*, 2005). Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen *et al.*, 2005). After stratification at 4ºC in the dark, seeds were exposed to 3 hours white light at room temperature to induce germination before placing them in the dark for additional 21 hours. The plates were then either placed in the dark or under specific wavelengths of light for additional 3 days. For quantitation of hypocotyl lengths and cotyledon area, a digital photograph was taken and at least 30 seedlings were measured using the publicly available software ImageJ (http://rsbweb.nih.gov/ij/). For lateral root number, seedlings were grown under white light at 22°C for nine days on a vertical plate. The number of lateral roots was counted by naked eye. The experiments were repeated at least three times.

Protein Extraction and CK2 Kinase Assays

Protein extraction and. CK2 phosphorylation assays were performed as described (Perales *et al.*, 2006; Dennis and Browning, 2009; Dennis *et al.*, 2009). Briefly, crude extracts were prepared by homogenizing 2-week-old green seedlings in buffer (40 mM HEPES, pH 7.4, 15 mM MgCl2, 1mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 0.1mM Sodium orthovanadate, 40 mM β-glycerophosphate and 10% Glycerol). The homogenate was centrifuged at 13,000 rpm for 15 min. Protein concentration was determined using the Bradford protein assay method (Bio-Rad Laboratories, Inc., Hercules, CA). 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-[^{32}P]ATP (~250 cpm/pmol), 100 mM KCl, ~1 pmol CK2 and ~10-20 pmol rWheIF4B. For the extracts from wild type and triple mutant plants, 0.5 (1x) and 1 (2x) µg of total protein

was added for each reaction. The reaction was incubated at 30ºC for 10 min and terminated by the addition of 6x SDS loading buffer. Samples were boiled 3 min and separated on 8% SDS-PAGE gel. The gels were dried and exposed to a phosphorImager.

To investigate phosphorylation of CO by CK2, Arabidopsis *CO* open reading frame (ORF) was cloned in frame with the maltose binding (MBP) tag in pMAL^{TM}-p2X vector and verified by sequencing. The vector was transformed into Arctic DE3 cell for protein expression and MBP-CO was purified using MBP-tag according to manufacturer's protocol (New England Biolabs, Ipswich, MA). His-PIF1 was expressed as described (Bu *et al.*, 2011*a*) and used as a positive control (Dennis and Browning, 2009; Dennis *et al.*, 2009; Bu *et al.*, 2011*a*). CK2 phosphorylation assays were performed as described (Dennis and Browning, 2009; Dennis *et al.*, 2009; Bu *et al.*, 2011*a*). Briefly, 20 μl kinase assay mixtures contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 0.2 mM γ-[^{32}P]ATP (~250 cpm/pmol), 100 mM KCl, ~1 pmol CK2 and ~10-20 pmol PIF1 or MBP-CO. The reaction was incubated at 30^{0}C for 30 min and terminated by the addition of 4x SDS loading buffer. Samples were boiled 3 min and separated on a 10% SDS-PAGE gel. The gels were dried and exposed to a phosphorImager.

Determination of Flowering Time under Long- and Short-Day Conditions

Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen *et al.*, 2005). After stratification at 4ºC in the dark, plates were placed either under Long Day (LD, 16h light/8h dark) or Short Day (SD, 8h light/16h dark) conditions for 7-10 days. Seedlings were transplanted to soil and grown under LD or SD conditions for evaluation of flowering time. For GA treatment, one-hundred μL of GA_3 (100 μM water-based

solution supplemented with 0.02% Tween 20) was sprayed directly on the meristem of both wild type and mutant plants twice a week until bolting. For each genotype, the number of days to flower and the total number of rosette leaves at the time of flowering was counted. For vernalization treatment, seeds were surface sterilized and plated on GM – Suc plates as described above and exposed to LD or SD for germination for 10 days. Then the seedlings were transferred to 4^0C for 40 days. After prolonged cold treatment, seedlings were transplanted on soil and then grown under LD or SD conditions at 21^0C until bolting.

Quantitative RT-PCR Assays for Floral Integrator Genes

The qRT-PCR was performed as previously described (Moon *et al.*, 2008). Briefly, samples were collected every 4 hours starting at ZT0 from 10 day-old wild type and CK2 α triple mutant seedlings grown under Short day and Long day conditions. Total RNA was extracted using Spectrum plant total RNA kit (Sigma-Aldrich Co., St. Louis, MO) and reverse transcribed using SuperScript III (Invitrogen Inc., Carlsbad, CA) as per the manufacturer's protocol. The qRT-PCR assays used the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems Inc., Foster City, CA). Real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). *PP2A* (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 ΔCT = CT(*PP2A*)-CT(specific gene). Primer sequences used for qRT-PCR are listed in supplementary table.

ABA and NaCl Response Assays

Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) or MS (GM-Suc)

supplemented with different concentrations of hormone (ABA) or NaCl. After stratification at 4ºC in the dark, seeds were placed under continuous light at 22°C. The number of germinated seeds and open, green cotyledons was recorded by observation under microscope. At least 50 seeds for each genotype were plated for each experiment.

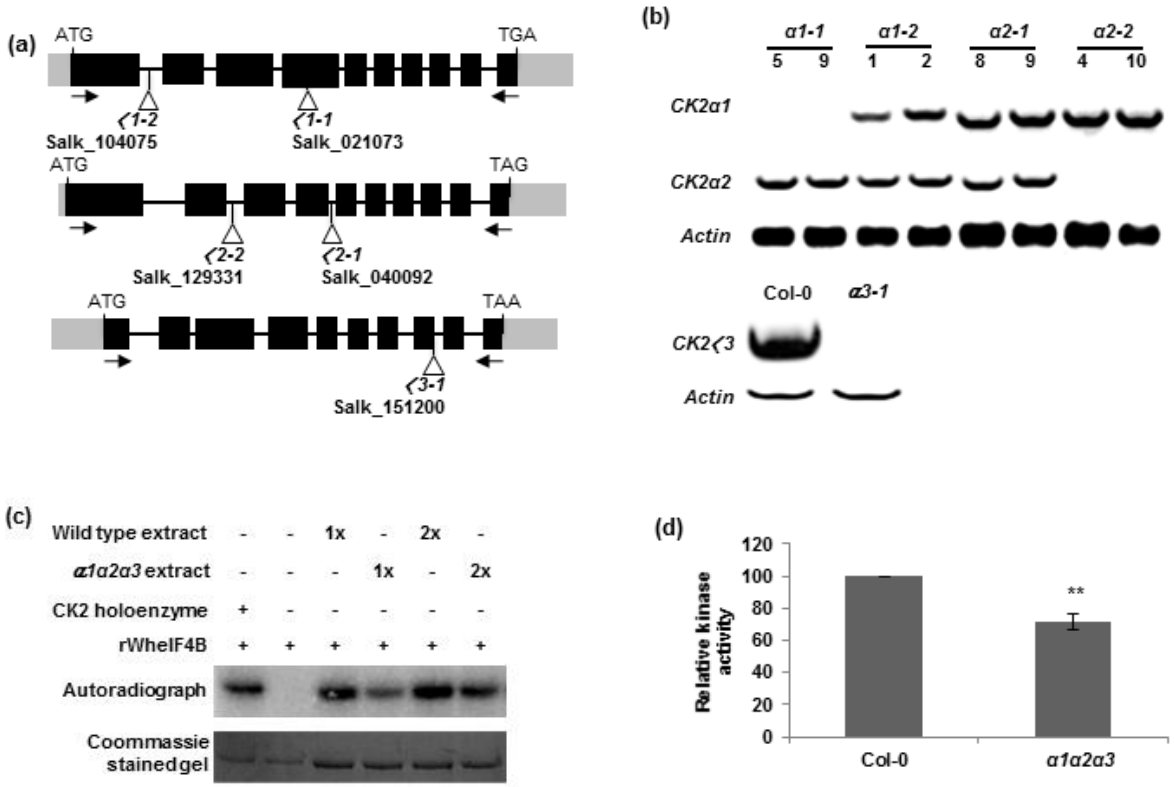

Figure 2.1: CK2 alpha subunit triple mutant displayed reduced kinase activity compared to wild type plant extracts *in vitro*. a) Isolation of T-DNA insertion mutants for α1, α2 and α3 subunit genes. Schematic representations of the exon (black rectangles) and intron (black line in between exons) structures are shown for all three α subunit genes. b) Semi-quantitative RT-PCR assays show the absence/level of transcripts expressed in various α subunit T-DNA insertion mutants. Primer positions (See supplementary table for sequences) for each gene used for PCR amplifications are indicated with forward and reverse arrows. The numbers under each genotype indicate individual plants. c) Gel photograph displaying reduced kinase activity for the *α1α2α3* triple mutant extracts compared to wild type extracts *in vitro*. Purified α1β1 holoenzyme was used as a control. 1x and 2x indicate 0.5 and 1 µg of total protein used from the wild type and mutant extracts, respectively. d) Bar graph showing the level of reduction in kinase activity of the *α1α2α3* triple mutant plants compared to wild type control plants. The experiment was repeated three times and the data are expressed as mean $\pm$ SE. **, indicates statistically significant difference with a p value <0.01. The mutant lines shown in panel a, and panel b were developed by Dr. Qingyun Bu.

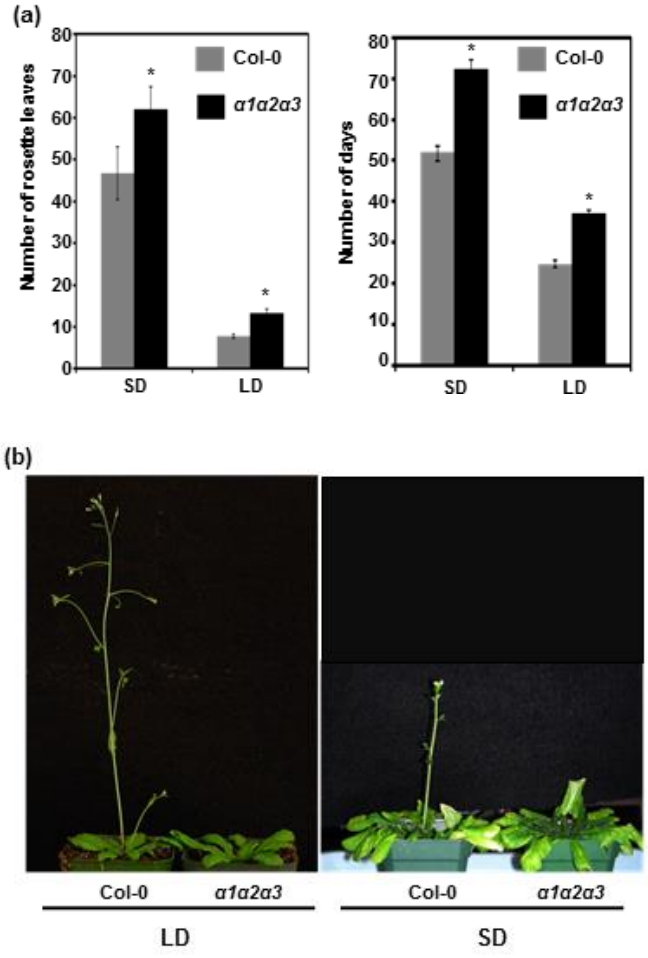

Figure 2.2: CK2 alpha subunit triple mutants displayed late flowering phenotype under both LD and SD conditions. a) Bar-graph showing average number of rosette leaves and days to flower at bolting under both LD and SD conditions. Plants were grown under LD (16h light/8h dark) and SD (8h light/16h dark) conditions. The experiment was repeated three times ($n \geq 15$) and the data are expressed as mean $\pm$ SE. *, indicates statistically significant difference with a p value <0.05. b) Photographs of wild type and *α1α2α3* triple mutant plants grown under LD and SD conditions as described above

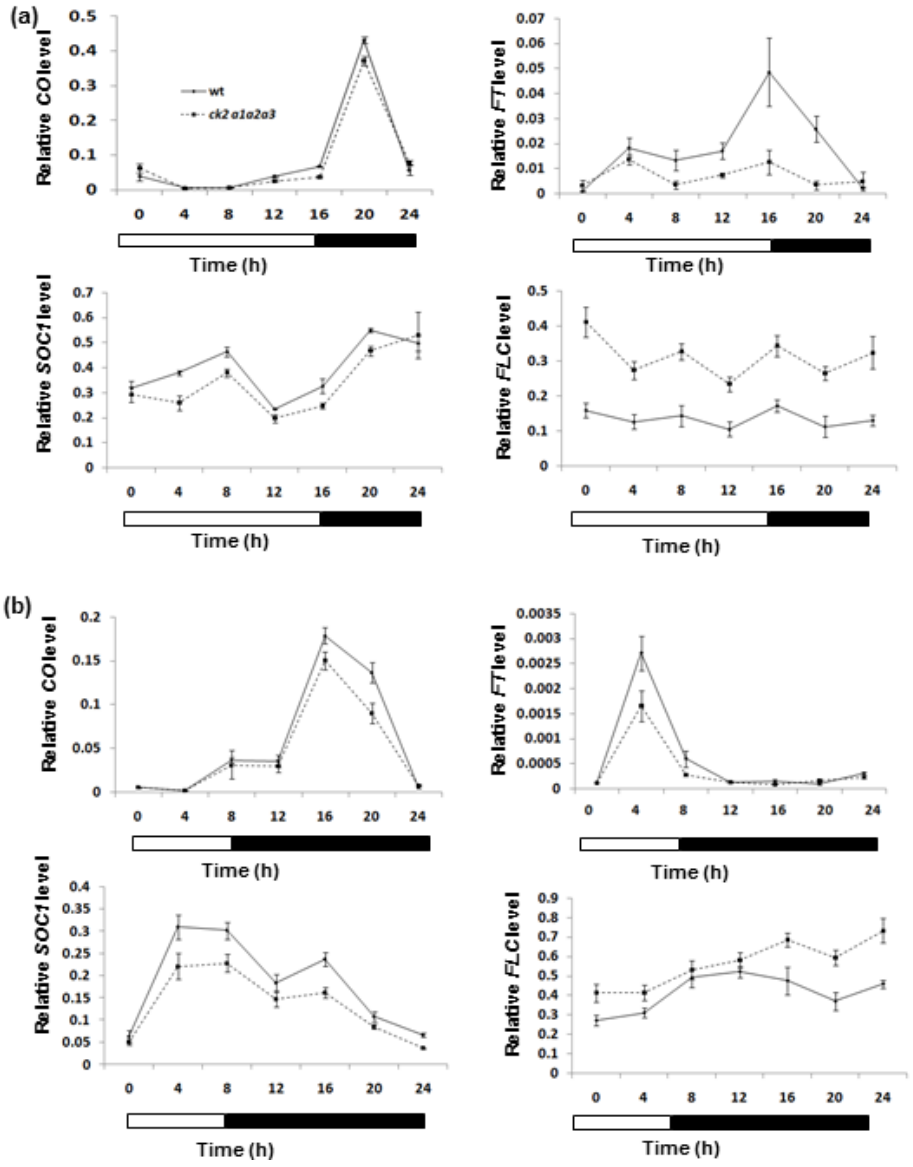

Figure 2.3: Flowering time genes are differentially regulated in CK2 alpha subunit triple mutant compared to wild type plants. Quantitative RT-PCR analyses for flowering time genes in *α1α2α3* triple mutant plants compared to wild type plants under LD (a) and SD (b) conditions. Samples were collected 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 $\pm$ St. Dev. This experiment was kindly performed in part by Dr. Fulu Chen.

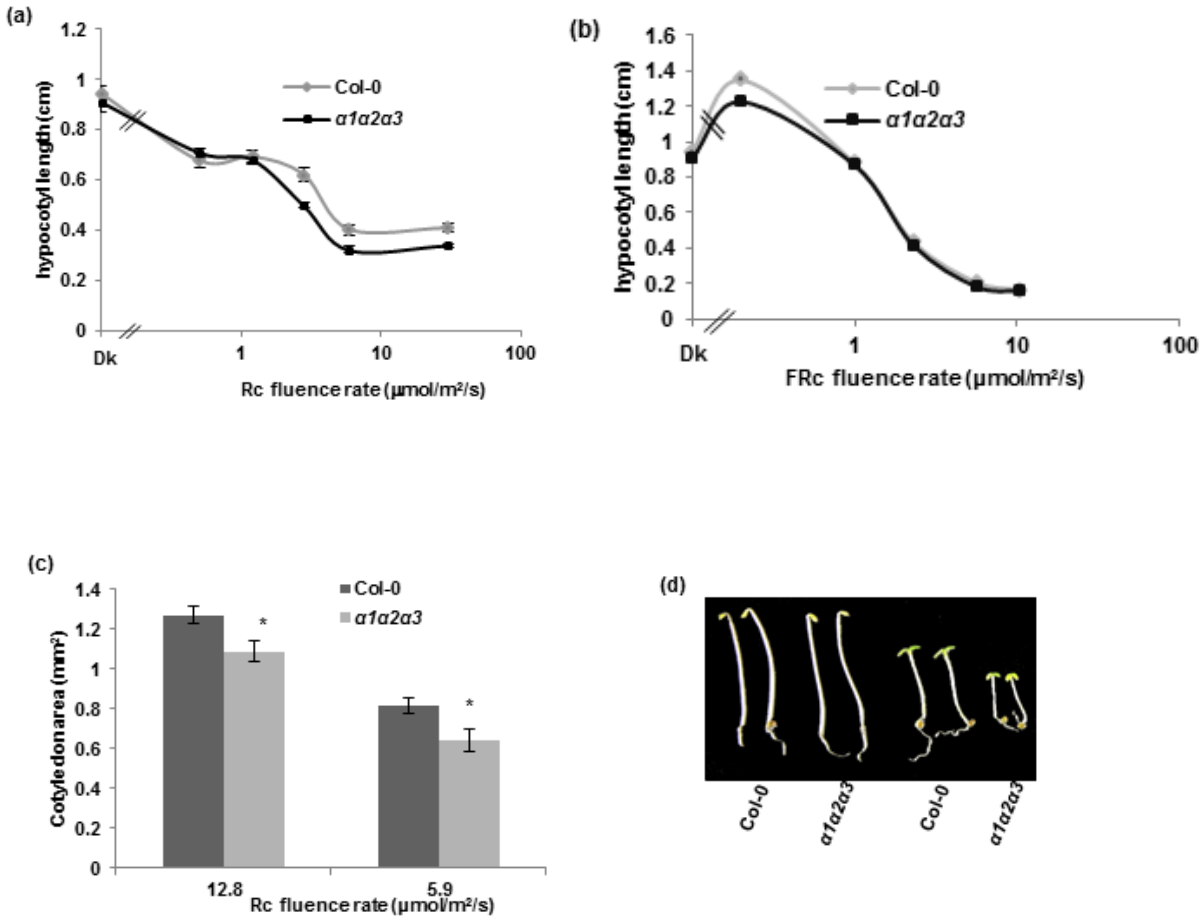

Figure 2.4: CK2 alpha subunit triple mutant displays reduced hypocotyl lengths and smaller cotyledons under red light. Hypocotyl lengths of the *α1α2α3* triple mutant and wild type seedlings grown in the dark and under red (a) or far-red (b) light conditions. Seedlings were grown either in the dark for four days or one day in the dark followed by three days under red or far-red light. The experiment was repeated three times and the data are expressed as mean ± SE. c) Cotyledon area for *α1α2α3* triple mutant and wild type seedlings grown under red light as described above. The experiment was repeated three times and the data are expressed as mean ± SE. d) Photographs of wild type and *α1α2α3* triple mutant seedlings showing shorter hypocotyls, smaller cotyledons and shorter roots for the *α1α2α3* triple mutant compared to wild type seedlings grown under red light (6 $\mu molm^{-2}s^{-1}$).

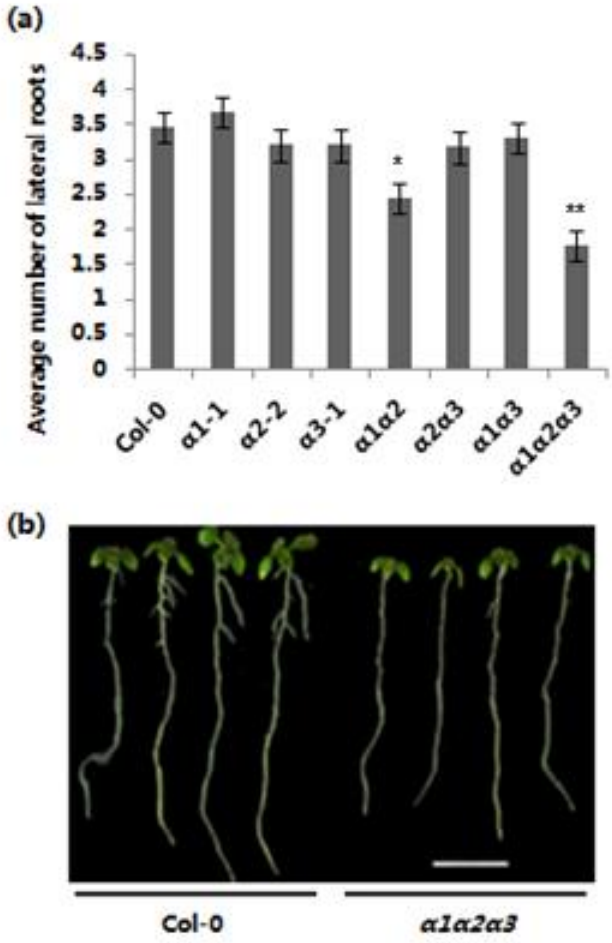

Figure 2.5: CK2 alpha subunits promote lateral root development. a) Bar-graph shows the average number of lateral roots for CK2 alpha single, double and triple mutants compared to wild type seedlings. Seedlings were grown on MS-agar plates for nine days at 22^0C under white light. The experiment was 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. b) Photographs of seedlings showing reduced number of lateral roots for the *α1α2α3* triple mutant compared to wild type seedlings.

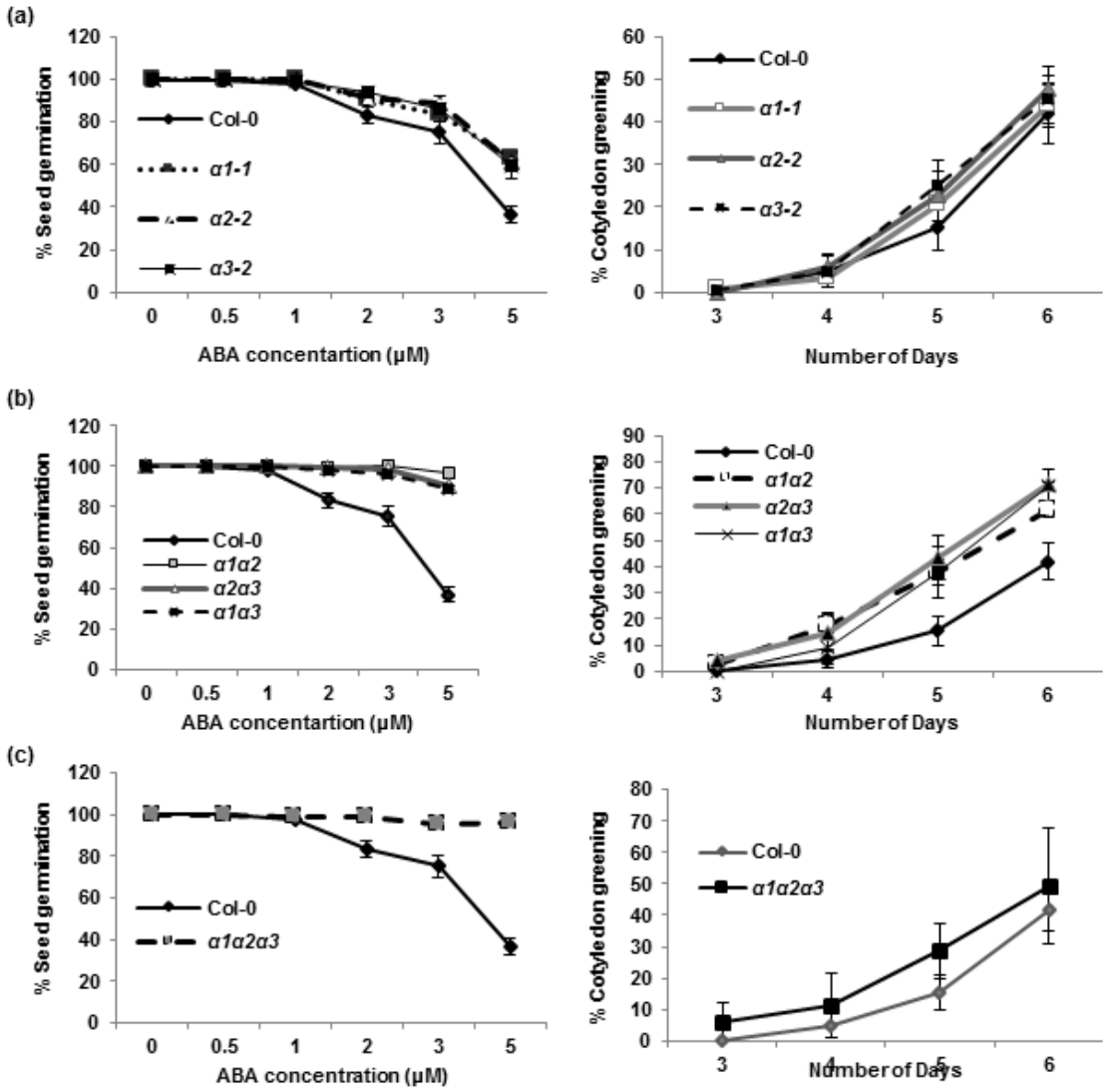

Figure 2.6: CK2 alpha subunit mutants are hyposensitive to ABA-induced block of seed germination and cotyledon greening in a synergistic manner. (Left panels) Concentration curves showing reduced seed germination for the single (a), double (b) and the triple mutant (c) compared to wild type in response to increasing concentrations of ABA. (Right panels) Cotyledon greening response for the single (a), double (b) and the triple mutant (c) compared to wild type after various days of growth in the presence of 1 µM ABA as indicated. The experiment was repeated three times ($n \geq 50$ for each genotype) and the data are expressed as mean $\pm$ SE.

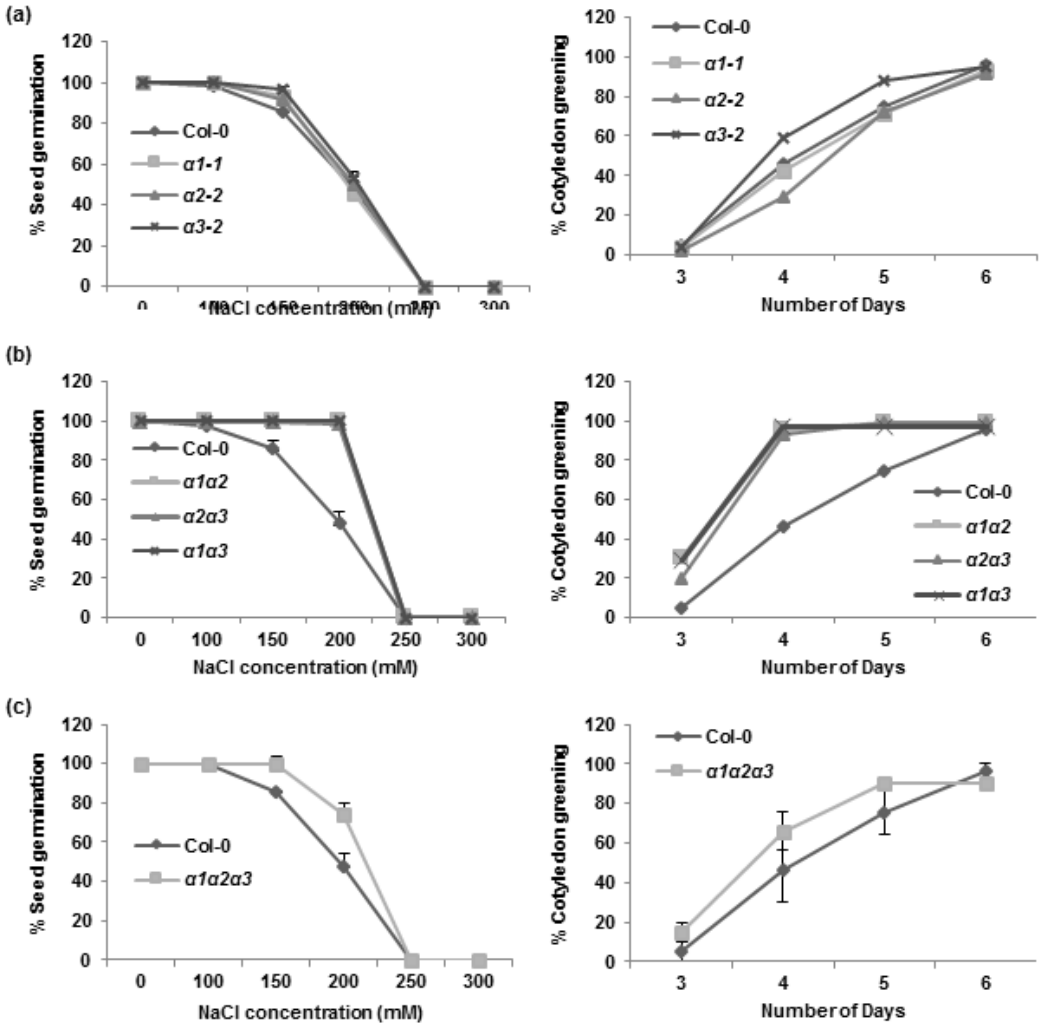

Figure 2.7: CK2 alpha subunit mutants are hyposensitive to NaCl-induced block of seed germination and cotyledon greening in a synergistic manner. (Left panels) Concentration curves showing reduced seed germination for the single (a), double (b) and the triple mutant (c) compared to wild type in response to increasing concentrations of NaCl. (Right panels) Cotyledon greening response for the single (a), double (b) and the triple mutant (c) compared to wild type after various days of growth in the presence of 150 mM NaCl as indicated. The experiment was repeated three times ($n \geq 50$ for each genotype) and the data are expressed as mean $\pm$ SE.

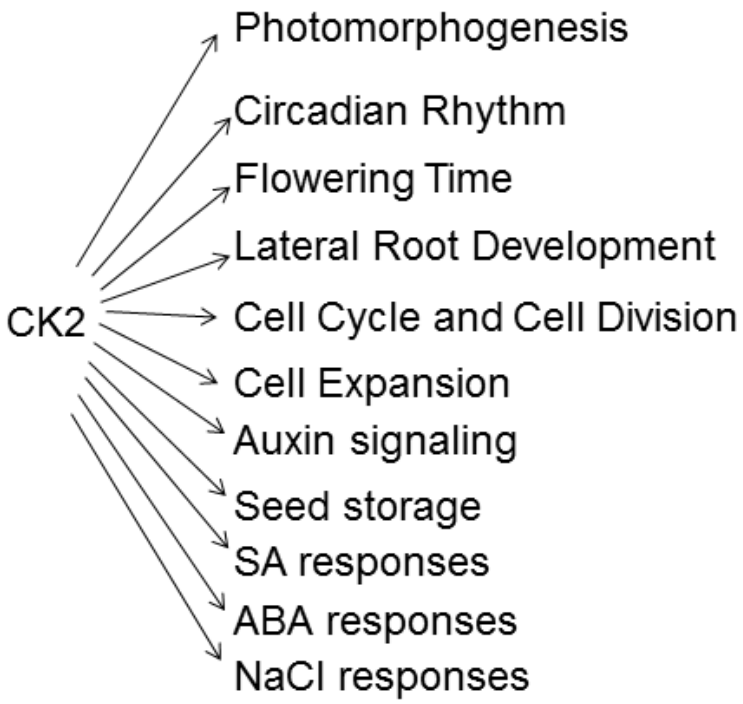

Figure 2.8: Model of CK2 functions in affecting plant growth and development. CK2 phosphorylates transcription factors, translation initiation factors and various other proteins involved in diverse pathways, and affects their stability, subcellular localization, ability to interact with other proteins and DNA, resulting in optimum growth and development and responses to stress conditions.

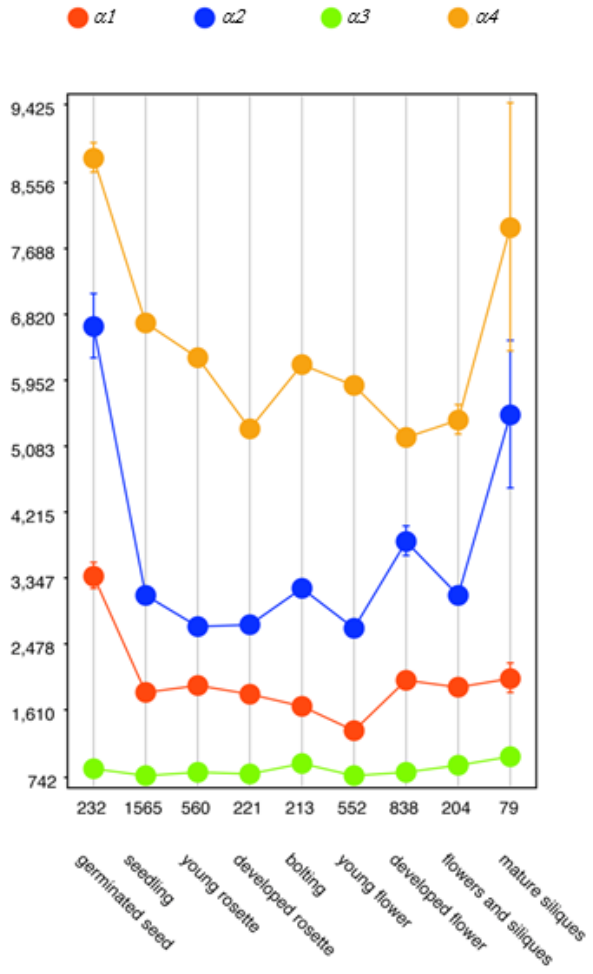

Figure 2.9: Developmental expression patterns for the four CK2 alpha subunit genes. Digital expression patterns for the four Arabidopsis CK2 alpha subunit genes α1 (At5g67380), α2 (At3g50000), α3 (At2g23080) and α4 (At2g23070) in various tissues were obtained from Genevestigator web site (Zimmerman et al., 2004).

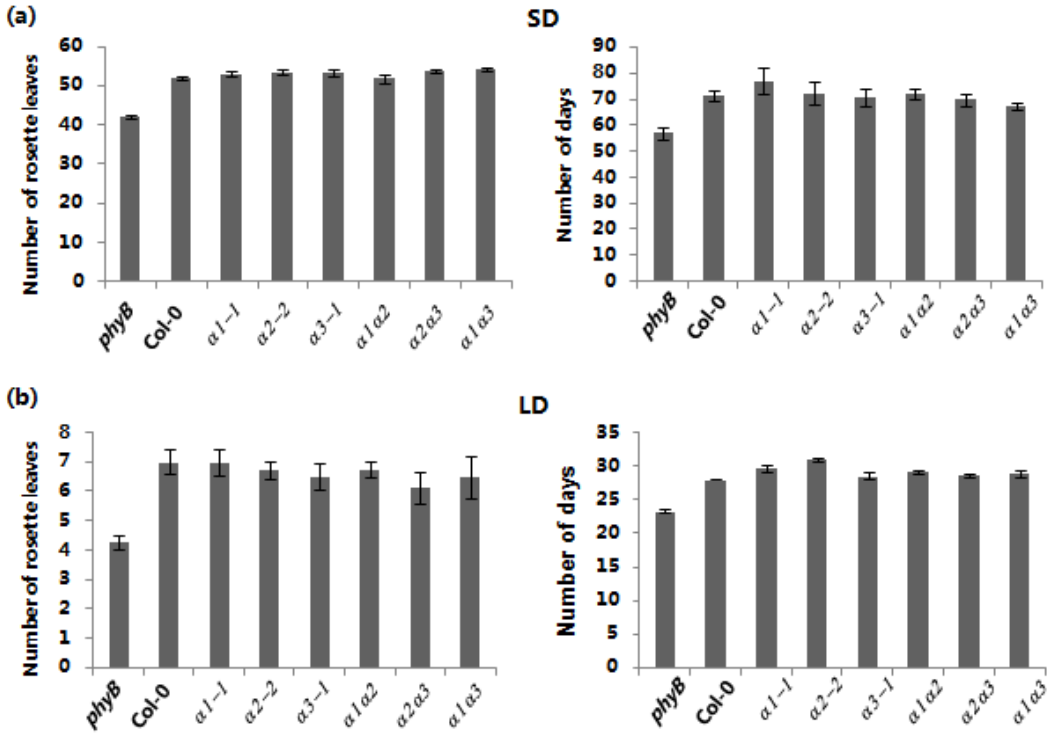

Figure 2.10: Flowering time phenotype for the CK2 alpha subunit single and double mutants under both LD and SD conditions. Bar-graphs show average number of rosette leaves (left) and days to flower (right) at bolting under both SD (a) and LD (b) conditions. Wild type, CK2 alpha subunit single and double mutant plants were grown under LD (16h light/8h dark) and SD (8h light/16h dark) conditions. *phyB* was included as a control.

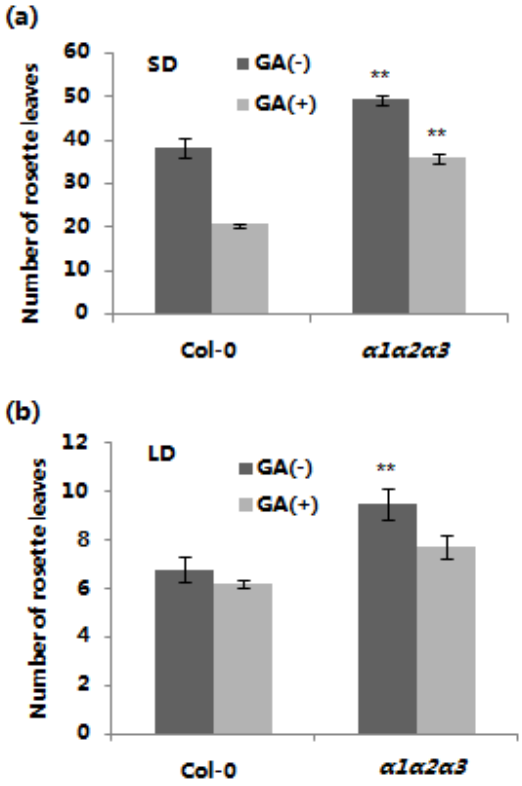

Figure 2.11: Flowering time phenotype for the wild type and CK2 *a1a2a3* triple mutant in the absence or presence of exogenous GA under both LD and SD conditions. Bar-graphs show average number of rosette leaves at bolting under both SD (a) and LD (b) conditions. Plants were grown under LD (16h light/8h dark) and SD (8h light/16h dark) conditions. **, indicates statistically significant difference with a p value <0.01.

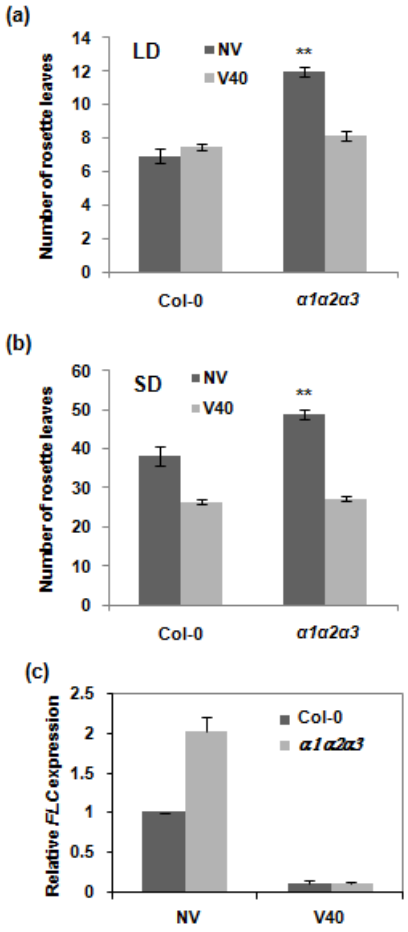

Figure 2.12: Flowering time phenotype for the wild type and CK2 *a1a2a3* triple mutant in response to vernalization treatment under LD and SD conditions. Bar-graphs show average number of rosette leaves at bolting under LD (a) and SD (b) conditions. Plants were vernalized for 40 days at 4^0C and then grown under LD (16h light/8h dark) and SD (8h light/16h dark) conditions at 21^0C until bolting. **, indicates statistically significant difference with a p value <0.01. (c) Expression of *FLC* is reduced to wild type level in the CK2 *a1a2a3* triple mutant after 40 days of vernalization. Plants were vernalized for 40 days at 4^0C and then grown under SD (8h light/16h dark) conditions at 21^0C until harvested for RNA extraction. NV, non-vernalized; V40, 40 days of vernalization at 4^0C.

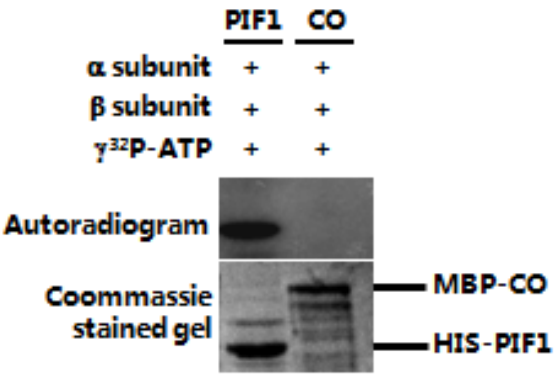

Figure 2.13: Arabidopsis CK2 does not phosphorylate Arabidopsis CO *in vitro*. Autoradiogram showing that MBP-CO was not phosphorylated by recombinant CK2 holoenzyme *in vitro*, while the positive control His-PIF1 is strongly phosphorylated by Arabidopsis CK2 under identical conditions. CK2 phosphorylation assays were performed in 20 µl kinase assay mixtures that contained 50 mM Hepes-KOH, pH 7.6, 5 mM $MgCl_2$, 2.4 mM DTT, 100 mM KCl, 0.2 mM γ-[^{32}P]ATP (~250 cpm/pmol), ~1 pmol CK2 a or ab holoenzyme and ~10-20 pmol MBP-CO or His-PIF1. The reaction was incubated at 30^{o}C for 30 min and terminated by the addition of 4x SDS loading buffer. Samples were boiled 3 min and separated on a 10% SDS-PAGE gel. The gels were dried and exposed to a phosphorImager. This experiment was kindly performed by Dr. Qingyun Bu.

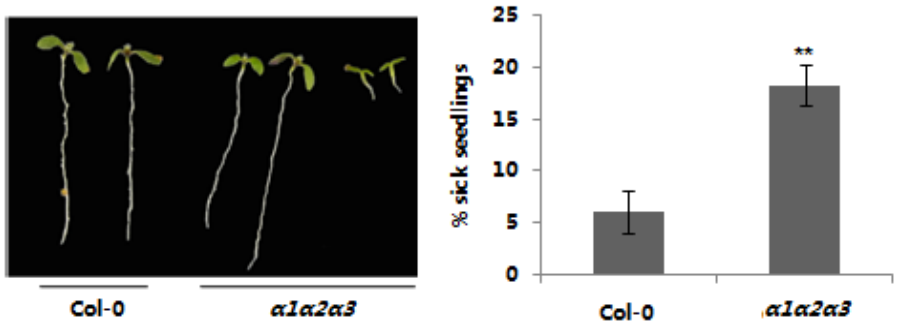

Figure 2.14: The CK2 *a1a2a3* triple mutant displayed developmental defects at the seedlings stage when grown in light. (Left) Photographs of wild type and *a1a2a3* triple mutant seedlings grown under white light for 5 days. The photograph shows two representative seedlings from healthy wild type Col-0, healthy *a1a2a3* triple mutant and sick *a1a2a3* triple mutant. (Right) Bar-graphs show average percentage of sick seedlings for wild type and *a1a2a3* triple mutants. **, indicates statistically significant difference with a p value <0.01.

Table 2.1 Primer sequences used in experiments described in Chapter 2

Gene	Forward	Reverse
For genotyping		
α1	CTCGGGTGAAACTAAGTGCTG	GTGAACAGCAAGGAGAAGTGC
α2	TGGCGCATTCTCTTATACCAC	CATGACATTGTGTGGCTTGAC
α3	GCAAGCCTAAGAGGGTCATTC	TCTCTGCGGCTTTAACTTGAG
For RT-PCR		
α1	caccATGTCGAAAGCTCGTGTGTAC	TTGACTTCTCATTCTGCTGGT
α2	caccATGTCGAAAGCTCGTGTTTATAC	TTGAGTCCTCATTCTGCTGCT
α3	caccATGTCGAAAGCTAGGGTTTATAC	CTGAGTTCGTAGTCTGCTGCTC
For qRT-PCR		
CO	AACTGCAGCGTACCACAGAC	GGATGAAATGTATGCGTTATGG
FT	GGAACAACCTTTGGCAATGAGAT	CTGCCAAGCTGTCGAAACAA
FLC	GCCAAGAAGACCGAACTCATGTTGA	CAACCGCCGATTTAAGGTGGCTA
SOC1	TGGGAGAAGGCATAGGAACATGC	CGCTTTCATGAGATCCCCACTTTTC
PP2A	TATCGGATGACGATTCTTCGTGCAG	GCTTGGTCGACTATCGGAATGAGAG

Chapter 3: Arabidopsis Casein Kinase 2 α4 subunit regulates various developmental pathways in a functionally overlapping manner[3]

ABSTRACT

Casein kinase 2 (CK2) is an essential and well-conserved Ser/Thr kinase that regulates proteins in a post-translational 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 alpha subunits in Arabidopsis act in a functionally redundant manner to regulate various developmental pathways. In this study we constructed two independent CK2 α4 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 responses, lateral root formation and flowering time. CK2 α4-GFP localizes to the chloroplast in transgenic Arabidopsis seedlings, consistent with the presence of a chloroplast localization signal at the amino-terminus of CK2 α4 subunit. Taken together, our results suggest a functionally overlapping role for the CK2 α4 subunit in regulating various developmental processes in plants.

INTRODUCTION

The group of enzymes termed protein kinases comprises an essential set of enzymes for all living organisms. Kinases add one/multiple 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

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reversibility of this post-translational 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 (Litchfield, 2003). Although, CK2 is primarily a Ser/Thr kinase, it has been shown to phosphorylate Tyrosine residues in a few cases (Vilk *et al.*, 2008; Basnet *et al.*, 2014). 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 the phosphate source (Pinna, 2003). 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 (Trembley *et al.*, 2010).

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) (Salinas *et al.*, 2001, 2006; Riera *et al.*, 2001*b*; Kato *et al.*, 2002; Ogrzewalla *et al.*, 2002; Ogiso *et al.*, 2010). Compared to over 300 targets in animal system, there are fewer known CK2 substrates in plants; however, the list is steadily growing (Litchfield, 2003; Mulekar and Huq, 2014). 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 α4 are nuclear localized. CK2 α4 subunit has an N-terminal chloroplast localization signal; however, it also contains the internal conserved nuclear localization signal (NLS) (Salinas *et al.*, 2006). This subunit also has the highest transcript level spatially and developmentally amongst the four alpha subunits (Mulekar *et al.*, 2012). The regulatory beta subunits are found both in the nucleus and cytoplasm. Both the subunits exists in a complex and as well as monomers in the cell (Salinas *et al.*, 2006).

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, cell expansion and root and shoot growth. However, prolonged induction of defective alpha subunit was lethal, supporting the essential nature of the enzyme (Moreno-Romero *et al.*, 2008). 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 (Lu *et al.*, 2011; Mulekar *et al.*, 2012). CK2 alpha subunits positively regulate responses to the hormone ABA, salt (NaCl) stress and lateral root development in an overlapping manner. They also redundantly regulate flowering time under both short- and long-day conditions (Mulekar *et al.*, 2012; Mulekar and Huq, 2012).

A recent study reported the characterization of T-DNA insertion knock-out lines of the chloroplast localized CK2 α4 subunit. The CK2 α4 single knock-out mutant was found to be hyposensitive to ABA and salt stress similar to the CK2 alpha triple mutant. The CK2 α4 knock-out mutant was also hypersensitive to heat stress and showed reduced expression of genes involved in retrograde signaling (Wang *et al.*, 2014). The 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 (Lu *et al.*, 2014).

In this study we characterized the function of the α4 subunit of CK2 in Arabidopsis thaliana using an 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 the CK2 α4 gene in both the wild type (Col-0) and CK2 alpha triple (*α123*) mutant background to better understand the function of α4 subunit.

RESULTS

Development of CK2α4 RNAi lines in wild type and alpha triple mutant background

In *Arabidopsis thaliana*, four genes encode for CK2 alpha subunits. Three of those are nuclear localized while the fourth subunit contains an N-terminal chloroplast localization signal (Salinas *et al.*, 2006). 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 (Mulekar *et al.*, 2012). Thus, to get an insight into the function of the fourth, chloroplast localized alpha subunit of CK2, we requested the CK2 α4 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 CK2α4 gene expression in both the wild type and CK2 alpha triple mutant background.

All four CK2 alpha subunit genes in Arabidopsis show a high level of conservation in the nucleotide sequence. Hence, we chose a 200 bp nucleotide sequence in the 3' UTR of the CK2α4 gene (AT2G23070) downstream of the stop codon. This nucleotide sequence is specific to the CK2α4 gene and is not found in the 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 backgrounds. However, based on the reduction in transcript as well as enzyme activity we chose two lines in each background for further characterization. All four selected lines show significant reduction in the CK2 α4 transcript (Fig. 3.1 A, B). We also checked the expression of the rest of the alpha subunit genes in the RNAi lines in wild type background (Fig. 3.1A). We found no significant difference in their expression confirming that the down regulation in the transcript was specific to CK2α4.

CK2 alpha subunit is the catalytic subunit of the CK2 holoenzyme. Hence, we examined the relative enzyme activity in the total plant extracts from two-week old seedlings of the RNAi lines using recombinant wheat eIF4B as a substrate (Fig. 3.1D). Wheat eIF4B is a verified CK2 substrate which is robustly phosphorylated by wild type Arabidopsis CK2 alpha subunit, and this phosphorylation is not affected by the presence of the CK2 beta subunit (Dennis and Browning, 2009). 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% (Fig. 3.1C). 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 a CK2 knock out in yeast is not viable (Padmanabha *et al.*, 1990). 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 may 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 or 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.

CK2α4 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 delayed flowering under long days (Takahashi *et al.*, 2001). In Arabidopsis, we found that the three nuclear CK2 alpha subunits redundantly promoted flowering under both short and long day conditions (Mulekar *et al.*, 2012). Only the CK2 alpha triple mutant plants flower later than the wild type while the alpha single and double mutant combinations flowered similarly 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 (Mulekar *et al.*, 2012; Mulekar and Huq, 2012). For the CK2α4 RNAi lines, we found that the RNAi lines in the wild type background flowered similarly to wild type under both SD and LD conditions (data not shown). However, the RNAi lines in the alpha triple mutant background (*α123-α4i*) showed delayed flowering under both SD and LD with respect to the alpha triple mutant. 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 (Fig. 3.2 A, B). Similar results were obtained under short day conditions, the two independent RNAi lines (*α123-α4i)* 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 (Fig. 3.3 A, B). Overall, these data suggest that CK2α4 regulates flowering time in a functionally overlapping manner with other alpha subunits.

CK2α4 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* (Imaizumi and Kay, 2006; Kim *et al.*, 2009). 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 (Mulekar *et al.*, 2012). 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 *α123-α4i* line than triple mutant (Fig 3.2 C, E). Both *SOC1* and *FT* have slightly reduced levels than the triple mutant (Fig. 3.2 D, F). Under SD conditions, the RNAi line shows a similar trend. The RNAi line shows a slight increase in the *FLC* expression and a 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 (Fig. 3.3 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.

CK2α4 promotes lateral root development

In mammals, the role of CK2 in regulating cell cycle and cell division is well documented (Trembley *et al.*, 2010). 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 (Moreno-Romero and Martínez, 2008;

Moreno-Romero *et al.*, 2008). The CK2 alpha triple mutant also shows significantly fewer lateral roots than the wild type (Mulekar *et al.*, 2012). To examine whether CK2α4 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 fewer lateral roots than the triple mutant (Fig. 3.4 A, B). This suggests that the CK2 α4 subunit also contributes to the lateral root development in a functionally overlapping manner.

CK2α4 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 (Riera *et al.*, 2004). In Arabidopsis, the nuclear CK2 alpha subunits contribute synergistically to the block of seed germination in response to the hormone ABA or salt (NaCl) in a concentration dependent manner (Mulekar *et al.*, 2012). Also, a recently published study shows that both CK2 *α4* single knock-out and CK2 *α3α4* double knock-out mutants are hyposensitive to the ABA-induced block of seed germination (Wang *et al.*, 2014). We analyzed the seed germination response to ABA in the CK2 α4 RNAi lines. We found 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 (Fig 3.5). 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).

CK2 α4 localizes primarily to chloroplasts in leaf tissue

Previous research indicates that CK2 α4 subunit contains a conserved N-terminal chloroplast targeting signal and has been shown to localize to chloroplasts in transient assays in tobacco leaves (Salinas *et al.*, 2006). To examine subcellular localization in transgenic plants *in vivo*, we constructed GFP tagged CK2 α4 over expression lines in the wild type background. We observed leaves and root samples from two-week-old seedlings under a fluorescent microscope. Results show that most of the CK2 α4-GFP is localized to the chloroplasts of the guard cells (Fig. 3.6). We examined extensively in root and leaf cells for nuclear staining; however, no consistent GFP fluorescence was observed in the nucleus of any cell types.

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 (Litchfield, 2003; Pinna, 2003). Similar to mammals, in plants, CK2 has been implicated in various developmental pathways supported by a small yet steadily growing list of substrates (Mulekar and Huq, 2014).

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-terminal chloroplast localization sequence and this subunit is shown to be chloroplast localized in transient assays in tobacco leaves (Salinas *et al.*, 2006). 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 the complete absence of any nuclear alpha subunits, the CK2 alpha triple mutant plants did not show any extreme developmental abnormalities under normal growth conditions (Mulekar *et al.*, 2012). Among all the CK2 alpha subunits, α4 is expressed at the highest level throughout Arabidopsis development (Zimmermann *et al.*, 2004). Thus, in the absence of a null mutant of CK2 α4 subunit, we used an RNAi approach to investigate the role of CK2α4 in the development of Arabidopsis. Analyses of transcript levels of all 4 subunits indicated that the CK2α4 subunit was specifically down regulated in the RNAi lines tested (Figs. 3.1A, B). Enzymatic assays also showed significant down regulation of CK2 activity in multiple independent transgenic lines (Fig. 3.1C, D), establishing that these lines can be used to analyze the function of the CK2α4 subunit. Using these lines, the phenotypic characterization and molecular evidence presented here suggest that CK2α4 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 (Mulekar *et al.*, 2012). This is consistent with the lack of any phenotype in the CK2α4 RNAi lines in the wild type background (data not shown). However, in the CK2α4 RNAi lines in the triple mutant background, flowering time was modestly but significantly delayed under both long and short day conditions (Figs. 3.2A, B, 3.3A, B). The gene expression analysis 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. 3.2E, F, 3.3E, F). Thus, it appears that

like the other three alpha subunits, CK2 α4 subunit also redundantly controls flowering in Arabidopsis possibly through the autonomous pathway as previously hypothesized (Mulekar *et al.*, 2012; Mulekar and Huq, 2012).

It is well known that CK2 controls cell cycle and cell expansion in mammals (Trembley *et al.*, 2010). The CK2 dominant negative mutant shows a strong defect in cell cycle as reflected in a significantly fewer lateral roots (Moreno-Romero *et al.*, 2008). CK2 is also speculated to affect auxin signaling which may also result in defects in the lateral root formation (Marquès-Bueno *et al.*, 2011; Armengot *et al.*, 2014). Consistent with these data, our results also show that the CK2 α4 subunit plays a functionally overlapping role in the pathway controlling development of lateral roots in Arabidopsis (Fig. 3.4).

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 (Fig. 3.5). This is consistent with the results published in a recent paper where they found that CK2 α4 single knock-out and *a3a4* knock out mutants are also hyposensitive to ABA treatment (Wang *et al.*, 2014). In our previous study we found that the nuclear CK2 alpha subunits control seed germination in the presence of ABA in a synergistic manner. All three double knock out combinations showed more enhanced hyposensitivity over any individual alpha subunit mutant (Mulekar *et al.*, 2012). The exact mechanism by which CK2 modulates this response is as yet unknown. However, it has been shown that the CK2 *α4* and *α3α4* double knock out plants contain significantly reduced endogenous ABA levels compared to the wild type (Col-0) plants (Wang *et al.*, 2014). Also, ABA treatment induces CK2 α4 expression and the *α4* and *α3α4* double knock outs show reduction in ABA responsive genes with respect to the wild type (Wang *et al.*, 2014). A

recent paper showed that CK2 phosphorylates maize OST1, which increases its affinity towards PP2C and triggers its degradation which modulates ABA responses such as stomata opening (Vilela *et al.*, 2015). 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 α4 RNAi lines in the CK2 alpha triple mutant background provides us important insights into the function of CK2 α4 in the development of Arabidopsis, it also presents a quandary about how a chloroplast localized enzyme regulates such diverse biological processes possibly controlled by nuclear genes. Although CK2α4 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 (Salinas *et al.*, 2006). In addition, there are other examples of proteins that are localized and function in both the nucleus as well as the chloroplast in Arabidopsis e.g. HEMERA (Chen *et al.*, 2010). To test this possibility, we made transgenic plants overexpressing CK2 α4 fused to GFP and expressed this 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 (Fig. 3.6). Thus, on the one hand, these data support a hypothesis in which chloroplast localized CK2 α4 subunit regulates these diverse biological process by controlling a retrograde signaling pathway as to regulate plant growth and development (Nott *et al.*, 2006; Koussevitzky *et al.*, 2007; Estavillo *et al.*, 2011; Chi *et al.*, 2013). Alternatively, it is still possible that a small undetectable amount of CK2 α4 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 α4 subunit.

MATERIALS AND METHODS

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 ± 0.5°C. Seeds were surface sterilized and plated on Murashige–Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen *et al.*, 2005). After stratification at 4°C in the dark, seeds were transferred to constant white light chamber at 22 ± 0.5°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.

Construction of CK2 α4 RNAi and CK2 α4-GFP over expression transgenic lines

A 200 bp region starting from the stop codon in the 3' UTR of Arabidopsis CK2 α4 gene (TAIR: AT2G23070) was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector pB7GWIWG2(II),0 (Karimi *et al.*, 2005). The recombinant construct was introduced into Wild type (Col-0) and CK2 alpha triple mutant plants via Agrobacterium mediated transformation (Clough and Bent, 1998). The reduction in the transcript level of CK2 α4 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 α4 gene was cloned into pENTR vector (Invitrogen Inc., Carlsbad, CA) and subsequently recombined into GATEWAY cloning vector called pB7FWG2 (Karimi *et al.*, 2005). The recombinant vector was introduced into wild type Col-0 plants via Agrobacterium mediated transformation as described (Clough and Bent, 1998).

Protein extraction and CK2 kinase assays

Protein extraction and CK2 kinase assays were performed as described (Perales *et al.*, 2006; Dennis and Browning, 2009; Dennis *et al.*, 2009). Briefly, crude extracts were prepared by homogenizing 2-week-old green seedlings in buffer [40 mM HEPES, pH 7.4, 15 mM MgCl2, 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 MgCl2, 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.

Quantitative RT-PCR assays for floral integrator genes

Quantitative RT-PCR was performed as previously described (Moon *et al.*, 2008). Briefly, samples were collected every 4 h starting at ZT0 from 12-day-old wild-type, CK2 alpha 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: 2ΔCt where ΔCt = Ct(PP2A))Ct (specific gene). Primer sequences used for qRT-PCR are listed in Table 1.

Abscisic 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.

Light fluorescence microscopy

Arabidopsis transgenic seedlings expressing CK2 α4-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.

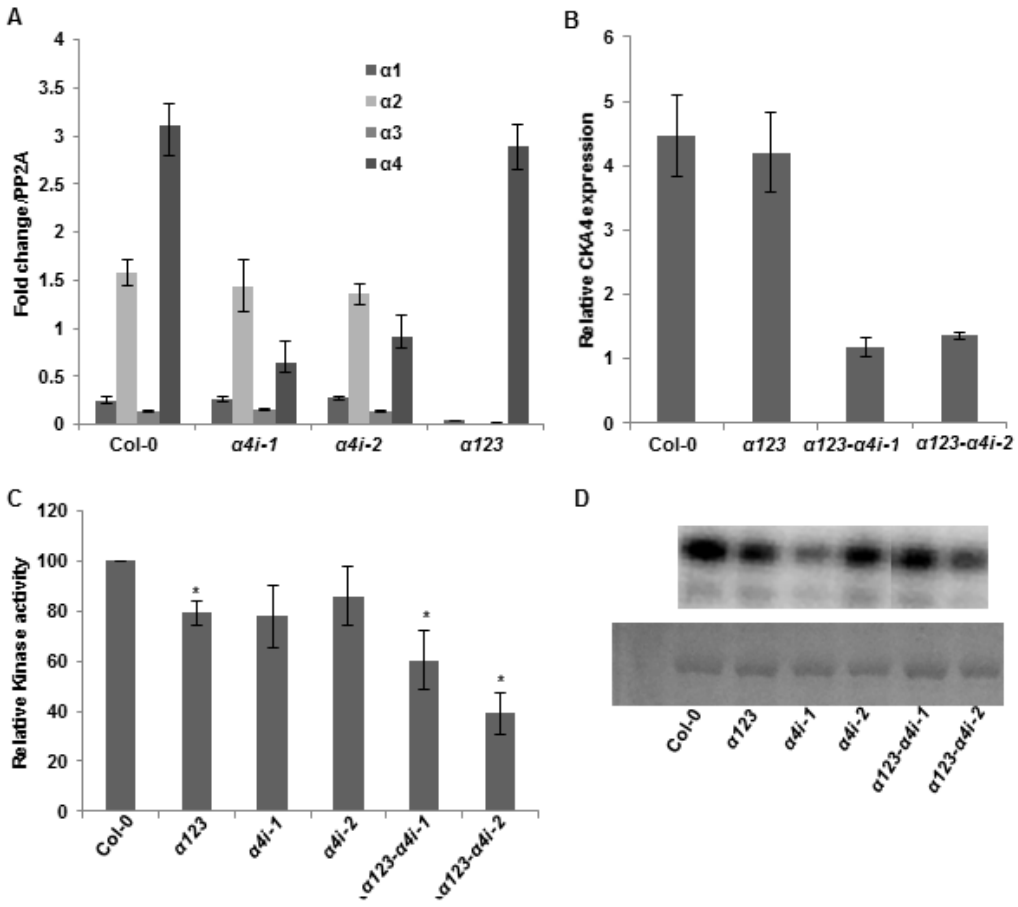

Figure 3.1: CK2α4 RNAi lines display reduced kinase activity compared to wild type plant extracts *in vitro*. A) Quantitative RT-PCR showing the transcript level of all four CK2 alpha subunit genes in Col-0, *α123* and two CK2α4 RNAi lines in the wild type background. *PP2A* was used as a reference gene B) Quantitative RT-PCR showing the transcript level of all four CK2 alpha subunit genes in Col-0, *α123* and two CK2α4 RNAi lines in the *α123* background. *PP2A* was used as a reference gene C) Bar graph showing the level of reduction in kinase activity of the CK2α4 RNAi lines compared to wild type and *α123* 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 α4 RNAi lines. D) Gel photograph displaying reduced kinase activity for CK2α4 RNAi lines compared to wild type and *α123* triple mutant plant extracts *in vitro*.

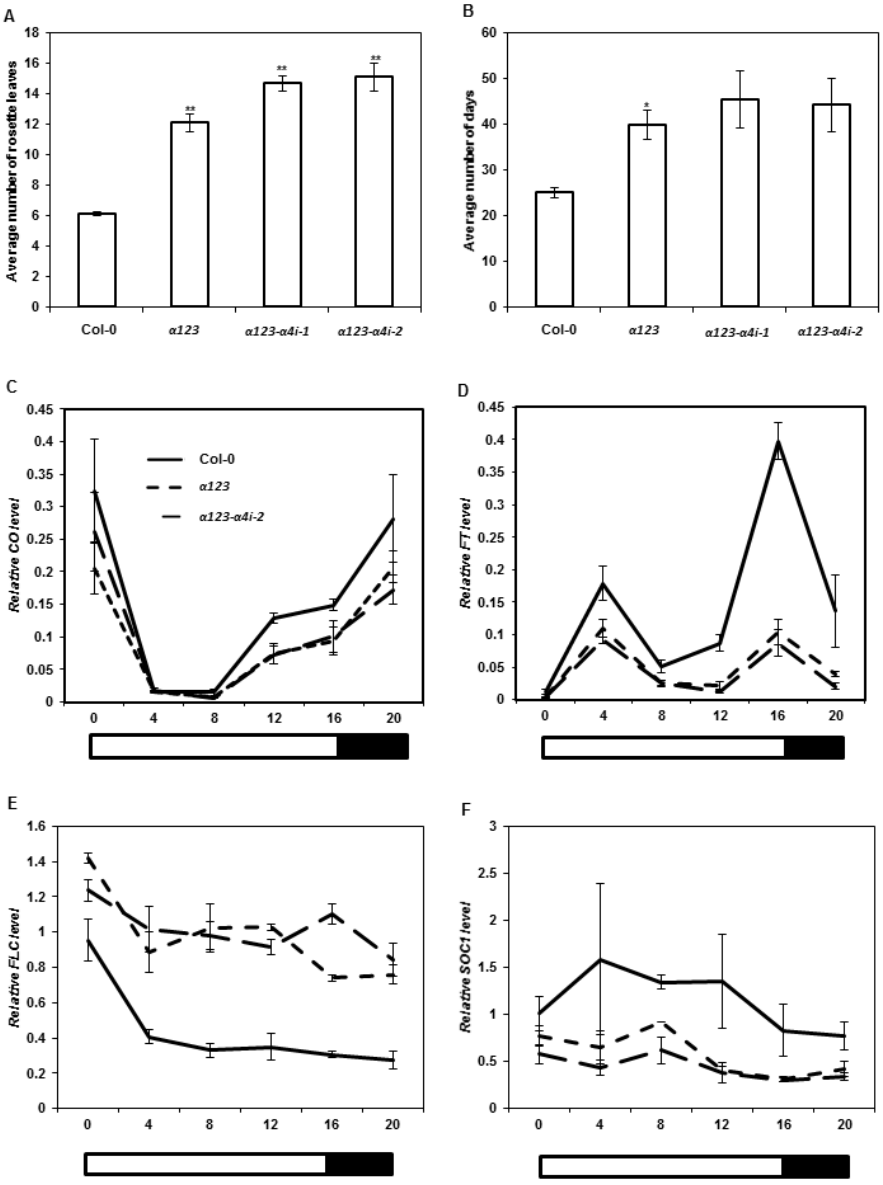

Figure 3.2: CK2 α4 RNAi lines display late flowering phenotype under long day (LD) conditions. Bar-graph showing average number of rosette leaves (A) and days to flower at bolting (B) under LD conditions. Plants were grown under LD (16h light/8h dark) conditions. The experiment was repeated three times ($n \geq 15$) and the data are expressed as mean ± 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 α4 RNAi lines. Quantitative RT-PCR analyses for flowering time genes *CO* (C), *FT* (D), *FLC* (E) and *SOC1* (F) in CK2α4 RNAi lines in *α123* mutant background compared to *α123* 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 ± SE.

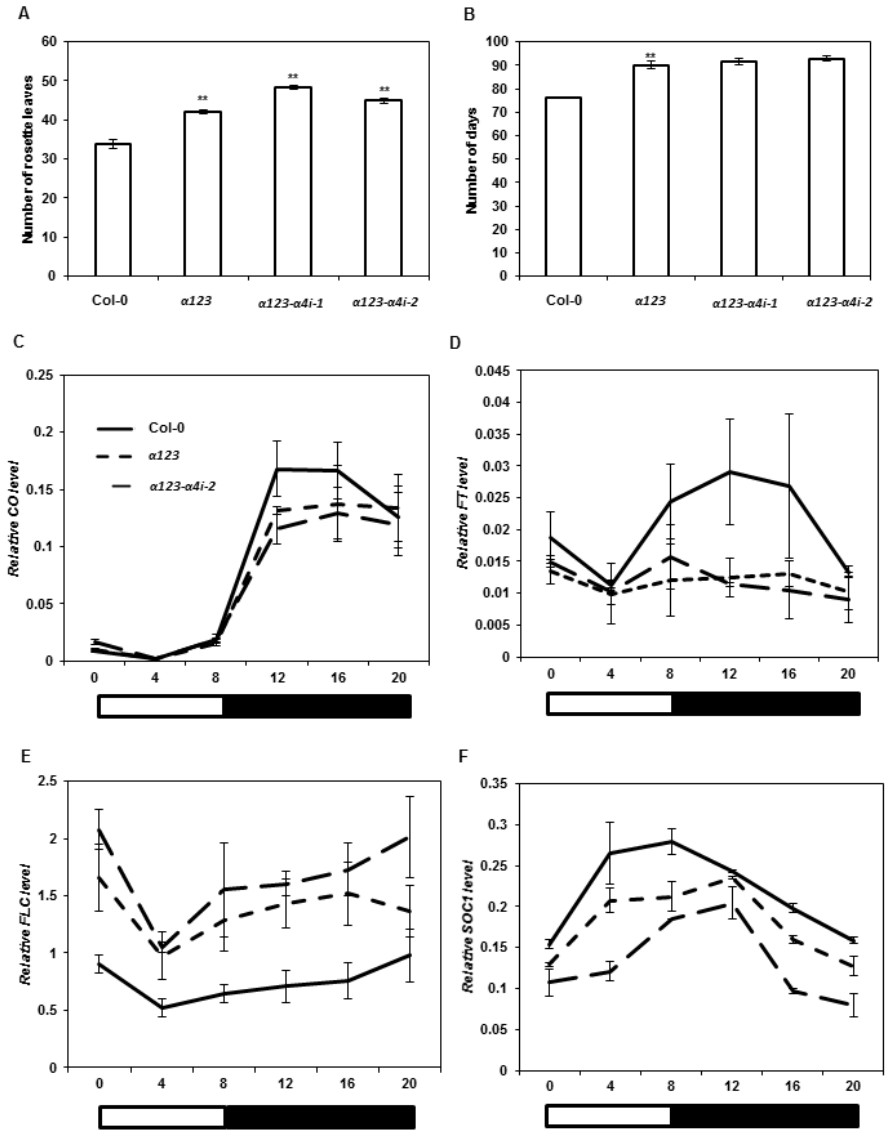

Figure 3.3: CK2 α4 RNAi lines display late flowering phenotype under short day (SD) conditions. Bar-graph showing average number of rosette leaves (A) and days to flower at bolting (B) under SD conditions. Plants were grown under SD (8h light/16h dark) conditions. The data are expressed as mean ± SE. * 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 α4 RNAi lines. Quantitative RT-PCR analyses for flowering time genes *CO* (C), *FT* (D), *FLC* (E) and *SOC1* (F) in CK2α4 RNAi lines in *α123* mutant background compared to *α123* 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 ± SE.

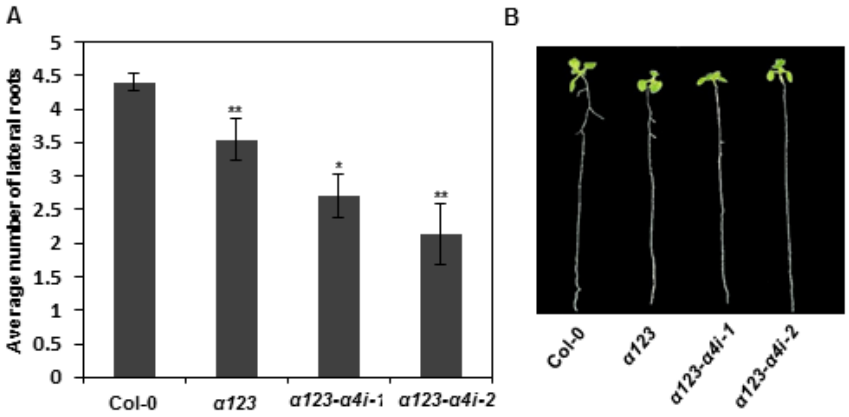

Figure 3.4: CK2 α4 subunit promotes lateral root development. A) Bar-graph shows the average number of lateral roots for Col-0, *α123* and CK2 α4 RNAi lines in *α123* mutant background. Seedlings were grown on MS-agar plates for nine days at 22^0C under white light. The experiment was repeated three 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 α4 RNAi lines. B) Photographs of seedlings showing reduced number of lateral roots for the Col-0, *α123* and CK2 α4 RNAi lines in *α123* mutant background.

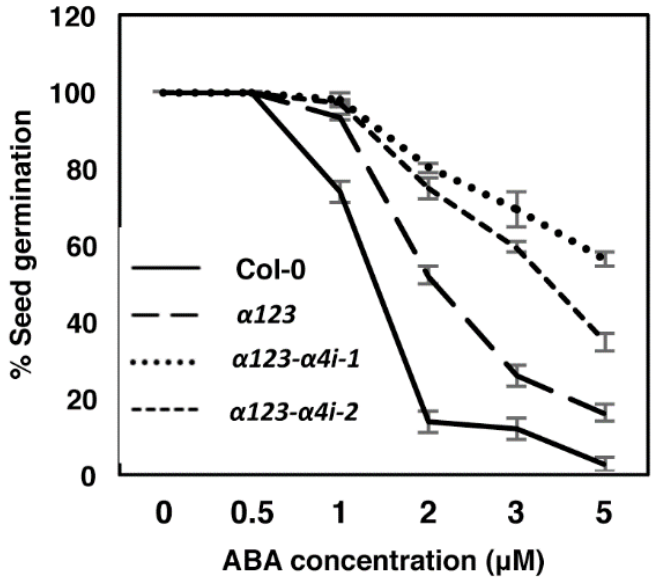

Figure 3.5: CK2 α4 subunit positively regulates ABA-induced block of seed germination. Line graph showing reduced seed germination of Col-0, *α123* and CK2 α4 RNAi lines in *α123* mutant background in response to increasing concentrations of ABA. The experiment was repeated three times ($n \geq 50$ for each genotype) and the data are expressed as mean $\pm$ SE.

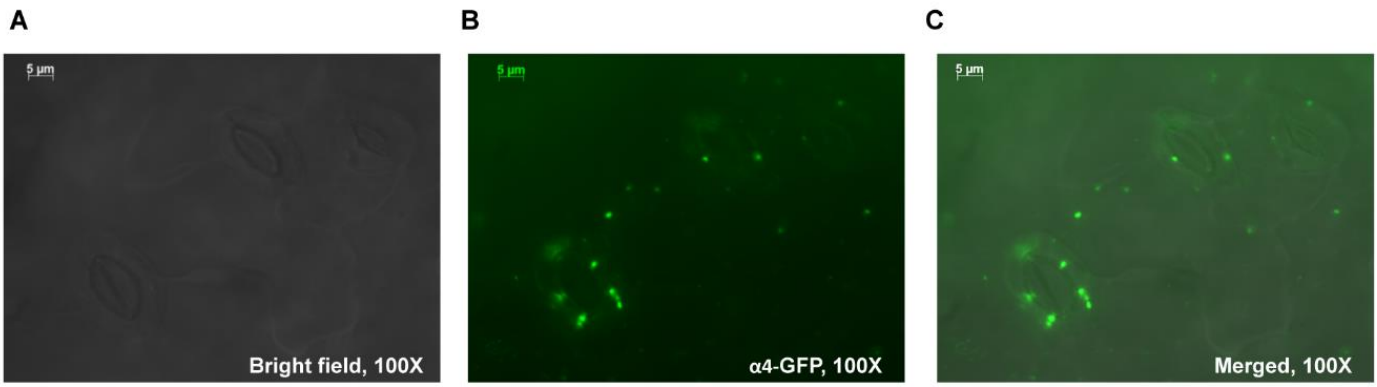

Figure 3.6: CK2 α4 subunit is localized to chloroplast in transgenic Arabidopsis seedlings. Photographs showing the guard cells expressing CK2 α4-GFP at 100X magnification under light fluorescence microscope. Guard cells under bright field (Panel A), guard cells expressing CK2 α4-GFP (Panel B) and merged Bright field and GFP image (Panel C). Bar = 5 µM.

Table 3.1: Primer sequences used for the experiments described in Chapter 3

Gene	Forward Primer	Reverse Primer
CK2α4i	TGATCTCGAAAACATTTGC	ACCTTTTGAAACCCGTCTTG
CK2α1	CAAAATCCTCAAACCTGTTAAGAAA	TGTCGTAATCAGTCAACGTAGGATA
CK2α2	GTTTATACCGATGTTAACGTGATCC	CTTCTTAACAGGCTTGAGAATCTTG
CK2α3	ATGTGGTGGACCAAATATTGTTAAG	TATGCATTATTCCTTGAGAGTGACA
CK2α4	ATATTCCGCAAGGAGCCGTTCTT	TGCCCACGAGGGATGTTAGATTAGG
CK2 α4 (ORF)	CACCATGGCCTTAAGGCCTTGTACTGG	CAGAGAGCAGCCGTACGCCGCGCAGCCAG
CO	AACTGCAGCGTACCACAGAC	GGATGAAATGTATGCGTTATGG
FT	GGAACAACCTTTGGCAATGAGAT	CTGCCAAGCTGTCGAAACAA
FLC	GCCAAGAAGACCGAACTCATGTTGA	CAACCGCCGATTTAAGGTGGCTA
SOC1	TGGGAGAAGGCATAGGAACATGC	CGCTTTCATGAGATCCCCACTTTTC
PP2A	TATCGGATGACGATTCTTCGTGCAG	GCTTGGTCGACTATCGGAATGAGAG

Chapter 4: Phosphorylation of Phytochrome Interacting Factors by CK2 and its functional significance

ABSTRACT

Light signaling through phytochromes, the red/far red light photoreceptors, involves the interaction with Phytochrome Interacting Factors (PIFs) in the nucleus followed by the rapid phosphorylation and degradation of PIFs through the 26S proteasome pathway. Casein Kinase 2, a ubiquitous Ser/Thr kinase has been shown to phosphorylate important molecular players in this signaling pathway such as HFR1, HY5 and PIF1. To test whether CK2 phosphorylates other the PIF family members, PIF3 through PIF7 were purified and *in vitro* kinase assays were performed. Results show that the CK2 holoenzyme phosphorylates PIF1, PIF3 and PIF5. Ten phosphorylation sites were mapped in PIF3. However, mutation of all 10 putative phosphorylation sites in the PIF3 protein could not completely eliminate CK2 phosphorylation, suggesting that there are additional sites in PIF3. Yeast two hybrid assays were performed to evaluate the effect of CK2 phosphorylation on the dimerization capability of PIF1. Results show that PIF1 phospho-deficient (PIF1-7M) mutant has a weaker homo-dimerization ability compared to the wild type PIF1 and PIF1 phospho-mimic (PIF1-3D) mutant. These preliminary results suggest an important role for CK2 phosphorylation for the regulation of PIF dimerization and function in light signaling pathways.

INTRODUCTION

Light is perceived as an important external cue by all organisms. Plants, being sessile organisms, use this cue for many important developmental events in their life cycle. A variety of photoreceptor protein families help in perceiving distinct wavelengths

of light in plants. This perceived signal is then relayed via a complex molecular signaling pathway that culminates in changes in the expression of genes, protein stability/localization/function (Rockwell *et al.*, 2006; Bae and Choi, 2008).

Our understanding of the molecular events involved in light signaling in higher plants comes mainly from the research done in the model plant *Arabidopsis thaliana*. There are 4 main photoreceptor protein families in Arabidopsis that perceive different wavelengths of light viz. cryptochromes (blue light), phototropins (blue light), phytochromes (red/far red) and UVR8 (UV-B light) (Jiao *et al.*, 2007). The molecular players involved in the signaling cascade downstream of each of these photoreceptors have been found to overlap to a certain extent adding another layer of complexity to the light signaling network (Galvão and Fankhauser, 2015).

Of the four photoreceptors, phytochromes play a crucial role in plant development as red light is the most favorable light for major developmental processes such as seed germination, early seedling development, as well as physiological processes such as photosynthesis. Since light is an important input to the circadian clock, phytochromes also play a vital role in setting the clock period (Briggs and Spudich, 2006).

There are five phytochromes in Arabidopsis (*PHYA-PHYE*). Structurally all phytochromes are synthesized as apoproteins attached to a chromophore which confers the photoreversibility crucial to relay the light signal. In the cell, phytochromes exist as soluble dimeric proteins. PhyA, classified as type I phytochrome, plays a central role in the perception of far-red light while phytochromes B through E are type II phytochromes and are involved in modulating responses to red light such as seed germination, seedling de-etiolation and shade avoidance response. PhyB serves as the principal receptor for red light while other phytochromes play some overlapping and a few distinct roles (Franklin and Quail, 2010).

The current simplified model of Phytochrome mediated light signaling pathway in Arabidopsis involves the following key events. Dark-grown seedlings display a skotomorphogenic phenotype characterized by an elongated hypocotyl, closed apical hook and closed cotyledons. Under this condition phytochromes exist in the Pr (inactive) form in the cytoplasm. In the dark, repressors of photomorphogenesis such as Phytochrome Interacting Factors (PIFs) are stable while the positive regulators (e.g., HY5, HFR1, LAF1 and others) are constantly degraded. Perception of red light changes the conformation of phytochrome molecules to the Pfr (biologically active) form, which translocates to the nucleus. Active phytochromes can interact with several proteins in the nucleus triggering changes in protein stability and gene expression. They interact with the PIF proteins and induce their rapid degradation (Huq, 2006). They also stabilize the above-mentioned positive regulators by repressing the activity of the E3 ligase COP1 in the nucleus. These changes lead to photomorphogenesis that involves de-etiolation of seedlings characterized by the shortening of the hypocotyl, straightened hook and open, green cotyledons (Fankhauser and Chen, 2008; Franklin and Quail, 2010).

Phytochrome Interacting Factors (PIFs) form a family of 7 bHLH transcription factors that interact with photoactivated phytochromes in the nucleus. There are 7 members of the PIF family in Arabidopsis, PIF1 and PIF3 through PIF8. As, bHLH transcription factors, all PIFs contain a basic DNA binding domain that recognizes and binds a G-box motif (CACGTG) and a helix-loop-helix domain that allows for both homo and hetero dimerization (Duek and Fankhauser, 2005; Castillon *et al.*, 2007; Leivar and Quail, 2011*a*). In addition, all PIFs contain an N terminal conserved APB (active Phytochrome B binding) motif required for interaction with the active (Pfr) form of PhyB. Two members, PIF1 and PIF3, contain an additional domain called APA for interacting with active form of PhyA. As transcription factors, PIFs modulate the

expression of light responsive genes both directly and indirectly to regulate photomorphogenesis (Leivar *et al.*, 2009). Recently, PIF1 has been shown to promote the degradation of HY5 by increasing its interaction with COP1 in the dark (Xu *et al.*, 2014). PIFs act as repressors of photomorphogenesis and promote skotomorphogenesis with a high functional redundancy. A PIF quadruple mutant, *pif1pif3pif4pif5* (*pifq*), shows constitutive photomorphogenic phenotype in the dark (Leivar *et al.*, 2008; Shin *et al.*, 2009). It has been established that the interaction with active form of phytochromes leads to rapid multisite phosphorylation of all PIFs (except PIF7) which are then ubiquitinated and degraded via the 26S proteasome pathway. Interaction with active Phytochrome is a prerequisite for phosphorylation and phosphorylation is necessary for the 26S proteasome mediated degradation of PIFs (Al-Sady *et al.*, 2006; Shen *et al.*, 2008*a*; Ni *et al.*, 2013). In the case of PIF3, LRB E3 ubiquitin ligase has been shown to promote polyubiquitination and degradation of both PhyB and phosphorylated PIF3 protein *in vivo* (Zhu and Huq, 2014; Ni *et al.*, 2014). Thus, rapid light induced phosphorylation followed by degradation is one major mechanism by which phytochrome mediated light signaling acts to remove the PIFs in light and ensue photomorphogenesis. Apart from degradation of PIFs, phytochromes also functionally inhibit PIFs from binding to their target promoters through sequestration (Park *et al.*, 2012). Light activated phytochromes also stabilize the positive regulators by interacting with SPA proteins in thus disrupting COP1/SPA complex responsible for degradation of positive regulators (Sheerin *et al.*, 2014). Hence, phytochromes induce photomorphogenesis by inhibiting the negative regulators as well as by stabilizing the positive regulators.

Several efforts have been made to identify the light specific kinase that phosphorylates PIFs upon interaction with phytochrome. The PRD and PKRD domains of Arabidopsis phytochromes show some sequence similarity to the histidine kinase

domain of bacterial phytochrome Cph1 (Yeh, 1997). Oat PhyA has been shown to exhibit Ser/Thr kinase activity *in vitro* however, convincing evidence showing *in vivo* kinase activity for phytochromes is still lacking (Li *et al.*, 2011). Recently, *in vivo* phosphorylation sites for PIF3 protein under both dark and light conditions were identified using mass spectrometry. Mutating up to 26 of these residues resulted in increased stability of PIF3 protein under light. Knocking out these sites also affected the negative feedback modulation of the phyB levels under prolonged light (Ni *et al.*, 2013). The high number of *in vivo* phosphorylation sites identified in PIF3 protein reaffirms the importance of this phosphorylation in light signaling. One of the PIF family members, PIF4 is phosphorylated by BIN2 promoting its degradation. This phosphorylation is required to restrict the transcriptional activity of PIF4 and is responsible for shaping the diurnal hypocotyl growth (Bernardo-García *et al.*, 2014). Thus, PIFs are regulated independent of light signal through a complex cross-talk between phytohormones wherein phosphorylation plays a critical role.

Casein kinase 2 (CK2) is a well conserved, ubiquitous Ser/Thr kinase that is known to be involved in light signaling pathway. CK2 antisense RNAi lines display differential regulation of various light regulated genes (Lee *et al.*, 1999). Two of the positive regulators of photomorphogenesis HY5 and HFR1 are CK2 substrates. Phosphorylation of HY5 by CK2 increases its stability by reducing its affinity towards COP1, an E3 ubiquitin ligase (Hardtke *et al.*, 2000). CK2 mediated phosphorylation also increases stability of HFR1 in light (Park *et al.*, 2008). Previous research from our lab showed PIF1, a major repressor of photomorphogenesis, is the direct target of casein kinase 2 in *in vitro* kinase assays. We also mapped the 7 major CK2 phosphorylation sites on PIF1 through mass spectrometry. Mutating these 6 of the 7 CK2 sites to alanine resulted in increased stability of PIF1 protein after red light pulse suggesting that CK2

mediated phosphorylation of PIF1 aids in its faster degradation upon light (Bu *et al.*, 2011*a*,*b*). These results indicate that CK2 plays a role in light signaling by modulating stability of both positive and negative factors. Thus, it is important to test whether all the PIF family proteins are substrates of CK2 and if so what is the biological significance of this phosphorylation.

RESULTS

In vitro kinase assay with purified PIF proteins and CK2 α1β1 holoenzyme

Previous results from our lab show that PIF1 is a direct target of enzyme CK2 and this phosphorylation is necessary for PIF1 degradation under light (Bu *et al.*, 2011*a*). PIF1 is a member of a family of proteins which has 7 other members. Through three different prediction programs we found that each of those members has some putative CK2 phosphorylation sites (Table 4.1). To test whether other PIF family proteins namely PIF3 through PIF7 are also CK2 substrates, these proteins were expressed and purified from *E.coli* cultures as recombinant proteins. To this end the open reading frames (ORFs) encoding all 5 PIF family proteins were cloned into expression vector pVP13 and transformed into *E. coli* DE3 cells. The over expression of PIF proteins in *E. coli* resulted in formation of insoluble protein bodies and required considerable optimization of the protein purification protocols in order to purify sufficiently concentrated quantities of all five PIF proteins (as described in the Materials and Methods as well as Discussion section).

The kinase assay was performed as described earlier using recombinant CK2 α1β1 holoenzyme and PIF1 protein was used as a positive control (Bu *et al.*, 2011*a*). The assay was repeated three times for consistent results. The kinase assay results show that PIF1, PIF3 and PIF5 are substrates of CK2 protein while PIF4, PIF6 and PIF7 proteins

are not (Fig. 4.1 A and B). Thus, CK2 does not appear to be a universal kinase for all the PIF family proteins.

Mapping CK2 phosphorylation sites on PIF3 protein

To map the CK2 phosphorylation sites, purified PIF3-MBP protein was phosphorylated in an *in vitro* kinase assay with cold ATP and CK2 α1β1 holoenzyme and subsequently subjected to mass spectrometry. Through the analysis of mass spec data we recovered 3 putative Ser residues (S316, S323, S380) as potential CK2 target sites. However, the recombinant mutant PIF3-3M (3S -> 3A) protein was robustly phosphorylated by CK2 in *in vitro* kinase assay (Fig. 4.2 A and B). We subsequently mutated more putative phosphorylation sites on PIF3 based on the phosphorylation site prediction programs and phosphorylation site saturation assays done with wild type PIF3. We tested all these mutant forms using *in vitro* kinase assays. Three higher order mutant versions of PIF3 proteins viz. 6M (S295, S297, S299, S301, S316, and S323), 8M (S283, S287, S295, S297, S299, S301, S316, and S323) and 10M (T119, S269, S283, S287, S295, S297, S299, S301, S316, and S323) were cloned, expressed, purified and tested. However, all these mutant forms are all significantly phosphorylated by CK2 (Fig. 4.2 C, D, E, F, G, and H). We decided not to pursue this aim to identify and mutate all putative CK2 sites in PIF3 any further at this stage.

Functional significance of CK2 phosphorylation of PIF1

Protein kinase CK2 phosphorylates PIF1 in *in vitro* kinase assays. CK2 mediated phosphorylation of PIF1 accelerates its degradation kinetics after a red light pulse (Bu *et al.*, 2011*a*). To find exactly how CK2 phosphorylation affects PIF1 protein function we tested both phospho-deficient (PIF1-7M) and phospho-mimic (PIF1-3D) forms of PIF1 in *in vitro* assays.

Effect of CK2 phosphorylation on dimerization of PIF1 protein in vitro

PIFs are bHLH family proteins and function as both homo and heterodimers. We checked whether CK2 phosphorylation affects PIF1 ability to dimerize in yeast two hybrid assays. We tested the interaction between a truncated (N terminal 150 amino acids deleted) form of PIF1 (WT/7M/3D) fused to GBD and full length PIF1 fused to GAD to avoid auto-activation. There is no significant difference between the wild type and PIF1-3D; however, the PIF1-7M mutant showed less β-galactosidase activity than both wild type and PIF1-3D in these yeast two hybrid assays (Fig. 4.3 A). These data suggest that the phospho-deficient PIF1 mutant is slightly affected in its dimerization ability. To confirm these results, we performed *in vitro* pull down assays with full length proteins. However, this experiment failed to show any significant difference between wild type PIF1 and its mutant versions with respect to the homo-dimerization capacity (Fig. 4.3 B). Hence, the results obtained from the yeast two hybrid data could not be corroborated by an independent experimental technique.

DISCUSSION

PIF family proteins are principal repressors of photomorphogenesis in Arabidopsis. One major mechanism through which phytochromes relay light signals involves their interaction with PIFs followed by rapid phosphorylation and degradation of PIFs through the 26S proteasome (Al-Sady *et al.*, 2006; Shen *et al.*, 2007, 2008*a*). The light regulated kinase that is responsible for rapid phosphorylation of PIFs has not yet been identified. Due to limited *in vitro* kinase activity and the presence of a histidine-kinase like domain, phytochromes have been long speculated to be the kinase (Li *et al.*, 2011). However, there is no strong *in vivo* experimental evidence to support this hypothesis. Casein kinase 2 (CK2) has been shown to phosphorylate both positive (HY5, HFR1) and negative factors (PIF1) involved in light signaling (Mulekar and Huq, 2014).

Hence, it is worth testing whether CK2 is a common kinase for all PIF family proteins. Although, CK2 is not known to be light regulated, it might be responsible for fine tuning light signaling. It is also likely that their interaction with Phytochromes alters the conformation of PIFs making unexposed Ser/Thr residues becoming more accessible for CK2 in the presence of light.

Phosphorylation of proteins has been shown to affect their function/stability/localization (Hardtke *et al.*, 2000; Daniel *et al.*, 2004). In the case of PIFs, phytochrome interaction post phosphorylation leads to their rapid degradation. It has been recently shown that PIF3 protein is heavily phosphorylated *in vivo* under both light and dark conditions (Ni *et al.*, 2013). Thus, apart from light induced phosphorylation that controls stability of PIFs, there might be another functional significance of phosphorylation, e.g. change in affinity with other proteins and/or DNA. Hence, we tested whether all PIF family proteins can be phosphorylated by CK2 in an *in vitro* kinase assay.

The PIF family proteins (PIF1, PIF3 through PIF7) were expressed and purified from *E. coli*. Over expression of PIFs with a small tag (His tag) in *E. coli* caused formation of aggregated insoluble protein which was very difficult to purify. Also, the proteins were very unstable and degraded rapidly during purification process. To overcome these problems, the protein purification process was modified and optimized for various parameters. To overcome protein insolubility issues, the protein was expressed with both His and MBP tags at the N-terminus. The MBP tag increases solubility of fusion proteins. To inhibit protein degradation a cocktail of protease inhibitors, PMSF and Roche Protease inhibitor complete (without EDTA) was used in the binding/wash buffer. To minimize formation of aggregates 10% glycerol was added to all the buffers. These modifications in the protocol significantly increased both solubility

and yield of protein. However, the purified protein was low in concentration. PIF proteins are very sticky; hence concentrating the protein through another column was unsuccessful. We found that dialysis worked in concentrating the protein with minimum loss. Hence, all the proteins were dialyzed against 50% glycerol buffer overnight to obtain purified protein of sufficient concentration.

The kinase assay results show that CK2 phosphorylates PIF1, PIF3 and PIF5 amongst all PIF family members *in vitro* (Fig. 4.1 A and B). Both PIF1 and PIF3 contain the Activated Phytochrome A (APA) binding domain, however PIF5 does not contain the APA domain (Castillon *et al.*, 2007). There is no amino acid or nucleotide sequence characteristic that can be used to group these three PIFs together amongst the PIF family proteins. However, functionally these three PIFs are repressors of chloroplast development and down regulate key chlorophyll biosynthetic genes (Leivar and Quail, 2011*b*). PIF7 is not phosphorylated after its interaction with activated phytochromes. The kinase assay shows that PIF7 is not a substrate for CK2. Due to its ubiquitous nature, CK2 is unlikely to be a light regulated kinase. Thus, CK2 mediated phosphorylation of these three PIFs could be a mechanism to fine tune their levels/function in both light and dark conditions. Regulation of PIFs is critical to achieve optimum survival in light hence, it is not surprising that there are several mechanisms to fine tune it.

Mapping CK2 phosphorylation sites on PIF3

PIF1 is the first PIF family member to be identified as a CK2 substrate. Through the *in vitro* kinase assays we found PIF3 and PIF5 are also CK2 targets. PIF3 has been extensively studied member of PIF family and has several striking similarities with PIF1. Both PIF1 and PIF3 contain a functional domain to interact with Phytochrome A (APA), both act as repressors of chloroplast development and undergo a light-induced rapid

phosphorylation which is dependent on interaction with active phytochrome form (Castillon *et al.*, 2007). PIF1 and PIF3 show different degradation kinetics and have different half-lives in light. PIF1 is degraded almost completely with a half-life of a minute while the half-life of PIF3 is around half an hour (Al-Sady *et al.*, 2006; Shen *et al.*, 2008*a*). Therefore PIF3 presents an ideal CK2 substrate to study the functional significance of CK2 mediated phosphorylation. However, mutating up to 10 putative CK2 sites in PIF3 to alanine did not result in a complete phospho-deficient mutant of CK2. Although, a rough phosphorylation site saturation curve indicated presence of up to 6 CK2 sites, we were not able to identify and knock out all of them. PIF3-10M did show significantly less phosphorylation indicating that some of the sites mutated were indeed true CK2 sites. However, PIF3 protein has a significantly high proportion of serine (71/524) and threonine (22/524) residues and few of the serines occur in tandem. Hence, it is hard to identify exactly which serine residue is the true CK2 site. Recently, PIF3 has been shown to have at least 26 phosphorylation sites *in vivo*. Abolishing all the 26 target sites did not completely abolish phosphorylation of PIF3 protein in light. It is plausible that in the absence of bona fide sites the kinase may choose alternate sites to modify the protein *in vivo* or multiple overlapping kinases phosphorylate PIF3 or both. These results certainly underscore the importance and complicated nature of PIF3 phosphorylation under both light and dark conditions.

In summary, our results indicate that PIF3 is heavily phosphorylated by CK2 at multiple residues *in vitro*. However, further work is required to map these sites and mutate them to create a phospho-deficient PIF3 mutant.

Functional significance of CK2 mediated phosphorylation of PIF1

CK2 phosphorylates three out of six PIF family proteins. To investigate the functional significance of this phosphorylation we focused on PIF1 and PIF3 proteins since both of them are heavily phosphorylated by CK2 in the *in vitro* kinase assay. CK2 phosphorylation has been shown to affect PIF1 stability after red light pulse (Bu *et al.*, 2011*a*).

Phosphorylation of proteins often changes affinity of proteins towards other proteins and also affects dimerization (Daniel *et al.*, 2004; Park *et al.*, 2008; Dennis *et al.*, 2009). We observe that phospho-deficient PIF1 mutant PIF1-7M consistently shows lower β- galactosidase activity than both wild type PIF1 and PIF1-3D in yeast two hybrid assays (Figure 3 A). Activated phytochromes have been shown to interact with PIF3 in an equimolar ratio (Zhu *et al.*, 2000). Thus, a dimer of Phytochrome B interacts with a PIF protein dimer. If CK2 phosphorylation affects the dimerization capacity of PIFs it could lead to change in affinity towards activated phytochrome molecules. As a bHLH transcription factor, PIF1 binds to promoter regions as a dimer (Moon *et al.*, 2008). If dimerization is affected then that may also affect DNA binding and its capacity to act as repressor. However, the difference observed in the yeast two hybrid assay was not observed in the *in vitro* pull down assays. In these assays PIF1-7M showed equal affinity as the wild type PIF1 and PIF1-3D. Hence, the observed difference in affinity in yeast two hybrid assays could be observed in the yeast system but was not seen in the *in vitro* pull-down assay. One likely explanation for this discrepancy is that yeast has an active CK2 enzyme which may phosphorylate the wild type PIF1 and PIF1-3D in yeast but is not able to phosphorylate PIF1-7M and hence we observe this difference in yeast.

In summary, the experiments performed were mostly inconclusive and not sufficient to uncover the functional significance of CK2 mediated phosphorylation of

PIF1. The experimental evidence from identification of *in vivo* phosphorylation sites for PIF3 strongly suggests that multiple kinases may phosphorylate PIFs under both light and dark conditions to fine tune their function and abundance since regulation under both the conditions is crucial for achieving optimum growth under light and dark conditions. Thus, it might be challenging to dissect the functional significance of one kinase *in vivo*. Novel strategies will be required to answer these questions.

MATERIALS AND METHODS

Cloning, expression and purification of recombinant PIF proteins

His-tagged PIF1 protein was purified as described earlier (Bu *et al.*, 2011*a*). For the rest of the PIF proteins (PIF3 through PIF7 and various PIF3 higher order mutants) following purification strategy was used. The full length cDNAs of all PIF proteins were cloned first into gateway vector pENTR (Invitrogen Inc., Carlsbad, CA) and subsequently into pVP13. Various phospho-deficient mutants of PIF3 were created through site directed mutagenesis as previously described using pENTR-PIF3 clone as template and then subsequently cloned into pVP13 vector. The constructs were confirmed by sequencing. All the primers are listed in Table 2. The correct clones were transformed into BL21 (DE3) *E. coli* cells for expression. Overnight cultures (75ml of LB media containing 100 μg/ml ampicillin) started from a single colony were used to inoculate 1.2 liter of LB media containing 100 μg/ml ampicillin (Invitrogen) and grown to an A_{600} of 0.5 at 30°C. Expression of protein was induced with the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.6 mM. Following isopropyl β-D-thiogalactoside (IPTG) induction, cells were grown for 3 h at 30 °C. Cells were harvested by centrifugation (6000 X g) for 15 min at 4 °C. The *E. coli* cell pellet was resuspended in 25 ml of Binding/Wash Buffer C (50 mM HEPES-KOH, pH 7.6, 600 mM KCl, 20 mM

imidazole, 2mM PMSF, and 10% glycerol) containing 1 Complete protease inhibitor tablet (EDTA-free, Roche Applied Science). The cells were disrupted by sonicating three times for 30 s at 70% power and two times for 30 s at 90% power using a Vibra Cell sonicator (Sonics & Materials Inc.). Lysed cells were centrifuged at 184,048 & g for 45 min at 4 °C. The supernatant was applied on a column containing 1 ml of Ni-NTA beads. The charged column was washed with Binding/Wash buffer. His6-tagged proteins were eluted from Ni-NTA with Elution Buffer (20mM HEPES-KOH, pH 7.6, 250 mM KCl, 250 mM imidazole and 10% glycerol). The fractions containing highest amount of protein were pooled and dialyzed overnight at 4°C in Dialysis buffer (20mM HEPES-KOH pH7.6, 250mM KCl, 0.1 mM EDTA, 50% glycerol). The dialyzed proteins were stored at -80°C.

***In vitro* kinase assay**

CK2 in vitro kinase assays were performed as described (Bu *et al.*, 2011*a*). Briefly, 25 μL of kinase assay mixtures contained 50 mM Hepes-potassium hydroxide (pH 7.6), 5mM MgCl2, 2.4mM DTT, 0.2mM γ- [32P]ATP (~250 cpm/pmol), 100 mM KCl, ~1 pmol CK2, and 2 μg of PIF protein. The reaction was incubated at 30 °C for 30 min and terminated by the addition of 6X SDS loading buffer. Samples were boiled 3 min and separated on 8% SDS-PAGE gels. The gels were dried and exposed to a phosphorImager.

Mapping CK2 Phosphorylation Sites in PIF3

The identification of CK2 phosphorylation sites in PIF1 was essentially performed as described (Dennis and Browning, 2009). Briefly, the phosphopeptides were enriched using an iron-based affinity resin (Phos-Select, Sigma), followed by analysis on a MALDI-TOF/TOF mass spectrometer (4700 proteomics analyzer, ABSciex, Foster

City, CA). Phosphopeptides were identified in the MS by mass shift using the MASCOT (Matrix Science) search algorithm, and the MS/MS were acquired for potential phosphopeptides. These were manually interpreted to assign the most probable location of the phosphorylation site.

Yeast two hybrid Assay

The full-length and truncated forms of PIF1, PIF1-7M and PIF1-3D were amplified by PCR using the primers listed in Table 4.2. These fragments were cloned into pGBT9 and pGAD434 using *EcoRI* - *SalI* restriction sites included in the primers to generate pGBT9-ΔPIF1, pGBT9-ΔPIF1-7M, pGBT9-ΔPIF1-3D and pGAD-PIF1. All the clones were verified by restriction enzyme digestion and sequencing. These vectors were transformed into yeast strain Y187 (Clonetech) and selected on a synthetic minimal medium without Leu and Trp for 3 days at 30°C. Colonies were cultured overnight in liquid synthetic medium without Leu and Trp supplemented with 2% (w/v) Glc. Aliquots of overnight cultures were then transferred to YPD medium supplemented with 2% (w/v) Glc and grown until OD_{600} was 0.8. A β-galactosidase activity assay was performed as described (Xu *et al.*, 2014)

TNT expression and *in vitro* pull down assay

In vitro co-immunoprecipitation experiments were performed as described (Huq and Quail, 2002). All proteins were expressed from T7 promoters in the TnT *in vitro* transcription/translation system (www.promega.com) in the presence of [^{35}S] Methionine. Full length ORFs for PIF1, PIF1-7M and PIF1-3D were PCR amplified and cloned into both pET17b-GAD and pET17b (Invitrogen), and confirmed by restriction digestion and sequencing. The binding buffer used contained 1× PBS pH 7.2, 0.1% (v/v) Tergitol NP-40 (Sigma), 0.1% BSA and 1× Complete protease inhibitor (Roche). The

same buffer was used for the first wash of the pellet, and the final wash was performed with the same buffer without BSA.

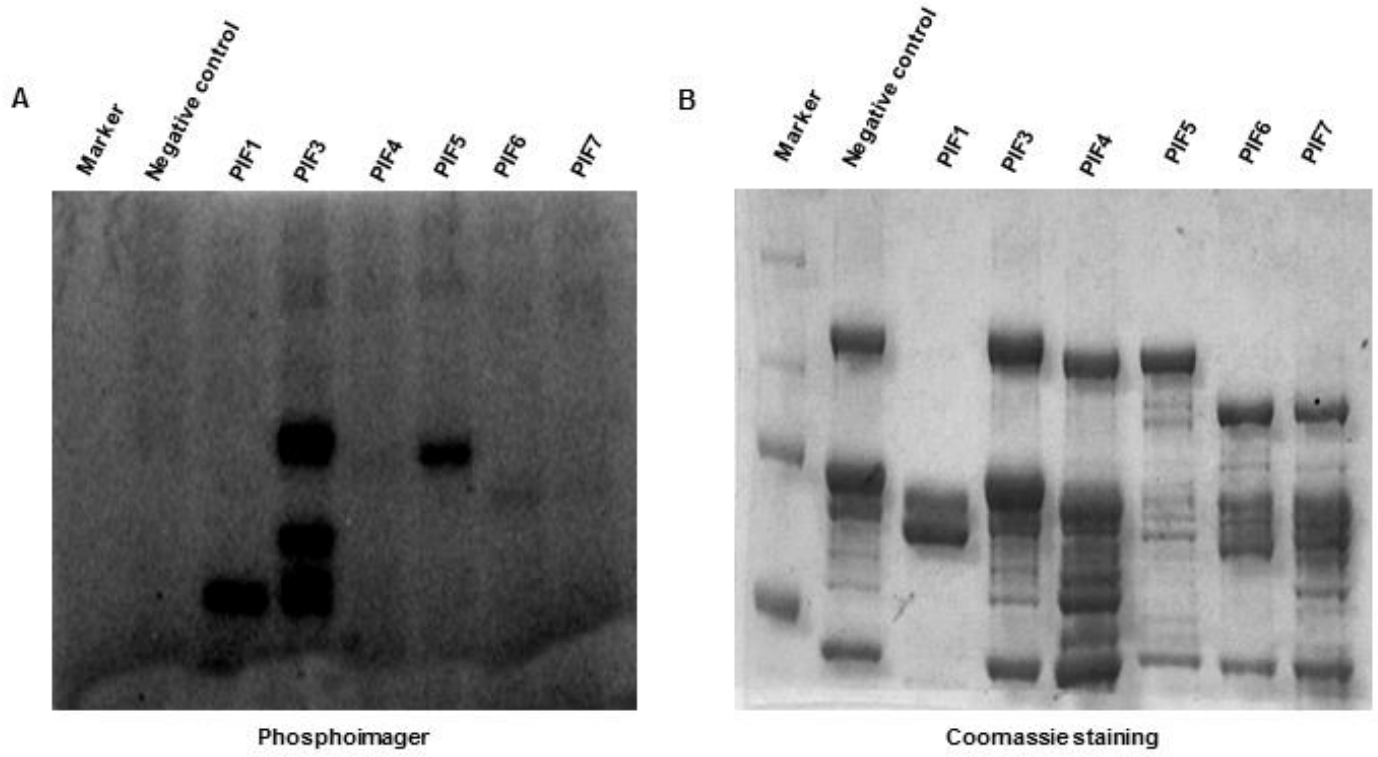

Figure 4.1: *In vitro* kinase assay for all PIF proteins using CK2 holoenzyme shows PIF1, PIF3 and PIF5 are CK2 substrate proteins. Autoradiograph showing the phosphorylated substrate proteins (A) and the same SDS-PAGE gel stained with coomassie blue showing amount of protein used in the assay (B). 2 µg of each substrate protein was phosphorylated *in vitro* by AtCK2 α1β1 holoenzyme using ^{32P}γ-ATP and was ran on 8.5% SDS-PAGE gel.

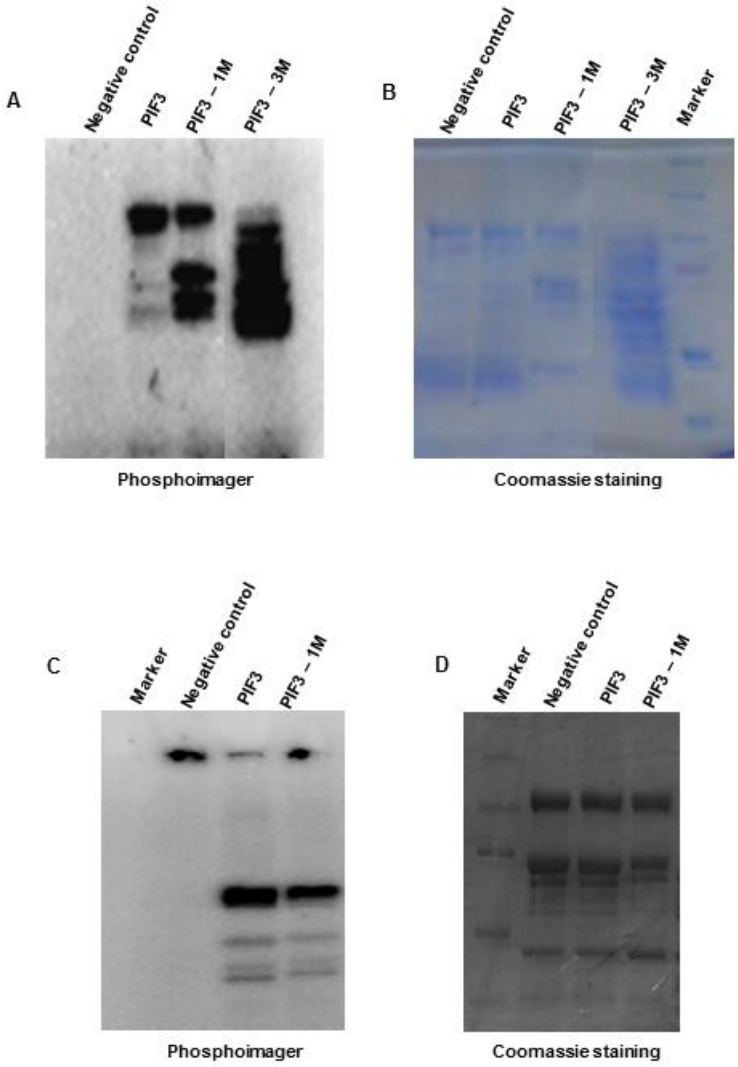

Figure 4.2: *In vitro* kinase assay for higher order CK2 phosphorylation site knockout mutant of PIF3 proteins using CK2 holoenzyme. Autoradiograph showing the phosphorylated WT-PIF3 and various mutant versions of PIF3; PIF3-3M (A), PIF3-6M (C), PIF3-8M (E) and PIF3-10M (G) and the same SDS-PAGE gel stained with coomassie blue showing the amount of protein used in the assay (B, D, F, H). Equal quantities of wild type PIF3 and mutant PIF3 proteins were phosphorylated *in vitro* by AtCK2 α1β1 holoenzyme using ^{32P}γ-ATP and run on 8.5% SDS-PAGE gel.

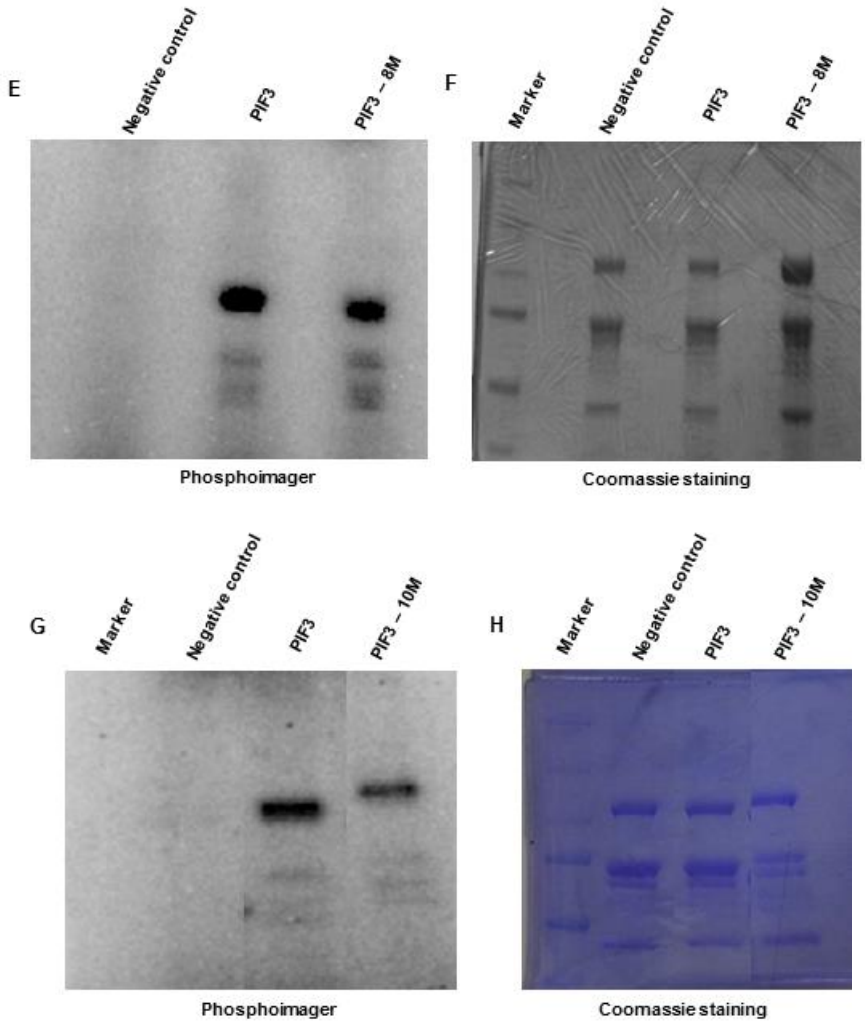

Figure 4.2: *In vitro* kinase assay for higher order CK2 phosphorylation site knockout mutant of PIF3 proteins using CK2 holoenzyme. Autoradiograph showing the phosphorylated WT-PIF3 and various mutant versions of PIF3; PIF3-3M (A), PIF3-6M (C), PIF3-8M (E) and PIF3-10M (G) and the same SDS-PAGE gel stained with Coomassie blue showing the amount of protein used in the assay (B, D, F, H). Equal quantities of wild type PIF3 and mutant PIF3 proteins were phosphorylated *in vitro* by AtCK2 α1β1 holoenzyme using $^{32P}\gamma$-ATP and run on 8.5% SDS-PAGE gel.

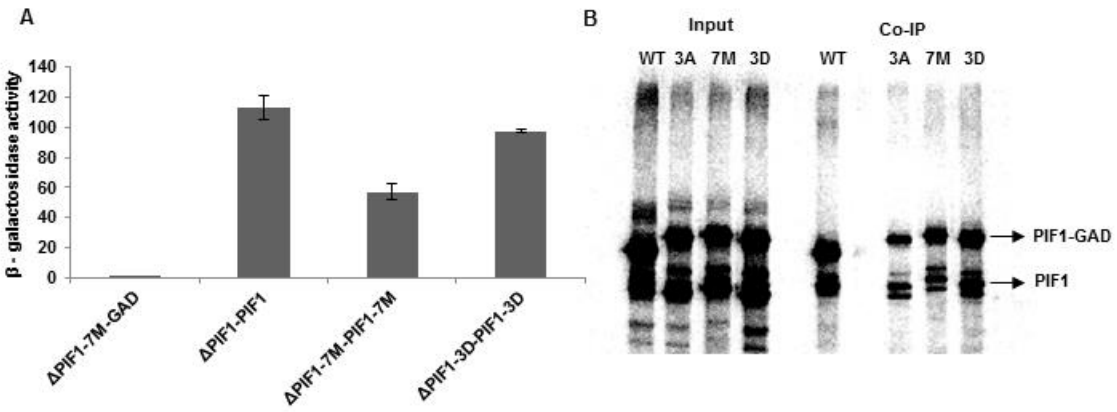

Figure 4.3: Homo-dimerization capability of PIF1-7M and PIF1-3D mutants. Graph showing β- galactosidase activity in yeast two hybrid assays for testing homo-dimerization of WT-PIF1, PIF1-7M and PIF1-3D (A) The experiment was repeated at least thrice. *In vitro* pull down assay with full-length GAD fusion and no tag versions of WT-PIF1, PIF1-7M and PIF1-3D proteins expressed using TnT system (www.promega.com) (B). *In vitro* pull down assay does not show any dimerization defect in PIF1-7M. PIF1-7M shows a slight defect in homo-dimerization in yeast two hybrid assay but not in the *in vitro* pull down assay.

Table 4.1: List of putative CK2 sites in PIFs

	Stringency	http://scansite.mit.edu	http://www.cbs.dtu.dk/services/NetPhosK/)	http://kinasephos.mbc.nctu.edu.tw/index.php
PIF3	Low	S16, S266, S316, S323, S380	T70, S108, T119, S125, S316, S323, S38	T41, S88, S276, S295, S323, S495
	Medium	S316, S323	S316, S323	S88, S276, S295, S323, S495
	High		S316	
PIF4	Low	T151, S294	S7, T64, T151, S184, S239	T50, S105, S197, S199, S235, S237, S256, S294, T422, S426
	Medium			T50, S197, S235, S256, S294
	High			S294
PIF5	Low	T130, T234, S293	S235, S293	T55, S174, S205, S227, S235, T236, S249, S255, S293, S386
	Medium		S235	T55, S174, S227, S235, S255, S293, S386
	High			S235, S293
PIF6	Low	S85, T87, T134, S137, S145, S146, S151, S208	T134, S144, S146, S151, S208	S85, S115, T134, S146, S151, S208
	Medium	T134, S146, S151, S208	S144, S151, S208	S115, T134, S151
	High	S151		
PIF7	Low	T143, T146, T153, S203	S139, T143, T146, T153	S139, S141, T146, S203
	Medium	T143, T146	S139, T143, T153	S139, S141, S203
	High	T146		S139

Table 4.2: List of primers used in Chapter 4

PRIMER	FORWARD	REVERSE
PIF3 ORF	CACCATGCCTCTGTTTGAGCTTTT	TCACGACGATCCACAAAACTG
PIF4 ORF	CACCATGGAACACCAAGGTTGGAG	CCGGTCGACCTAGTGGTCCAAACGAGAAC
PIF5 ORF	CACCATGGAACAAGTGTTTGCTG	CCGGTCGACTCAGCCTATTTTACCCATATG
PIF6 ORF	CACCATGATGTTCTTACCAACCG	CCGGTCGACTCATCTGTTAGTTTTCCTTG
PIF7 ORF	CACCATGTCGAATTATGGAGTTAAAG	CCGGTCGACCTAATCTCTTTTCTCATGATTCG
PIF3 S316A	GACATTGACTGTCATGCTGAAGATGTGGAAGA	TCTTCCACATCTTCAGCATGACAGTCAATGTC
PIF3 S323A	GATGTGGAAGAAGAAGCAGGAGATGGAAGAAAG	CTTTCTTCCATCTCCTGCTTCTTCTTCCACATC
PIF3 S380A	CAAGGTGGACAAAGCTGCGATGCTAGATGAAG	CTTCATCTAGCATCGCAGCTTTGTCCACCTTG
PIF3 4S>4A S295, S297, S299, S301	CGATGGCCCAGCCGAAGCTCCTGCACTTGCTTTAAAGAGA	TCTCTTTAAAGCAAGTGCAGGAGCTTCGGCTGGGCCATCG
PIF3 S283A	AAAGCAGTTGTATGTGCTTCTGTTGGCGCGGGT	ACCCGCGCCAACAGAAGCACATACAACTGCTTT
PIF3 S287A	TGTTCTTCTGTTGGCGCGGGTAATAGTCTCGAT	ATCGAGACTATTACCCGCGCCAACAGAAGAACA
PIF3 T119A	GTCGTCTCCTGTTGCTGTCAACGAGCAAGAGAG	CTCTCTTGCTCGTTGACAGCAACAGGAGACGAC
PIF3 T269A	CCTAATATCAGAGGACGCATGTAGAAAAGACC	GGTCTTTTCTACATGCGTCCTCTGATATTAGG
PIF1-7M	GCAGGGTGGCTGCTGCTAAGGAAGCTGAGG	CCTCAGCTTCCTTAGCAGCAGCCACCCTGC
PIF1-3D	TCGAGCAGGGTGGATGATGATAAGGAATCTGAG	CTCAGATTCCTTATCATCATCCACCCTGCTCGA

Chapter 5: Future directions

Role of Casein Kinase 2 in light signaling in Arabidopsis

Phosphorylation of the molecular players is one important regulatory mechanism in the red/far red light signaling pathway. The phosphorylation of PIFs upon interaction with light-activated phytochromes is a key event required for rapid degradation of PIFs at the onset of photomorphogenesis (Al-Sady *et al.*, 2006; Shen *et al.*, 2007, 2008*b*). Several efforts have been made in the past to identify the light-regulated kinase(s) that is responsible for this rapid phosphorylation. We found that three PIF proteins (PIF1, PIF3 and PIF5) are phosphorylated by CK2 in the *in vitro* kinase assay (Fig. 4.1). Recent studies indicate that PIFs are phosphorylated even in the dark (Ni *et al.*, 2013). It is likely that phosphorylation does not merely act as a marker for degradation of PIFs but also controls other functions/activity/interaction with other proteins. Apart from functioning as a transcriptional repressor in the dark, PIFs also seem to regulate the stability of transcription factors such as HY5 to suppress photomorphogenesis in the dark (Xu *et al.*, 2014). We find that a phospho-deficient PIF mutant shows reduced dimerization capability in the yeast two hybrid assay (Fig. 4.3A). Thus, CK2 phosphorylation of PIFs might be a mechanism to fine tune their functions in addition to rapid degradation.

CK2 is a ubiquitous kinase and is not known to be light regulated. Also, it does not seem to phosphorylate all the PIF family proteins. Hence, the search for a light regulated kinase still continues. Since phosphorylation is essential to the survival of the seedling, it is plausible to have a high level of functional redundancy at this step. Several kinases may be actively phosphorylating PIFs in the dark as well as in the light. When in light, interaction with active Phytochrome certain residues in the PIF proteins may become more accessible and prone to phosphorylation than in the dark. Efforts to co-crystallize the Phytochrome-PIF complex can provide the necessary evidence to support

this hypothesis. Also, *in vivo* pull-down of active Phytochrome-PIF complexes before degradation, followed by mass spectrometry analysis can furnish a more accurate estimate of the light-specific phosphorylation sites in PIFs. This may provide clues to identify the light regulated kinase. Overall, our understanding of role of kinases in degrading PIFs in light signaling is still very limited due to the functional redundancy, which seems to be crucial for this important developmental event.

Role of Casein Kinase 2 in control of flowering time in Arabidopsis

One of the previously unreported biological functions for CK2 alpha subunits is their involvement in regulating flowering time. The CK2 alpha triple mutant flowered much later than the wild type under both long- and short-day conditions, while the single and double mutants flowered similarly to wild type (Fig. 2.2). The triple mutant responded to both exogenous GA treatment as well as prolonged vernalization, suggesting that the alpha subunit triple mutants are not defective in these pathways (Fig. 2.11, 2.12). The triple mutant also showed reduced expression of the floral integrators, *FT* and *SOC1*, and higher expression of *FLC* compared to wild type under both long- and short-day conditions (Fig. 2.3). The reduced expression of *FT* and *SOC1* under long day can be partly explained by a reduced expression of *CO,* suggesting a defect in the photoperiod pathway. This is supported by another recent report that showed CK2 alpha triple mutant is also defective in the circadian clock. However, the higher expression of *FLC* resulting in reduced expression of *FT* and *SOC1* under both long- and short-day conditions is a hallmark characteristic for the autonomous pathway that regulates flowering time. The results from the α4 RNAi indicate that this subunit shows functional redundancy with the nuclear alpha subunits and is involved in regulating flowering time

in spite of being chloroplast localized. Taken together, these data suggest that CK2 alpha subunits regulate flowering time through both photoperiod and autonomous pathways.

The involvement of CK2 in regulating circadian clock and photoperiod pathway is better understood. Both alpha and beta subunits regulate the circadian clock by phosphorylating the central clock components, CCA1 and LHY (Sugano *et al.*, 1998, 1999; Daniel *et al.*, 2004). Also, over-expression of two of the CK2 regulatory subunits (CKB3 and CKB4) in Arabidopsis leads to defects in the circadian clock and altered flowering under both long- and short-day conditions (Sugano *et al.*, 1999; Perales *et al.*, 2006). The CKB4 level is also regulated post-translationally by the circadian clock resulting in altered flowering time (Perales *et al.*, 2006).

Despite the above progress on involvement of CK2 in regulation of circadian clock and photoperiod pathway, how CK2 subunits control the *FLC* expression through the autonomous pathway remains unknown. The autonomous pathway in Arabidopsis consists of internal signals that regulate floral induction (Amasino, 2010). The genes implicated in this pathway are known to act by repressing the *FLC* levels; however, the underlying molecular mechanisms are not completely elucidated. Of those, FPA, FCA and FLK contain an RNA-binding domain, while FLD is a histone demethylase and LD is a homeodomain protein that might bind to both DNA and RNA (Simpson, 2004; Michaels, 2009). These genes are not known to be regulated at the level of transcription and are preferentially expressed in shoot and root apical regions (Simpson, 2004). One possibility is that CK2 phosphorylates any or all of these autonomous pathway components, and regulates their activity. Interestingly, three different phosphorylation site prediction programs (Scansite 2.0, NetPhosK 1.0 and KinasePhos 2.0) showed that all five proteins have putative CK2 phosphorylation sites (data not shown). Since phosphorylation by CK2 has been shown to alter protein function as well as stability, it is

possible that these proteins are substrates of CK2 and are regulated post-translationally. Regulation of the activity and/or stability of the autonomous pathway components by CK2 mediated phosphorylation may explain the higher expression of *FLC* in the CK2 alpha triple mutant compared to wild type, resulting in delayed flowering under both long- and short-day conditions.

In summary, our data and those of others show involvement of CK2 in multiple pathways in plants. Until 2003, more than 300 CK2 substrates have been identified in animal system, and more are predicted to be identified (Litchfield, 2003). In plants, the number of CK2 substrates is still less than 50 (Riera *et al.*, 2001*b*; Mulekar and Huq, 2014). Given the range of physiological processes CK2 has been shown to affect, there is a high probability of finding new substrates, which will improve our current understanding of CK2's diverse roles in plant growth and development.

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