UNIVERSITY OF CALIFORNIA, SAN DIEGO

Higher Order Calcium-Dependent Protein Kinases (CPKs) Mutant Lines Elucidate Roles of CPKs within Abscisic Acid Signal Transduction in *Arabidopsis*

and

In Vivo Interactions of Calcium-Dependent Protein Kinases, CPK6 and CPK23, with PP2C Protein Phosphatases within Abscisic Acid Signaling

A Thesis submitted in partial satisfaction of the requirements
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by

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ABSTRACT OF THE THESIS

Higher Order Calcium-Dependent Protein Kinases (CPKs) Mutant Lines Elucidate Roles of CPKs within Abscisic Acid Signal Transduction in *Arabidopsis* and *In Vivo* Interactions of Ca^{2+}-Dependent Protein Kinases, CPK6 and CPK23, with PP2C Protein Phosphatases within Abscisic Acid Signaling

by

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Stomata, present in the aerial epidermis of land plants, provide gateways for regulating carbon dioxide and water exchange between plants and the atmosphere. The

stress-induced phytohormone abscisic acid (ABA) reduces transpirational water loss crucial for the fitness of plants by inducing stomatal closure. Cytosolic Ca^{2+} has been reported to play a major role in ABA-induced stomatal closure through Ca^{2+} sensors, such as Calcium Dependent Protein Kinases (CPKs). Plants lacking transcripts of *CPK3* and *CPK6* (*cpk3cpk6*) have shown impaired ABA- and Ca^{2+}-induced stomatal closing. Double knockout mutants of CPK4 and CPK11 (*cpk4cpk11*) exhibit similar ABA-hyposensitive phenotypes in ABA-induced stomatal closing response as well as a reduced ABA response in seed germination and seedling growth. Single mutant *cpk23*, however, demonstrated increased sensitivity to ABA, indicating a possible negative regulatory role in ABA signaling for CPK23. To improve the understanding of these proteins and their functions, two quadruple mutant plant lines, *cpk3cpk4cpk6cpk11* and *cpk5cpk6cpk11cpk23*, were established. In Chapter 1, the quadruple mutant lines are phenotypically characterized by examining a variety of ABA-dependent biological responses.

Other proteins important in ABA-dependent stomatal responses are Protein Phosphatases 2C (PP2Cs). Group A PP2Cs are known negative regulators within the Ca^{2+}-independent ABA signaling pathway; however, experimental evidence suggests other regulatory roles exist for PP2Cs within the Ca^{2+}-dependent branch of this signaling network which includes CPKs. In Chapter 2, yeast two-hybrid system is used to evaluate whether PP2Cs interact with CPKs. Observed interactions can provide additional insight to these proteins' functions within the Ca^{2+}-dependent ABA signaling.

I.

Higher Order Calcium-Dependent Protein Kinases (CPKs) Mutant Lines Elucidate Roles of CPKs within Abscisic Acid Signal Transduction in *Arabidopsis*

1.1. Abstract

Plants are subjected to a variety of abiotic stresses, including drought, salinity, flooding, extreme temperatures, and extreme atmospheric carbon dioxide levels. To respond to these environmental changes, the endogenous hormone abscisic acid (ABA) is produced and released within the plant, where it plays a major role in different mechanisms involved in abiotic stress tolerance responses. A model of ABA signal transduction has been proposed in guard cells; however, many key regulatory features within ABA-induced signaling leading to stomatal closure remain unclear. Also, the role of Ca^{2+} as second messenger in ABA-dependent developmental responses such as seed germination and seedling establishment remains uncertain. Previous studies have indicated that Ca^{2+}-Dependent Protein Kinases (CPKs) CPK3, CPK4, CPK6 and CPK11 are associated with ABA-induced closing of stomata and that a disruption in these genes gave rise to an ABA-hyposensitive phenotype. Double mutants *cpk3cpk6* and *cpk4cpk11* in *Arabidopsis thaliana* resulted in a more pronounced lack of response to ABA than single mutants, adding to the rationale that CPKs' actions are of synergistic nature. Surprisingly, a loss-of-function mutation for CPK23 showed an ABA-hypersensitive phenotype. Determining specific biological roles for CPKs have been difficult due to redundancies and overlapping CPK functions. Therefore, two different quadruple mutant lines *cpk3cpk4cpk6cpk11* and *cpk5cpk6cpk11cpk23* have been isolated and were tested for their responses to ABA further helping to understand the roles of CPKs within ABA-dependent stress adaption mechanisms. Seed germination rates and stomatal closing in response to ABA indicate that actions of these proteins occur in a synergistic manner.

1.2. Introduction

Plants have developed physiological and biochemical mechanisms to counteract extreme environmental changes, which are generally referred to as abiotic stresses. However, despite attempts to promote adaptation and survival in crop plants, abiotic stresses cause major declines in crop yield, especially drought and salt stresses, which are recognized as two of the most serious threats to crop production (Boyer, 1983; Epstein et al., 1980; Ingram and Bartels, 1996).

Water transpiration and gas exchange in plants are facilitated by specialized cells called guard cells, which are located in the epidermis of the aerial parts of plants and modulate the opening and closing of pores known as stomata. Open stomata allow for the uptake of carbon dioxide (CO_2) from the atmosphere, but result in water loss through transpiration. Stomatal movements are achieved by altering the content of osmotically active substances in the guard cells which is mediated by the regulation of ion channels and proton pumps in response to various stimuli, including carbon dioxide concentrations, water status, and plant hormones including abscisic acid (ABA) (Kim et al., 2010; Pandey et al., 2007; Raschke et al., 1988). During drought stress, for example, the stomata close to conserve water. The second messenger, free cytosolic calcium (Ca^{2+}), plays a major role in the regulation of these ion channels and proton pumps, such that an increase in $[Ca^{2+}]_{cyt}$ induces stomatal closure (Siegel et al., 2009) and prevents light-induced stomatal opening (Garcia-Mata et al., 2007).

Besides triggering stomatal closure, the phytohormone ABA is essential for many crucial processes within plants such as regulating seed germination, root development, flowering, and seed maturation (Acharya et al, 2009; Himmelbach et al., 1998; Kim et al.,

2010; Santner et al., 2009). Studies have shown that ABA promotes seed dormancy and root growth inhibition during abiotic stress (Deak and Malamy, 2005; Rodriguez-Gacio et al., 2009). Furthermore, the increase in ABA synthesis triggered by drought and high salinity stress stimulating stomatal closure protects plants from dehydration (Wilkinson and Davies, 2002). Proteins that regulate stomatal closure in the presence of ABA include pyrobactin resistance (PYR) or regulatory components of ABA receptor (RCAR) proteins, negative regulators class A Protein Phosphatases 2Cs (PP2Cs), subclass III Snf1-Related Protein Kinase 2 (SnRK2s) and Slow Anion Channel Associated 1 (SLAC1) (reviewed in Kim et al., 2010) (**Figure 1**). In brief, in the presence of ABA, PP2C phosphatases are inhibited leading to the activation of SnRK2 protein kinases, which, in turn, phosphorylate and activate SLAC1 ultimately leading to stomatal closure, representing the Ca^{2+}-independent branch of this ABA signaling pathway (Kim et al., 2010; **Figure 1**).

A Ca^{2+}-dependent pathway also exists in parallel to the Ca^{2+}-independent ABA signal transduction previously described (**Figure 1**). Recent studies have suggested crosstalk between these two pathways, indicating that they do not function independently of each other (reviewed in Laanements et al., 2013); however, additional investigation is necessary to fully characterize this crosstalk. In the presence of ABA, intracellular Ca^{2+} levels are elevated in guard cells and function as an important second messenger in ABA signaling (De Silva et al., 1985; Grabov et al., 1998; Hamilton et al., 2000; Webb et al., 2001). Besides others, Calcium Dependent Protein Kinases (CPKs) are thought to play a major role in Ca^{2+}-dependent ABA signaling mechanisms in guard cells (reviewed in Laanements et al., 2013). CPKs are serine/threonine kinases (Harper et al., 1994) and are predicted to be encoded by 34 genes in *Arabidopsis thaliana* (Boudsocq et al., 2013).

CPKs have been studied extensively and many have been identified as key regulators in a variety of signaling pathways involved in plant growth and development as well as homeostatic maintenance (Boudsocq et al., 2013). These protein kinases act as cytosolic Ca^{2+} sensors and upon calcium binding, most CPKs are activated (Boudsocq et al., 2012), subsequently phosphorylating downstream targets, such as SLAC1 in guard cells (Geiger et al., 2010; Scherzer et al., 2012; Brandt et al., 2012). Mutants in the *Arabidopsis CPK4* and *CPK11* genes displayed lower expression levels of *ABF1, ABF2, ABI4, ABI5, RD29A, RAB18, KIN, KIN2* and *ERD10* upon ABA treatment (Zhu et al., 2007), suggesting the involvement in gene expression regulation. When compared to wild-type plants, the single mutants *cpk4* and *cpk11* also demonstrated an ABA-hyposensitivity and a salt hypersensitivity in seed germination and root growth experiments (Zhu et al., 2007). Furthermore, stomatal closure was inhibited in the loss-of-function single mutant plants *cpk4* and *cpk11*, which correlates with the ABA hyposensitive phenotype exhibited in other tests (Zhu et al., 2007). The *cpk4cpk11* double mutant showed an even weaker response to ABA than the single mutants as seen in the expression levels of ABA-inducible genes, seed germination and lateral root growth rates, and stomatal movement (Zhu et al., 2007). These data suggest that these CPKs act in a synergistic manner in signaling pathways potentially through overlapping functions (Zhu et al., 2007). The ABA-hyposensitive phenotype in plants with disrupted *CPK4* and *CPK11* genes supports the indication that these two CPKs are positive regulators of the ABA signaling pathway.

Other known positive regulators of ABA signal transduction are CPK3 and CPK6 in *Arabidopsis*. CPK3 and CPK6 are expressed in guard cells and mesophyll cells, and

when these genes are disrupted, an ABA-hyposensitive phenotype is observed (Mori et al., 2006). ABA activation of S-type anion currents mediated by SLAC1 and ABA regulation of plasma membrane Ca^{2+}-permeable (I_{Ca}) channels were impaired in *cpk3cpk6* double mutant lines in patch clamp experiments (Mori et al., 2006). As mentioned above, CPK6 and CPK3 have been shown to phosphorylate and activate SLAC1 (Brandt et al., 2013; Scherzer et al., 2012) in line with the impaired S-type anion current activation seen for single and double *cpk3cpk6* mutants. It is important to note that activation of R-type anion channels, which are also regulated by ABA, was not affected in the double mutant lines (Mori et al., 2006). This is consistent with the reduced sensitivity to ABA observed in downstream responses and not an absolute ABA-insensitivity in these mutants. Also, CPK21, CPK3, and CPK23 have been reported to activate SLAC1 further explaining why *cpk3cpk6* double mutant plants do not exhibit complete insensitivity in stomatal closure responses (Geiger et al., 2010; Scherzer et al., 2012; Mori et al., 2006). As mentioned above, CPK3 and CPK6 are SLAC1 activators, which raise the question of the phenotype of the knockout mutants in ABA dependent stomatal closure responses. Measurements of *cpk3* and *cpk6* stomata treated with ABA showed decreased closure in these loss-of-function mutant lines similar to that of plants with *CPK4* and *CPK11* knockouts (Mori et al., 2006; Zhu et al., 2007). Double *cpk3cpk6* mutants showed even stronger phenotypes compared to the single mutants (Mori et al., 2006). However, *cpk3* and *cpk6* do not display the same ABA-hyposensitive phenotype in seed germination and seedling growth assays as *cpk4* and *cpk11* (Mori et al., 2006). Similar to CPK3, CPK4, CPK6, and CPK11, CPK10 has also been reported to be involved in plant responses to drought stress via ABA- and Ca^{2+}-mediated stomatal movements. Disruption of the *CPK10* gene in the

cpk10 mutants resulted in impaired stomatal closure when ABA- and Ca^{2+}-induced (Zou et al., 2010).

Unlike the CPKs previously discussed, *cpk23* knockout mutants exhibit an ABA-hypersensitive phenotype when subjected to drought conditions and salt stress (Ma and Wu, 2007), suggesting that CPK23 acts as a negative regulator in stress signaling. In stomatal movement experiments, the *cpk23* mutant line showed increased stress tolerance as indicated by stronger stomatal closure when subjected to darkness compared to wild-type plants (Ma and Wu, 2007). The increased sensitivity to ABA in the *cpk23* mutant plants could be explained by an upregulation of other CPK genes to compensate for the loss of *CPK23* (Franz et al., 2011). This rationalization was proposed when increased expression levels of other *CPK* genes were seen in the *cpk21* knockout mutant line, which is the closest homolog of CPK23 (Franz et al., 2011). *cpk21* also demonstrated comparable hypersensitivity to ABA as that exhibited by *cpk23* (Franz et al., 2011). In addition to CPK21 and CPK23, CPK12 also demonstrates possible negative regulatory roles in seed germination and post-germination growth as seen by ABA-hypersensitive phenotypes in knockout mutant *cpk12* (Zhao et al., 2011).

CPKs CPK3, CPK4, CPK6, CPK11, CPK5 and more play fundamental roles in plant innate immune signaling and defense mechanisms against pathogen attack or biotic stress (Kim et al., 2011). Although biotic and abiotic stress signaling has been studied independently of each other, studies reveal that a crosstalk between these two pathways does in fact exist (Klusener et al., 2002). For example, the small molecule [5-3,4-dichlorophenyl) furan-2-yl]-piperidine-1-ylmethanethione (DFPM) has been found to activate a plant immune response pathway, which in turn negatively regulates ABA

signal transduction either downstream of ABA-activated SnRK2s or at the level of Ca^{2+} signaling (Kim et al., 2011).

Due to the redundancy of the CPK genes within ABA signaling, it has been difficult to distinguish a specific biological role for each CPK. By knocking out more than one *CPK* gene to eliminate possible overlap in CPK functions, it might be possible to observe which CPKs act synergistically. For instance, studies with *cpk3cpk6* and *cpk4cpk11* double mutant lines have shown enhanced ABA-hyposensitivity in guard cells compared to individual mutants, which confirms the idea that the actions of these protein kinases have an additive effect (Mori et al., 2006; Zhu et al., 2007).

To further elucidate the role of CPKs in guard cell ABA signaling as well as in other ABA responses, homozygous quadruple mutant lines *cpk3cpk4cpk6cpk11* and *cpk5cpk6cpk11cpk23* have been established by crossing *cpk3cpk6* with *cpk4cpk11* and *cpk5cpk6cpk11* with *cpk23*, respectively. While *cpk4cpk11* double mutants have been reported to have reduced sensitivity to ABA in seed germination and root growth, very little is known about global ABA responses in other CPK mutant backgrounds. The objective of this study was to determine ABA-dependent phenotypes in stomatal movement, seed germination, and root growth in higher order CPK mutant lines to further understand the importance of CPKs and therefore, roles of calcium in plant stress signaling.

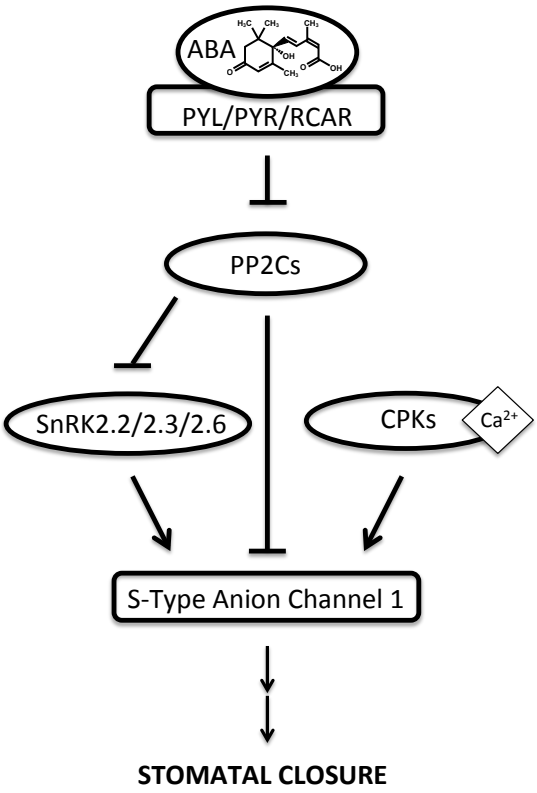

Figure 1: Proposed model for abiotic stress-activated ABA signaling pathway in guard cells.

1.3. Results

1.3.1. Identification of Quadruple T-DNA Insertion Mutants cpk3/4/611 and cpk5/6/11/23

Prior to establishing quadruple mutant lines, genomic DNA of *Arabidopsis* plants *cpk3-2cpk6-1* (short *cpk3/6*), *cpk4-2cpk11-2* (short *cpk4/11*), *cpk5cpk6-3cpk11-2* (short *cpk5/6/11*), and *cpk23* (short *cpk23*) was extracted and the T-DNA insertion sites (**Figure 2A**) for the mutants were confirmed by PCR analysis. Primers specific to the T-DNA insertion sites and *CPK* genes are listed in **Table 2** (see Chapter 1.5.3.). T-DNA insertions disrupt the expression of the *CPK* genes of interest and ecotype Columbia (Col0) is the genetic background for all mutants. After all T-DNA insertions were confirmed, flowering *cpk3/6* plants were crossed with *cpk4/11* and *cpk5/6/11* were crossed with *cpk23* (**Figure 3**). The F1 generation was confirmed to be heterozygous for all *CPK* genes, signifying a successful cross. The F1 plants were left to self-fertilize and the F2 generation was screened for homozygosity for all mutant genes of interest using PCR analysis. The screening process that led to identifying the homozygous quadruple mutant lines is outlined in **Figure 3**. Reverse Transcription-Polymerase Chain Reactions or RT-PCR analyses of mutant lines *cpk3/4/6/11* and *cpk5/6/11/23* revealed no detectable mRNA transcript levels of the *CPK* genes, confirming that in the two quadruple mutant lines, all *CPK* genes were indeed knocked out (**Figure 2B**).

Table 1: T-DNA names and AGI number to corresponding mutant lines.

Gene	Mutant	T-DNA Name	AGI Number
CPK3	*cpk3-2*	SALK_022862	At4g23650
CPK4	*cpk4-2*	SALK_000685	At4g09570
CPK5	*cpk5*	SAIL_657C06	At4g35310
CPK6	*cpk6-1*	SALK_093308	At2g17290
	cpk6-3	SALK_025460	At2g17290
CPK11	*cpk11-2*	SALK_054495	At1g35670
CPK23	*cpk23*	SALK_007958	At4g04740

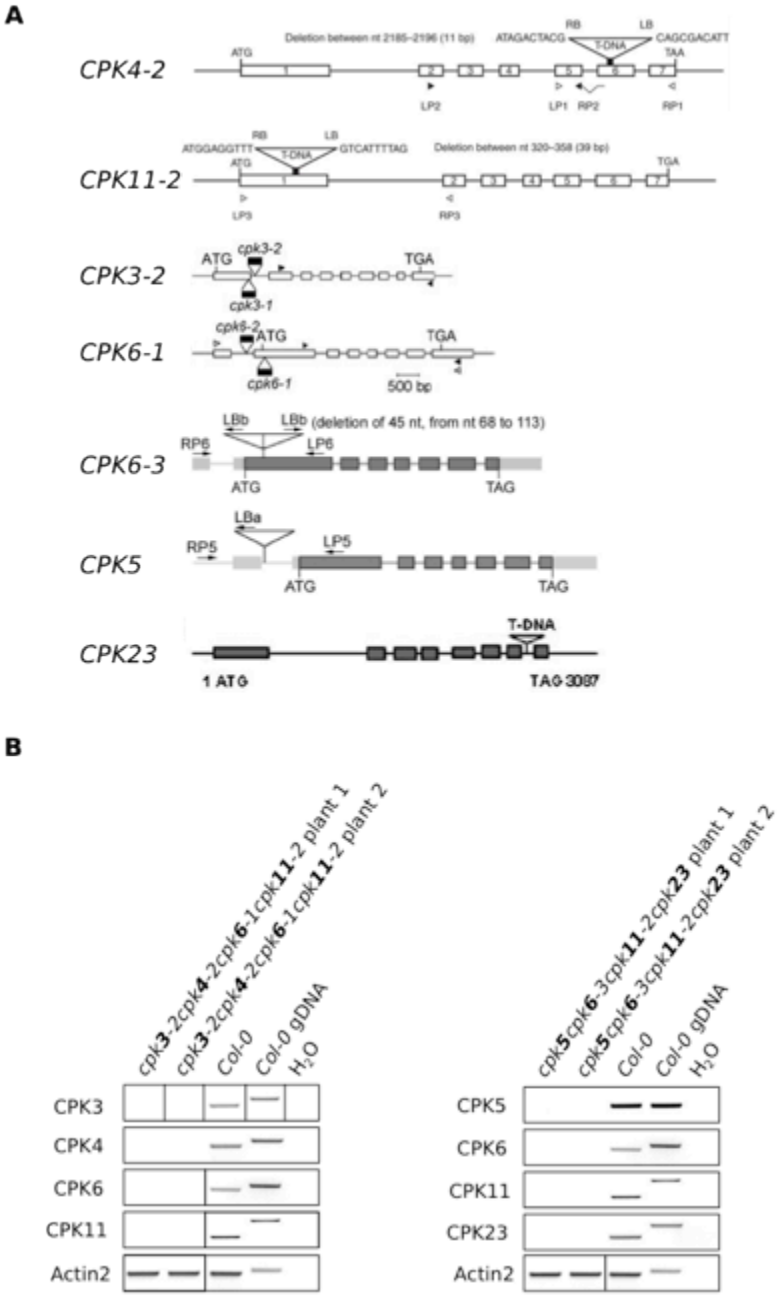

Figure 2: Analysis of T-DNA insertion mutants. A) Mapped T-DNA insertion sites for *cpk4-2, cpk11-2*, *cpk3-2*, *cpk6-1, cpk6-3, cpk5,* and *cpk23* (Images of T-DNA insertion sites: *CPK4-2* and *CPK11-2* from Hubbard et al., 2012; *CPK3-2* and *CPK6-1* from Mori et al., 2006; *CPK6-3* and *CPK5* from Boudsocq et al., 2010; *CPK23* from Ma and Wu, 2007). Lines and boxes represent introns and exons, respectively. ATG represents the start codon and TAA, TAG, or TGA represents the stop codon. Positions of arrows indicate the location of the primers used to identify the mutants. (**B**) *CPK* expression in higher order mutants. RT-PCR analyses (provided by Brandt, unpublished) confirmed that the *cpk* alleles of interest were disrupted.

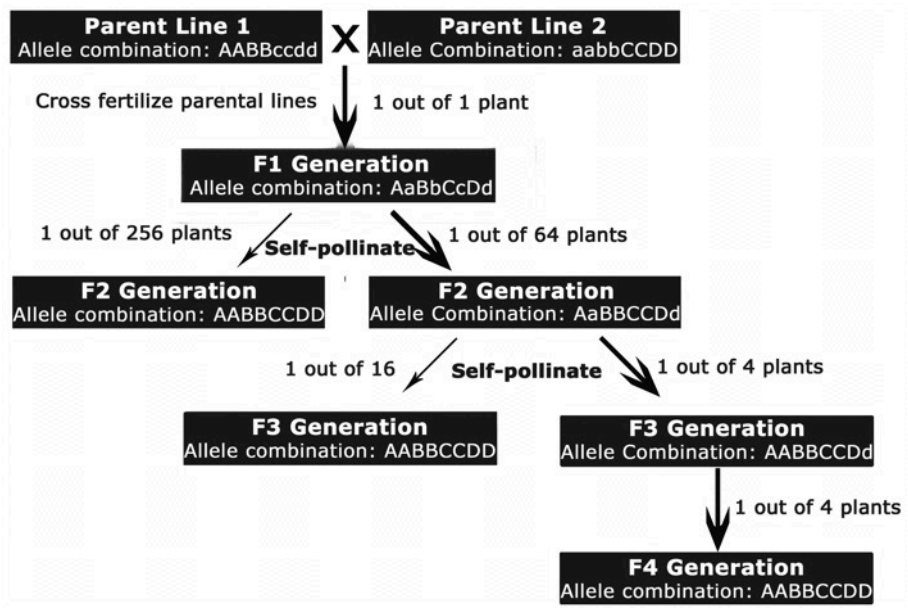

Figure 3: Schematic of genotyping up to 4 filial generations with probability of finding quadruple homozygous mutant genes included. Bolded arrows indicate path chosen when isolating the quadruple mutant. Upper case letters stand for mutant alleles and lowercase letters stand for wild-type alleles, such that AABBCCDD represents a quadruple mutant.

1.3.2. ABA-induced stomatal responses in cpk3/4/6/11 and cpk5/6/11/23

Two different methods were used to test stomatal response to ABA in mutant lines *cpk3/4/6/11* and *cpk5/6/11/23*. The first method involved tracking individual stomata over a time course, using epidermal peals of rosette leaves on a glass slide (Hubbard et al., 2012). However, wild-type (WT) plants showed little to no response to ABA after several trials and variability of stomatal aperture width was broad for all lines tested (**Figure 4A-B**). Reasons for why this method was ineffective could include inconsistent amount of adhesive used or varying pressure used to place the leaves on the glass slide.

Another method was employed (as described in Materials and Methods) and was revealed to be more successful for this particular test. Since ABA was dissolved in ethanol, leaves subjected to 10 μM ABA treatment was compared to those with addition of ethanol alone (mock treatment), which acted as the solvent control and the baseline for aperture measurements. A single experiment was performed double blind with genotype and treatment unknown and was conducted in triplicate within the same day, where 35-45 stomata were measured for each genotype. Prior to measurements, leaves were bathed in a buffer containing 5 mM KCl, 50 μM $CaCl_2$, 10 mM MES at a pH 5.6 adjusted with Tris base (opening buffer) and incubated under light to promote stomatal opening. After a 2-hour incubation, treatment was added to the buffer and the leaves were left to incubate for an additional hour before finally measuring stomatal opening length. A total of 3 experiments have been completed with similar results.

Wild-type or *Col0* plants showed ~60% stomatal closure upon ABA addition compared to those treated with ethanol. Parental lines *cpk3/6* and *cpk4/11* displayed

lower sensitivity to ABA compared to WT in the stomatal closing response, similar to previous findings (Mori et al., 2006; Zhu et al., 2007. Additionally, quadruple mutant lines *cpk3/4/6/11* showed an even less sensitive phenotype in comparison with the parental lines *cpk3/6* and *cpk4/11* as determined by an observed stomatal closing of ~21% compared to ~35-40% in response to ABA, respectively (**Figure 5A-B**). Aperture measurement data for *cpk5/6/11/23* and parental lines *cpk5/6/11* and *cpk23* showed similar stomatal response to ABA. Average percentage of aperture closure in *cpk5/6/11/23* was ~3.5% compared to ~32% closure in parental lines (**Figure 5C-D)**. These observations are consistent with a suggested stronger alteration in ABA sensitivity in stomata as more *CPK* genes are disrupted.

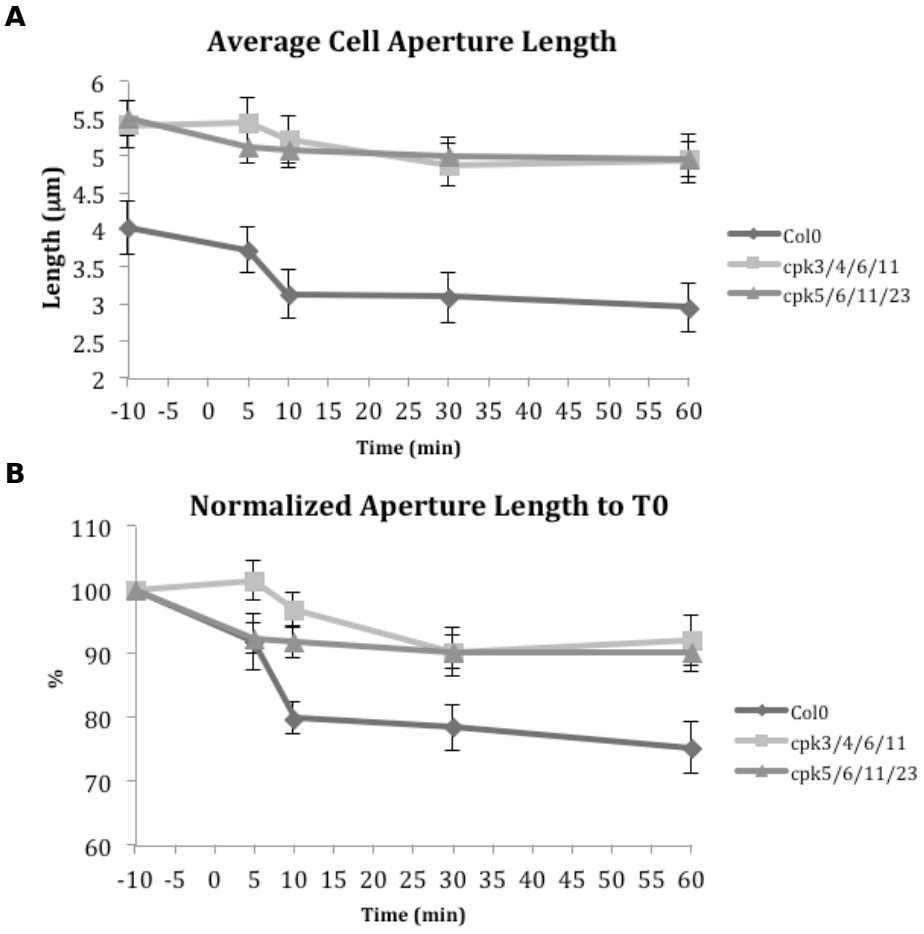

Figure 4: ABA-induced stomatal closing measurements by tracking individual stomata. Prepared slides were incubated in opening buffer and light for 2 hours prior to assay. **A**) Average aperture length of *Col0*, *cpk3/4/6/11*, and *cpk5/6/11/23* in the presence of 10 μM ABA. At least 15 individual stomata were tracked for each genotype. 10 μM ABA was added at T=0 min. **B**) Normalized average aperture lengths to measured lengths at T=0 min. This experiment was repeated three times with similar results. Data shown above represents a single experiment. Error bars depict means ± SE. (n=15-16)

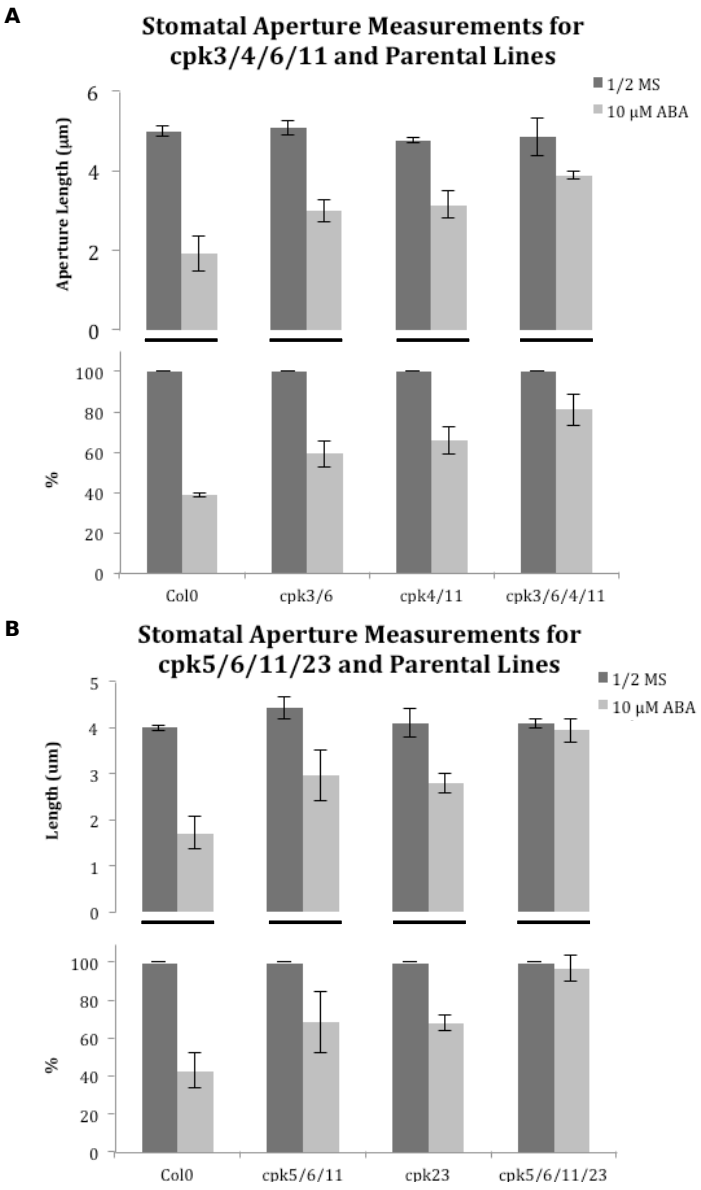

Figure 5: ABA-induced stomatal closure experiments with quadruple mutants and respective parental lines (Blending method). Rosetta leaves were pre-incubated in opening buffer with light for 2 hours prior to treatment. Subsequently, leaves were subjected to either ethanol (solvent control) or 10 µM ABA 1 hour before measuring the aperture length. (**A**) Averaged aperture widths for *Col0*, *cpk3/6*, *cpk4/11*, and *cpk3/4/6/11*. (**B**) Averaged aperture measurements for *Col0*, *cpk5/6/11*, *cpk23*, and *cpk5/6/11/23*. Upper panels (A and B) show raw aperture lengths and lower panels show normalized closing responses. To normalize data, stomatal aperture relative to the mock treatment is plotted. Data shown represents a single experiment (3 technical replicates per experiment). Error bars depict means ± SE. (n=3 with 35 to 45 stomata analyzed per condition). This experiment has been repeated 3 times with similar results.

1.3.3. Effects of ABA in seed germination in disrupted cpk3/4/6/11 and cpk5/6/11/23 plants

Germination rates of seeds of both quadruple mutant lines and their respective parental were comparable to WT seeds when plated on medium without ABA (**Figure 6A** and **7A**). Once 2 μM ABA was added to the medium, however, a dramatic decrease in radicle emergence and cotyledon expansion was evident in all genotypes tested (**Figure 6A** and **7A**). In addition, when subjected to 2 μm ABA, sprouting of cotyledons in all genotypes displayed an abnormal coloration or loss compared to seeds germinated without ABA (**Figures 6A** and **7A**). Signs of chlorosis due to ABA have not been reported in previous studies of loss-of-function *CPK* mutants; although, chlorosis has been shown to occur in seedling growth of single mutants *cpk4* and *cpk11* and double mutant *cpk4/cpk11* under salt stress conditions (Zhu et al., 2007).

Both radicle and cotyledon emergence were monitored for a total of 7 days after plating on ½ MS plates with or without 0.8 or 2 μm ABA. Ethanol was added in plates without ABA as a control. The genotype of the seeds was unknown in the experiments. A total of 3 experiments were performed yielding similar results. Within each experiment, seed germination analyses were made for each genotype three times. Seeds with a known ABA-insensitive phenotype, *abi1-1* (for ABA-Insensitive 1), were used as the positive control (Gosti et al., 1999).

When examining the number of radicles and cotyledons counted on the third day post-plating, it appears that the quadruple mutant *cpk3/4/6/11* exhibits a decreased sensitivity to ABA in seed germination rates compared to parental lines *cpk3/6* and *cpk4/11* (**Figure 6B**, upper panel)**.** Seed germination for *cpk5/6/11/23*, however,

illustrates a more ambiguous response to ABA. While the mutants *cpk5/6/11* and *cpk23* show a decreased ABA response, this could not be seen for *cpk5/6/11/23* mutant plants (**Figure 7B**, upper panel); thus, conclusions regarding a possible additive effect in *CPK* functions cannot be made here.

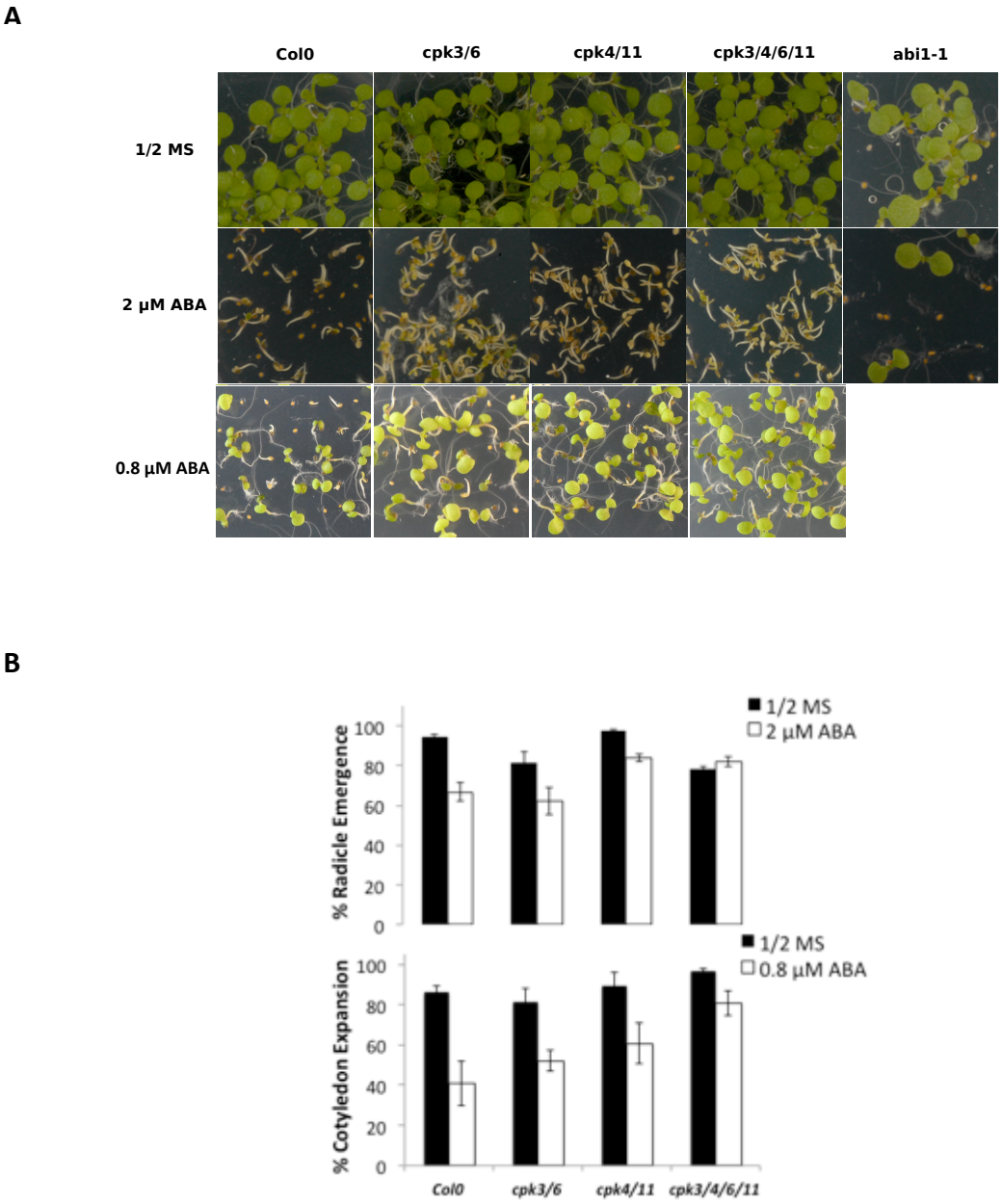

Figure 6: Seed germination analyses in loss-of-function mutants *cpk3/4/6/11*, *cpk3/6*, and *cpk4/11*. Number of radicles and cotyledon appearance was counted for every 24h period for 7 days following stratification of 72 hrs without light. Seeds were plated in triplicate and assay was performed with genotype unknown. (**A**) Post-germinational growth on ½ MS alone or with 0.8 or 2 µm ABA on the 6th day following the stratification period. (**B**) Quantification of radicle emergence (top panel) and cotyledon expansion (bottom panel) grown on media ± ABA on the 3rd day and 4th day, respectively, after transferring the plates into the growth chamber. Error bars depict mean ± SE (n=3 with at least 50 seeds per genotype and treatment).

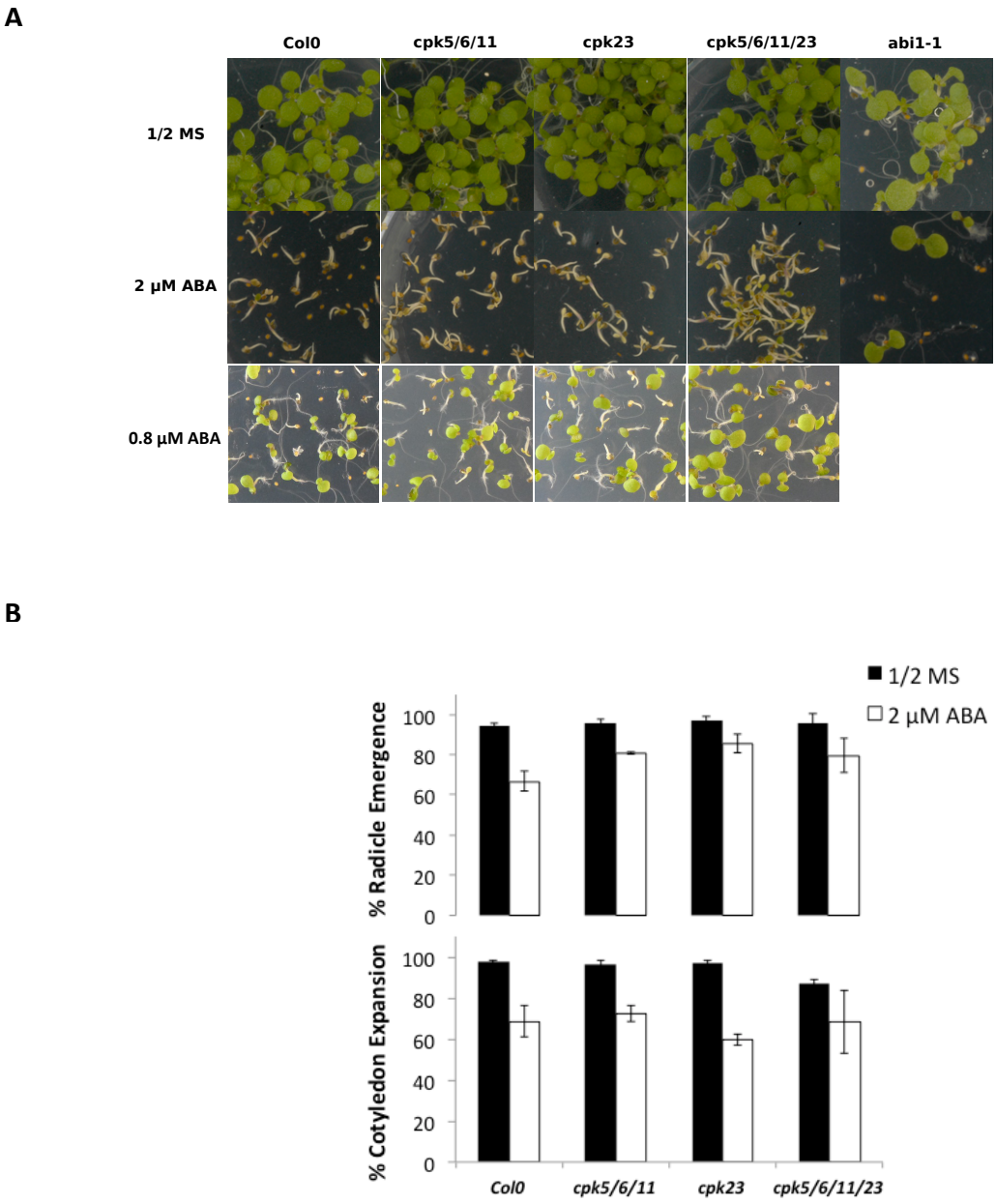

Figure 7: Seed germination analyses in loss-of-function mutants *cpk5/6/11/23*, *cpk5/6/11*, and *cpk23*. Number of radicles and cotyledon appearance was counted for every 24h period for 7 days following stratification of 72 hrs without light. Seeds were plated in triplicate and assay was performed with genotype unknown. (**A**) Post-germinational growth on ½ MS alone or with 0.8 or 2 µm ABA on the 6th day following the stratification period. (**B**) Quantification of radicle emergence (top panel) and cotyledon expansion (bottom panel) grown on media ± ABA on the 3rd day and 4th day, respectively, after transferring the plates into the growth chamber. Error bars depict mean ± SE (n=3 with at least 50 seeds per genotype and treatment).

1.3.4. Effects of ABA in root growth in cpk3/4/6/11 and cpk5/6/11/23 mutants

ABA is known to inhibit root growth in plants; however, the molecular mechanism involved in this response is poorly understood. Previous studies have shown that loss-of-function single mutants *CPK4* and *CPK11* have increased seedling growth compared to that of WT seedlings in ABA, which is consistent with their ABA-hyposensitive phenotype. Double mutants *cpk4/11* demonstrated even less response to ABA in seedling growth measurements (Zhu et al., 2007). While root growth effects due to ABA has yet to be determined in *CPK* mutants, it is worthy to explore this effect in the quadruple *CPK* mutant lines *cpk3/4/6/11* and *cpk5/6/11/23*.

Quadruple mutant lines along with their parental lines and a WT control were grown on ½ MS medium for one week before they were transferred to medium supplemented with or without 10 μM ABA. The blue marks indicate the initial root length when transferred to (**Figure 8-9A)**. The seedlings were grown vertically in order to assess root lengths. Root lengths are measured in centimeters (cm).

After repeating the experiment three times, no interpretable or clear differences in root lengths among wild-type, parental lines, and both quadruple mutant lines were apparent (**Figure 8-9)**.

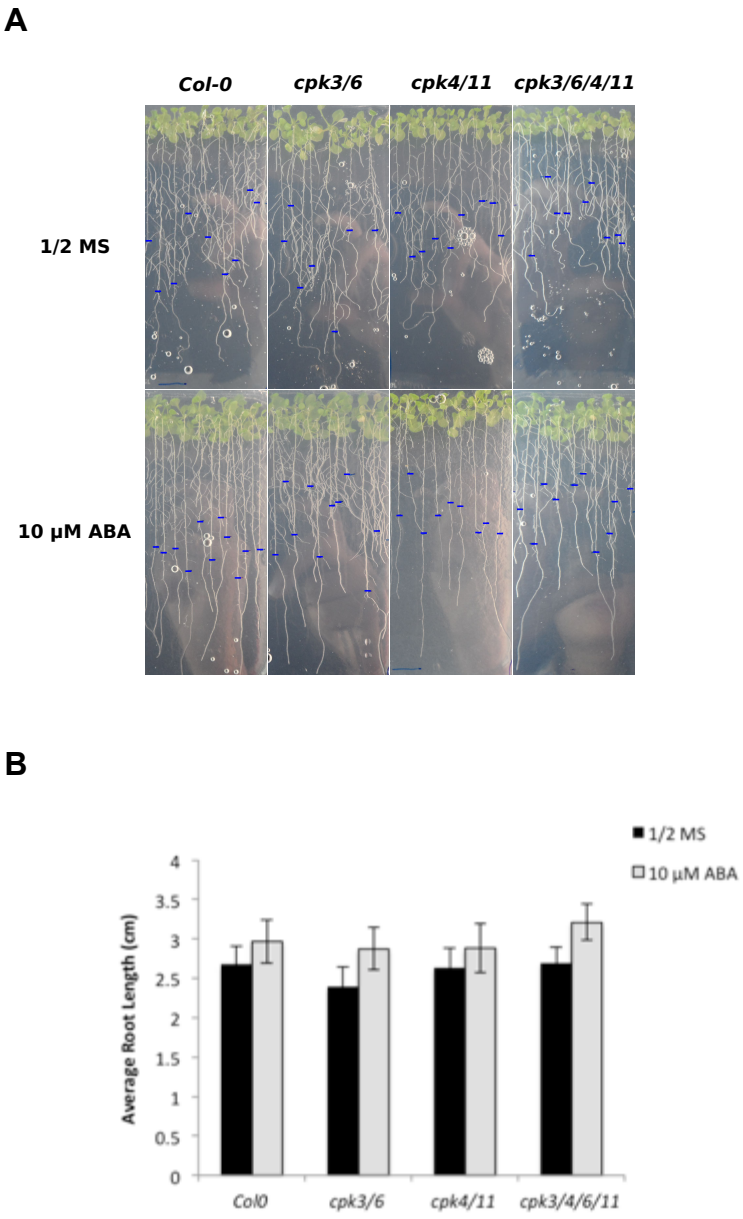

Figure 8: Root growth analyses of *cpk3/4/6/11*, *cpk3/6*, and *cpk4/11*. Seedlings were transferred after germinating 2 weeks on ABA-free medium to plates with or without ABA. **(A-B)** Analysis of root elongation response to ABA six days after transfer. Root lengths measured in cm. Blue marks indicate initial root length after transfer to plates ± ABA. Error bars depict means ± SE (n=12-16 root lengths measured)

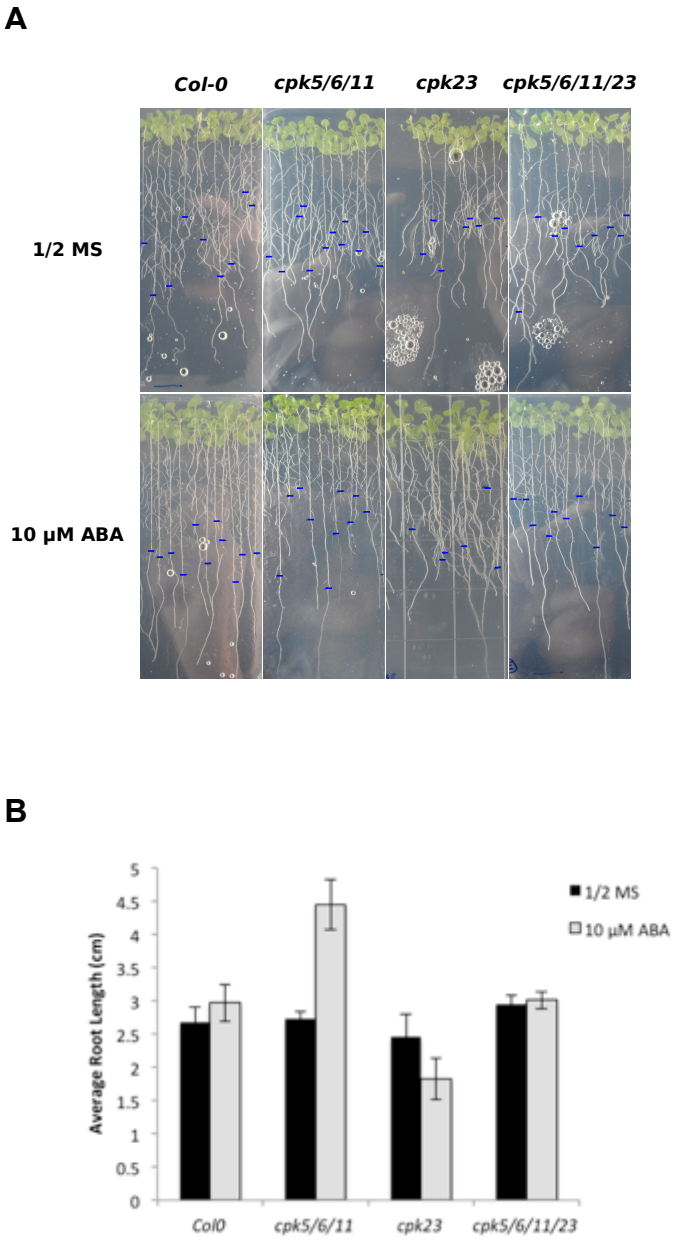

Figure 9: Root growth analyses of *cpk5/6/11/23*, *cpk5/6/11*, and *cpk23.* Seedlings were transferred after germinating 2 weeks on ABA-free medium to plates with or without ABA . **(A-B)** Analysis of root elongation response to ABA six days after transfer. Root lengths measured in cm. Blue marks indicate initial root length after transfer to plates ± ABA. Error bars depict means ± SE (n=12-16 root lengths measured)

1.4. Discussion

A plethora of evidence gathered within the past two decades has implicated important roles of calcium as second messenger in modulating diverse physiological processes important for stress tolerance (Laanements et al., 2013; Reddy et al., 2011). For example, during drought stress, the presence of ABA results in increased intracellular Ca^{2+} levels (Siegel et al., 2009). Integral to the Ca^{2+}-dependent ABA signaling within stomata are CPKs, calcium sensors found to be involved in a number of ABA responses, including stomatal closing by activation of the SLAC1 anion channel as well as upregulation of relevant ABA-responsive genes (Boudsocq et al., 2013; Kim et al., 2010).

While these CPKs have been of great interest due to their connection to abiotic stress tolerance mechanisms in plants, determining a distinct biological function for the CPKs has been challenging due to the redundant functions of the 34 CPKs encoded within the *Arabidopsis* genome (Boudsocq et al., 2013); nevertheless, attempts have been made to understand the roles of the CPKs. Past experiments have shown that CPK3, CPK4, CPK6, and CPK11 act as positive regulators and CPK23 acts as a negative regulator in ABA signal transduction (Ma and Wu, 2007; Mori et al., 2006; Zhu et al., 2007). As explained in detail in the introduction, CPK3, CPK4, CPK6, CPK11, and CPK23 were reported to be involved in ABA-dependent stomatal closure (Ma and Wu, 2007; Mori et al., 2006; Zhu et al., 2007). CPK4 and CPK11 have been implicated to have roles in ABA responses in seed germination and seedling growth as well (Zhu et al., 2007). To further explore the additive effects and eliminate functional redundancies of CPKs, the quadruple mutant lines *cpk3/4/6/11* and *cpk5/6/11/23* were established by crossing mutant lines *cpk3-2cpk6-1* with *cpk4-2cpk11-2* and *cpk5cpk6-3cpk11-2* with

cpk23, respectively. It is important to note that while CPK5's role within ABA signaling has not been investigated in detail, CPK5 is the closest homolog to CPK6 (Boudsocq et al., 2010; Boudsocq et al., 2013), a known positive regulator in guard cell signal transduction (Brandt et al., 2012; Mori et al., 2006; Xu et al., 2010). For this reason, CPK5 was included in this study.

Based on Mendelian genetics, a probability of 1 out of 256 plants within the F2 generation was expected to contain the quadruple mutant genes. Using PCR-based genotyping, the two quadruple mutant lines were identified within the F4 generation (**Figure 3**). After confirming that the homozygous quadruple mutant lines were truly isolated (**Figure 4B)**, a series of experiments were conducted in order to phenotypically characterize the quadruple mutants.

Observations found in higher order mutants in a variety of ABA-induced biological responses largely confirmed that CPKs act synergistically, as seen in reduced responses to ABA in quadruple mutants compared to their parental mutants (**Figures 4-9)**. Assessing stomatal movements in the presence of ABA showed that quadruple mutants *cpk3/4/6/11* and *cpk5/6/11/23* revealed strongly decreased sensitivity to ABA for a number of responses in relation to the parental mutant lines (**Figure 4)**. *cpk5/6/11/23* mutant plants demonstrate a complete ABA-insensitive phenotype as they failed to close stomata when ABA was added (**Figure 5)**. The results seen point towards synergistic CPK action within ABA signaling, with more pronounced phenotypes for an increasing number of genes knocked out. To date, *cpk5* and *cpk23* mutant plants have not been tested for ABA-induced stomatal movements. While *cpk23* single mutant was revealed to have a ABA-hypersensitive phenotype due to increased inhibition of stomatal opening in

drought conditions (Ma et al., 2006), our studies show that the quadruple mutant *cpk5/6/11/23* exhibited an ABA-hyposensitive phenotype in stomatal closing responses compared to parental lines. This implies that *cpk23* could actually respond less to ABA than what was initially reported (Ma and Wu, 2007). Additional stomatal movement studies would need to be conducted between single mutant *cpk23* and the quadruple mutant *cpk5/6/11/23* with the parental lines to further validate this finding.

Similarly, a reduced response to ABA in seed germination was observed for *cpk3/4/6/11* when compared to parental lines *cpk3/6* and *cpk4/11* (**Figure 6**). In relation to seeds germinating on media without ABA, seeds of all genotypes display a slowed radicle emergence and cotyledon expansion when grown in the presence of 2 μM ABA. However, quantifying the number of radicles over a course of 7 days demonstrated an increased germination rate for the mutant lines compared to wild-type, with the quadruple mutant line *cpk3/4/6/11* having the most pronounced effect. This effect was also apparent in the rate of cotyledon expansion on ½ MS with 0.8 μM ABA. Conversely, *cpk5/6/11/23* did not exhibit similar ABA-hyposensitive phenotypes, as noted by the ambiguity of radical emergence and cotyledon expansion results (**Figure 7**). It was decided that conclusions could not be made given the high variability of germination rates for the quadruple mutant seeds. Therefore, while the premise of additive functions for CPKs was seen in germination rates for the quadruple mutant *cpk3/4/6/11*, the same conclusion cannot be made for the second quadruple mutant *cpk5/6/11/23* due to non-interpretable results. The unclear difference in germination rates between the genotypes tested could be due to the high ABA concentration used in this assay. Additional experiments with

lower ABA concentrations for *cpk5/6/11/23* are needed to further characterize an ABA-response phenotype in seed germination.

ABA concentrations below 1 μM have been implicated in stimulating root growth in plants that need to increase their ability to extract water from soil during abiotic stress (Ghassemian et al., 2000). However, at higher concentrations of ABA, ABA inhibits root growth (Antoni et al., 2013; Ghassemian et al., 2000). CPKs have not yet been reported to play a role in ABA-dependent root growth inhibition. Testing the quadruple mutant lines along with their parental lines for root growth on media supplemented with 10 μM ABA, however, showed no apparent differences in root growth rates compared to the wild-type control. Therefore, whether CPKs are important in root growth during stress remain unclear.

This study further suggested that CPKs CPK3, CPK4, CPK5, CPK6, CPK11, CPK23 have important regulatory functions in ABA signal transduction in stomatal movement and seed germination. Furthermore, because these protein kinases exhibit similar ABA-induced biological responses, it is probable that they are functioning redundantly within the same pathway, as seen by less pronounced ABA-response phenotypes in lower order mutants. Moreover, as emphasized in this study, the quadruple mutant displayed a more pronounced ABA-hyposensitive phenotype compared to double mutants, suggesting that the CPKs not only work synergistically, but could also be involved in several different pathways in parallel, especially since these CPKs are known to localize in both the cytoplasm and nucleus (Dammann et al., 2003). CPKs have a wide range of sub-cellular distributions, existing as both soluble forms and as different membrane bound forms at the plasma membrane or on membranes of different organelles

(Harper and Harmon, 2005). Together, the extensive distribution of isoforms allows access to potentially hundreds of proteins for interactions and/or as potential substrates, while maintaining isoform specific responses. For instance, CPK3 and CPK4 occur as soluble proteins with nuclear and cytosolic localization whereas CPK6 and CPK21 are membrane-associated due to the presence of a membrane-directed myristoyl anchor at the Glycine residue at the second position (Dammann et al., 2003; Benetka et al., 2008).

While the actions of the discussed CPKs within the cytoplasm of guard cells have been mentioned, CPKs have also been reported to phosphorylate different targets within the nucleus. For example, CPK4 and CPK11 induce ABA-responsive genes indirectly by activating transcription factors, such as ABF1 and ABF4 (Zhu et al., 2007). Thus, not only do the CPKs function directly in plant drought tolerance by inducing stomatal closure, but they also behave indirectly by inducing genes that promote drought tolerance. However, in order to speculate on how CPKs function within different pathways, additional experiments to test induction of ABA-responsive genes have yet to be carried out for the quadruple mutants.

Additionally, since ABA regulates adaptation in plants to drought stress and therefore controls water deficit, additional studies regarding osmotic stress should be made with the quadruple mutants to further consider the CPKs' functions in regulating water balance and cellular dehydration tolerance. Of the CPKs included in this study, only CPK4, CPK11 and CPK23 have been attributed to salt tolerance, where single mutants and double mutant *cpk4cpk11* display increased tolerance to salt (Ma and Wu, 2007; Zhu, et al., 2007).

In conclusion, the cooperation of the many CPKs within the ABA signaling pathway has proven to be extremely complex and thus, difficult to assess. Yet, using reverse genetic approaches by testing higher order mutants allows us to eliminate redundancies in the *CPK* functions and study these proteins' roles in ABA signaling. These experiments suggest that some of these CPKs could function as positive regulators in ABA signal transduction in stomatal movement and seed germination, as reported in previous studies. Moreover, the CPKs were shown to act synergistically, which prompted additional speculation of exactly how many other CPKs act redundantly in ABA response. The next approach to answer some of these questions would be to create a higher order sextuplet mutant *cpk3/4/5/6/11/23* and phenotypically characterize it. The results of these studies that prompt further questioning thereafter emphasize how much there is still left to investigate and understand about plant adaptation to environmental challenges. All of these efforts are imperative to confer more drought-resistance in plant crops.

1.5. Methods and Materials

1.5.1. Plant Growth Conditions

Arabidopsis thaliana seeds of the Columbia-0 ecotype were sterilized by incubating in 70% ethanol and 0.04% SDS in a rotation shaker for 15-20 min and plated on solid ½ MS medium [1/2 strength Murashige and Skoog basal medium (Sigma-Aldrich, M0404), 1 mM MES (pH 5.7), 1% sucrose, and 1.0% phytoagar]. After plates were stratified in the dark at 4ºC for 48 hours, they were transferred to a growth cabinet with a cycle of 16-hour light at 21ºC and 8-hour dark at 18ºC (200 μmol $m^{-2}s^{-1}$, 70% Hr). After approximately 14 days, the seedlings were transferred to autoclaved soil (Sungro Special blend Professional Growing Mix, Seba Beach, Alberta, Canada) and grown in a growth chamber (16-hr-light/8-hr-dark, 21ºC, 75 μmol $m^{-2}s^{-1}$).

1.5.2. Genomic DNA Extraction

One or two mature leaves were removed from plants and placed into an eppendorf tube. Leaves were grinded in 400 μl extraction buffer (0.2 M Tris-HCl, pH 7.5, 0.250 M NaCl, 25 mM EDTA, 0.5% SDS) and tube was centrifuged at full speed for 5 minutes. The supernatant was transferred into a new tube containing an equal volume of isopropanol. After centrifugation at full speed for 10 minutes, the supernatant was discarded and the pellet was washed with 400 μl 70% ethanol. Following another spin at full speed for 5 minutes, the ethanol was removed and the pellet was left to dry completely. 50 μl of millipore (MQ) water was used to re-suspend the pellet containing genomic DNA.

1.5.3. Establishing and Screening of Loss-of-Function Mutant Lines

Mutant lines *cpk3-2 cpk6-1*, *cpk4-2 cpk11-2*, *cpk5-1 cpk6-3 cpk11-2*, and *cpk23-1* seeds (refer to **Table 1** for T-DNA name and accession numbers) were grown using conditions previously stated (**Section 1.5.1**). An initial cross was made between double mutants *cpk3-1 cpk6-1* and *cpk4-1 cpk11-2* and between *cpk5-1 cpk6-3 cpk11-2* and *cpk23-1*. PCR-based genotyping using the genomic DNA extracts (**Section 1.5.3**) with GreenTaq™ polymerase (Thermo Scientific, www.thermoscientific.com) confirmed heterozygosity of the mutant alleles, indicating a successful cross. F1 plants were left to grow and self-fertilize. Seeds of F1 plants were then collected, grown, and screened. Homozygous quadruple mutants *cpk3 cpk4 cpk6 cpk11* and *cpk5 cpk6 cpk11 cpk23* were identified using primers listed in **Table 2**.

Table 2: Sequences of oligonucleotide primers used for genotyping of *cpk* mutants.

Gene	Mutant	Primers used (5' → 3')
CPK3	*cpk3-2*	F: CATCGTGTCTGATCTCAGAC R: GAATGACAACTATGCACAACC
CPK4	*cpk4-2*	F: TTACTTTGGTGAATCATCAGATTTAG R: CGCGGAGAGACTCTCGGAGGAAG
CPK5	*cpk5*	F: TCGTTCCAAATTGACCTTGAC R: GAGGAAACAGCGGAGAGAGAC
CPK6	*cpk6-1* *cpk6-3*	F: AATTACCCAACCAGAAACAGC R: ACCAACAAGAAATTCTCAGGC
CPK11	*cpk11-2*	F: ATGGAGACGAAGCCAAACCC R: AGTACGGACTTCCAACTACG
CPK23	*cpk23*	F: CAGTGGAATGGATACTGTTTCC R: AACATCCTTGGATCAAAGGG
T-DNA Sail (Lb)	----	TTCATAACCAATCTCGATACAC
T-DNA Salk (LBa1)	----	TGGTTCACGTAGTGGGCCATCG

1.5.4. Total RNA Isolation for cDNA Synthesis

Mature leaves (about 4 weeks following germination) were harvested and immediately flash frozen in liquid nitrogen. An RNA extraction kit (RNeasy Plant RNA Isolation Kit; Qiagen, http://qiagen.com) was used to extract total RNA of the leaf samples, following manufacturer's instructions. To remove DNA contaminations, 1 μg of RNA sample was incubated at room temperature for 15 minutes with 1 μl 10X DNase I Reaction Buffer, 1 μl DNaseI and DEPC-treated H_2O to 10 μl. After adding EDTA to inactivate DNaseI, the RNA sample was incubated for an addition 10 minutes at 65ºC.

For cDNA synthesis the First Strand cDNA kit (GE Healthcare, http:///www.gehealthcare.com) was used to reverse-transcribe 1 μg of the treated total RNA with NotI-d(T)18 primers according to the manufacturer's manual.

1.5.5. RT-PCR Analysis

To determine *cpk* mRNA abundance, cDNA (prepared as mentioned in **Section 1.5.4**) of the two quadruple mutant lines was subjected to RT-PCR analyses using 35 cycles (performed by Benjamin Brandt). To control equal loading, actin2 primers (see **Table 3**) were used (25 cycles).

Table 3: Sequences of oligonucleotide primers for RT-PCRs.

Gene	Mutant	Primers used (5' → 3')
CPK3	*cpk3-2*	F: CATCGTGTCTGATCTCAGAC R: GGAAGTGCTAAATCATCCGTG
CPK4	*cpk4-2*	F: CAGAGATAGACGTGTGGAGCG R: CTATATCCGCCGCATCCATAAG
CPK5	*cpk5*	F: TCGTTCCAAATTGACCTTGAC R: GAGGAAACAGCGGAGAGAGAC
CPK6	*cpk6-1* *cpk6-3*	F: GTTCTTTTAAGGACAAAATCTACGAG R: CAGCATCAAATATTCCTTGCTG
CPK11	*cpk11-2*	F: CAAAAGTAATCGTTCCGCTG R: CTATAAGCCAGGACAATATTTATATG
CPK23	*cpk23*	F (RP1): CAGTGGAATGGATACTGTTTCC F (RP2): CTAGTTATGTGCCCATTTTTGTC R: CTAGTTATGTGCCCATTTTTGTC

1.5.6. Stomatal Movement Assays

Stomatal aperture movement assays were performed double blind, with both genotype and treatment unknown, where three trials were conducted within a single experiment. One or two rosette leaves of 4-week-old plants were floated in opening buffer solution (5 mM KCl, 50 μM $CaCl_2$, 10 mM MES, pH 5.6 with Tris base) and incubated for 2-2.5 hours at 21ºC under a cold-light source at 150 μmol $m^{-2}s^{-1}$. After the incubation, either mock treatment (ethanol) or ABA (dissolved in ethanol) was added to the opening buffer followed by an additional hour under light. Subsequently, the leaves were blended in water with some of the opening buffer using a commercial blender (Waring commercial blender, Torrington, Connecticut) for 20 seconds. The blended mixture was filtered through 100 μm nylon mesh (EMD Millipore, http://www.millipore.com) and plant material from the mesh was dabbed directly onto a microscope slide and covered with a glass coverslip. For each sample, about 35-50 stomata were recorded using an inverted light microscope (exact number of stomata is given in the figure legends for each measurement) and pictures were taken. Measurements of stomatal apertures and guard cell lengths were determined using program ImageJ (http://rsb.info.nih.gov/ij/).

1.5.7. Seed Germination Assays

Seeds from wild-type (Col), parental lines and quadruple mutant lines were sterilized using method previously mentioned (section number). About 50 seeds each were then plated in triplicate on ½ MS medium (section number), supplemented with or without treatment (± ABA). The seed germination assays were performed single blind,

with genotype unknown. The plates were stratified without light for at least 72 hours at 4ºC and subsequently moved to the growth cabinet (light/dark cycle at 16 hr 21ºC/8 hr 18ºC; 300 μmol $m^{-2}s^{-1}$, 70% Hr). Images were taken every 24 hours. Emergence of radicles (germination) and cotyledons was counted using the Fiji software (http://fiji.sc/Fiji).

1.5.8. Root Growth Assays

About 15-20 sterilized seeds from wild-type (Col), parental lines and quadruple mutant lines were sown horizontally across the top edge of large plates containing MS medium [1/2 strength Murashige and Skoog basal medium (Sigma-Aldrich, M0404), 1 mM MES (pH 5.7) and 1.0% phytoagar]. After stratification for >72 hours at 4 ºC without light, the plates were transferred in a growth cabinet (light/dark cycle at 16 hr 21ºC/8 hr 18ºC; 300 μmol $m^{-2}s^{-1}$, 70% Hr) where they were placed vertically. Following ~2 weeks, the seedlings were transferred using sterile forceps onto ½ MS plates supplemented with or without ABA. Root lengths of the transferred seedlings were monitored for 10 days by marking the initial position of the root tips and taking pictures of the plates every 24h. ABA effects on root growth of the plants tested were assessed by measuring root lengths at different time points using the Fiji program (http://fiji.sc/Fiji).

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II.

In Vivo Interactions of Calcium-Dependent Protein Kinases, CPK6 and CPK23, with PP2C Protein Phosphatases within Abscisic Acid Signaling

2.1. Abstract

The plant phytohormone abscisic acid (ABA) is critical for mediating stomatal closure to prevent cellular dehydration as well as control carbon dioxide uptake in plants when faced with environmental stress, such as during drought or at extreme atmospheric carbon dioxide levels. Based on previous studies, a molecular signaling pathway for the stomatal closing event has been proposed; however, many key regulatory details within the pathway remain elusive. ABA signaling transduction is known to diverge into two pathways that supposedly run in parallel to each other. One pathway does not depend on increased intracellular levels of Ca^{2+} that follow ABA activation. Central to this pathway are group A Protein Phosphatases 2s (PP2Cs), which are known negative regulators of the pathway and promotes stomatal opening. The other ABA signaling pathway is Ca^{2+}-dependent, where Calcium-Dependent Protein Kinases (CPKs) play an essential role in phosphorylating downstream targets, such as ion channels that regulate guard cell volume and turgor. A functional relation between the PP2Cs and CPKs has been implicated, which suggests cross-regulation between the two known pathways. In this study, a yeast 2-hybrid system was utilized to test for interactions between CPKs CPK6 and CPK23 with PP2Cs ABI1, ABI2, and PP2CA. An interaction was observed *in vivo* between CPK6 and PP2CA, confirming our hypothesis that the PP2Cs and CPKs are somehow regulating each other. Our studies also show that disrupting membrane localization of the CPKs by mutating the myristoylation site increased the likelihood for the proteins to interact in yeast. This finding could therefore improve future interaction studies with CPKs in yeast 2-hybrid systems, which have been challenging in the past.

2.2. Introduction

Abscisic acid (ABA) is a known phytohormone involved in abiotic stress tolerance in plants (Ingram and Bartel, 1996; Jakab et al., 2005). During drought conditions, it is important for the plant to conserve water by reducing transpirational water loss that occurs through its stomata. This is achieved in an ABA-dependent process in guard cells resulting in a decrease of cell volume and turgor and thus, stomatal closure (reviewed in Kim et al., 2010).

Based on our current understanding of the ABA signaling pathway, ABA signaling is initiated by ABA binding to one or more proteins belonging to a family of ABA receptors called pyrobactin resistance (PYR) proteins or regulatory components of ABA receptor (RCAR) proteins (Ma et al., 2009; Park et al., 2009). ABA-bound PYR/RCAR proteins form complexes with Group A Protein Phosphatase Type 2C (PP2C), which inhibits the PP2Cs' negative regulatory activity (Ma et al., 2009; Nishimura et al., 2009; Park et al., 2009; Santiago et al., 2009). Group A PP2Cs, whose members include ABI1 (ABA-Insensitive 1), ABI2 (ABA-Insensitive 2), and PP2CA, are known negative regulators of ABA signaling (Merlot et al., 2001; Sheen 1998). Loss or reduction of PP2Cs ABI1, ABI2, and PP2CA activity, for instance, lead to enhanced ABA sensitivity in ABA-dependent responses, such as those in stomatal regulation and seed germination (Gosti et al., 1999; Kuhn et al., 2006; Merlot et al., 2001; Yoshida et al., 2006). Without ABA, PP2Cs directly inhibit subclass III SnRK2s (Snf1-Related Protein Kinase 2) (Umezawa et al., 2009; Vlad et al., 2009; Yoshida et al., 2006). Subclass III SnRKs, including SnRK2.6, SnRK2.3, and SnRK2.2, are known to be positive regulators of ABA signal transduction (Fujii et al., 2009; Mustilli, et al., 2002; Nakashima et al.,

2009; Yoshida, et al., 2002). PP2Cs have been shown to regulate SnRK2 activity by de-phosphorylating residues in the activation loop in the kinase domain, which renders these kinases inactive (Vlad et al., 2010).

In the presence of ABA, PP2C phosphatases are inhibited and the SnRK2 kinases in turn are active, phosphorylating downstream targets such as transcription factors regulating ABA-responsive genes, including ABF1, ABE2, ABF3 and ABF4 (Furihata et al., 2006; Johnson et al., 2002; Uno et al., 2000). Within guard cell signaling, SnRK2.6 or Open Stomata 1 (OST1) has been implicated as a necessary component in positively regulating stomatal closure (Mustilli et al., 2002; Yoshida et al., 2002). OST1 acts on ion channels that are relevant in osmotic homeostasis and are known to down-regulate inward rectifying potassium (K^+) channels (KAT1) (Sato et al., 2009) and activate SLAC1 (S-Type Anion Channel 1) (Lee et al., 2009; Geiger et al., 2009). SLAC1 is a guard cell anion channel that has been shown to be critical for stomatal closure by regulating cell volume and turgor (Negi et al., 2008; Vahisalu et al., 2008) (**Figure 10**).

An alternate Ca^{2+}-dependent pathway for SLAC1 activation also occurs. Levels of intracellular [Ca^{2+}] increase in the presence of ABA (DeSilva et al., 1985; Grabov et al., 1998; McAinsh et al., 1990), which then activate some CPKs (Calcium-Dependent Protein Kinases) (reviewed in Boudsocq et al., 2012). CPKs CPK3, CPK6, CPK21, and CPK23 have specifically been identified as key players in SLAC1 activation in guard cells and stomatal closure (Brandt et al., 2012; Geiger et al., 2010; Scherzer et al., 2012). There is evidence suggesting that these two pathways for SLAC1 activation do not occur independently of each other. Specifically, a functional relation between PP2Cs and CPKs has been observed, but has yet to be studied in detail (Geiger et al., 2010; Brandt et al.,

2012). Recent findings have demonstrated that the PP2C ABI1 can directly dephosphorylate the N terminus of SLAC1 (Brandt et al., 2012). Additionally, ABI1 and ABI2 were found to down-regulate CPK21- and CPK23-mediated SLAC1 anion currents while ABI1, ABI2 as well as PP2CA were reported to inhibit CPK6-mediated SLAC1 activation (Brandt et al., 2012; Geiger et al., 2010). Whether PP2Cs directly regulate CPK activities and thereby negatively regulate SLAC1 activation remains unknown. To date no evidence for this direct regulation *in vivo* or *in vitro* has been published. *In vivo* interaction studies in yeast may provide further insight as to whether these two proteins do in fact directly interact with each other.

In this study, variants of CPK6 and CPK23 with the PP2Cs ABI1, ABI2, and PP2CA were tested for possible interactions *in vivo*. Of the different protein variants tested, the non-myristoylatable mutant versions of the CPKs were included in this study. Myristolation is a post-translational modification that targets the protein to membranes by enabling interaction with membranes with a myristoyl anchor, which affects subcellular localization. While few CPKs have been experimentally tested for N-myristoylation, 29 out of 34 CPKs in *Arabidopsis thaliana* contain a myristoylatable glycine at position 2, indicating that most CPKs harbor this motif (Boudsocq et al., 2013). A glycine to alanine substitution at position 2 (G2A) disrupted membrane localization of CPK6, a known membrane-associated protein, in *in vitro* and *in vivo* experiments (Benetka et al., 2008).

The goal of this study was to identify possible protein-protein interactions *in vivo* using different variants of CPK6 and CPK23 with the PP2Cs ABI1, ABI2, and PP2CA in yeast. These interaction studies aim to clarify some of the complex details underlying

ABA signal transduction and highlight any potential cross-regulation of the Ca^{2+}-independent and Ca^{2+}-dependent ABA signaling pathways.

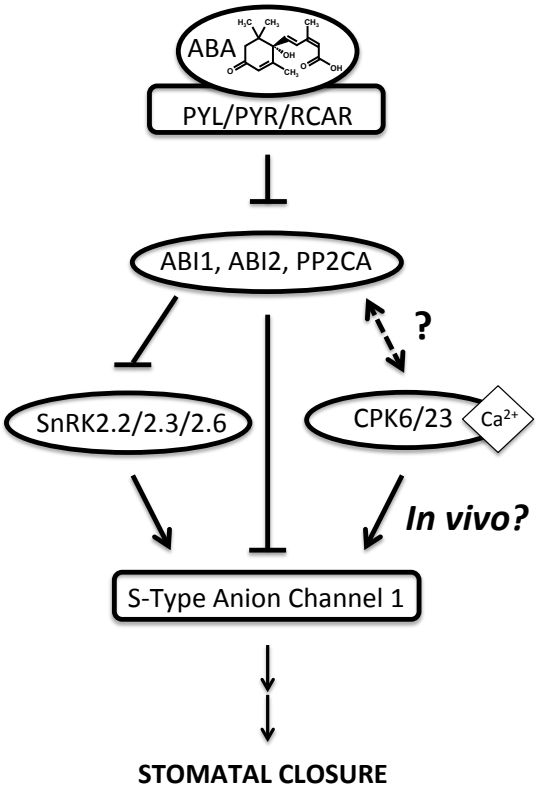

Figure 10: Proposed model for abiotic stress-activated ABA signaling pathway. Dotted line indicates possible interaction. Question marks "?" depict interactions tested in this study.

2.3. Results

2.3.1. Setting up the yeast 2-hybrid system to test for protein-protein interactions

Prior to performing the actual assays, DNA constructs to express the proteins of interest in yeast were prepared. Amino acid substitutions were introduced by amplifying the genes using specific primers resulting in point mutations in the respective codons (**Table 4).** As mentioned above, G2A mutations were introduced to mutate the myristoylation site of CPK6 and CPK23. Glutamate to alanine substitutions at sites 209 and 193 for CPK6 and CPK23, respectively, rendered the kinases inactive. Point mutations at D413L, D140L, and D380L for ABI1, ABI2, and PP2CA, respectively, led to phosphatase inactive PP2C proteins.

The CPKs were cloned into the pGBT9.BS plasmid, such that the expressed protein will be fused with the Gal4 DNA-binding domain (BD), which recognizes the Gal promoter in yeast. In this setup, the CPKs act as the "bait". On the other hand, the PP2Cs act as the "prey", where the *PP2C* genes were cloned into the pGAD.GH plasmid containing the Gal4 activating domain (AD). The expressed phosphatases are therefore fused to the Gal4 AD. The general principle of the yeast 2-hybrid system is if the proteins of interest (bait and prey) are indeed interacting with each other, the interacting proteins with their respective binding and activating domains will activate transcription of reporter genes (**Figure 11**). The yeast *S. cerevisiae* strain PJ69-4A used in this study was genetically manipulated and is unable to synthesize essential amino acids leucine (L), tryptophan (W), and histidine (H) and therefore will only be able to grow on media supplemented with these amino acids (James et al., 1996). Therefore, maintenance of

plasmids and reporter genes in the yeast cells is dependent on converting this auxotrophic yeast strain to an autotrophic state. Genes allowing the conversion to autotrophy are encoded on the plasmid, where one of the genes is only expressed exclusively when proteins interact . In this system, leucine, tryptophan, and histidine biosynthesis served as selectable autotrophic markers.

The prepared plasmids were co-transformed into yeast strain PJ69-4A using the high-efficiency lithium acetate method (outlined in Materials and Methods) (James et al., 1996). To select for yeast containing both bait and prey plasmids, the transformed yeast was plated on synthetically defined (SD) media without leucine and tryptophan (SD-LW), also known as double dropout media. Yeast growth on double dropout media indicated successful transformations of the bait and prey plasmids, since they have the ability to encode for leucine and tryptophan biosynthesis, respectively.

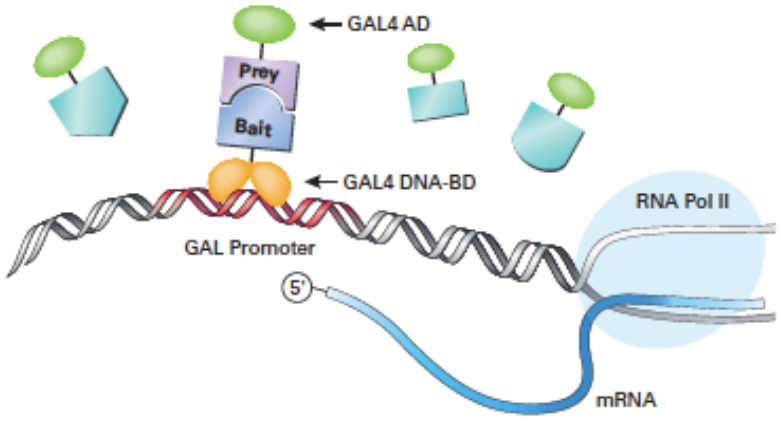

Figure 11: General principle of a yeast 2-hybrid assay. Image provided by Clontech Laboratories, Inc.

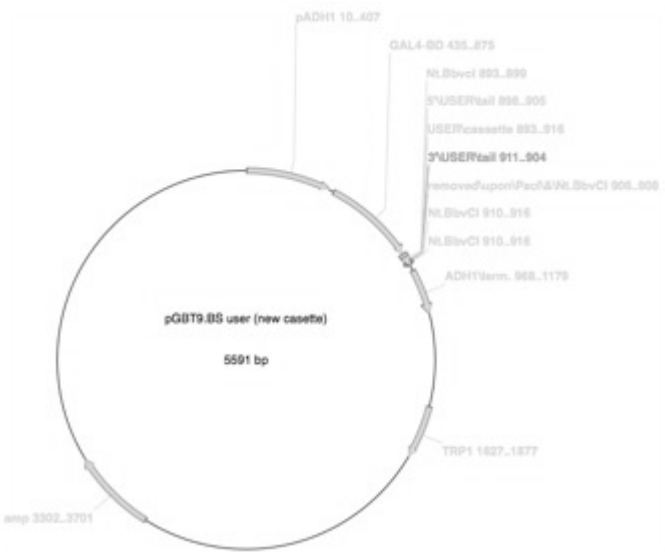

Figure 12: Vector map of pGBT9.BS

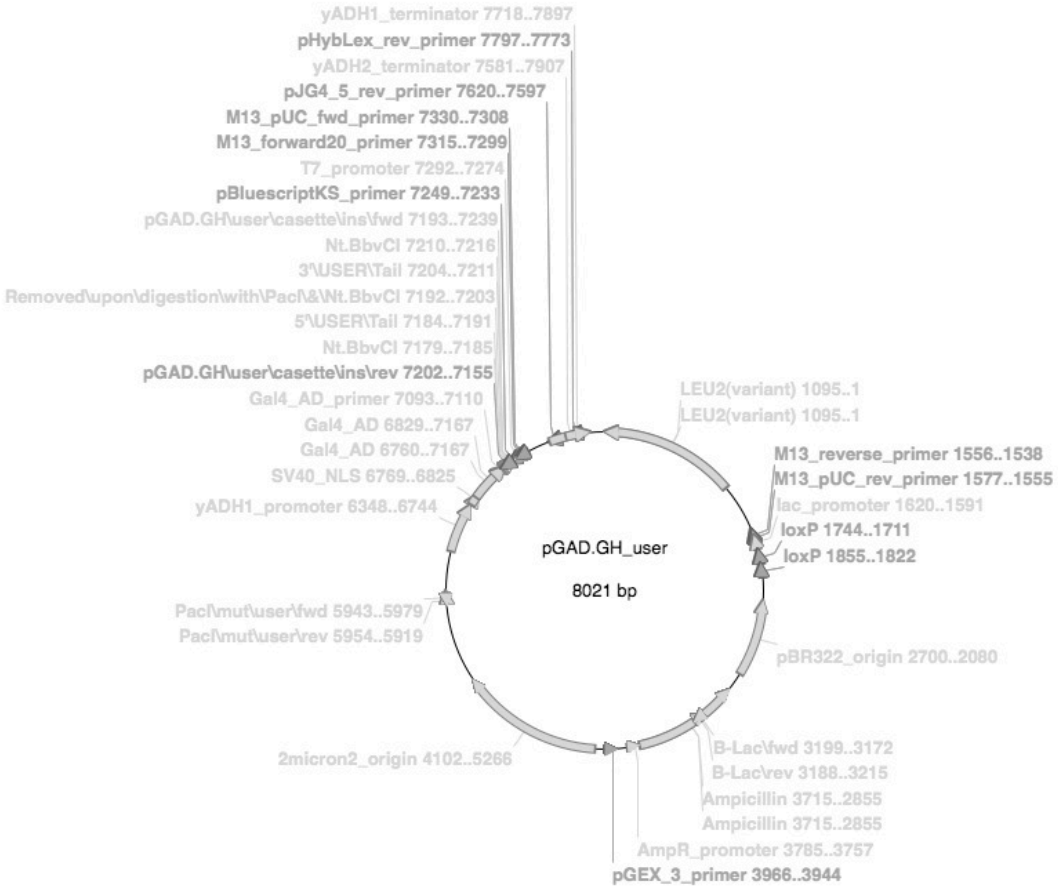

Figure 13: Vector map of pGAD.GH

2.3.2. In vivo interaction studies between CPK6 and CPK23 with PP2Cs

After co-transformation of the plasmids into yeast, the yeast was then spotted on to SD media lacking leucine, tryptophan, and histidine (SD-LWH, or triple dropout media). 5 µl of yeast in 2% glucose was spotted following a 1:10 dilution series (O.D. 1, 1:10, 1:100, and 1:1000). Histidine biosynthesis acted as the autotrophic marker indicating protein-protein interaction.

Pilot yeast 2-hybrid experiments between wild-type variants of CPK6 and CPK23 with wild-type PP2Cs ABI1, ABI2, and PP2CA did not reveal any interactions (**Table 5**). Inactive, or dead, kinase variants of CPK6 and CPK23 were tested next with wild-type and dead phosphatase versions of PP2Cs; however, no notable or reproducible interactions were detected (**Table 5).** Testing with constitutively active versions of the CPKs was considered; however, the G2A mutation, which disrupts protein localization to the plasma membrane, in CPK6 resulted in a detectable and reproducible interaction with PP2CA (**Figure 14).** Out of the 8 times that this assay was repeated, the interaction between CPK6 G2A and PP2CA was detected the later 6 times. Negative and positive results for all combinations tested were consistent, with each plasmid combination tested in at least 3 separate assays.

Table 4: Experimental attempts for protein-protein interactions. Highlighted row indicates interaction that was most readily detected and reproducible.

BD (Bait)	CPK variant	AD (Prey)	PP2C variant	Interaction?
CPK6	Wild-type	PP2CA	Wild-type	No
		PP2CA D380L	Inactive phosphatase	No
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No
CPK6 D209A	Inactive kinase	PP2CA	Wild-type	No
		PP2CA D380L	Inactive phosphatase	No
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No
CPK6 G2A	Non-myristoylatable	PP2CA	Wild-type	Yes; Strong
		PP2CA D380L	Inactive phosphatase	Yes; Weak
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No
CPK23	Wild-type	PP2CA	Wild-type	No
		PP2CA D380L	Inactive phosphatase	No
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No
CPK23 D193A	Inactive kinase	PP2CA	Wild-type	No
		PP2CA D380L	Inactive phosphatase	No
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No
CPK23 G2A	Non-myristoylatable	PP2CA	Wild-type	No
		PP2CA D380L	Inactive phosphatase	No
		ABI1	Wild-type	No
		ABI1 D413L	Inactive phosphatase	No
		ABI2	Wild-type	No
		ABI2 D402L	Inactive phosphatase	No

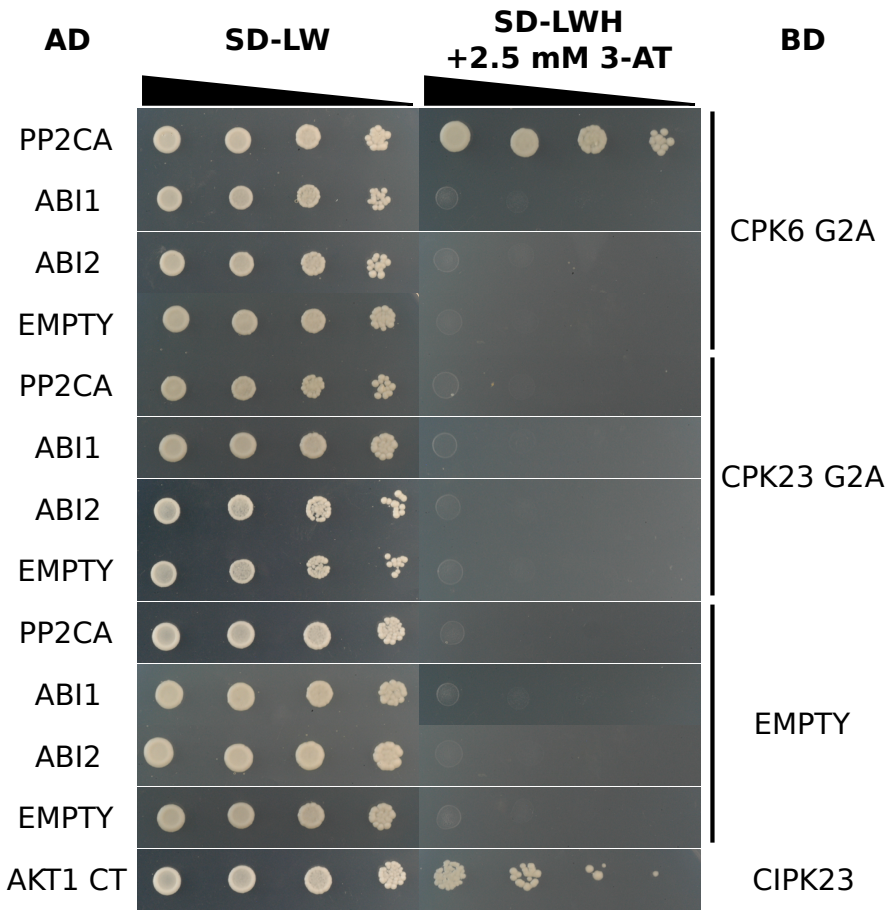

Figure 14: Interaction studies of CPK6 G2A and CPK23 G2A with PP2Cs ABI1, ABI2, and PP2CA in yeast. The yeast strain PJ69-4A containing the indicated plasmid combinations was grown on medium without leucine (L) and tryptophan (W). 5 µl of a 10-fold dilution series were spotted onto selective media (SD-leucine-tryptophan-histidine) supplemented with 2.5 mM 3-AT and also spotted on nonselective media (SD-LW). Negative controls include empty AD and BD vectors. AKT1-C terminus with CIPK23 is the positive control (Geiger et al., 2009). Images were taken 6 days after plating. Yeast growth on SD-LWH + 2.5 mM 3-AT indicates protein-protein interaction.

2.4. Discussion

A large number of evidence has pointed to group A Protein Phosphatases 2C (PP2Cs) as negative regulators in ABA signaling (reviewed in Kim et al., 2010). Without stress, PP2Cs promote stomatal opening by preventing SnRK2.6 or OST1 protein kinases from auto-phosphorylating to activate the SLAC1 anion channel (Lee et al., 2009; Ma et al., 2009; Umezawa et al., 2009). The PP2Cs' ability to inhibit stomatal closing was implicated by studying abnormal stomatal regulation in double mutants of intragenic revertants of *abi1-1* and *abi2-1* and mutants with the disrupted *PP2CA* gene (Gosti et al., 1999; Kuhn 2006; Merlot et al., 2001).

Recent findings have indicated that the PYL/RCAR-PP2C-OST1-SLAC1 pathway does not occur linearly. It was discovered that by inhibiting OST1 activity (Belin et al., 2006; Umezawa et al., 2009; Vlad et al., 2009), PP2Cs ABI1, ABI2, and PP2CA are able to downregulate SLAC1-mediated anion currents (Geiger et al., 2009; Geiger et al., 2010; Lee et al., 2009). ABI1, in particular, has been found to directly dephosphorylate the N terminus of SLAC1, providing another method of control over SLAC1 activation (Brandt et al., 2012). Moreover, ABI1 and ABI2 were shown to inhibit CPK23 and, to a lesser degree, CPK21 activations of SLAC1 in the *Xenopus laevi*s oocyte system (Geiger et al., 2010). It has also been reported that PP2Cs ABI1, ABI2, as well as PP2CA down-regulated CPK6-activated SLAC1 anion currents (Brandt et al., 2012). These studies suggest that the PP2Cs could function competitively with CPK6 in deactivating or activating SLAC1, implying further control for stomatal opening.

This prompts us to question whether the PP2Cs are able to regulate CPK functions or vice versa, but whether regulation between the PP2Cs and the CPKs occurs

has not been reported. In this study, interactions between CPK6 and CPK23 with PP2Cs ABI1, ABI2, and PP2CA were tested in yeast 2-hybrid assays. We found that a non-myristoylatable variant of CPK6 strongly interacted with PP2CA in a majority of the trials conducted (**Figure 14**). While the detailed mechanistic relationship between the two proteins remains unclear, this study provides evidence for an *in vivo* interaction.

Wild-type versions of CPK6 and CPK23 with wild-type versions of PP2Cs were tested initially with no interactions. It was thought that no interactions in yeast could be seen due to the transient nature of the kinase-substrate interaction and the brief phosphorylation event. Therefore, testing with inactive kinase versions of CPK6 and CPK23 and inactive phosphatase versions of the PP2Cs were attempted next, considering that disruption of phosphorylation or dephosphorylation activities, respectively, the potential CPK-PP2C complex is of a less transient nature and therefore an interaction could be observed indicated by yeast growth. However, these variants resulted in no yeast growth, and therefore, no interactions of the proteins were observed (**Table 5**). Again, this could be attributed to a fleeting CPK-substrate interaction event and if the interaction was not stable enough, this would prevent the reporter gene from being expressed. The next attempt involved establishing non-myristoylatable versions of CPK6 and CPK23, with the idea that by preventing membrane localization, the CPKs could remain soluble in the cytosolic space or more crucially, within the nucleus (Benetka et al., 2008). As mentioned above, interactions in yeast 2-hybrid assays have to occur in the nucleus; thus, this approach might increase reporter gene expression. By establishing a single mutation coding for alanine at position 2, the CPKs cannot be post-translationally modified by N-myristoylation, which causes impaired membrane localization (Benetka et al., 2008). As

previously emphasized, the non-myristoylatable variant of CPK6 with wild-type PP2CA intriguingly resulted in robust yeast growth 6 out of 8 repeats of the assay (the 2 failed assays is most likely attributed to the fact that they were pilot experiments) (**Figure 14).**

This approach to eliminate the myristoylatable site of CPKs has profound implications in future *in vivo* interaction studies with CPKs and their substrates. Interaction studies with CPKs have been unsuccessful in the past, perhaps due to the highly transitory interaction CPKs have with their substrates. Additionally, a positive signal in a yeast 2-hybrid assay requires the interaction to occur in the nucleus; therefore, CPKs localizing at the membrane could be problematic in determining possible CPKs' interactions with substrates. Eliminating the myristoylation site could thus improve possible positive readouts in yeast 2-hybrid experiments.

In conclusion, this study demonstrates that out of all the protein-protein interactions tested, only the non-myristoylatable variant of CPK6 resulted in a clear interaction with PP2CA. However, whether the CPKs CPK6 or CPK23 interact with more PP2Cs remains an open question, since they could still interact in other protein interaction studies. Furthermore, additional experiments will need to be performed to confirm the interaction between CPK6 and PP2CA, such as co-immunoprecipitation or co-localization experiments. As more details emerge about ABA signaling transduction, it becomes clear that the pathways are much more intricate than originally understood. Yet, it is crucial to further pursue and uncover these mechanistic regulatory details to complete our comprehension of ABA signaling, which will subsequently provide a better understanding of how plants resist extreme changes in the environment in order to engineer more stress tolerant plants.

2.5. Methods and Materials

2.5.1. Construction of Plasmids and Cloning

Point mutations at G2A site for CPK6 and CPK23 were generated by amplifying these genes using primers carrying point mutations in the respective codons (**Table 4**). DNA templates of the CPKs and protein phosphatases 2Cs (PP2Cs) were amplified using a mutant Pfu polymerase, PfuX7 (Norholm et al., 2010). To achieve fusion of the genes of interest to either the activation domain (AD) or binding domain (BD) the PCR products were inserted in modified yeast vectors (pGAD.GH and pGBT9.BS, respectively) using the Uracil-Specific Excision Reagent (short USER) cloning system introduced by New England Biolabs (New England Biolabs Inc., Ipswich, MA, USA). This system was further developed by Nour-Eldin, et al (2010). Accordingly primers were designed (**Table 4**) and plasmids were prepared with PCR-generated DNA fragments coding for PP2Cs and CPKs cloned into pGAD.GH and pGBT9.BS vectors, respectively. The plasmids were transformed into Top10 *E. Coli* strain and plated on LB medium (1.5% agar, 5 g/L NaCl, 0.2% lysogeny broth) with 50 μg/ml carbenicillin. After inoculation in a liquid culture (0.2% lysogeny broth + 50 μg/ml carbenicillin), the plasmids were isolated using QIAprep Spin MiniPrep Kit (QIAGEN Sciences, http://www.qiagen.com). Successful cloning was confirmed by sequencing the recovered plasmids.

Table 4: Primers used for site-directed mutagenesis.

Protein	Variant	Primer Sequence (5'→ 3')
CPK6	D209A	F: AGAGCTTUAAAGCCTGAGAATTTCTTGTTG R: AGAGCTTUAAAGCCTGAGAATTTCTTGTTG
	G2A	F: GGCTTAAUATGGCCAATTCATGTCGTG R: GGTTTAAUCTACACATCTCTCATGCTGATGTTTAG
CPK23	D193A	F: ATCGAGCTCUCAAGCCTGAGAATTTCTTGTTC R: AGAGCTCGAUGAATCACACCATTCAAATGACAAATC
	G2A	F: GGCTTAAUATGGCTTGTTTCAGCAGTAAAC R: GGTTTAAUTCAGTGGAATGGATACTGTTTCC
ABI2	D402L	F: AGCAAACTCAAUATAAGTGTGGTAGTGGTTGATTTG R: ATTGAGTTTGCUTCCTTTTTGCAAAGCCATC
PP2CA	D380L	F: AGCTCCCTTAACGUGAGCGTCGTTGTGGTTGAC R: CGTTAAGGGAGCUCTGTCTTGCTAGAGCCAACTTTG

2.5.2. Yeast strain and co-transformation

The yeast strain PJ69-4A used in these experiments had genotype *MAT***a** *trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* (James et al., 1996).

Prepared constructs were transformed into yeast using the high efficiency LiAc method of Gietz and Schiestl with minor modifications (Gietz and Schiestl, 1995). To prepare for the transformations, the yeast strain PJ69-4A was streaked onto YPDA medium [2% Bactopeptone (BD Biosciences, http://www.bd.com), 1% yeast extract, 0.02% adenine, 2% glucose, and 2% agar] and incubated at 30ºC for 2 days. Depending on the number of co-transformations done, 1-2 single yeast colonies were re-streaked onto YPDA plates and incubated at 30ºC for one more day. The fresh yeast was added to 500 μl of 2% glucose and incubated on a shaker for 30 minutes at 30ºC. After a quick spin, the glucose was discarded and the yeast was re-suspended in a transformation mix, containing 1 μg of each plasmid, 1 mg/ml of salmon sperm DNA (Invitrogen, http://www.invitrogen.com), which was boiled at 95ºC and rapidly cooled, 50% polyethylene glycol (MW 3350), and 1M LiAc. The yeast underwent two incubation periods afterwards – the first for 30 minutes at 30ºC on a shaker and the second for 20 minutes at 42ºC in a water bath. After incubation periods, the yeast was centrifuged at full speed and the supernatant was discarded. The transformed yeast was re-suspended in 75 μl of 20% Glucose, plated on CSM medium (3.35% YNB with ammonium, 0.32% CSM-Leu-Trp-His, 20% agar, 20% glucose) selected for leucine and tryptophan and incubated for 2 days at 30ºC.

2.5.3. In vivo protein-protein interaction studies in yeast

Once the yeast was successfully transformed, concentrations of each yeast transformation were measured at OD_{600}. Yeast concentrations were adjusted to OD 1 and ten-fold dilution series were prepared. 5 µl of the ten-fold dilution series were spotted onto synthetically defined or SD plates (3.35% YNB with ammonium, 0.32% SD-Leu-Trp-His, 20% agar, 20% glucose) selected for leucine, tryptophan, and histidine. Additionally, the triple dropout media was supplemented with 2.5 mM of 3-amino-1,2,4-triazole (3-AT). 3-AT is a known competitive inhibitor of imidazole glycerol phosphate dehydratase, a histidine biosynthetic enzyme (Hilton et al., 1965; Klopotowski and Wiater, 1965; Wiater et al., 1971). By inhibiting this enzyme, 3-AT restricts histidine biosynthesis and therefore, yeast growth on SD-LWH. However, a strong interaction would activate the HIS3 reporter gene, which encodes for the enzyme inhibited by 3-AT. Hence, increased expression of this enzyme due to a protein-protein interaction is able to exceed the growth inhibitory effects of 3-AT. Therefore, the addition of 3-AT to the media functions to increase the stringency of the assay and to positively select for successful interactions. Plates incubated for 6 days at 30ºC prior to analysis.

2.6. References

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Appendix

Other ABA-induced stomatal bioassays attempted

Leaves were treated with or without 10 μM ABA and individual stomata were tracked.

- *cml9-1* with *Col0* control
- *cml9-2* with *Col0* control
- With DFPM: *cpk3/4/6/11* with *Col0* control

Other yeast 2-hybrid experiments attempted

- BD-CPK6 G2A and BD-CPK23 G2A with AD-SLAC1-N terminus
    - Interaction test was repeated 3 times. No interactions detected.
- BD-OST1 with AD-PP2CA, -ABI1, -ABI2 (both wild-type and inactive phosphatase variants)
    - Each interaction test was repeated 3 times; very weak or no interactions were detected
- BD-CPK6 G2A and BD-OST1 with AD-14-3-3-chi/epsilon/mu/nu/omicron/phi/psi/upsilon
    - OST1 was found to interact with all 14-3-3 proteins
    - CPK6 G2A was found to interact with 14-3-3-chi and 14-3-3-phi