THE ARABIDOPSIS CALCINEURIN B-LIKE10 CALCIUM SENSOR COUPLES
ENVIRONMENTAL SIGNALS TO DEVELOPMENTAL RESPONSES

by

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A Dissertation Submitted to the Faculty of the
SCHOOL OF PLANT SCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

2011
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ACKNOWLEDGEMENTS

Karen Schumaker – Thank you for helping me through every step of this process; reading papers with me to increase my understanding of basic plant development and physiology, helping me to identify projects that are of interest, discussing and designing experiments with me, training me in how to do those experiments, and finally analyzing the results. I have enjoyed being in your lab and appreciate all that you have done to help me acquire my Ph.D.

Ramin Yadegari, Brian Larkins, Frans Tax, Ravishankar Palanivelu – Thank you for serving on my committee and thank you for your advice and helpful suggestions.

YongSig Kim – Thank you for starting these projects and for always being there to answer my questions and teach me how to do some of these experiments.

Rocío Álvarez-Aragón – Thank you for helping me screen all of the transgenic lines on salt and isolating RNA. You are always so cheerful and it has been a pleasure working with you.

Courtney A. Magness – Thank you for helping me screen lines, generate mutants, and count seeds. I look forward to continuing to work with you.

My Parents – Thank you for all the love and encouragement you have given me. I appreciate all the sacrifices that you have made to help me succeed and am blessed to have such wonderful parents.

My God – Thank you for the grace you give me to accomplish anything!
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ABSTRACT

Calcium is a component of signal transduction pathways that allow plants to respond to numerous endogenous and environmental signals during growth and development. Calcium-mediated signaling involves multiple components including: 1) channels, pumps, and exchangers that act in concert to generate a change in cytosolic calcium, 2) calcium-binding proteins that sense the calcium change, and 3) downstream target proteins that modify enzyme activity and gene expression needed for the subsequent response. One method for achieving specificity during calcium signaling is through regulation of the calcium-binding proteins that perceive changes in cytosolic calcium. These proteins can be regulated through differences in expression in response to stimuli, localization within the cell or plant, affinity for calcium, and interaction with downstream target proteins; all of which can result in specific cellular responses.

My projects have focused on the Arabidopsis thaliana (Arabidopsis) CALCINEURIN B-LIKE10 (CBL10) calcium-binding protein, and specifically on understanding: 1) how post-transcriptional regulation of the CBL10 gene is used to modulate seedling growth in saline conditions (salinity), and 2) CBL10’s function in the flower during growth in salinity. In addition, 3) I have examined the roles of two putative CBL10-interacting proteins in plant growth and development.

CBL10 is alternatively spliced into two transcripts; CBL10 encoding the characterized, full-length protein and CBL10 LONG A (CBL10LA) encoding a putative truncated protein
due to a pre-mature termination codon within a retained intron. When seedlings are grown in the absence of salinity, both alternatively spliced transcripts are detected; however, in response to salinity, levels of the \textit{CBL10LA} transcript are reduced. My data suggest a model in which the relative abundance of the two transcripts regulates the \textit{SALT-OVERLY-SENSITIVE} (SOS) pathway involved in maintaining cellular sodium ion homeostasis. The presence of CBL10LA in the absence of salinity ensures that the SOS pathway is inactive. The removal of CBL10LA in response to saline conditions results in CBL10 activation of the SOS pathway to prevent sodium ions from accumulating to toxic levels in the cytosol.

Successful fertilization during flowering requires the coordinated development of stamens and pistils. Stamens must elongate and anthers dehisce to release pollen onto the stigma while the pistil prepares to receive the pollen and promote growth and targeting of the female gametophyte. When the \textit{cbl10} mutant is grown in salinity, flowers are sterile due to decreased stamen elongation, reduced anther dehiscence, and abnormal pistil development. My studies demonstrated that the SOS pathway is not involved in maintaining flower development in salinity and indicate that CBL10 functions in different pathways to regulate vegetative and reproductive development during growth in saline conditions.

An \textit{in silico} search for Arabidopsis proteins that might interact with CBL10 resulted in the identification of two components of the Mediator complex involved in the regulation of transcription in eukaryotes. While additional studies I carried out suggest
that interaction with CBL10 is unlikely, I have shown that these proteins are important for plant growth in high levels of chloride and in maintenance of growth in short-day conditions.
CHAPTER 1

CALCIUM-MEDIATED SIGNAL TRANSDUCTION IN PLANTS

Introduction

As sessile organisms, plants have unique and complex signaling processes that enable them to adjust growth in response to environmental signals to ensure successful development and reproduction. For example, dormant seeds perceive temperature and light to determine germination timing (Penfield and King, 2009). Seedlings perceive numerous signals including: gravity to determine the direction of root growth; soil nutrient status and water availability to determine root branching patterns and root hair development; light to determine the direction of leaf growth and leaf orientation; and light, humidity, and CO$_2$ to regulate stomatal apertures on the surface of the leaf (Kim et al., 2010; Monshausen and Gilroy, 2009; Muller and Schmidt, 2004; Stamm and Kumar, 2010). Mature plants perceive photoperiod and temperature to determine the switch between vegetative and reproductive development and light and soil nutrient status to initiate senescence programs (Amasino, 2010; Guiboileau et al., 2010). In addition, plants perceive and respond to environmental conditions that can limit growth including inadequate water (drought), accumulation of salts in the soil (soil salinity), and extremes of temperature (Reddy et al., 2011).
Signal transduction is the process by which a plant perceives an environmental signal and transduces it into a physiological or developmental response. Signal transduction pathways begin with perception of a signal which leads to biochemical or molecular changes that initiate specific responses. Calcium is a key component of many pathways, coupling perception of a signal with downstream cellular responses. Plant cells have developed mechanisms to maintain low cytosolic calcium levels to avoid the toxic effect of this ion which, at high concentrations in the cytosol, will precipitate phosphate and disrupt phosphate-based metabolism (Reddy et al., 2011). To prevent toxicity, calcium is chelated, compartmentalized within cellular organelles, or extruded from the cell. The regulation of calcium generates a large gradient between the cytosol (100-200 nM) and the compartments storing calcium (cell wall, endoplasmic reticulum, vacuole, 1-10 mM) (Reddy and Reddy, 2004). This gradient allows cells to use calcium as a signaling molecule so that small, transient changes in cytosolic calcium concentration can be perceived and can serve as a source of information.

In plants, a diverse range of stimuli induce changes in cytosolic calcium levels including drought, soil salinity, extremes of temperature, light, pathogen elicitors, nodulation factors, and hormones (Baum et al., 1999; Benjamins et al., 2003; Ehrhardt et al., 1996; Gilroy and Jones, 1992; Kiegle et al., 2000; Vadassery and Oelmuller, 2009). With so many stimuli inducing changes in cellular calcium levels, questions arise as to how specific cellular pathways are initiated and how specific responses are achieved. Specificity is thought to reside in the temporal and spatial characteristics of the calcium
change (signature) and in the regulation of proteins that perceive changes in calcium (Reddy and Reddy, 2004).

Specificity in the calcium signature occurs through the coordination of channels that allow calcium into the cytosol and pumps and exchangers that actively transport calcium out of the cytosol. The activity and regulation of these proteins determines the amplitude (how much calcium moves into the cytosol), the duration (how long calcium remains), and the frequency (lag time between calcium changes) of calcium in the cytosol and generates signal-specific spikes or oscillations (Allen et al., 2000; Kosuta et al., 2008).

Once calcium levels change, calcium-binding proteins (calcium sensors) perceive and transduce those changes into the appropriate cellular response. The majority of calcium sensors have one or more highly conserved calcium-binding helix-loop-helix motifs called EF-hands (Reddy et al., 2011). This 29 amino acid motif consists of an alpha helix E (residues 1-10), a loop (residues 10-21), and a second alpha helix F (residues 19-29) (Tuteja and Mahajan, 2007). The affinity of the EF-hand for calcium depends on the residues within the loop; specific residues, often aspartate and glutamate, are present at precise positions to stabilize the positive charge of the calcium ion (Tuteja and Mahajan, 2007). Changes in the amino acids present at a given position in the EF-hand will not necessarily abolish binding, but instead may modulate binding affinity allowing calcium sensors to respond to different calcium signatures (Kolukisaoglu et al., 2004). In
Arabidopsis thaliana (Arabidopsis) there are approximately 250 EF-hand-containing calcium sensors (~1% of the predicted proteome) (Reddy and Reddy, 2004).

Calcium sensors contribute specificity through differences in expression in response to stimuli, localization within the cell or plant, affinity for calcium, and interaction with downstream proteins; all of which can result in specific cellular responses (Reddy and Reddy, 2004). In plants, calcium sensors are divided into two main groups, sensor responders and sensor relays. Sensor responders have a catalytic or functional domain in addition to the EF-hand motifs. Ca\(^{2+}\)-dependent protein kinases (CDPKs/CPKs) and Ca\(^{2+}\)- and Ca\(^{2+}\)/CALMODULIN (CaM)-dependent protein kinases (CCaMKs) are examples of sensor responders (Reddy and Reddy, 2004). Sensor relays have no enzymatic activity or other functional domains (Reddy and Reddy, 2004). Upon binding calcium, the conformation of these proteins is changed to allow them to interact with and regulate the activity of downstream target proteins. CaM, CaM-Like (CML), and CALCINEURIN B-Like (CBL) proteins are categorized as sensor relays.

One environmental signal to which calcium mediates responses is soil salinity. Highly saline environments affect plant growth in two primary ways, the presence of salt in the soil lowers the water potential of the soil, reducing water and mineral uptake into the plant (osmotic stress) and accumulation of sodium in the cytosol can inhibit metabolic and photosynthetic processes (ionic toxicity) (Munns and Tester, 2008). The primary strategies for protection against ionic toxicity are to reduce uptake of sodium,
transport it from the cytosol out of the cell, or sequester it in the vacuole (Serrano and Rodriguez-Navarro, 2001).

The identification of salt-overly-sensitive (sos) mutants and their underlying genes elucidated a calcium-mediated signal transduction pathway involved in the efflux of sodium out of the cell during plant growth in saline conditions. Cellular calcium changes elicited by sodium are perceived by the SOS3/CBL4 EF-hand calcium sensor (Knight et al., 1997; Liu and Zhu, 1998). After binding calcium, SOS3 undergoes a conformational change that alters the surface properties of the protein activating a downstream serine/threonine protein kinase, SOS2/CBL-INTERACTING PROTEIN KINASE24 (CIPK24) (Sánchez-Barrena et al., 2005). SOS3 interacts with the FiSL motif located within an auto-inhibitory domain of SOS2 that blocks kinase activity (Guo et al., 2001; Halfter et al., 2000; Liu et al., 2000). Calcium binding to SOS3 releases the inhibitory domain from the kinase domain. The SOS3-SOS2 complex phosphorylates the plasma membrane Na’/H’ exchanger, SOS1, at a serine within an auto-inhibitory domain in the SOS1 C-terminal tail (Qiu et al., 2002; Quintero et al., 2011; Shi et al., 2000). Phosphorylation relieves inhibition and activates transport of sodium; sodium is then extruded using the energy stored in the proton gradient.

In addition to SOS3, another family member, CBL10/ SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCaBP8), plays a role in plant responses to soil salinity. Mutations in CBL10 result in decreased leaf growth and chlorosis when seedlings are grown in the presence of sodium (Kim et al., 2007; Quan, 2007). This is in contrast to mutations in SOS3 which
result in decreased root growth (Quan, 2007). CBL10 has also been shown to interact with SOS2 to regulate SOS1 activity (Lin et al., 2009; Quan, 2007).

My dissertation research has focused on understanding how regulation of the CBL10 calcium sensor generates specificity in calcium-mediated signaling. Specifically I explored:

1. How alternative splicing of CBL10 is used to regulate signal transduction pathways

Amplification of CBL10 from wild-type seedling RNA produced two bands representing alternatively spliced variants of the CBL10 gene (Y.S. Kim, unpublished (Quan, 2007). There is no evidence for alternative splicing of SOS3 indicating that this regulation is unique to the role of CBL10 in salt responses. My experiments examined the function of each variant and, based on my data, I have developed a model for how alternative splicing of CBL10 regulates the SOS pathway. In this model, CBL10 variants with antagonistic functions ensure a quick response to salinity. During growth in saline conditions, the negative regulator, CBL10LA, is degraded increasing the effective concentration of the positive regulator, CBL10, which signals a salt response through activation of SOS2.

2. The role of CBL10 in leaf and flower development during growth in saline conditions

In addition to defects in leaf development, mutations in the CBL10 gene result in sterility during growth in saline conditions. My studies focused on the role of CBL10 in flower development to understand which processes are disrupted by sodium and to
determine if \textit{CBL10} functions similarly in flowers and in leaves. I found that, when the \textit{cbl10} mutant is grown in saline conditions, stamen and pistil development are disrupted leading to sterility and that \textit{CBL10}'s function in flowers is independent of the SOS pathway.

3. The proteins with which \textit{CBL10} functions

The CBL family of calcium-binding proteins is thought to interact mostly with the CIPK family of protein kinases; however, there are reports of novel CBL-interacting proteins (Nozawa et al., 2001; Oh et al., 2008). To identify other potential CBL targets, an \textit{in silico} search for proteins containing a SOS2-like CBL10-interaction domain (FISL motif) was carried out. Two proteins were identified and I examined their role in plant growth and development and responses to the environment. These proteins are components of the Mediator complex involved in regulation of gene expression. Although they are unlikely to interact with the CBL family, my studies demonstrate that they play a role in plant growth in high levels of chloride and in maintenance of growth in short-day conditions.
CHAPTER 2

POST-TRANSCRIPTIONAL REGULATION OF CBL10 MEDIATES PLANT RESPONSES TO SALINITY

Introduction

The build-up of salt in agricultural fields (soil salinity) is a widespread problem that limits the growth and yield of important crop species. Salts, particularly sodium chloride, are present in soils irrigated with poor quality water and, with time, can accumulate to levels that prohibit plant growth. The Food and Agricultural Organization has estimated that 20% of irrigated land worldwide (which produces over 40% of all food) is affected by salt and that 1-2% of irrigated land is lost yearly due to the build-up of salt (FAO, 2002).

When plants are growing in saline conditions, sodium ions accumulate in cells and interfere with metabolic reactions (Serrano and Rodriguez-Navarro, 2001). To prevent the toxic build-up of salt, sodium is transported out of the cytosol across the plasma membrane or into the vacuole. In Arabidopsis, several proteins have been identified that function in the Salt Overly Sensitive (SOS) pathway to remove sodium from the cytosol. In this pathway, the perception of sodium causes an influx of calcium into the cytosol which is perceived by SOS3/CALCINEURIN B-LIKE4 (CBL4), a calcium-binding
protein, which interacts with and activates the serine/threonine kinase SOS2/CBL-INTERACTING PROTEIN KINASE24 (CIPK24, (Halfter et al., 2000)). The SOS3-SOS2 complex phosphorylates SOS1, a plasma membrane Na$^+$/H$^+$ exchanger, leading to transport of sodium out of the cell (Qiu et al., 2002). In addition to SOS3, another family member, CBL10/SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCaBP8), functions to activate the SOS pathway during growth in saline conditions (Quan, 2007). Mutations in CBL10 result in decreased leaf growth and chlorosis when seedlings are grown in the presence of NaCl whereas mutations in SOS3 result in decreased root growth (Quan, 2007). These tissue-specific phenotypes suggest that SOS3 and CBL10 function to protect roots and leaves, respectively, during growth in saline conditions.

When CBL10 was amplified from wild-type seedling RNA, two bands were observed. These bands were isolated, the DNA was cloned and sequenced and found to represent alternatively spliced variants of the CBL10 gene. Alternative splicing is the differential processing of pre-mRNAs to produce different transcripts from a single gene due to the inclusion of introns, exclusion of exons, or alternative acceptor and donor sites for exons. These variants can expand the proteome by coding for proteins with different domains, activities, localization, interactions with other proteins, and post-translational modifications (Simpson et al., 2008). It is estimated that approximately 80% of human genes and 20% of Arabidopsis and rice genes (likely a conservative estimate due to the lack of RNA sequence data) are alternatively spliced (Barbazuk, 2008). In plants, changes in alternative splicing patterns are observed in response to the environment,
but the functions of specific variants and the mechanisms underlying their role in plant adaptation are not well understood (Barbazuk, 2008).

The two predominant variants produced from the CBL10 gene were the characterized CBL10 transcript and an alternatively spliced variant, CBL10LA, with the seventh intron retained in the final mRNA. To understand how alternative splicing of CBL10 regulates the SOS pathway, the roles of CBL10 and CBL10LA during plant growth in saline conditions were examined.

**Materials and Methods**

**Plant material**

*Arabidopsis thaliana* Col-0 was used as wild type for this study. A CBL10 T-DNA insertion line (SALK_056042) was obtained from the Arabidopsis Biological Resource Center (ABRC) and backcrossed to wild type three times to remove insertions in other genes. Genomic DNA from the mutant was used as a template to identify wild-type and mutant alleles. A homozygous cbl10 mutant was identified in the first self-pollinated generation of backcross three (BC3F2). To test for the presence of a T-DNA insertion, the LBa1 (5’-TGGTTCACGTAGTGCCATC-3’) and 056042_L (5’-TCTGCTATTCTGGAATCTGA-3’) primers were used. To identify alleles without a T-DNA insertion (wild-type alleles), the 056042_L primer was used with 056042_R (5’-CTGCCATAGACGCAAGATGA-3’).
RNA isolation and cDNA synthesis

Cloning

To clone the *CBL10* and *CBL10LA* cDNA transcripts, RNA was isolated from 17 day-old wild-type plants using Trizol (Invitrogen, Carlsbad, CA). To synthesize cDNA, total RNA (5 µg; volume adjusted to 11 µl with distilled water) was incubated with 1 µl of 100 µM oligo dT primer at 65°C for 5 min and then transferred to ice for 2 min to anneal the primer to the RNA. A reaction mix containing 1 µl SuperScript III Reverse Transcriptase (Invitrogen), 4 µl 5X first strand buffer, 1 µl 10 mM dNTPs, 1 µl 0.1 M DTT, and 1 µl RNase Inhibitor (Invitrogen) was added to the RNA for a total volume of 20 µl. This mixture was incubated at 50°C for 60 min followed by 70°C for 15 min. After incubation, 1 µl of RNaseH (Invitrogen) was added along with 3 µl of 10X buffer and 6 µl of water for a total volume of 30 µl. The reaction was incubated at 37°C to allow the RNaseH to degrade the RNA strand of the RNA-DNA duplex.

Transcript analysis

To monitor the *CBL10* and *CBL10LA* transcripts, RNA was isolated in a 50 µl volume using the Qiagen RNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD) and treated with 2 µl TURBO DNase (Ambion, Austin, TX) to remove any DNA present. RNA was purified using the RNeasy MinElute Cleanup Kit from Qiagen prior to cDNA synthesis. cDNA synthesis was carried out as described above for cloning with the following modifications; the concentration of RNA was decreased to 1.5 µg and the Qiagen
MinElute Kit was used to purify the cDNA after synthesis was complete. For the PCR reactions, 1 µl of cDNA was used in a 20 µl volume.

**Primers for amplifying CBL10 and CBL10LA transcripts**

To amplify only CBL10, the CBL10-qRT-F2 (5’- TTGCATTAGGTCTATGATCTAAGAC-3’) and CBL10-qRTPCR-R2 (5’- CAGAATCTGCATCAGCAATGTGTTT-3’) primers were used. To amplify only CBL10LA, the following primers were used: CBL10LA-qRT-F2 (5’-TGCATTAGGTCTATGATCTAAGAC-3’) and CBL10LA-qRT-R5 (5’-TATACATCACACTAAGAGATGGGA-3’) (detection of the full-length transcript) or CBL10LA-qRT-R4 (5’- GAACTCACTTTATCAATAATCATGG-3’) (detection of the transcript prior to the pre-mature termination codon). To amplify both transcripts, the CBL10.2F (5’- TGAGCTATTCAAGAAATTGAGCTG-3’) and CBL10.2R (5’-ACTAATTTTACGTCTTTGTCAGAA-3’) primers were used. For loading controls, the following primers were used: TUBULIN, 5Tub (5’-TYATGGATYNGAGCCTGG-3’) and 3Tub (5’-TCAGAGTGNAGYWCCMGG-3’) and GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC), GAPC-F2 (5’-CACTTGAAGGGTGCCCAAG-3’) and GAPC-R (5’-CCTGTTGTGCAGAACGACATC-3’).

**Constructs for complementation and over-expression**

**CBL10 genomic C-terminal Green Fluorescent Protein (GFP) fusion lines**

A 3,790 base pair CBL10 genomic fragment containing the CBL10 gene and 2,383 base pairs upstream of the translational start site was amplified from genomic DNA
using Phusion High Fidelity DNA Polymerase (Thermo Scientific, Lafayette, CO) and the primers: PM5-CBL10 (5’-CAGCGACGATAAAATGGTT-3’) and PM3-AtCBL10-3Xhol (5’-CATCTCGAGGTCTTCAACCTCAGTGGT-3’). The product was cloned into the pCR-XL-TOPO vector (Invitrogen) and subcloned into the plant binary vector pBN-GFP containing a C-terminal Green Fluorescent Protein (GFP) fusion using the BamHI and Xhol restriction sites. The binary vector was transformed into *Agrobacterium tumefaciens* strain GV3101 followed by transformation into the *cbl10* mutant using the standard floral dip method (Clough and Bent, 1998). Transformed seed was selected on kanamycin and T2 lines with 75% resistance (single insertion) were chosen. Homozygous seed was obtained by screening T3 seed on kanamycin to identify lines with 100% resistance.

**CBL10 genomic N-terminal 6X Myc fusion lines**

A stepwise cloning was used to generate an N-terminal-tagged *CBL10* genomic construct. A 318 base pair PCR fragment containing a 6X Myc-tag was amplified from the vector pCM1307:6XMyC (provided by Dr. Yan Guo, China Agricultural University, Beijing) using the BamHI-1307-F(5’-ACTGGATCCGATTTAAGCTATGGAGCA-3’) and 1307-R (5’-GTGATTTTTGCAGAGTACC-3’) primers and cloned into pCAMBIA2300 using the BamHI and Xba1 restriction sites. A 2,200 base pair fragment containing the *CBL10* promoter region was amplified from genomic DNA using Phusion High Fidelity DNA Polymerase and the AtCBL10p5 (5’-GGCGAATTCCAGCGACGATAAAATGGTT- 3’) and BspHI-CBL10p-R (5’-CCGTCATGAGTTCATTCAAATCACA-3’) primers. The product was cloned into the pGEM-T EASY vector (Promega, Madison, WI) and subsequently
subcloned into the pCAMBIA2300 vector containing the 6X Myc fragment using the EcoRI site. A 2,600 base pair fragment containing the CBL10 genomic sequence and 3’UTR was cloned into the vector pFGC5491 (provided by Dr. Richard Jorgensen, University of Arizona). The CBL10 genomic sequence, 3’UTR and the Octopine Synthase (OCS3) terminator were digested with XhoI and PstI and subcloned into the pCAMBIA2300 vector containing the CBL10 promoter and 6X Myc tag using the SalI and PstI restriction sites. Plant transformation and selection were as described above for the CBL10 genomic C-terminal GFP construct.

**CBL10 and CBL10LA cDNA lines with the native CBL10 promoter**

A 2,200 base pair fragment containing the CBL10 promoter region was amplified from genomic DNA using Phusion High Fidelity DNA Polymerase and the PM5-AtCBL10-3PstI (5’-CGCCTGCAGCGGCCAGCGACGATAAAATGGTT-3’) and PM3-AtCBL10-5XhoI (5’-CGCCTCGAGGAGTTCATTCAAAATCACAATCACAG-3’) primers. The product was cloned into the pCR-XL-TOPO vector and subsequently subcloned into the pBN-GFP vector using the PstI and XhoI restriction sites. The CBL10 cDNA (738 base pairs), containing the protein coding sequence of CBL10 minus the stop codon, was synthesized from seedling RNA using Taq DNA polymerase (Invitrogen) and the N5CBL10B (5’-GGCGGATCCATGGAACAAGTTTCCTAGAT-3’) and AtCBL10p3 (5’-GGCGGATCCTCAGTCTTCAACCTCAGTGGT-3’) primers. The product was cloned into the pBlueScript (pBS) SK(-) vector and subcloned into the pBN-GFP vector containing a C-terminal GFP fusion using the BamHI restriction site. In a similar manner, the CBL10LA
fragment (582 base pairs), containing the protein coding sequence of *CBL10LA* minus the stop codon, was amplified from a plasmid containing the full-length *CBL10LA* transcript using Taq DNA polymerase and the N5CBL10B (5'-GGCGGATCCATGGAACAAGTTTCCTCTAGAT-3') and 3CBL10L4-BamHI (5'-GGCGGATCGAACTCACTTTATCAATAATCATG-3') primers. The product was cloned into the pBS SK(-) vector and subcloned into the pBN-GFP vector using the BamHI restriction site. Plant transformation and selection were as described above for the *CBL10* genomic C-terminal GFP construct.

**CBL10 and CBL10LA cDNA over-expression lines**

*CBL10* over-expression lines were generated by amplifying the 738 base pair cDNA fragment (protein coding sequence of *CBL10* minus the stop codon) from cDNA synthesized from seedling RNA using Taq DNA polymerase and the 5CBL10XhoI (GCGCTCGAGATGGAACAAGTTTCCTCTAGAT) and 3CBL10B (5'-GGCGGATCCCTACACCTCAGTTGG-3') primers. The product was cloned into the pBS SK(-) vector, digested with XhoI and BamHI restriction enzymes, and subcloned into the corresponding site of the plant binary vector pEZT-NL (Cutler and Ehrhardt, Carnegie Institution of Washington, Stanford, CA) containing the Cauliflower Mosaic Virus 35S promoter and a C-terminal GFP fusion. *Agrobacterium tumefaciens* stain LBA4404 containing the binary vector was used to transform wild type and the *cbl10* mutant. Transformed seed was selected on 10 µg/ml glufosinate ammonium (Crescent Chemical Company, Inc., Islandia, NY). PCR using 056042_L and 056042_R primers (see Plant
Material) was performed to confirm the presence of the transgene in T1 plants. T2 seed was screened on glufosinate ammonium and lines with 75% resistance (single insertion) were chosen. Homozygous seed was obtained by screening T3 seed on glufosinate ammonium to identify lines with 100% resistance.

*CBL10LA* over-expression lines were generated by amplifying a 582 base pair cDNA fragment (protein coding sequence of *CBL10LA* minus the stop codon) from a plasmid containing the full-length *CBL10LA* transcript using Taq DNA polymerase and the 5CBL10Xhol (GCGCTCGAGATGGAACAAGTTTCCTCTGAT) and 3CBL10L4-BamHI (5’-GGCGGATCCGAACTCACTTTATCAATAATCATG-3’) primers. The product was cloned into the pBS SK(-) vector. Subcloning, plant transformation, and selection were as described above for *CBL10*.

**Screen for seedling salt sensitivity**

Seeds were germinated on vertical plates with 1/2X Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 2% sucrose, 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES), and 1% agar (pH adjusted to 5.7 with KOH), stratified for 2 days at 4°C in the dark, and then transferred to a growth chamber at 21°C under a 16 hour light/8 hour dark photoperiod. Five day-old seedlings were transferred to solid media containing 1/2X MS or 1/2X MS with NaCl. Photographs were taken 12 days after transfer.
**Immunoprecipitation of Myc-tagged-CBL10 and -CBL10LA proteins**

Soluble and membrane-bound proteins were isolated from control (*cbl10*) and *CBL10* genomic N-terminal 6X Myc fusion lines using a protocol adapted from Dr. Yan Guo (China Agricultural University, Beijing). Approximately 1 g of 10 day-old seedlings were homogenized in 2 ml cold homogenization buffer (10 mM Tris-HCl pH7.5, 2 mM EDTA, 150 mM NaCl, 0.5% (v/v) igepal CA-630, 1 mM PMSF, 3 µg/ml leupeptin, and 1 µg/ml pepstatin A) using pre-cooled mortars and pestles. The homogenate was centrifuged using an Eppendorf 5402 tabletop microcentrifuge at 12,000 rpm (12000g) for 10 min at 4°C. The supernatant was centrifuged again using a Beckman TL tabletop ultracentrifuge at 57,000 rpm (140,000g) for 60 min at 4°C. The supernatant was collected as the soluble fraction and the pellet was resuspended in 2 ml of homogenization buffer and collected as the microsomal membrane fraction. Protein was stored at -80°C. Protein was quantified using the BioRad protein assay dye reagent concentrate (BioRad, Hercules, CA). To immunoprecipitate proteins, 3 mg of soluble and membrane-bound proteins were incubated with anti-Myc- HORSERADISH PEROXIDASE (HRP) conjugate antibodies (Invitrogen) pre-bound to Protein G-coated agarose beads (Sigma, St. Louis, MO). To pre-bind the antibody to the beads, the antibody was incubated with beads for 5 h at 4°C on a nutator. Beads were centrifuged using a microcentrifuge at 2,000 rpm for 1 min. The supernatant was removed and the beads were washed with NP-40 buffer (50 mM tris-HCl pH7.5, 150 mM NaCl, and 1% igepal CA-630). The proteins were incubated with the antibody-beads overnight on a
nutator at 4°C. Beads were centrifuged and washed three times as described above. To release the proteins from the beads, 2X sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) gel loading buffer (5X loading buffer – 60 mM tris-HCl pH6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) was added to beads and incubated in a 90°C water bath for 5 min. Beads were pelleted with a microcentrifuge at 14,000 rpm for 5 min and supernatant was loaded onto a 12% SDS-PAGE gel and run at 90 volts for 2 h. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) in transfer buffer (20% methanol, 25 mM Tris, 1 M glycine, pH 8.3) at 90 volts for 50 min. The membrane was incubated overnight in blocking buffer (1X PBS with 0.05% tween-20 and 5% powdered milk) followed by 2 washes with PBST (1X PBS with 0.05% tween-20) and incubated with antibody (1:50,000 dilution) for 2 h at room temperature. Anti-Myc-HRP conjugate antibodies were used to detect Myc-tagged proteins. The GE Healthcare Amersham Enhanced Chemiluminescence (ECL) Advance Western Blotting detection kit (Amersham) was used to detect bound antibody. Blue autoradiography film (ISC BioExpress, Kaysville, UT) was used to detect luminescence.

**Purification of recombinant proteins from E. coli**

The cDNA for SOS2 was amplified using the 5SOS2N-BamHI (5’-
GGCGGATCCATGACAAAGAAAATGAGAAGAGT-3’) and 3SOS2N-EcoRI (5’-
GGCGAATTCTTGATTCCTTGAAATTTGAATA-3’) primers and cloned into the pGEX-2TK vector (GE Healthcare, Piscataway, NJ) in frame with the *Glutathione S-Transferase*
(GST) coding sequence using the BamHI and EcoRI restriction sites. The SOS2-GST construct was introduced into *E. coli* BL21 codon*+* cells (Stratagene/Agilent Technologies, Santa Clara, CA) and transformants were selected for with 50 µg/ml ampicillin (AMP). To isolate protein, 2 ml Lysogeny Broth (LB) was inoculated with cells from a single colony and grown at 200 rpm overnight at 37°C. 1 ml of the overnight culture was used to inoculate 500 ml of LB. This culture was incubated at 37°C and 200 rpm until OD600 was approximately 0.6. Expression of the SOS2-GST fusion protein was induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 200 rpm overnight at room temperature. After induction, cells were harvested and resuspended in 1X phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) with 0.1 mg/ml leupeptin and 0.1 mg/ml pepstatin A. After sonication, 1% triton X-100 was added and the mixture was incubated on ice for 30 min with gentle shaking followed by centrifugation twice at 11,000g to separate the soluble and pellet fractions. The soluble fraction was incubated with glutathione sepharose beads (GE Healthcare Life Sciences, Sweden) for 1 hour at 4°C on a nutator. The soluble fraction was then placed in a Poly-prep chromatography column (Bio-Rad, Hercules, CA) and the beads were allowed to settle for 30 min. The column was washed 3 times with 1X PBS and bound protein was eluted with glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0).

The cDNAs of *CBL10* and *CBL10LA* were cloned into the pBAD-Myc-HIS A vector (Invitrogen) in frame with the *Myc* coding sequence followed by six histidine residues.
**CBL10** was amplified using the 5CBL10Ncol (5’- GCGCCATGGAAACAAGTTTCTCTCTAGAT-3’) and N3CBL10BamHI (5’- GCGGATCCCCGTTCTTTCAACCTCAGTGTTGAA-3’) primers and cloned into the pBAD vector using the Ncol and BglII sites. **CBL10LA** was amplified using the same 5’ primer, 5CBL10Ncol, with the 3CBL10L4-PstI (5’-GCGCTGAGAACTCAGTTATCAATAATCATG-3’) 3’ primer and cloned into the pBAD vector using the Ncol and PstI sites. The Myc-HIS tagged protein constructs were introduced into *E. coli* Top10 cells (Invitrogen) and transformants were selected using 50 µg/ml AMP. Protein purification was as described for SOS2-GST with the following modifications: expression was induced with 0.02% L-arabinose, cells were resuspended in 1X native purification buffer (50 mM sodium phosphate monobasic, 0.5 M NaCl, pH 8.0), the soluble fraction was incubated with Ni-NTA Agarose beads (Invitrogen), and the column was washed with native purification buffer containing 20 mM imidazole followed by 100 mM imidazole before protein elution with 200 mM imidazole.

**Calcium-binding assay**

Recombinant CBL10-Myc-HIS and CBL10LA-Myc-HIS proteins (1 µg) were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane as described above. After transfer, the membrane was washed with 5 mM EGTA, pH 7.0 for 1 min to remove calcium and then 3 times with wash buffer (60 mM KCl, 2.5 mM MgCl₂, 5 mM imidazole, pH 7.0) for 30 min each to remove excess transfer buffer and re-nature the proteins. For calcium binding, the membrane was incubated in wash buffer with 1 µCi ⁴⁵Ca²⁺ per ml for 15 min followed by 2 washes with 50% ethanol for 2 min. The blot was exposed
to Kodak XAR-5 film for 24 h at -80°C. After the film was developed, the membrane was
stained with 0.2% Ponceau S to monitor protein levels.

**In vitro pull-down assay**

Recombinant SOS2-GST (5 µg) and CBL-Myc-HIS (4 µg)-tagged proteins were
incubated with glutathione sepharose beads in binding buffer (50 mM Tris-HCl at pH 6.7,
100 mM NaCl, 0.05% tween-20, 1 mM PMSF) at 4°C overnight on a nutator. The
proteins were centrifuged in a microcentrifuge at 2,000 rpm for 5 min to pellet beads
and washed 3 times with binding buffer before proteins were eluted with glutathione
elution buffer as described above. Protein separation, transfer, blocking, and washes
were as described for the immunoprecipitation protocol. The membrane was incubated
with antibody (a 1:5000 dilution) for 2 h at room temperature. Anti-GST- HRP (GE
Healthcare) and anti-Myc-HRP conjugate antibodies were used to detect GST- and Myc-
tagged proteins, respectively. The GST Western Blotting Enhanced Chemiluminescence
(ECL) detection kit was used to detect bound antibody. Blue autoradiography film was
used to detect luminescence.
Results

CBL10 is alternatively spliced into two predominant transcripts, CBL10 and CBL10LA

To understand the role of CBL10 in plant growth and development, a T-DNA mutant line was obtained from the SALK Institute (Figure 2.1A). As observed previously (Kim et al., 2007; Quan, 2007), growth of cbl10 on NaCl resulted in reduced leaf growth and chlorosis compared to growth of wild-type seedlings (Figure 2.1B). When the CBL10 gene was transformed into the cbl10 mutant, growth on media containing NaCl was restored to wild type-like levels (Figure 2.1B).

RNA was isolated from wild-type and cbl10 seedlings to examine expression of CBL10; no full-length CBL10 transcript was present in the cbl10 mutant (Figure 2.1C). In the process of amplifying CBL10 from wild-type seedling RNA, two bands were observed (Figure 2.1C). The bands were isolated, the DNA was cloned and sequenced and found to represent alternatively spliced variants of the CBL10 gene (Appendix Figure 2.1). In the first round of cloning, the two bands were combined and 16 clones were sequenced. Six of the clones encoded the CBL10 transcript while the other 10 clones represented four variants with alternative exon donor and acceptor sites and a retained intron (Appendix Figure 2.1A). The transcripts were labeled as short (S) or long (L) followed by an identifying letter. Further sequencing was performed using RNA from seven day-old seedlings grown in the absence and presence of 200 mM NaCl for one hour. A fragment of CBL10 was amplified to focus on the region where alternative splicing was observed and DNA within the upper and lower bands was cloned individually. The lower
Figure 2.1 CBL10 is involved in plant responses to growth in saline conditions

A. CBL10 genomic structure indicating the position of the T-DNA insertion. Black boxes, exons; lines, introns; triangle, T-DNA; red arrows, position of primers used for RT-PCR in panel C.

B. The cbl10 mutant is hypersensitive to growth in saline conditions. Seeds from wild type (WT), cbl10, and cbl10 transformed with the CBL10 genomic clone under the control of the native promoter (cbl10;pCBL10:CBL10) were germinated on solid MS for five days and transferred to solid MS without or with increasing concentrations of NaCl. Photographs were taken 12 days after transfer. Seedlings from one of ten homozygous, independently transformed lines are shown. One representative image of three replicates.

C. No full-length CBL10 transcript was detected in the cbl10 mutant and two bands were amplified in wild type. RNA was isolated from wild-type and cbl10 seedlings and used to synthesize cDNA. The primers indicated in panel A were used to detect the presence of a full-length CBL10 transcript. TUBULIN was used as a loading control. One representative image of three replicates.
Figure 2.1

A.

B.

C.

WT  cbl10  cbl10:pCBL10::CBL10

MS

50 mM NaCl

100 mM NaCl

150 mM NaCl

CBL10

TUBULIN
band (short transcripts) predominantly contained the *CBL10* variant (16 of the 21 sequenced transcripts; Appendix Figure 2.1B). The upper band (long transcripts) predominantly contained the LA variant (13 of the 22 sequenced transcripts, five of which were the *CBL10* variant; Appendix Figure 2.1B). These results indicate that *CBL10* and *CBL10LA* represent the predominant variants present in the two bands; these variants were characterized in subsequent assays.

The *CBL10* variant is 741 base pairs while *CBL10LA* is 841 base pairs due to retention of the seventh intron in the final mRNA (Figure 2.2A). The *CBL10LA* transcript likely encodes a truncated protein due to a pre-mature termination codon within the first 12 base pairs of the intron.

**Levels of *CBL10LA* are reduced during plant growth in saline conditions**

To understand the dynamics of post-transcriptional regulation of *CBL10*, *CBL10* and *CBL10LA* transcript accumulation was monitored during seedling growth in saline conditions. For these studies, reverse transcriptase polymerase chain reaction (RT-PCR) was used with primers designed to amplify both variants and primers designed to amplify each variant individually. To examine both transcripts, primers to the third and eighth exons were used (Figure 2.2A, red arrows). To monitor the *CBL10* transcript, a 3’ primer was generated that spanned the seventh and eighth exons, annealing to three and 25 base pairs, respectively (Figure 2.2A, blue arrows). To verify that the *CBL10LA* transcript is not amplified by the *CBL10*-specific primers, several assays were performed. First, the primers were tested on wild-type cDNA. A single band was amplified
Figure 2.2  *CBL10LA* transcript levels decrease within hours of seedling exposure to NaCl

A. *CBL10* and *CBL10LA* genomic structures. Black boxes, protein coding exons; grey boxes, non-protein coding exons; lines, introns; red arrows, primers to amplify both variants; blue arrows, primers to amplify *CBL10*; green arrows, primers to amplify *CBL10LA*.

B. The *CBL10LA* transcript is reduced during seedling exposure to NaCl. Five day-old wild-type seedlings germinated on solid MS media were placed in liquid MS without or with 200 mM NaCl. Seedlings were harvested at the indicated times; RNA was isolated and used to synthesize cDNA. The primers indicated in panel A (red arrows) were used to amplify both *CBL10* variants. *TUBULIN* was used as a loading control. Image of one of two replicates.

C. The *CBL10LA* transcript is reduced with increasing concentrations of NaCl. Five day-old wild-type seedlings germinated on solid MS media were placed in liquid MS without or with increasing concentrations of NaCl. Seedlings were harvested after 5 h; RNA was isolated and used to synthesize cDNA. Primers indicated in panel A were used to amplify *CBL10LA* (green arrows) and *CBL10* (blue arrows). *TUBULIN* was used as a loading control. Image of one replicate.
Figure 2.2

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<td>TUBULIN</td>
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suggesting that the 3’ portion of the primer was unable to anneal to the eighth exon in \textit{CBL10LA} to produce a second, longer band; subsequent sequencing verified these results. To demonstrate that the 5’ portion of the reverse primer is not able to anneal to initiate reverse transcription of both variants (which would produce one band), the primers were tested on plasmid DNA that contained either the \textit{CBL10} or \textit{CBL10LA} cDNAs. The primers amplified the \textit{CBL10} but not the \textit{CBL10LA} template indicating that the three base pairs in the 5’ portion of the reverse primer are unable to anneal to just the seventh exon to amplify the \textit{CBL10LA} transcript. To examine just the \textit{CBL10LA} transcript, a 3’ primer was generated to anneal to the \textit{CBL10LA} intron (Figure 2.2A, green arrows). When tested on wild-type cDNA, a single band was amplified, sequenced, and found to correspond to the \textit{CBL10LA} transcript.

To determine if NaCl affects \textit{CBL10} alternative splicing, wild-type seedlings germinated on solid MS media were incubated in liquid MS containing 200 mM NaCl for 0, 30 minutes, one, and six hours or liquid MS for six hours. The \textit{CBL10LA} transcript decreased after one hour of salt stress and remained low after six hours (Figure 2.2B). To determine if the concentration of NaCl affects \textit{CBL10} alternative splicing, wild-type seedlings were exposed to liquid MS containing 100, 200, and 300 mM NaCl for five hours. The decrease in the \textit{CBL10LA} transcript was most apparent when seedlings were exposed to 200 and 300 mM NaCl (Figure 2.2C). In contrast, accumulation of the \textit{CBL10} transcript was unaffected by time of exposure to or the concentration of NaCl (Figure 2.2B and C).
When *CBL10LA* transcript levels were examined in wild-type seedlings germinated on solid MS media and transferred to solid MS without or with 100 mM NaCl for 12 days, only a subtle decrease in *CBL10LA* was observed. To better understand the dynamics of *CBL10LA* transcript accumulation in response to NaCl, wild-type seedlings were germinated on solid MS media for five days and transferred to MS media without or with 200 mM NaCl for one day. Under these conditions, a decrease in *CBL10LA* was observed (Figure 2.3). These results indicate that a decrease in *CBL10LA* is observed when plants are exposed to high concentrations of NaCl for short periods of time.

**Putative CBL10 and CBL10LA proteins were detected in planta**

While transcripts with pre-mature termination codons are often targets of non-sense mediated decay (NMD) (Lareau et al., 2007a), the abundance of the *CBL10LA* transcript suggests that it is not a target of this degradation process even though it contains a pre-mature termination codon. To determine if *CBL10LA* is translated into a protein, the *CBL10* gene (genomic sequence) was fused to an N-terminal 6X Myc-tag. This construct would tag both CBL10 and CBL10LA proteins which could be distinguished by size on an acrylamide gel. The constructs were transformed into the *cbl10* mutant under the control of the native *CBL10* promoter and two homozygous, independently transformed lines were screened on NaCl; the salt sensitive phenotype of the mutant was complemented in both lines (Figure 2.4A). These lines were used to detect the CBL10 isoforms by cellular fractionation and immunoblotting. To increase protein levels
Figure 2.3 The \textit{CBL10LA} transcript is reduced when seedlings are grown on 200 mM NaCl

Five day-old wild-type seedlings germinated on solid MS media were transferred to solid MS without or with 200 mM NaCl. Seedlings were harvested after one day; RNA was isolated and used to synthesize cDNA. Primers indicated in Figure 2.2A were used to amplify \textit{CBL10LA} (green primers), \textit{CBL10} (blue primers), or both transcripts (red primers). \textit{TUBULIN} was used as a loading control. Image of one of two replicates.
Figure 2.3
Figure 2.4 Putative CBL10 and CBL10LA proteins were detected \textit{in planta}

A. The \textit{CBL10} N-terminal-tagged genomic construct complements the \textit{cbl10} salt-sensitive phenotype. Seeds from wild type, \textit{cbl10} transformed with a 6X \textit{Myc}-N-terminal-tagged \textit{CBL10} genomic clone under the control of the native promoter (\textit{cbl10;pCBL10:Myc-CBL10}, \textit{cbl10} + \textit{Myc-CBL10}, T2 segregating population), and \textit{cbl10} were germinated on solid MS for five days and transferred to solid MS without or with 100 mM NaCl. Photographs were taken 12 days after transfer. Seedlings from one of three T2 segregating, independently transformed lines are shown. Image of one replicate.

B. Putative CBL10 and CBL10LA proteins were detected in the soluble fraction of protein isolated from transgenic plants. Soluble and membrane bound proteins were isolated from \textit{cbl10} and \textit{cbl10} transformed with a 6X-Myc-N-terminal-tagged \textit{CBL10} genomic clone under the control of the native promoter (\textit{cbl10;pCBL10:MyC-CBL10}). Tagged-proteins were immunoprecipitated with an anti-\textit{Myc} antibody linked to Protein G-coated agarose beads, separated on an SDS-PAGE gel, and transferred to a PVDF membrane. An anti-\textit{Myc}-HRP antibody was used to detect the presence of the tagged-proteins. Two homozygous, independently transformed lines are shown (1 and 2) along with a non-transformed \textit{cbl10} control (C).
Figure 2.4

A.  

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B.  

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for detection, soluble and microsomal membrane proteins (golgi, endoplasmic reticulum, vacuolar, and plasma membranes) were first immunoprecipitated using an anti-Myc antibody linked to Protein G-coated agarose beads and separated on an SDS-PAGE gel, transferred to a PVDF membrane, and probed with an anti-Myc-HRP conjugated antibody. In the soluble fraction, a band corresponding to the size of CBL10 was observed in both of the transformed lines that were examined and a band corresponding to the size of CBL10LA was observed in one of the lines (Figure 2.4B). In the membrane fraction, a band corresponding to CBL10 was observed in one of the lines (Figure 2.4B). Experiments are underway to further increase protein levels for detection and examine protein levels when plants are exposed to NaCl.

CBL10LA does not bind calcium

Sensor relays like CBL10 bind calcium and undergo a conformational change enabling them to interact with and regulate downstream targets. A common structural motif that binds calcium in these sensors is an EF-hand (helix-loop-helix). The loop, positioned between two alpha helical structures, consists of 12 residues, six of which are critical for binding calcium by providing oxygen atoms to stabilize the positive charge of the calcium ion (Tuteja and Mahajan, 2007). CBL10 has four putative EF-hand motifs (Kolukisaoglu et al., 2004); the fourth (C-terminal most) represents a canonical EF-hand (Kolukisaoglu et al., 2004). The second and third EF-hands have basic amino acid substitutions at key positions and the first has non-oxygen containing amino acid substitutions at key positions. These changes most likely decrease the affinity of
Figure 2.5 The predicted CBL10LA protein does not contain the fourth EF-hand found in CBL10 and does not bind calcium

A. **Amino acid alignment of the predicted CBL10 and CBL10LA proteins.** Black letters, amino acids; red letters, EF-hand motifs; blue circled amino acid, phosphorylated serine (Lin et al., 2009); *, stop codons.

B. **CBL10LA does not bind calcium.** Recombinant CBL10 and CBL10LA proteins purified from *E. coli*, were separated on an SDS-PAGE gel, transferred to a PVDF membrane, and incubated with radio-labeled calcium (*^{45}\text{Ca}^{2+}*). Autoradiograph, bound calcium; Ponceau S stain, protein. One representative image of three replicates.
Figure 2.5

A.

CBL10  MEQVSSRSSS  LVQEOFPGAV  PIPPPAIDV  LVSSVQCFD  CRSTSPTTCQ  HADLERLAB  SQPSVNBVBA
CBL10LA  MEQVSSRSSS  LVQEOFPGAV  PIPPPAIDV  LVSSVQCFD  CRSTSPTTCQ  HADLERLAB  SQPSVNBVBA

CBL10  LYLEPKRLSC  SIIDQGLINE  EELRALPQA  FNGFNLFD  VFDLDEEEN  GVIEFEEFH  ALSVFHPTAS
CBL10LA  LYLEPKRLSC  SIIDQGLINE  EELRALPQA  FNGFNLFD  VFDLDEEEN  GVIEFEEFH  ALSVFHPTAS

CBL10  IQERTDPFR  LYDDQTGPI  EREEVQQVS  AILLESMDH  SDLLTMID  KTPADADSR  DQKISDEMN
CBL10LA  IQERTDPFR  LYDDQTGPI  EREEVQQVS  AILLESMDH  SDLLTMID  KVSS*

CBL10  VYVIEHPSSL  ERMDTLPYLD  VTTASPSIF  NIEVED*

B.

**Autoradiograph**

**Ponceau S stain**
the EF-hand for calcium and may prevent binding at this site (Kolukisaoglu et al., 2004).

The predicted CBL10LA protein is truncated containing only the first three EF-hands, and lacking the last, canonical EF-hand (Figure 2.5A). As a first step in understanding the function of the CBL10LA protein, its ability to bind calcium was monitored. *CBL10* and *CBL10LA* transcripts were amplified from wild-type cDNA and cloned into the pBAD-Myc-HIS vector. The plasmids encoding the tagged proteins were expressed in *E. coli* and recombinant *CBL10-Myc-HIS* and *CBL10LA-Myc-HIS* proteins were purified. The proteins were separated on an SDS-PAGE gel, transferred to a PVDF membrane and incubated with radiolabeled calcium (\(^{45}\text{Ca}^{2+}\)); CBL10 bound calcium but CBL10LA did not (Figure 2.5B). Since binding calcium is critical for the function of a calcium sensor, these results suggest that CBL10 is likely functional while CBL10LA is not.

**CBL10LA interacts with the SOS2 protein kinase**

In response to plant growth in sodium, CBL10 interacts with and activates SOS2 to regulate SOS1 (Lin et al., 2009; Quan, 2007). To determine if CBL10LA also interacts with SOS2, an *in vitro* pull-down assay was performed. Recombinant *CBL10-Myc-HIS*- and *CBL10LA-Myc-HIS*-tagged proteins were incubated with GST-tagged SOS2. GST-SOS2 was pulled down using glutathione-coated sepharose beads and an anti-Myc antibody was used to monitor the presence of CBL10 and CBL10LA. Both CBL10 and CBL10LA were detected after the pull-down indicating that both variants interact with SOS2 (Figure 2.6).
Figure 2.6  CBL10LA interacts with SOS2

Recombinant Myc-tagged CBL10 (Lane 1) and CBL10LA (Lane 2) proteins were incubated with recombinant GST-tagged SOS2 proteins in an *in vitro* pull-down assay. SOS2, bound to glutathione sepharose beads, was pulled-down and eluted. SOS2 and any interacting proteins were separated on an SDS-PAGE gel and transferred to a PVDF membrane. An anti-Myc-HRP antibody was used to detect CBL10 and CBL10LA and an anti-GST-HRP antibody was used to detect SOS2. Protein input, pre-pull-down protein diluted 0.1X; Pull-down, proteins present after pull-down. Two replicate pull-downs are shown (panels A and B). Lane 3 in panel B shows an assay with Calmodulin 4 (CaM4), a negative control.
Figure 2.6

A.

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<th>Pull-down</th>
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<tr>
<td>1</td>
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<tr>
<td>CBL10-Myc</td>
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<tr>
<td>CBL10LA-Myc</td>
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<td>GST-SOS2</td>
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B.

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<thead>
<tr>
<th>Protein Input</th>
<th>Pull-down</th>
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<td>1</td>
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<td>CaM4-Myc</td>
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<td>GST-SOS2</td>
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To determine if SOS2 interacts preferentially with CBL10 or CBL10LA, competitive pull-down assays were performed. In these assays, fixed amounts of SOS2 and CBL10 proteins were incubated with increasing concentrations of CBL10LA. SOS2 was pulled-down and an anti-Myc-HRP antibody was used to monitor the presence of CBL10 and CBL10LA. While some interaction with CBL10LA was observed, CBL10 interaction with SOS2 did not decrease in the presence of increasing concentrations of CBL10LA (Appendix Figure 2.2) suggesting that CBL10LA does not compete with CBL10 for interaction with SOS2 in an in vitro pull-down assay.

**CBL10 alone is needed to activate responses to growth in saline conditions**

The CBL10LA transcript is reduced during seedling growth in salt (Figure 2.2) and recombinant CBL10LA protein cannot bind calcium (Figure 2.5B) suggesting that CBL10LA is not functional and, therefore, is not required for the response of the plant to salinity. To experimentally determine the function of the two CBL10 alternatively spliced transcripts, transgenic cbl10 mutant lines expressing only CBL10 or CBL10LA were generated and their ability to complement the cbl10 salt-sensitive phenotype was monitored. The CBL10 transcript was amplified from cDNA, without introns, to prevent the formation of the CBL10LA variant. The CBL10LA transcript was also amplified from cDNA with primers designed to amplify just the protein coding sequence to avoid generating the CBL10 transcript. For the CBL10LA construct, the 3’ primer anneals just before the pre-mature termination codon within the retained intron. The CBL10 and CBL10LA transcripts were cloned into the pBN-GFP vector and transformed into the
cbl10 mutant under the control of the native CBL10 promoter (2,200 base pairs). For the CBL10 transcript, two of the 10 lines analyzed showed some complementation of the salt-sensitive phenotype (Appendix Figure 2.3A). For the CBL10LA transcript, none of the 10 lines examined showed complementation (Appendix Figure 2.4). To understand why only two of the CBL10 transformed lines complemented, the levels of CBL10 were examined and found to be reduced or absent in the lines that did not complement (Appendix Figure 2.3B). This reduced expression could be due to: 1) the promoter region chosen for the construct being insufficient to drive expression, 2) the seven base pair linker sequence between the promoter and cDNA interfering with expression, or 3) a requirement of introns for CBL10 expression. We were able to rule out the possibility that the construct promoter region was insufficient to drive CBL10 expression based on the fact that the same promoter was used to successfully generate the C- and N-terminal CBL10 genomic constructs (Figure 2.1 and Appendix Figure 2.3). The second possible explanation for reduced CBL10 expression, that the seven base pair linker sequence between the promoter and the cDNA interfered with expression, is also unlikely because a similar seven base pair linker sequence inserted between the promoter and cDNA in the N-terminal-tagged constructs did not affect their ability to rescue the mutant phenotype (Figure 2.4). Several factors suggest that possibility three, that introns are required for CBL10 expression, most likely explains the reduced expression observed. Introns have been identified as important for the full expression of a number of genes in mammals, nematodes, insects, fungi, and plants (Hir et al.,
Most importantly, the major difference between the C-and N-terminal genomic constructs (which complement the mutant phenotype) and the cDNA constructs (which do not complement) is the presence of introns within the CBL10 gene.

Based on the presence of a motif identified in introns of Arabidopsis genes known to increase transcription (Rose et al., 2008), an algorithm was developed to predict the likelihood that a given intron affects expression (IMEter; http://korflab.ucdavis.edu/cgi-bin/web-imeter2.pl) (Rose et al., 2008). Each CBL10 intron was tested using the IMEter program to determine the likelihood that it enhances transcription (indicated by a high score). CBL10 contains eight introns; the scores for introns 5’ to 3’ are: 6.17, 12.14, 8.47, 2.04, 1.20, 0.00, 1.74, and 8.09. These results indicate that the second intron within the CBL10 gene is the one most likely to affect expression.

To express CBL10 and CBL10LA at sufficient levels to determine function, cDNA for each transcript was transformed into the cbl10 mutant under the control of the Cauliflower Mosaic Virus 35S promoter, a strong constitutive promoter. Eight homozygous, independently transformed lines were generated for each construct and RNA was isolated from two lines of each to monitor CBL10 and CBL10LA transcript levels. The CBL10 transcript was present in wild type and the two CBL10 transgenic lines (Figure 2.7A). The CBL10LA transcript was present in wild type and the two CBL10LA transgenic lines and at a low level in the cbl10 mutant and the two CBL10 transgenic lines (Figure 2.7A). The T-DNA insertion disrupts expression of the full-length CBL10 gene but a portion of the transcript 5’ to the insertion is still transcribed (Quan, 2007). To amplify
**Figure 2.7** The *CBL10* variant complements the *cbl10* salt sensitive phenotype, *CBL10LA* is not functional

**A.** *CBL10* and *CBL10LA* were expressed in the *cbl10* mutant using the Cauliflower Mosaic Virus 35S promoter. The protein-coding sequences of *CBL10* and *CBL10LA* were cloned and transformed into the *cbl10* mutant under the control of the 35S promoter. RNA was isolated from wild-type, *cbl10*, and transgenic seedlings and used to synthesize cDNA. Primers indicated in Figure 2.2A were used to amplify *CBL10* (blue arrows) and *CBL10LA* (green arrows). *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT* (*GAPC*) was used as a loading control. Lane 1, wild type; Lane 2, *cbl10*; Lanes 3 and 4, two homozygous, independently transformed lines of *cbl10;p35S:CBL10*; Lanes 5 and 6, two homozygous, independently transformed lines of *cbl10;p35S:CBL10LA*. Image of one replicate.

**B.** *CBL10* complements the *cbl10* salt-sensitive phenotype. Seeds from wild type (WT), *cbl10* transformed with the *CBL10* protein-coding sequence under the control of the 35S promoter (*cbl10;p35S:CBL10*, 10 OE 10), and *cbl10* were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken after 12 days. Seedlings from two of eight homozygous, independently transformed lines are shown growing on 125 mM NaCl. One representative image of three replicates.
C. **CBL10LA does not complement the cbl10 salt-sensitive phenotype.** Seeds from wild type (WT), *cbl10* transformed with the *CBL10LA* protein-coding sequence under the control of the 35S promoter (*cbl10;p35S:CBL10LA, 10 OE LA*), and *cbl10* were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken after 12 days. Seedlings from two of eight homozygous, independently transformed lines are shown growing on 125 mM NaCl. One representative image of three replicates.
Figure 2.7

A.

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<td>CBL10LA</td>
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<td></td>
</tr>
<tr>
<td>GAPC</td>
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B.

```
WT 10 OE 10  cbl10
MS
125 mM NaCl
125 mM NaCl
```

C.

```
WT 10 OE LA  cbl10
MS
125 mM NaCl
125 mM NaCl
```
the \textit{CBL10LA} transgene, the 3’ primer anneals to the beginning of the retained intron which is 5’ to the T-DNA insertion site explaining why there is a low level of \textit{CBL10LA} transcript in the mutant. To determine if \textit{CBL10} and/or \textit{CBL10LA} complement the salt-sensitive mutant phenotype, the eight transgenic lines for each construct were screened for growth on salt. \textit{CBL10} complemented the salt-sensitive phenotype in all lines while \textit{CBL10LA} did not (Figure 2.7B and C). This suggests that \textit{CBL10} is functional and sufficient to activate responses to NaCl in the \textit{cbl10} mutant and that \textit{CBL10LA} is not functional during growth in saline conditions.

\textbf{Removal of \textit{CBL10LA} is important for plant responses to salinity}

Reduced levels of \textit{CBL10LA} during plant growth in saline conditions suggested that this variant might have a negative role during salt signaling. To investigate this possibility, the \textit{CBL10LA} protein-coding sequence was expressed in wild type under the control of the 35S promoter with the idea that over-expression might prevent the removal of the transcript during growth in saline conditions. Over-expression of \textit{CBL10LA} did cause wild type to become hypersensitive to growth on NaCl, similar to what is observed in the \textit{cbl10} mutant (Figure 2.8A). Ten homozygous, independently transformed lines were examined and had varying degrees of sensitivity to growth on NaCl. Each transgenic line was screened five times on 125 mM NaCl and the number of salt-sensitive seedlings was scored (Figure 2.8B). In three of the lines (5, 7, and 10), virtually all of the seedlings were completely sensitive to growth on NaCl. In three other lines (3, 8, and 9), over three quarters of the seedlings were sensitive. In four lines (1, 2,
Figure 2.8 Over-expression of *CBL10LA* in wild type leads to a salt-sensitive phenotype

**A. Wild type expressing *CBL10LA* under the control of the 35S promoter is sensitive to high concentrations of NaCl.** Seeds from wild type (WT), wild type transformed with the *CBL10LA* protein-coding sequence under the control of the 35S promoter (WT;35S:*CBL10LA*, WT OE LA), and *cbl10* were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken 12 days after transfer. Seedlings from two of 10 homozygous, independently transformed lines (lines 7 and 8) are shown growing on 125 mM NaCl. One representative image of five replicates.

**B. Sensitivity to NaCl varied among the wild type over-expressing *CBL10LA* lines.**

Wild type was transformed with the *CBL10LA* protein-coding sequence under the control of the 35S promoter (WT;35S:*CBL10LA*) and growth of sixteen seedlings for each of the 10 homozygous, independently transformed lines was examined and scored as wild-type-like or hypersensitive to NaCl (like *cbl10*). The percentage of sensitive seedlings for each line is shown. Standard deviation of each mean is shown, n = 5.
**Figure 2.8**

A.  

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<tr>
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<th>WTOE LA</th>
<th>cbl10</th>
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<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
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<td><img src="image6.png" alt="Image" /></td>
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<tr>
<td>125 mM NaCl</td>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
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<tr>
<td>125 mM NaCl</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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B.  

![Bar chart: % sensitive vs Lines](image13.png)
4, and 6), less than half of the seedlings were sensitive. When the lines were grown on higher concentrations of NaCl, all showed an increased percentage of salt-sensitive seedlings (data not shown). CBL10LA transcript levels are being examined in each of the lines to determine if those with a higher degree of salt sensitivity have higher levels of CBL10LA. To verify that the salt-sensitive phenotype is specific to CBL10LA, CBL10 was also transformed into wild type under the control of the 35S promoter. Five independent transformed lines were examined; none showed a salt-sensitive phenotype (Figure 2.9).

An alternative explanation for the salt-sensitive phenotype in the CBL10LA over-expressing lines is that over-expression silences the endogenous CBL10 transcript. To determine if CBL10 expression was reduced in the transgenic lines, RNA was isolated from wild-type and transgenic plants grown without or with NaCl. In the transgenic lines, the CBL10LA transcript was expressed at significantly higher levels than wild type (Figure 2.10). No change in the level of the CBL10 was observed in these lines suggesting that silencing of endogenous CBL10 transcripts does not account for the salt-sensitive phenotype (Figure 2.10).
Figure 2.9  Over-expression of *CBL10* in wild type does not lead to a salt-sensitive phenotype

Seeds from wild type (WT), wild type transformed with the *CBL10* protein-coding sequence under the control of the 35S promoter (WT;*p*35S:*CBL10*, WT OE 10), and *cbl10* were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken 12 days after transfer. Seedlings from one of five homozygous, independently transformed lines are shown. One representative image of three replicates.
Figure 2.9
Figure 2.10  *CBL10* transcript levels do not decrease in the wild type over-expressing *CBL10LA*

Wild-type (WT) and wild-type seedlings expressing the protein-coding sequence of *CBL10LA* under the control of the 35S promoter (WT;*p*35S:*CBL10LA*) were germinated on solid MS for five days and transferred to solid MS without or with 100 mM NaCl. Tissue was harvested 12 days after transfer; RNA was isolated and used to synthesize cDNA. *CBL10* and *CBL10LA* transcript levels from two of 10 homozygous, independently transformed lines are shown. Primers indicated in Figure 2.2A were used to amplify *CBL10LA* (green primers) and *CBL10* (blue primers). *TUBULIN* was used as a loading control. Image of one replicate.
Figure 2.10

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<tr>
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<td>MS</td>
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<td></td>
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<td></td>
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<td>MS</td>
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- CBL10LA
- CBL10
- TUBULIN
Discussion

Alternative splicing of *CBL10*: a mechanism for regulating the SOS pathway

The *CBL10* gene is transcribed into two major variants, the characterized *CBL10* transcript and an alternatively spliced transcript, *CBL10LA*, with a retained intron. Several observations suggest *CBL10LA* plays a negative role during plant growth in saline conditions: 1) when seedlings are grown in the presence of salt, the *CBL10LA* transcript is reduced (Figure 2.2); 2) *CBL10LA* likely encodes a truncated protein due to a premature termination codon within the retained intron (Figure 2.5); 3) the encoded recombinant protein is unable to bind calcium (Figure 2.5); 4) the *CBL10LA* transcript is unable to complement the *cbl10* salt-sensitive phenotype (Figure 2.7); and 5) if over-expressed in wild-type, *CBL10LA* causes sensitivity to growth on high levels of NaCl (Figure 2.8).

Based on these data, a model for how post-transcriptional regulation of *CBL10* might affect the activity of the SOS pathway was developed (Appendix Figure 2.5). Alternative splicing of *CBL10* produces two proteins with antagonistic functions. When seedlings are grown in optimal conditions, CBL10, CBL10LA, and all the components of the SOS pathway are present. CBL10LA, a non-functional protein, inhibits activation of the pathway. In response to sodium, *CBL10LA* levels decrease and the SOS pathway is activated. This activation involves an increase in intracellular calcium levels, CBL10 binding to calcium followed by interaction with and activation of SOS2 to initiate SOS1 \( \text{Na}^+/\text{H}^+ \) exchange. Reduction in *CBL10LA* levels during plant growth in salt could serve as
part of a mechanism for generating rapid and regulated responses. For example, if the full activation of SOS1 Na⁺/H⁺ exchange is not necessary or beneficial during growth in low concentrations of or transient exposure to salt, CBL10LA could be used to modulate the activation of SOS1. During growth in high concentrations of or prolonged exposures to salt, CBL10LA decreases and the activity of the SOS pathway would increase. Evidence supporting this model includes: 1) the greater decrease in CBL10LA levels when seedlings were exposed to high concentrations of NaCl (Figure 2.2) and 2) the fact that wild-type seedlings over-expressing CBL10LA did not display a salt-sensitive phenotype unless the plants were exposed to high concentrations of NaCl (Figure 2.8).

Phosphorylation of CBL10 likely provides an additional mechanism to regulate the SOS pathway in response to low concentrations of or short exposures to salt. In the SOS pathway, CBL10 interacts with and activates SOS2 (Quan, 2007). SOS2, in turn, phosphorylates CBL10, strengthening the interaction between the two proteins (Lin et al., 2009). SOS2 phosphorylation is specific for CBL10; SOS3 is not phosphorylated and the predicted CBL10LA protein is missing the SOS2-specific phosphorylation site (Lin et al., 2009). CBL10 phosphorylation might be sufficient to initiate responses to salt even in the presence of CBL10LA, but upon exposure to higher concentrations of salt, the removal of CBL10LA might be critical for full activation of SOS2 and SOS1.

While the mechanism underlying CBL10LA’s regulation of the SOS pathway is unknown, there are several ways that CBL10LA could inhibit the SOS pathway: 1) CBL10LA could interfere with SOS2 activity by forming a heterodimer with CBL10 and
preventing CBL10 from activating SOS2. Experiments have shown that SOS3/CBL4 forms a homodimer upon binding calcium (Sánchez-Barrena et al., 2005). To determine if CBL10LA dimerizes with CBL10, interaction between CBL10 and CBL10LA could be tested with recombinant protein in an in vitro pull-down assay. 2) Because CBL10LA is able to interact with SOS2 in an in vitro pull-down assay, it might directly compete with CBL10 for interaction with SOS2. In this model, CBL10 would bind calcium and activate SOS2 while CBL10LA would interact with SOS2 but would be unable to activate SOS2 possibly because CBL10LA cannot bind calcium. As the levels of CBL10LA decrease, the activity of SOS2 would increase due to more frequent interaction with CBL10. To test this model, recombinant CBL10 and CBL10LA proteins were incubated with recombinant SOS2 protein in an in vitro pull-down assay to determine if the two proteins compete for interaction with SOS2. CBL10 appears to preferentially bind to SOS2 (Appendix Figure 2.2) suggesting that either this model is incorrect or that it cannot be tested using this assay. Another approach to determine if CBL10LA interferes with activation of SOS2 would be to measure SOS2 phosphorylation of a target protein in the presence of CBL10 and in the absence and presence of CBL10LA using an in vitro kinase assay. 3) CBL10LA could inhibit SOS1 activity either by interfering with CBL10-SOS2 activation of SOS1 or perhaps through a direct interaction with SOS1. Recent experiments demonstrated that recombinant CBL10 protein added to wild-type and cbl10 mutant plasma membrane vesicles increased Na\(^+\)/H\(^+\) exchange activity suggesting that CBL10 might directly regulate SOS1 (Lin et al., 2009). To determine if CBL10LA reduces SOS1 activity, Na\(^+\)/H\(^+\)
exchange could be measured in plasma membranes vesicles isolated from wild type, the cbl10 mutant, wild-type over-expressing CBL10LA, and wild-type over-expressing CBL10 to determine if activity is lower in the plants expressing CBL10LA. CBL10 and CBL10LA recombinant protein could be added to plasma membrane vesicles to determine if CBL10LA directly affects SOS1 activity.

**How are CBL10LA levels reduced during seedling growth in salt?**

There are several mechanisms that could reduce the CBL10LA transcript during growth in saline conditions. The first is that CBL10LA is targeted for decay by the NMD pathway. This pathway targets transcripts with pre-mature termination codons for decay, preventing the generation of truncated proteins that could be destructive to the cell. In mammals, a termination codon is recognized as pre-mature if it is located at least 50 nucleotides upstream of an exon-exon junction (Lareau et al., 2007b). The criteria for NMD targeting of transcripts in plants is unclear (van Hoof and Green, 2006). While CBL10LA has a premature termination codon 201 base pairs upstream of the last exon-exon junction, it appears to evade NMD due to the presence of the transcript and putative protein (Figure 2.4) when seedlings are grown in optimal conditions. There are other examples of transcripts that contain pre-mature termination codons that are able to evade NMD; however, the mechanisms underlying this evasion are unknown (Lareau et al., 2007b). In humans, proteins that bind to the 3’UTR of transcripts can either target a transcript for or protect a transcript from NMD by recruiting or blocking the recruitment of proteins involved in degrading the transcript (Singh et al., 2008). Since
the CBL10LA transcript is reduced during growth in salt, the mechanism allowing
CBL10LA to evade detection by NMD during growth in optimal conditions is likely
removed. To determine if NMD is involved in the removal of CBL10LA in response to
salinity, alternative splicing of CBL10 could be examined in the NMD mutants upf1 or
upf3 (Arciga-Reyes et al., 2006; Hori and Watanabe, 2005). If CBL10LA is a target,
transcript levels would not be reduced during growth in salt in the upf mutants and
seedlings would show increased sensitivity to salt relative to wild type.

A second mechanism for the removal of CBL10LA might involve a change in the
splicing pattern so that the CBL10LA intron is spliced out, generating only the CBL10
transcript. The selection of alternative splice sites is regulated by RNA-binding proteins
that enhance or suppress activity at a particular site (Simpson et al., 2008). The
serine/arginine-rich (SR) protein family regulates alternative splicing and members of
the family are themselves alternatively spliced. The splicing patterns of the SR proteins
change in response to low temperature, high light, and salinity (Hirayama and Shinozaki,
2010; Tanabe et al., 2006). This suggests that environmental conditions can affect the
alternative splicing of the SR proteins which in turn affect the alternative splicing of
downstream target pre-mRNAs. If the alternative splicing of CBL10 changes during
growth in salt so that only the CBL10 variant is generated, an increase in CBL10
transcript levels would be expected as CBL10LA levels decrease. Using RT-PCR, levels of
CBL10 do not appear to dramatically increase in response to salt (Figure 2.2 and Figure
2.3). However, quantitative RT-PCR should be used to determine if there is a significant increase in the level of \textit{CBL10}.
Appendix Figure 2.1  Multiple alternatively spliced \textit{CBL10} transcripts are present in Arabidopsis

\textbf{A. Genomic structure of \textit{CBL10} and four other variants.} Two bands were present when \textit{CBL10} was amplified from wild-type RNA (Figure 2.1B). The two bands were isolated together and the DNA was cloned and sequenced. Boxes, exons; lines, introns; grey boxes, changes in final mRNA structure compared to \textit{CBL10}; numbers indicate clones identified for each variant.

\textbf{B. \textit{CBL10} and \textit{CBL10LA} represent the major transcripts present in the lower (short transcripts) and upper (long transcripts) bands, respectively.} Seven day-old wild-type seedlings germinated on solid MS media were placed in liquid MS without or with 200 mM NaCl for one hour. RNA was isolated and used to synthesize cDNA. \textit{CBL10} was amplified using RT-PCR and the two bands were cloned and sequenced separately. Boxes, exons; lines, introns; grey boxes, changes in the final mRNA structure compared to \textit{CBL10}; numbers indicate clones identified for each variant.
Appendix Figure 2.1

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**Short transcripts - no NaCl**

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**Short transcripts - NaCl**

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Appendix Figure 2.2  CBL10LA does not compete with CBL10 for interaction with SOS2

Recombinant GST-tagged SOS2 protein was incubated with Myc-tagged CBL10 protein in the presence of increasing concentrations of Myc-tagged CBL10LA protein or Myc-tagged CaM4 protein (negative control). SOS2, bound to glutathione-coated sepharose beads, was pulled down and eluted along with interacting proteins. SOS2 and interacting proteins were separated on an SDS-PAGE gel and transferred to a PVDF membrane. An anti-Myc-HRP antibody was used to detect CBL10, CBL10LA, and CaM4 and an anti-GST-HRP antibody was used to detect SOS2. Protein input, pre-pull-down protein diluted 0.1X; Pull-down, proteins present after pull-down.

One representative image of three replicates.
Appendix Figure 2.2
Appendix Figure 2.3  The native promoter is not sufficient to drive expression of the \textit{CBL10} cDNA in \textit{cbl10} mutant seedlings

\textbf{A. The} \textit{CBL10} cDNA \text{expressed under the control of the native promoter does not complement the salt-sensitive phenotype of the} \textit{cbl10} \text{mutant.} Seeds from wild type (WT), \textit{cbl10} transformed with the \textit{CBL10} protein-coding sequence under the control of the \textit{CBL10} promoter (\textit{cbl10};\textit{pCBL10:}$\textit{CBL10}$, \textit{cbl10} + \textit{CBL10}), and \textit{cbl10} were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken after 12 days. Seedlings from two of 10 homozygous, independently transformed lines (lines 2 and 4) are shown growing on 100 mM NaCl. One representative image of three replicates.

\textbf{B. The} \textit{CBL10} cDNA \text{is not fully expressed under the control of the native promoter.} RNA from wild-type and \textit{cbl10} expressing \textit{CBL10} under the control of the native promoter (\textit{cbl10} + \textit{CBL10}) was isolated from 17 day-old seedlings grown on solid MS. Primers indicated in Figure 2.2A were used to amplify both transcripts (red arrows) in 10 homozygous, independently transformed lines. \textit{TUBULIN} was used as a loading control. Five of ten lines are shown (Lanes 1-5). One representative image of two replicates.
Appendix Figure 2.3

A.

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B.

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- CBL10
- CBL10LA
- TUBULIN
**Appendix Figure 2.4** The *CBL10LA* cDNA expressed under the control of the native promoter does not complement the salt-sensitive phenotype of the *cbl10* mutant

Seeds from wild type (WT), *cbl10* transformed with the *CBL10LA* protein-coding sequence under the control of the *CBL10* promoter (*cbl10;pCBL10:CBL10LA, cbl10 + CBL10LA*), and *cbl10* were germinated on solid MS for five days and transferred to solid MS without or with 125 mM NaCl. Photographs were taken after 12 days. Two of five homozygous, independently transformed lines are shown on 100 mM NaCl. One representative image of three replicates.
Appendix Figure 2.4

WT  cbl10 + CBL10LA  cbl10

MS

100 mM NaCl

100 mM NaCl
Appendix Figure 2.5  Model for post-transcriptional and post-translational regulation of CBL10

Alternative splicing of CBL10 produces two proteins with antagonistic functions. The full-length CBL10 protein activates the SOS pathway while the truncated CBL10LA protein inhibits activation of the pathway. During growth in control conditions (no NaCl), the presence of CBL10LA ensures that the SOS pathway is inactive. In response to sodium, an influx in intracellular calcium is perceived by CBL10. CBL10 interacts with and activates SOS2. SOS2 phosphorylates CBL10 to strengthen the interaction. The CBL10-SOS2 complex phosphorylates SOS1 to initiate Na⁺/H⁺ exchange. CBL10LA is removed to prevent it from inhibiting the activity of the pathway.
Appendix Figure 2.5
CHAPTER 3

CALCINEURIN B-LIKE 10 (CBL10) MEDIATES FLOWER ORGAN MATURATION

Introduction

Plant growth, development, and reproduction are highly sensitive to environmental extremes including drought, salinity, flooding, and high and low temperatures which result in reductions in growth leading to decreases in crop yields (Bray et al., 2000). Therefore, an understanding of the mechanisms that enable plants to adjust their growth in extreme environmental conditions is essential for maintaining and improving crop productivity.

As sessile organisms, plants have highly complex signaling pathways that enable them to respond to and adjust their growth in diverse environmental conditions. To identify the genes involved in responses to salinity, a mutagenesis screen was performed in Arabidopsis and mutants with increased salt sensitivity relative to wild type were selected (Liu et al., 2000; Liu and Zhu, 1998; Wu et al., 1996). Cloning of the genes and characterization of the proteins led to the identification of the Salt Overly Sensitive (SOS) pathway which functions to remove sodium from the cytoplasm preventing its toxic effect. In this pathway, the perception of sodium triggers an influx in cytosolic calcium levels. Increased calcium is perceived by a calcium-binding protein, CALCINEURIN B-LIKE10 (CBL10)/SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCaBP8) which
interacts with and activates a serine/threonine protein kinase, SOS2/CBL-INTERACTING PROTEIN KINASE24 (CIPK24 (Quan, 2007)). SOS2 phosphorylates SOS1, a Na⁺/H⁺ exchanger, initiating transport of sodium out of the cell (Qiu et al., 2002).

Most research has focused on the molecular pathways operating in leaves and roots during growth in saline soils but salinity has also been shown to affect reproductive development in plants including rice, wheat, and Arabidopsis (Asch and Wopereis, 2001; Jenks et al., 2007; Khatun and Flowers, 1995; Sun et al., 2004). For example, in rice, panicle initiation (the beginning of the reproductive phase) is more sensitive to salinity than all other developmental stages (Asch and Wopereis, 2001). Under saline conditions, sterility and reduced spikelet (floral unit) number resulted in decreased yields (Asch and Wopereis, 2001). In Arabidopsis, addition of 200 mM NaCl to hydroponic medium for 12 hours reduced fertility (seed set) by 90% compared to control plants (Sun et al., 2004). This reduction in fertility was due to decreased stamen elongation, collapse of pollen grains, the presence of callose in ovules (an early sign of ovule abortion), and disrupted divisions within the female gametophyte (Sun et al., 2004).

Arabidopsis flowers include four organs arranged in whorls; four sepals, four petals, six stamens, and two fused carpels referred to as the pistil (Smyth et al., 1990). The stamen, the male reproductive organ, includes a pollen-containing anther and a stalk-like filament (Scott et al., 2004). Pollen grains are the mature male gametophyte consisting of a tube cell with two sperm cells. When pollen grains are fully developed,
anthers dehisce and undergo programmed cell death to release pollen while the filament elongates to position the anther above the female reproductive organ, the pistil (Scott et al., 2004). The pistil includes the stigma, composed of elongated cells (the papillae) that function in pollen capture, recognition, hydration, and germination; the style, through which the pollen tube elongates; and the ovary, a hollow cylinder with a central tissue, the septum, giving rise to multiple ovules. Within each ovule is the seven-celled mature female gametophyte consisting of two synergid cells, one egg cell, a central cell, and three antipodal cells (Kagi and Gross-Hardt, 2007).

Fertilization begins when pollen grains on the stigma hydrate and germinate through the style and the transmitting tract within the septum of the ovary (Crawford and Yanofsky, 2008). Upon perception of guidance cues, pollen tubes exit the transmitting tract and enter into the ovule to release two sperm cells whose nuclei fuse with the egg cell nucleus and the two polar nuclei of the central cell forming the embryo and endosperm, respectively (Higashiyama, 2010; Kagi and Gross-Hardt, 2007).

In addition to its role during seedling growth in salinity, the CBL10 calcium sensor is important for flower development during growth in saline conditions. To understand CBL10’s role in reproductive development, the floral processes disrupted in the mutant grown in saline conditions are being characterized and interacting proteins that contribute to CBL10’s function are being identified.
Materials and Methods

Plant material

*Arabidopsis thaliana* Col-0 was used as wild type for this study. A *CBL10* T-DNA insertion line (SALK_056042) was obtained from the Arabidopsis Biological Resource Center (ABRC) and backcrossed to wild type three times to remove insertions in other genes. Genomic DNA from the mutant was used as a template to identify wild-type and mutant alleles. A homozygous *cbl10* mutant was identified in the first self-pollinated generation of backcross three (BC3F2). To test for the presence of a T-DNA insertion, the LBa1 (5′-TGGTTCACGTAGTGGGCCATC-3′) and 056042_L (5′-TCTGCTATTCTCTGGAATCTGA-3′) primers were used. To identify alleles without a T-DNA insertion (wild-type alleles), the 056042_L primer was used with 056042_R (5′-CTGCCATAGACGCAAGATGA-3′).

Plant growth

Soil

Seeds were germinated on 1/2X Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 2% sucrose, 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES), and 0.6% agar (pH adjusted to 5.7 with KOH), stratified for 2 days at 4°C in the dark, and transferred to a growth chamber at 21°C under a 16 h light/8 h dark photoperiod. After approximately 14 days, seedlings were transferred to SunGro MetroMix360 with vermiculite in a 3:1 ratio. Seedlings were placed in a growth
chamber at 21°C under a 16 h light/8 h dark photoperiod. Growth was monitored and photographs were taken at the indicated times.

**Hydroponics**

Plants were grown in an hydroponics system (Araponics, Belgium) consisting of a 1.8 L container, a cover with 35 holes, 1.5 ml seed holders, an air stone, and a clear plastic lid. Each seed holder was filled with 0.65% agar made in 1/2X Hoagland’s media (Hoagland and Arnon, 1938) with cobalt chloride in place of cobalt nitrate and a final pH of 5.7 (adjusted with KOH). The agar was allowed to solidify, stirred to form small pieces, and then injected into seed holders with a 10 ml syringe without a needle. Seeds were sown onto the agar and the container was filled with 1.3 L of 1/2X Hoagland’s media, pH 5.7. The container with the lid in place was incubated at 4°C for two days followed by transfer to a growth chamber at 21°C with a 16 h light/8 h dark photoperiod. At the time of transfer, the container was connected to a pump to supply roots with oxygen through an air stone. After one week, roots emerged from tubes into the liquid media at which time the lids were removed. Media was changed weekly.

**Histochemical analyses**

**Pollen tube growth through pistils**

To examine pollen tube growth in wild-type and *cbl10* pistils (Johnson et al., 2004), pre-anthesis flowers (stage 12; Smyth et al., 1990) were emasculated and pistils were allowed to mature for 20 h. Pistils were pollinated by brushing anthers from wild-type
flowers expressing β-glucuronidase (GUS) under the control of the pollen-specific Lat52 promoter (Lat52p:GUS) against the pistil to release pollen onto the stigma. After 18 h, pistils were detached and cut along the replum to remove the ovary wall. The pistil consisting of the stigma, style, and central tissue of the ovary with the ovules attached was submerged in 80% acetone and incubated for 30 min. The pistils were then dipped in GUS buffer (500 mM NaPO₄ buffer pH7, 50 mM ferrocyanide, 50 mM ferricyanide) before being placed in GUS staining solution (GUS buffer containing 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in DMSO (X-Gluc; Gold Biotechnology, St Louis, MO)) and were incubated in a 37°C water bath overnight. Pistils were mounted on slides using 50% glycerol. Photographs were taken using bright field microscopy.

**CBL10 promoter activity**

To determine the temporal and spatial activity of the CBL10 promoter, the 5’ upstream region of CBL10 was transcriptionally fused to GUS as a reporter gene in pCAMBIA1381. A 2,214 base pair fragment representing the CBL10 promoter was amplified using Col-0 genomic DNA as a template, Phusion High Fidelity DNA Polymerase (Thermo Scientific, Lafayette, CO), and the PM5-AtCBL10-3PstI (5’- CGCCTGCAGCGCCAGCGACGATAAAATGGTT-3’) and PM3-AtCBL10-5XhoI (5’- CGCCTCGAGGAGTTCATTCAAAATCACAATCACAG-3’) primers. The promoter-GUS construct was introduced into Agrobacterium tumefaciens strain GV3101 and subsequently into wild-type Arabidopsis via the floral dip method (Clough and Bent, 1998). Transformed seed was selected on 25 μg/ml hygromycin and T2 lines with 75%
resistance (single insertion) were chosen. Homozygous seed was obtained by screening T3 seed on hygromycin to identify lines with 100% resistance. Flowers along a single inflorescence were removed and a small incision was made in each pistil to facilitate substrate entry into the ovary. Sepals and petals were removed and pistils and stamens were immersed in 90% acetone for 20 min and transferred to a GUS staining solution. Flowers were vacuum infiltrated for 15 min and then incubated in staining solution overnight at 37°C. Flowers were removed from stain and cleared in 70% ethanol for 5 h before mounting on slides with 50% glycerol. Photographs were taken using bright field microscopy.

**Analysis of CBL10 expression during flower development**

Flowers from five week-old wild-type plants grown under long-day conditions were staged (Smyth et al., 1990) and collected in the following pools: 1) meristem development, 2) early flower development, 3) anthesis (stages 11, 12, and 13), and 4) fertilization (stages 14 and 15). RNA was isolated in a 50 µl volume using the Qiagen RNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD) and treated with 2 µl TURBO DNase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was purified using the RNeasy MinElute Cleanup Kit from Qiagen prior to cDNA synthesis. To synthesize cDNA, RNA (1.5 µg, volume adjusted to 11 µl with distilled water) was incubated with 1 µl of 100 µM oligo dT primer at 65°C for 5 min and then transferred to ice for 2 min to anneal the primer to the RNA. A reaction mix containing 1 µl SuperScript III Reverse Transcriptase (Invitrogen), 4 µl 5X first strand buffer, 1 µl 10 mM
dNTPs, 1 µl 0.1 M DTT, and 1 µl RNase Inhibitor (Invitrogen) was added to the RNA for a total volume of 20 µl. This mixture was incubated at 50°C for 60 min followed by 70°C for 15 min. After incubation, 1 µl of RNaseH (Invitrogen) was added along with 3 µl of 10X buffer and 6 µl of water for a total volume of 30 µl. The reaction was incubated at 37°C to allow the RNase to degrade the RNA. The Qiagen MinElute Kit was used to purify the cDNA after synthesis was complete. For the PCR reaction, 1 µl of cDNA was used in a 20 µl volume. To monitor the CBL10 transcript, the CBL10-RT-F1 (5’-GATCAAGCTCTCTACTGTC-3’) and CBL10-RT-R1 (5’-GCGCCTGACAATCTCCTC-3’) primers were used. To monitor transcript levels of control genes, the following primers were used: TUBULIN, loading control, 5TUB (5’-TYATGGATYTGAGCTGG-3’) and 3TUB (5’-TCAGAGTTYTGCCCMGGG-3’); ABORTED MICROSPORES (AMS), early flower development marker, AMS_F (5’-TCGCTTGTCCCAGGATAACC-3’) and AMS_R (5’-TTCCAGCAACGATCGTTACG-3’); and MYB21, late flower development marker, MYB21_F (5’-TAAAACGAAACCGGGAAAAGTT-3’) and MYB21_R (5’-GCGGCCGAATAGTACCAGTAG-3’).
Results

cbl10 mutant flowers are sterile; stamens do not fully elongate and anther dehiscence is reduced

To understand the role of CBL10 in Arabidopsis growth and development, a T-DNA mutant line was obtained from the SALK Institute. When cbl10 was grown in soil in standard growing conditions, early flowers on the inflorescence were sterile and did not produce seed (Figure 3.1A). However, later in inflorescence development, some cbl10 mutant flowers recovered fertility and produced siliques with a reduced seed set (Figure 3.2). To determine the basis of the sterility, flower development in wild type and the cbl10 mutant was compared. During anthesis, wild-type anthers dehisce and filaments elongate to position the anther above the pistil to release pollen onto the stigma. In cbl10 flowers, early stamen differentiation and development appear normal but during anthesis, filaments do not elongate and anthers do not fully dehisce (Figure 3.1B and C). cbl10 mutant flowers display two phenotypes based on the development and morphology of the stamens and petals. In one group of flowers (phenotype one) no stamen or petal elongation is observed; both organs remain covered by sepals and only the pistil elongates above the sepals (Figure 3.3). In a second group of flowers (phenotype two), stamens and petals partially elongate but stamens do not reach above the pistil and petals are smaller than wild-type petals (Figure 3.3). Both phenotypes can be found along a single inflorescence. An example of this is seen in Figure 3.1B; the oldest cbl10 flower (far right) is a phenotype one flower while the next five are
Figure 3.1 *cbl10* flowers are sterile due to decreased stamen elongation and reduced anther dehiscence

A. *cbl10* produces short siliques during early inflorescence development. Siliques and flowers were removed from the primary inflorescence of seven week-old wild-type (WT) and *cbl10* mutant plants. Bars, 3 mm.

B. *cbl10* stamens do not fully elongate. Enlarged images of the flowers in A. Bars, 1 mm.

C. *cbl10* anthers have reduced dehiscence. Images of anthers from wild-type (WT) and three representative *cbl10* flowers. Bars, 1 mm.
Figure 3.1

A.

WT

cb110

B.

WT

cb110

C.

WT cb110 cb110 cb110
Figure 3.2 The *cbl10* mutant recovers fertility later in inflorescence development

A. The *cbl10* mutant has an increased number of secondary inflorescences.

Photographs of representative seven week old wild-type (WT) and *cbl10* mutant plants. Bars, 5 mm.

B. Silique size increases in the *cbl10* mutant later in development. Siliques were removed from the primary inflorescence of a seven week-old *cbl10* mutant plant.

C. Seeds develop in the apical portion of a *cbl10* silique. One representative silique from a wild-type (WT) and *cbl10* mutant plant. Bars, 1 mm.
Figure 3.2
Figure 3.3 *cbl10* flowers have multiple phenotypes

Comparison of wild-type (WT) and *cbl10* flowers. Images of *cbl10* flowers show the two sterile phenotypes observed (Phenotype 1 and Phenotype 2) and flowers that recover fertility (Recovered). Bars, 1 mm.
Figure 3.3

WT

*cbl10*

Phenotype 1  Phenotype 2  Recovered
phenotype two flowers. In flowers that recover fertility, stamens fully elongate above the pistil to release pollen and initiate fertilization (Figure 3.3).

**cbl10 mutant pistil development is abnormal**

Stamens of *cbl10* mutant flowers do not fully elongate to release pollen onto the pistil in either of the flower phenotypes observed. To determine if this alone is responsible for sterility in the mutant, *cbl10* pistils were manually pollinated with wild-type pollen and fertilization was examined. No fertilization occurred indicating that there is also a problem with development of the pistil. To determine which processes (pollen tube germination, growth in the transmitting tract, targeting to the ovule, or fusion of the gametes) are affected during fertilization in the mutant, *cbl10* pistils were manually pollinated with wild-type pollen (to monitor pollen tube targeting of the female gametophyte by examining seed set) or wild-type pollen expressing β-glucuronidase (GUS) under the control of the *Lat52* pollen-specific promoter (*Lat52p:GUS*; to monitor pollen tube growth through the transmitting tract). In wild-type pistils pollinated with wild-type pollen expressing *Lat52p:GUS*, pollen tube growth was observed in the transmitting tract of all four pistils examined (Figure 3.4). In wild-type pistils pollinated with wild-type pollen, approximately 60 seeds developed in each of the five pollinated pistils examined and no unfertilized ovules (Figure 3.4). In *cbl10* phenotype one pistils pollinated with wild-type pollen expressing *Lat52p:GUS*, pollen tubes moved through the transmitting tract in five out of the 10 pistils examined (Figure 3.4). In the other five pistils, pollen tubes grew through the style but were unable to
Figure 3.4 *cbl10* has defects in pollen tube growth and targeting

Representative images of wild-type and *cbl10* flowers (left panels). Representative pistils pollinated with wild-type pollen expressing *GUS* driven by the pollen specific promoter, *LAT52*, to monitor pollen tube growth (middle panels). Representative siliques from pistils pollinated with wild-type pollen to monitor targeting by examining seed set (right panels). Bars, 1 mm.
Figure 3.4

Wild type

\[ \text{cbl10} \]

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<th>Phenotype 2</th>
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   :alt: Image of wild type

.. figure:: image2.png
   :width: 600px
   :align: center
   :alt: Image of cbl10 phenotypes

Scale bars for reference.
enter the transmitting tract. However, pollen tube targeting of ovules was reduced in all four pistils pollinated with wild-type pollen; three of the four siliques produced less than 10 seeds, some of which were brown indicating seed abortion, and the fourth silique did not produce any seed (Figure 3.4). In phenotype two flowers pollinated with wild-type pollen expressing Lat52p:GUS, pollen tubes were unable to enter the transmitting tract in any of the 20 pistils examined (Figure 3.4). In nine of the pistils analyzed, pollen grains did not germinate; in the other 11 pistils, pollen grains germinated and pollen tubes grew through the style but stopped before entering the transmitting tract. Seed set was examined in 11 pistils from phenotype two flowers; seven had no seeds, three had one to three fertilized seeds which aborted, and one had 12 fertilized seeds (Figure 3.4).

**CBL10 is expressed throughout flower development**

To determine if **CBL10** is present during flower development, **CBL10** expression was monitored using reverse transcriptase polymerase chain reaction (RT-PCR) and promoter GUS fusions. For expression analysis, wild-type flowers were divided into four pools representing specific hallmarks of flower development: meristem development, early flower development, anthesis (stages 11-13), and fertilization (stages 14-15). Both **CBL10** and an alternatively spliced variant, **CBL10LA**, were found to be expressed throughout flower development (Figure 3.5). **ABORTED MICROSPORES** and **MYB21** were amplified as positive controls for early and late flower development, respectively (Mandaokar et al., 2006; Sorensen et al., 2003).
Figure 3.5 *CBL10* and an alternatively spliced variant, *CBL10LA*, are expressed throughout flower development

RNA was isolated from wild-type flowers collected in four pools representing different stages of development. 1, meristem formation; 2, early flower development; 3, anthesis (stages 11 - 13); 4, fertilization (stages 14 - 15). *TUBULIN* loading control; *AMS, ABORTED MICROSPORES*, marker for early flower development; *MYB21*, marker for late flower development. Image of one replicate.
Figure 3.5
To determine in which floral tissues *CBL10* is expressed, flowers from wild-type plants expressing GUS under the control of the *CBL10* promoter were examined. To generate the promoter GUS construct, 2 kb of sequence upstream of the *CBL10* start of translation was amplified, transcriptionally fused to the GUS reporter gene, and transformed into wild-type plants. Three homozygous, independently transformed lines were identified and *CBL10* expression was examined in flowers at stages spanning the developmental time frame in which the mutant phenotype appears. Two staining patterns were observed: in two of the lines, GUS expression was observed in stamen filaments and the style and medial tissue of the pistil (Figure 3.6A) and in the third line, GUS expression was observed in pollen and the style and lateral tissue of the pistil (Figure 3.6A). To determine the predominant expression pattern for *CBL10*, stage 12 flowers (the stage at which the *cbl10* mutant phenotype is observed) were examined from 20 independently transformed lines (Figure 3.6B). Expression was observed in most floral organs except for the stigma, anther, and ovules. The predominant pattern is expression throughout the flower including the vascular tissue of sepals and petals, the style and ovary of the pistil, and the filament and pollen of stamens.

**NaCl induces the *cbl10* mutant phenotype**

The *cbl10* sterility phenotype is variable depending on the growing conditions. When the *cbl10* mutant was grown in soil without additional vermiculite, the flowers were fertile. Vermiculite is a clay used to improve aeration and moisture retention and to prevent leaching of fertilizer. Clays have negative charges which attract and hold
Figure 3.6 *CBL10* is expressed in stamens and pistils

A. **CBL10 is expressed in stamen filaments and in pistils.** Expression patterns of the *GUS* reporter gene under the control of the *CBL10* promoter were examined in wild-type flowers (three independent lines, top to bottom) at different stages of development (stages 14, 13, 12 and 11, left to right). To detect GUS staining in pistils, all other organs were removed and an incision was made in pistils to allow the stain to penetrate the ovary wall. Bars, 1 mm.

B. **CBL10 is expressed throughout the flower.** Stage 12 wild-type flowers expressing *GUS* under the control of the *CBL10* promoter were examined in 20 independently transformed lines to determine the predominant *CBL10* expression pattern. Check marks indicate tissues in which expression was observed. The numbers recorded above each column indicate the number of lines with that particular pattern. Transmitting tract (tt).
Figure 3.6

A.

B.

<table>
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positively charged ions (including magnesium, calcium, potassium, sodium, hydrogen, and aluminum) that are released for uptake by the plant (CornellCE, 2007). To determine if the cbl10 mutant is sensitive to a particular ion being released into the soil by the vermiculite, the cbl10 mutant was grown in hydroponics to precisely control ion concentrations. In seedlings, CBL10 has been shown to regulate sodium ion homeostasis and cbl10 mutants are hypersensitive to growth in the presence of excess sodium. To determine if sodium might be responsible for the sterile flower phenotypes, wild type and the cbl10 mutant were grown in hydroponics and, at the time of bolting, plants were treated with 10 or 20 mM NaCl. cbl10 mutant plants grown in control conditions were fully fertile but in the presence of NaCl, the cbl10 mutant produced siliques with no or very few seeds (Figure 3.7).

**CBL10 functions independently of the SOS pathway to promote flower maturation during growth in saline conditions**

The presence of CBL10 in flowers and the importance of its function during flower development when plants are grown in saline conditions suggest that it might regulate ion homeostasis in flowers in the same way that it regulates ion homeostasis in leaves.

If this is the case, mutations in SOS pathway genes like SOS2 and SOS1 would be expected to manifest a similar flowering phenotype when grown in the presence of NaCl. To determine if CBL10, SOS2, and SOS1 function in a pathway to regulate ion homeostasis in flowers, wild type and the three mutants were grown in hydroponics and treated with 10 mM NaCl at the start of bolting. In control conditions (no NaCl),
Figure 3.7 *cbl10* is sterile when plants are grown in saline conditions

Wild-type (WT) and *cbl10* plants grown in hydroponics were left untreated (Control) or treated with 10 or 20 mM NaCl at the time of bolting (3 weeks of growth). After 2 weeks of treatment, siliques along the primary inflorescence were removed and photographed. Bar, 2 cm.
Figure 3.7

Control

WT

$cbl10$

10 mM NaCl

WT

$cbl10$

20 mM NaCl

WT

$cbl10$
silique size and seed number were similar in all plants (Figure 3.8 and Figure 3.9). When plants were treated with 10 mM NaCl, silique size was unaffected in wild type and sos2, but reduced in cbl10 and sos1 (Figure 3.8). When siliques at positions 5, 10, 15, and 20 in each genotype were opened, wild type and sos2 had similar numbers of green, developing seeds and almost no aborted seed or empty positions (Figure 3.9). The sos1 mutant had 80% green seed and 20% unfertilized or aborted seed (Figure 3.9). The small size of sos1 siliques appears to be due to a lack of elongation; the total number of positions in a silique (27) was only slightly reduced compared to wild type (31) but most of the seed was in pairs instead of single files (Figure 3.9). In contrast, the cbl10 mutant produced siliques with only 25% green seed and 75% unfertilized or aborted seed (Figure 3.9).
Figure 3.8 *cbl10* and *sos1* silique size is reduced when plants are grown in saline conditions

Wild-type (WT), *cbl10*, *sos1*, and *sos2* plants were grown in hydroponics. When inflorescence development began, plants were left untreated (Control) or treated with 10 mM NaCl. After 3 weeks of treatment, siliques were removed from a primary inflorescence from wild type (upper panels), *cbl10* (second panels), *sos1* (third panels), or *sos2* (bottom panels) and fertilization was monitored. Bar, 5 mm.
Figure 3.8

 WT

 cbl10

 sos1

 sos2
Figure 3.9 CBL10 acts independently of SOS1 and SOS2 to maintain fertility during growth in saline conditions

A. Seed set is reduced in the cbl10 mutant. Representative siliques from wild-type (WT), cbl10, sos1 and sos2 plants grown in hydroponics and left untreated (Control) or treated with 10 mM NaCl at the time of bolting were opened to monitor seed set. Bar, 5 mm.

B. cbl10 has a greater number of unfertilized ovules while sos1 and sos2 are fertile. Four siliques (at positions 5, 10, 15, and 20) were opened from three primary inflorescence stems for each genotype. The number of green, healthy, seed (blue bars) and aborted seed/unfertilized ovules (green bars) from one side of the silique were counted. Standard deviation of each mean is shown, n = 5.
Figure 3.9

A. 

Control  |  NaCl
---------|---------
WT       |         
cbl10    |         
sos1     |         
sos2     |         

B. 

Control

<table>
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<th>cbl10</th>
<th>sos1</th>
<th>sos2</th>
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<tr>
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</table>

10 mM NaCl

<table>
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<th>WT</th>
<th>cbl10</th>
<th>sos1</th>
<th>sos2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy seed</td>
<td>30</td>
<td>5</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Unfertilized ovules, aborted seed</td>
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During plant growth in saline conditions, CBL10 protects flower development independently of the SOS pathway

CBL10 is a calcium-binding protein originally characterized for its role in leaf responses during growth in saline conditions. CBL10 has been shown to function in the removal of cytosolic sodium by interacting with and activating the SOS2 protein kinase which, in turn, phosphorylates the SOS1 Na⁺/H⁺ exchanger. When the cbl10 mutant was treated with salt at bolting, developing flowers were sterile. To determine if CBL10 functions in the SOS pathway to important for flower development, the sos2 and sos1 mutants were treated with salt at bolting and fertility was examined. The presence of salt did not affect fertility in the sos1 and sos2 mutants as it did in the cbl10 mutant suggesting that CBL10 does not function in the SOS pathway to maintain flower development during growth in saline conditions (Figure 3.8 and Figure 3.9).

Two mechanisms have been identified for the removal of cytosolic sodium, transport of sodium out of the cell due to the activity of the SOS1 Na⁺/H⁺ exchanger and compartmentation of sodium in the vacuole driven by the activity of the tonoplast-localized NHX family of Na⁺/H⁺ exchangers (Serrano and Rodriguez-Navarro, 2001). Since most ovules in the sos1 mutant are fertile it suggests that transport of sodium out of the cell is not the primary strategy to protect flower development during growth in saline conditions. As a result, sequestration of sodium in the vacuole is likely the major strategy for sodium regulation during flowering. In support of this model, several of the
NHX genes including *NHX1*, *NHX2*, and *NHX3* are expressed in flowers (Aharon et al., 2003; Apse et al., 2003; Shi and Zhu, 2002). To determine if NHX proteins are involved in maintaining flower development during growth in saline conditions, fertility is being examined in single and double *nhx* mutants.

While SOS2 has been shown to interact with both CBL10 and NHX1 (Qiu et al., 2004; Quan, 2007), the lack of a flower phenotype in the *sos2* mutant suggests that it does not function in flowers (Figure 3.8 and Figure 3.9). Instead, CBL10 might interact with another member of the SOS2 family of protein kinases to regulate NHX during flower development. SOS2 belongs to the CBL-Interacting Protein Kinase (CIPK) family, consisting of 25 protein kinases. Two members of the CIPK family that might be involved in flower development are *CIPK12* and *CIPK20*. Expression of these genes is down-regulated in *receptor-like protein kinase2* mutant flowers which have defects in anther dehiscence, stamen elongation, and pollen maturation similar to the *cbl10* mutant (Mizuno et al., 2007). Single and double, *cipk12* and *cipk20* mutants could be grown in saline conditions and fertility examined to determine if these proteins function together in a pathway with CBL10.

In leaves, *CBL10* is alternatively spliced into two transcripts, *CBL10* and *CBL10LA* which appear to have antagonistic functions in activation of the SOS pathway. *CBL10LA* functions as a negative regulator of the SOS pathway ensuring that the pathway is not activated in control conditions (in the absence of sodium). In response to acute salt stress, *CBL10LA* is removed allowing the plant to activate responses to salt through
CBL10. Both variants are present in flowers (Figure 3.5) and studies are underway to determine if they have opposing functions in flower development as well. For these studies, the cbl10 mutant has been transformed with each variant separately and fertility during growth in saline conditions is being monitored. During seedling growth in salt, removal of CBL10LA is critical for a salt response; wild-type seedlings expressing CBL10LA under the control of the constitutive 35S promoter are sensitive to salt. To determine if reducing CBL10LA is also important during flowering, fertility will be examined in transgenic wild-type plants expressing CBL10LA under the control of the 35S promoter.

**Which processes are disrupted during flower development in the cbl10 mutant?**

cbl10 mutant flowers have reduced stamen elongation and reduced pollen tube growth through the transmitting tract (Figure 3.1 and Figure 3.4). However, the mechanisms underlying these defects are not known. The final stages of stamen maturation involve the coordination of three processes: pollen maturation, dehiscence and programmed cell death of the tissues in the anther to release the pollen, and elongation of the filament to position the anther above the pistil (Scott et al., 2004). To determine which processes are disrupted by NaCl in the cbl10 mutant, pollen viability and development could be examined with Lugol’s solution (I2-KI) which stains viable pollen black and DAPI to monitor cell division during microsporogenesis and microgametogenesis (Cheng et al., 2004; Chhun et al., 2007). Anther development and function could be monitored in transverse sections of wild-type and cbl10 anthers at
different stages of development to determine if cbl10 anther tissues undergo normal programmed cell death (Cheng et al., 2004; Ishiguro et al., 2001). Stamen elongation could be examined with scanning electron microscopic images of epidermal cells in stamen filaments to determine if cell division or cell elongation is responsible for reduced stamen length in the cbl10 mutant (Cheng et al., 2004).

Fertilization begins when pollen grains on the stigma hydrate and germinate through the style and the transmitting tract of the ovary. In the cbl10 mutant, pollen tubes were unable to enter the transmitting tract of the ovary suggesting that the development or function of the pistil is disrupted. The transmitting tract consists of cells that secrete a mixture of polysaccharides, glycoproteins, and glycolipids into the extra cellular matrix (ECM) providing nutrients and guidance cues to growing pollen tubes (Crawford and Yanofsky, 2008). Alcian blue, which stains acidic polysaccharides (a major component of the ECM; (Crawford et al., 2007; Gremski et al., 2007)), could be used to monitor ECM development in the cbl10 mutant.

Coinciding with the release of ECM compounds is programmed cell death of transmitting tract cells to facilitate pollen tube movement through the ovary (Crawford et al., 2007; Crawford and Yanofsky, 2008). To determine if programmed cell death is altered in the cbl10 mutant, cross-sections of wild-type and cbl10 pistils at different stages of development could be examined (Crawford et al., 2007; Gremski et al., 2007).

In addition to its role in the transmitting tract of the pistil, programmed cell death plays a role in the function of tapetal cells within the stamen. Proper ion homeostasis
might be important for the initiation of programmed cell death in these organs and
disruption of CBL10 function during growth in saline conditions could prevent these
tissues from undergoing normal cell death. Evidence in support of this model comes
from studies of programmed cell death in germinating seeds of cereal crops including
rice, barley, and oat. These studies have demonstrated that there are similarities
between the development and function of aleurone cells within seeds of these crop
plants and tapetal cells within the anther. For example, tapetal and aleurone cells
release compounds important for pollen and embryo development and maturation,
respectively before undergoing programmed cell death (Bethke et al., 1999; Parish and
Li, 2010). Prior to cell death, cells in both tissues go through a process of vacuolation
(Bethke et al., 1999; Parish and Li, 2010). The involvement of a barley and rice CBL in
the vacuolation of aleurone cells suggests that CBL10 might function in the vacuolation
of tapetal cells.

Do hormones link CBL10 to flower organ maturation?

When the cbl10 mutant is grown in the presence of NaCl, two flower phenotypes are
observed (Figure 3.3). Analysis of these phenotypes suggests that organ development
within the flowers might be uncoupled with one organ preferentially developing over
the other. In phenotype one flowers, where there is no stamen elongation beyond
initial development and no anther dehiscence, some pistils were able to support pollen
tube growth through the transmitting tract (Figure 3.4). Phenotype two flowers have
the opposite phenotype; stamens and petals partially elongate and anthers have
reduced dehiscence but pistils do not support pollen tube growth through the transmitting tract (Figure 3.4). One explanation for the two cbl10 mutant phenotypes is that a hormone coordinates the development and maturation of stamens and the pistil. In the cbl10 mutant, there might be a reduction in the level of this hormone so that amounts are insufficient to coordinate the maturation of both organs, leading to the preferential development of one organ (the pistil in phenotype one flowers, the stamen in phenotype two flowers). As discussed below several hormones have been shown to be involved in organ maturation during flower development (Appendix Figure 3.1).

**Does CBL10 link to GA signaling?**

During flower development, Gibberelic Acid (GA) is important for the removal of DELLA nuclear growth repressor inhibition of floral genes including AGAMOUS, MYB33 and MYB65 (Cheng et al., 2004; Millar and Gubler, 2005; Yu et al., 2004). Flowers of the GA biosynthetic mutant ga1-3 are sterile due to a similar but more severe phenotype than cbl10 (decreased stamen elongation and reduced anther dehiscence) (Cheng et al., 2004). The ga1-3 phenotype can be suppressed by mutating multiple DELLA repressors. If the function of two DELLA proteins (RGA and RGL2) is removed, sterile flowers are produced early during flowering but fertility recovers when plants are approximately 50 days-old (Cheng et al., 2004). If three DELLA repressors (RGA, RGL2, and GAI) are removed, fertility recovers completely. These studies demonstrate that GA is important for flower development and that the level of GA or GA signaling can affect fertility.
There is emerging evidence that the DELLA proteins also coordinate plant growth in response to the environment. When plants are grown in the presence of salt, seedling growth is reduced and time to flowering is increased in a DELLA-dependent manner (Achard et al., 2006). This is due, in part, to a decrease in the level of GA and stabilization of the DELLA proteins (Achard et al., 2006). This reduction in seedling growth in the presence of salt seems to be important for prolonged seedling survival. GA biosynthetic mutants are more tolerant to high concentrations of salt whereas DELLA mutants are less tolerant compared to wild type (Achard et al., 2007). These studies suggest that DELLAs promote survival in adverse environments by restraining growth.

Since GA is important for fertility and growth in the presence of salt can lead to decreases in the level of GA, the sterile flower phenotype of the cbl10 mutant could be due to decreased levels of GA and/or increased stability of the DELLA proteins. The amount of GA could be compared in wild type and the cbl10 mutant using gas chromatography/ mass spectrophotometry (Achard et al., 2006). If GA levels are reduced in cbl10 mutant flowers or DELLA proteins are more stable, another approach would be to increase GA signaling in the cbl10 mutant by removing the activity of the DELLA proteins which should allow for proper stamen development even during growth in saline conditions. For these studies, the DELLA mutants, rga and rgl2, are being crossed with the cbl10 mutant and fertility will be examined during growth in salt.
A decrease in the level of GA might explain the stamen phenotype of *cbl10*, but it does not explain the pistil phenotype. There is no known role for GA in pistil development (Balanza et al., 2006). GA is important for pollen tube growth in Arabidopsis and rice (Chhun et al., 2007; Swain et al., 2004) and exogenous GA stimulates pollen tube growth in *Torenia fournieri* L.; however, it would appear that the stimulation is from pollen synthesizing GA, not GA present within the pistil (Wu et al., 2008).

**Does CBL10 link to auxin signaling?**

The auxin signaling mutant, *arf6;arf8*, has defects in both stamen and pistil development (Nagpal et al., 2005; Wu et al., 2006). Some of the phenotypes of the double mutant are similar to the *cbl10* mutant including reduced anther dehiscence, decreased stamen elongation, and the absence of pollen tube growth in the transmitting tract (Nagpal et al., 2005; Wu et al., 2006). In addition, the level of *ARF6* and *ARF8* expression seems to control the severity of the stamen phenotype. In a homozygous *arf8*; heterozygous *ARF6* (*arf8;arf6/+*) mutant, stamens elongate to just below the stigma and petal elongation is similar to that in wild type (Wu et al., 2006), very similar to what is observed in *cbl10* phenotype two flowers. In the double homozygous *arf8;arf6* mutant, no stamen and petal elongation is observed (Wu et al., 2006), very similar to *cbl10* phenotype one flowers. This suggests that the two sterile stamen phenotypes observed in the *cbl10* mutant could be due to differences in auxin levels. The amount of auxin could be compared in wild type and the *cbl10* mutant using
gas chromatography/mass spectrophotometry (Barkawi et al., 2010). Another approach would be to use the DR5:GUS reporter gene to visualize auxin levels in stamens and pistils of wild type and cbl10 mutant flowers (Aloni et al., 2006).
Appendix Figure 3.1 Model for plant hormone and gene interactions during stamen maturation

Plant hormones and genes with known roles in filament elongation and anther dehiscence were placed in a pathway. Genes indicated in black have characterized mutant phenotypes; genes indicated in blue are part of hormone biosynthetic pathways. Arrow, activation; bar, repression; grey bar, removal of repression.

Mutant and over-expression phenotypes are written as symbols. (Achard et al., 2004; Cheng et al., 2004; Fleet and Sun, 2005; Ishiguro et al., 2001; Ito et al., 2007; Mandaokar et al., 2006; Millar and Gubler, 2005; Nagpal et al., 2005; Park et al., 2002; Sanders et al., 2000; Singh et al., 2002; Wu et al., 2006; Yu et al., 2004)
Appendix Figure 1.3

**Hormone**
- Pathway gene (genetic evidence)
- Biosynthetic gene
- M = mutant
- OE = over-expression line

**Phenotypes**
- ★ Normal pistil development
- • Abnormal pistil development
- ○ No anther dehiscence
- ✱ Filament elongation
- ✡ No filament elongation

---

**Diagram**

- **GA**
  - GA2ox
  - GA3ox
  - GA20ox
  - KAO
  - KO
  - KS
  - CPS (ga1-3)
  - M = ★

- **JA**
  - COI1
  - JAZ

- **OPR (dde1)**
  - AOC
  - AOS
  - LOX

- **Stamen development**

- **Auxin**
  - ARF6, ARF8
  - miR167
  - OE = ★

- **KNOX (STM)**
  - M = ★

- **MYB21, MYB24**
  - M = ★

- **Delphi**
  - (RGA, RGL1, RGL2)

- **MYB33, MYB65**
  - M = ★

- **miR159**

- **Stamen development**
CHAPTER 4

TWO COMPONENTS OF MEDIATOR, IDENTIFIED AS CBL10-INTERACTING PROTEINS, ARE INVOLVED IN PLANT RESPONSES TO HIGH LEVELS OF CHLORIDE AND GROWTH IN SHORT-DAY CONDITIONS

Introduction

Correct temporal and spatial gene expression patterns are important for plant growth and development and responses to the environment. Gene expression and activity are regulated at multiple levels including chromatin remodeling, initiation of transcription, processing of RNA, translation, and post-translation. Transcription begins when RNA polymerase II (Pol II) and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, assemble into a pre-initiation complex (PIC) at the promoters of active genes (Casamassimi and Napoli, 2007; Sikorski and Buratowski, 2009). TFIIH then phosphorylates Pol II on the carboxy-terminal domain releasing Pol II from the PIC to initiate the elongation phase of transcription (Sikorski and Buratowski, 2009).

Mediator is a large complex consisting of 20-30 proteins and is involved in processes that regulate transcription in eukaryotes (Casamassimi and Napoli, 2007; Sikorski and Buratowski, 2009). For example, Mediator enhances transcription by interacting with activators and recruiting Pol II to the promoter, accelerates elongation by stimulating the phosphorylation of Pol II by TFIIH, and promotes the reassembly of the PIC after Pol
II has been released during the elongation phase of transcription (Casamassimi and Napoli, 2007; Sikorski and Buratowski, 2009). Mediator can also repress transcription through interaction with the Med12-Med13-CDK8-CycC complex causing a conformational change in Mediator that prevents it from interacting with Pol II and blocks Pol II incorporation into the PIC (Taatjes, 2010).

While Mediator is thought to be involved in the transcription of most genes, there is evidence that it can contribute to specificity of gene regulation. This evidence includes the fact that the Mediator complex can be composed of different subunits, that expression of these subunits can be differentially regulated, and that activators interact with specific subunits of the complex (Sikorski 2009, D’Alessio 2009). An example of Mediator’s role in the regulation of specific genes comes from studies with yeast where the recruitment of Mediator did not always correlate with the presence of Pol II and transcriptional activity indicating that Mediator is not needed for transcription of every gene. In response to environmental stress, the presence of Mediator at actively transcribed genes increased suggesting that it might be important for enhanced transcription of genes involved in yeast responses to environmental stresses including heat, salt, and heavy metals (Fan et al., 2006).

In Arabidopsis, 21 conserved and six plant-specific Mediator proteins have been identified (Backstrom 2007). The Arabidopsis Mediator has been shown to have a role in cell division during leaf and embryo development, the transition to flowering, and the activation of genes involved in jasmonic acid (JA)- and salicylic acid (SA)-mediated
defense responses to fungi (Autran et al., 2002; Dhawan et al., 2009; Gfeller et al., 2010; Gillmor et al., 2010; Kidd et al., 2009).

Two components of the Mediator complex were identified in an in silico search for proteins that interact with the CALCINEURIN B-LIKE (CBL) family of calcium-binding proteins. In Arabidopsis, the CBL family consists of 10 proteins that act as calcium sensors, binding to calcium and undergoing a conformational change that permits interaction with and activation of the CBL-Interacting Protein Kinase (CIPK) family (Batistic and Kudla, 2009). The CBLs activate CIPKs by binding to the FISL motif within a CIPK regulatory domain releasing inhibition of CIPK kinase activity (Albrecht et al., 2001; Guo et al., 2001). The FISL motif is a 21 amino acid region that is necessary and sufficient for binding to the CBLs. Sequence analysis of all twenty-five Arabidopsis CIPKs indicates that, within this 21 amino acid motif, six residues (A, F, I, S, L, and F) are conserved (Guo et al., 2001). To uncover novel functions for the CBL proteins and to understand the mechanisms underlying their roles in Arabidopsis growth and development, the FISL motif of one member of the CIPK family, SALT OVERLY SENSITIVE2 (SOS2)/CIPK24, was used in an in silico search with the Arabidopsis proteome to identify novel CBL-interacting proteins. In vivo interaction assays and mutational analyses were used to determine if the CBLs interact with components of Mediator and to uncover the role of Mediator subunits in plant development and responses to the environment.
Materials and Methods

Plant material and gene expression analyses

*Arabidopsis thaliana* Col-0 was used as wild type for this study. A *MED33a*, *MED33b*, *CBL10* and *CBL7* insertion line was obtained from the Arabidopsis Biological Resource Center (ABRC) and backcrossed to wild type three times to remove insertions in other genes; *med33a*-1 (SALK_119561), *med33a*-2 (SALK_022477), *med33b*-3 (SALK_100877), *med33b*-4 (SALK_140098), *cbl10* (SALK_056042), and *cbl7* (SAIL_201_A01). The reduced epidermal fluorescence4-1 (*ref4*-1) and *ref4*-3 ethyl methanesulfonate (EMS) mutants were provided by Dr. Clint Chapple (Department of Biochemistry, Purdue University, West Lafayette, IN) and were previously described (Ruegger and Chapple, 2001; Stout et al., 2008). Each SALK line was backcrossed to Columbia-0 (wild type) three times. Homozygous single *med33* and *cbl* alleles were crossed to generate double mutants.

Genomic DNA from the insertion lines was used as a template to identify wild-type and mutant alleles. To test for the presence of a T-DNA insertion, the following primers were used in combination with the LBa1 primer (5’-TGGTTCAGTAGTGGGCCATC-3’): *med33a*-1 (5’-AGAGGTAAAGGAAGCGTTGC-3’), *med33a*-2 (5’-
GGAAATTGATGCTCCTGAGAG3’), *med33b*-3 (5’-TTTGTAATGGCGGAGATGAAC-3’),
*med33b*-4 (5’-GGAAGCTTGGTACGTCAAC3’), and *cbl10* (5’-
AACAAAGCAAAGTGCTTGAC-3’). To verify the presence of a T-DNA in *cbl7*, the p745 (5’-AACGTCGCAGTGTGGTTATTAAGTTGTC-3’) primer was paired with *cbl7* (5’-
GGCGGATCCGGGTATCTTCCACTTGAG-3’) primer. To identify alleles without a T-DNA
insertion (wild-type alleles), the gene-specific primers indicated above were paired with the following primers: med33a-1 (5’-TGGTTTGACTGTGTTGATCAGTTG-3’), med33a-2 (5’-GCCACAAGTGAAAGAAGATGG-3’), med33b-3 (5’-GCGTTATTGCTTTTCATGC-3’), med33b-4 (5’-TGGAGGATGATGACTCTGTA-3’), cbl10 (5’-TCTGCTATTCTTCTCTCTCCTCCTG-3’), and cbl7 (5’-GGCGAATTCTGATGGAACACAGAAATTCAGC-3’). To confirm the position of the T-DNA, PCR products from reactions performed to verify the presence of the T-DNA were cloned into the pGEM-T EASY TA cloning vector (Promega, Madison, WI) and sequenced.

To confirm that the T-DNA disrupted expression of full-length transcripts, RNA was isolated from 9 day-old wild-type, med33a-1;med33b-4 (med33-14), and med33a-2;med33b-3 (med33-23) seedlings in a 50 µl volume using the Qiagen RNeasy Plant Mini Kit (Qiagen Sciences, Germantown, MD) and treated with 2 µl TURBO DNase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA was purified using the RNeasy MinElute Cleanup Kit from Qiagen prior to cDNA synthesis. To synthesize cDNA, RNA (1.5 µg; volume adjusted to 11 µl with distilled water) was incubated with 1 µl of 100 µM oligo dT primer at 65°C for 5 min and then transferred to ice for 2 min to anneal the primer to the RNA. A reaction mix containing 1 µl SuperScript III Reverse Transcriptase (Invitrogen), 4 µl 5X first strand buffer, 1 µl 10 mM dNTPs, 1 µl 0.1 M DTT, and 1 µl RNase Inhibitor (Invitrogen) was added to the RNA for a total volume of 20 µl. This mixture was incubated at 50°C for 60 min followed by 70°C for 15 min. After incubation, 1 µl of RNaseH (Invitrogen) was added along with 3 µl of 10X buffer and 6 µl
of water for a total volume of 30 µl. The reaction was incubated at 37°C to allow the RNase to degrade the RNA. The Qiagen MinElute Kit was used to purify the cDNA after synthesis was complete. For the reverse transcriptase polymerase chain reaction (RT-PCR), 2 µl of a 1/16th dilution of cDNA was used in a 10 µl volume. The Med33a transcript was amplified with 5’-AGCTGCTTGTCGTAAGACC-3’ and 5’-AGCTGCTTGTCGATTAGG-3’ primers and the Med33b transcript was amplified with 5’-CGGTAAGCGTGACTCGTCTG-3’ and 5’-GAACCAGAGGAGACAAATCGC-3’ primers. The full-length transcript was monitored using the following primers: med33a-1 (5’-CAGCACCAGATGCATAGC-3’ and 5’-TTGAGAAACCGCTCTGAGGC-3’), med33a-2 (5’-CGGGAATTCCTCAGAAATAC-3’ and 5’-GACTTTCCAACATTGCCACC-3’), med33b-3 (5’-CACGACATGGATTACGACCG-3’ and 5’-TTTGTAATGCGACGATGAC-3’), and med33b-4 (5’-TGGAGGTGATGACTCGTCTG-3’ and 5’-GAGTAACGGGACCCAAAGCC-3’).

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC) was amplified as a control using 5’-CACTTGAAGGGTGGTCCCAAG-3’ and 5’-CCTGTTGTCGCAAACGAAGTCT-3’ primers.

**Plant growth conditions**

**Long-day**

Seeds were germinated on 1/2X Murashige and Skoog (MS) media (Murashige and Skoog, 1962) containing 2% sucrose, 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES), and 0.6% agar (pH adjusted to 5.7 with KOH), stratified for 2 days at 4°C in the
dark, and transferred to a growth chamber at 21°C in a 16 h light/8 h dark photoperiod. After approximately 14 days, the seedlings were transferred to soil (either SunGro MetroMix360 with vermiculite (4:1) (Figure 4.4) or SunGro Sunshine LC1 mix (Figure 4.6)). Seedlings were placed in a growth chamber at 21°C in a 16 h light/8 h dark photoperiod. Growth was monitored and photos were taken at the indicated times.

**Short-day**

Seeds were sown directly onto soil (SunGro MetroMix360 with vermiculite (4:1) (Figure 4.4) or SunGro Sunshine LC1 mix (all other figures)) and stratified for 2 days at 4°C before being transferred to a growth chamber at 21°C in an 8 hour light/16 hour dark photoperiod. Growth was monitored and photographs were taken at the indicated times.

**Protein interaction - yeast two hybrid assays**

The FISL motif from the SOS2/CIPK24 protein sequence (NAFEMITLSQGLNLSALFD) was aligned with Med33a and Med33b protein sequence using the Clustal X program. Two websites ([http://www.vivo.colostate.edu/molkit/hydropathy/index.html](http://www.vivo.colostate.edu/molkit/hydropathy/index.html) and [http://blanco.biomol.uci.edu/hydrophobicity_scales.html](http://blanco.biomol.uci.edu/hydrophobicity_scales.html)) were used to generate hydropathy plots for each protein to avoid the inclusion of highly hydrophobic regions. The FISL fragments were amplified with Taq Polymerase (Invitrogen) using RNA isolated from 2 week-old seedlings. The following primers were used to amplify the Med33 and SOS2/CIPK24 FISL motifs for cloning into the pGADT7 vector of the MATCHMAKER Two-
Hybrid System (Clonetech, Mountain View, CA) using the Nde1 and BamH1 restriction sites: Med33aFISL (5’- GACAGAGTTGTTCCAACCCG-3’ and 5’- ACCAGGTTTGGCTTGTCGCA-3’), Med33bFISL (5’- TCCCCAAGGGATAATCCCAA-3’ and 5’- ACTTCCCCGGTTTCTTGA-3’), and SOS2FISL (5’- CCCCTGATGATGAATGCCT-3’ and 5’- TCCCTCGAGCCTTGCTTGTA-3’).

Full-length CBL sequences were cloned into the pGBK7 vector. The pGADT7 clones were transformed into *Saccharomyces cerevisiae* strain Y187 and the pGBK7 clones were transformed into *Saccharomyces cerevisiae* strain AH109. Yeast were mated and grown on synthetic complete medium (SD) minus leucine and tryptophan (SD-LW) to select for the presence of both constructs. To determine interaction, diploid yeast was grown on SD minus leucine, tryptophan, adenine, and histidine (SD-LWAH).

**Analysis of seedling sensitivity to ions and heavy metals**

Germination conditions and MS media were the same as those indicated for long-day growth except that seeds were germinated on vertical plates and the MS media contained 1% agar. Five day-old seedlings were transferred to solid 1/2X MS, solid 1/2X MS with 100 mM NaCl, KCl, NaNO₃, or KNO₃, or solid 1/2X MS with 75 μM CuSO₄. Photographs were taken at the indicated times.

**Nutrient complementation assay**

Nutrient solution used to fertilize wild-type and *med33* mutant plants was prepared with 1 tablespoon of Peter’s professional 20-20-20 all-purpose plant food (Scotts-Sierra Horticultural Products Company, Marysville, OH) containing 20% total nitrogen, 20%
available phosphate, 20% soluble potash, 0.5% total magnesium, 0.02% boron, 0.05% copper, 0.10% iron, 0.05% manganese, 0.0005% molybdenum, and 0.05% zinc dissolved in 2 gallons of distilled water. Plants were fertilized with 1/2X nutrient solution once a week and growth was monitored for 10 weeks.

**Analysis of dark-induced senescence in detached leaves**

Leaves were detached from soil-grown 3 week-old wild-type, med33-14, med33-23, ref4-1, and ref4-3 plants and placed in petri dishes on filter paper soaked in 1/2X MS containing 2.5 mM MES (pH adjusted to 5.7 using KOH). Petri dishes were wrapped in foil and incubated at 23°C. Photographs were taken at the indicated times.

**Results**

**In silico identification of two putative CBL-interacting proteins**

To identify novel proteins that interact with the CBL-family of calcium-binding proteins, an in silico search was performed using the SOS2/CIPK24 FISL motif (the CBL-interaction domain) to query the Arabidopsis proteome. SOS2/CIPK24 has six amino acids (A, F, I, S, L, and F) within the FISL motif that are present in all twenty-five CIPKs (Guo et al., 2001). Two Arabidopsis proteins, encoded by At3g23590 and At2g48110, were found to contain FISL-like motifs which are divided into two parts (Figure 4.1A and B). At3g23590 contains five of the six conserved amino acids while At2g48110 contains
Figure 4.1 Identification of two putative CBL-interacting proteins

A. Med33a and Med33b genomic structure indicating the position of the FISL-like motif. Black boxes, exons; lines, introns; red bar, position of the FISL-like motif.

B. Amino acid alignment of Med33a and Med33b with the SOS2/CIPK24 FISL motif. Sequences were aligned using the Clustal X program. Symbols above the amino acids represent the strength of conservation. ***, identical residues; :,” and “.,” conserved and semi-conserved substitutions, respectively. The strength of conservation is based on the amino acid scoring system used by Clustal X. The amino acids in the SOS2FISL domain, highlighted in red, are the conserved residues among all 25 Arabidopsis CIPKs.

C. The Med33 proteins interact with CBL10 and CBL7. Med33 cDNAs encoding the protein sequence surrounding the putative FISL domain indicated in panel B were cloned into the pGAD vector and fused to the GAL4 activation domain (Med33aFISL-AD and Med33bFISL-AD). Full-length CBL1, CBL3, CBL7, and CBL10 cDNAs were cloned into the pGBK vector and fused to the GAL4 DNA binding domain (CBL-BD). Diploid yeast harboring both constructs were spotted onto synthetic complete medium minus leucine and tryptophan (SD-LW) and synthetic complete medium minus leucine, tryptophan, adenine, and histidine (SD-LWAH) to select for the presence of the two constructs and determine interaction, respectively. Serial dilutions of yeast cells were plated to determine
the strength of interaction. Positive controls for this experiment were full-length
\textit{SOS2}/\textit{CIPK24} cDNA (SOS2-AD) and a fragment of \textit{SOS2}/\textit{CIPK24} cDNA containing
the FISL domain (SOS2FISL-AD) cloned into the pGAD vector. The cDNA of the
calcium-binding protein, \textit{CALMODULIN4 (CaM4)}, was cloned into the pGBK
vector (CaM4-BD) and used as a negative control. Yeast shown in the top panels
were grown for 6 days, yeast shown in the middle and bottom panels were
grown for 8 days. One representative image of two replicates.
Figure 4.1

A.

Med33a

Med33b

0.5 kb

B.

Med33a (upper) / SOS2FISL (lower)

** **
DRVFPTRSTQQAAYRIYELLRKRNMTIKIDHISPQHQRVMISVSNILRLSELFIDLDTSRPG
NAF

** **:
EMITLSQGLNLALFD

Med33b (upper) / SOS2FISL (lower)

** **:
SPRVIFNRKLHPAA YRLYELLRKRAFSFPLIRAPG YHTMNSIDDILHSETGQDQPES

NAF

** **:
EMITLSQG

** **:
LNLSALFD

C.

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<th>CBL10-BD</th>
<th>CBL7-BD</th>
<th>CBL3-BD</th>
<th>CBL1-BD</th>
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four (Figure 4.1A and B). Since this search was conducted, these genes were identified in two other screens. The first involved a biochemical purification of the Mediator complex in Arabidopsis using antibodies to two known Mediator subunits followed by mass spectrophotometry to identify co-precipitating proteins (Backstrom et al., 2007). At3g23590 and At2g48110 co-precipitated with both subunits and were subsequently named Med33a and Med33b, respectively, due to their sequence homology (52% identity/68% similarity over 1336 amino acids) (Backstrom et al., 2007).

In a second study, At2g48110 was identified in an EMS mutagenesis screen for genes that have a role in the phenylpropanoid pathway (Ruegger and Chapple, 2001). The At2g48110 mutant identified in this screen was significantly reduced in size and had lower quantities of phenylpropanoid-pathway end products; the gene was subsequently named REDUCED EPIDERMAL FLUORESCENCE4 (REF4) (Stout et al., 2008). Heterozygous ref4 mutants have intermediate phenotypes compared to wild type and homozygous mutants indicating that the mutation is semi-dominant. At3g23590 was identified as a gene highly homologous to REF4 and was named REF4-RELATED1 (RFR1). In this dissertation, these genes are referred to as Med33a and Med33b.

**Med33a and Med33b interact with CBL10 and CBL7 in a yeast-two hybrid assay**

To determine if the Med33 proteins interact with the CBLs, a yeast two-hybrid assay was performed. Before these proteins were identified as components of Mediator, they had not been characterized. Several membrane topology programs, including SOSUI (Nagoya University, Japan, http://bp.nuap.nagoya-u.ac.jp/sosui/) and HMMTOP
(Hungarian Academy of Science, http://www.enzim.hu/hmmtop/), were used to identify transmembrane domains; depending on the program, 0 to 11 domains were predicted. Based on the possibility that they were transmembrane proteins, hydropathy plots were used to determine how much of the protein surrounding the FISL motif could be used in the assay before encountering regions with a high number of hydrophobic amino acids. cDNA fragments of 186 base pairs for Med33a and 192 base pairs for Med33b encoding 62 and 64 amino acid peptides, respectively (shown in Figure 4.1B), were amplified from seedling RNA using RT-PCR and cloned into the two-hybrid vector in-frame with the activation domain of the yeast GAL4 protein. All 10 full-length CBL cDNAs were amplified using RT-PCR from seedling RNA and cloned into the two-hybrid vector in-frame with the DNA binding domain of the yeast GAL4 protein. The full-length SOS2/CIPK24 cDNA and a 210 base pair fragment containing the SOS2/CIPK24 FISL motif (a 70 amino acid peptide) were used as positive controls. SOS2/CIPK24 interacted strongly with SOS3/CBL4, but interaction was reduced if only the FISL motif was used (Figure 4.1C). Both Med33 peptides interacted strongly with CBL7 and CBL10 but not with the other CBL proteins (Figure 4.1C and Appendix Figure 4.1). Med33b also interacted with CBL3, but the interaction was not confirmed in a subsequent assay (Appendix Figure 4.1). As a negative control, the CALMODULIN4 (CaM4) calcium-binding protein was fused to the GAL4 DNA binding domain; no interaction was detected between the Med33 proteins and CaM4. Yeast-two hybrid assays in the reverse orientation, where the Med33 peptides were fused to the GAL4 binding domain and the
CBLs were fused to the GAL4 activation domain, were performed, but no yeast growth was observed indicating that interaction is directional (data not shown). Directional interaction was also observed for SOS2/CIPK24 and SOS3/CBL4 (Appendix Figure 4.1).

The med33 double mutant is sensitive to chloride ions

To understand the role of Med33a and Med33b during Arabidopsis growth and development, several T-DNA mutants were obtained from the SALK Institute (Figure 4.2A). Two allelic double mutant combinations were generated, med33a-1;med33b-4 (med33-14) and med33a-2;med33b-3 (med33-23). The transcript 5’ to the T-DNA insertion was detected for both genes in the double mutant; however, no full-length transcript was detected in either (Figure 4.2B).

In the process of amplifying and cloning the Med33b transcript, the expressed regions did not correspond with what is reported on The Arabidopsis Information Resource (TAIR) website. In the cloned cDNA, exon 6 is not present and the start site for exon 7 is earlier in the sequence (Figure 4.2A). Additional experiments will be needed to confirm this observation and determine if this is the correct gene structure.

Because CBL10 is involved in plant responses to salinity (Kim et al., 2007; Quan, 2007) and the Med33 proteins interacted with CBL10 in vitro, the growth of the double mutant on NaCl was examined. Wild-type, single, and double med33 mutant seeds were germinated on solid MS media and five day-old seedlings were transferred to solid MS without or with 100 mM NaCl. Compared to the growth of wild-type seedlings, single mutant seedlings did not show increased sensitivity; in contrast, the double
Figure 4.2 Med33a and Med33b gene structure and T-DNA insertions

A. Med33a and Med33b genomic structure indicating positions of SALK T-DNA insertions and primers used for RT-PCR. Black boxes, exons; lines, introns; triangles, insertion of the T-DNAs; arrows, position of the primers used in RT-PCR in panel B. Med33a and Med33b structures were determined by cloning and sequencing the transcripts. Med33b TAIR represents the genomic structure of Med33b reported on the TAIR website.

B. No full-length transcripts were detected in med33 double mutant. RNA was isolated from wild-type (WT) and the med33a-1;med33b-4 (med33-14) and med33a-2;med33b-3 (med33-23) double mutants and used to synthesize cDNA. The primers indicated in panel A were used to amplify portions of the Med33 transcripts. GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE C SUBUNIT (GAPC) was used as a loading control. Image of one replicate.
Figure 4.2

A.

![Diagram of Med33a and Med33b genes with markers and primers](image)

B.

<table>
<thead>
<tr>
<th>WT med33-14</th>
<th>WT med33-23</th>
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<tbody>
<tr>
<td>F1/R1</td>
<td>F1/R1</td>
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<td>GAPC</td>
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0.5 kb
mutant was hypersensitive to NaCl, with decreased leaf size and increased accumulation of anthocyanins (Appendix Figure 4.2 and Figure 4.3). To identify the ion causing this sensitivity, seeds were germinated on solid MS media and five day-old seedlings were transferred to solid MS without or with 100 mM NaCl, KCl, KNO₃, or NaNO₃. Double mutant seedlings were hypersensitive to NaCl and KCl but showed only a slight sensitivity to NaNO₃ and KNO₃ indicating that chloride was the major ion responsible for the increased sensitivity in the double mutant (Figure 4.3). NaCl affects plant growth and development through ionic, osmotic, and oxidative changes. To determine if the chloride hypersensitivity in the med33 double mutant is the result of oxidative changes, growth of the double mutant was compared to wild type on low concentrations of copper sulfate (CuSO₄). Heavy metals like CuSO₄ have been shown to increase the accumulation of reactive oxygen species (ROS) leading to oxidative stress (Keilig and Ludwig-Muller, 2009; Maksymiec et al., 2005). In an initial assay, the roots of the double mutant were shorter than wild type when exposed to 75 µM CuSO₄; however, this phenotype was not reproducible in two subsequent replications suggesting that the double mutant is not sensitive to this form of oxidative stress induction (Appendix Figure 4.3).

**The med33 double mutant has an early senescence phenotype**

In the process of growing the med33 double mutant, some plants accumulated anthocyanins in their leaves and senesced earlier than wild type, although this did not affect their ability to flower and produce seed (Figure 4.4A). This early senescence
**Figure 4.3 The med33 double mutant is sensitive to chloride ions**

Seeds from wild type (WT) and med33 double homozygous mutant (med33-14 and med33-23) were germinated on solid MS media for five days and transferred to solid MS without or with 100 mM NaCl, KCl, KNO$_3$, or NaNO$_3$. Photographs were taken 12 days after transfer. One representative image of four replicates.
Figure 4.3

<table>
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Figure 4.4 The *med33* double mutant senesces early but single mutants do not have a phenotype

A. **The double mutant accumulates anthocyanins and senesces early.** Wild type (WT) and the *med33-14* double mutant were germinated on MS and transferred to soil after 10 days. Plants were grown in long-day conditions (16 h light/8 h dark). Images of five week-old wild-type and two double mutant plants are shown. Enlarged images of wild type and one of the double mutants are shown below. Bars, 2.5 cm.

B. **The early senescence phenotype is enhanced in short-day conditions.** Wild type (WT) and single (*med33a-1* and *med33b-4*) and double (*med33-14*) mutants were germinated and grown in soil in short-day conditions (8 h light/16 h dark). Photographs were taken after 11 weeks of growth. Bars, 1 cm.
Figure 4.4

A.

WT  med33-14

B.

WT  WT  WT

med33a-1  med33b-4  med33-14
phenotype suggested that, if the vegetative phase was prolonged, the double mutant would be unable to flower and set seed. When growth was prolonged in short-day conditions (8 hours light/16 hours dark), the double mutant, but not the single mutants, was unable to complete its life cycle as the plants senesced before flowering (Figure 4.4B and Figure 4.5). The growth of the double mutant was very similar to wild type until week seven when spontaneous lesions developed on older leaves; subsequently, the area surrounding the lesions became chlorotic, and the leaves eventually died (Figure 4.5). Both double mutant combinations, med33-14 and med33-23, senesced before flowering; however the severity and timing of the phenotype was different. The med33-14 mutant showed the earliest signs of lesions and the lesions spread more quickly; after 12 weeks, this mutant was dead. In contrast, at 12 weeks, the older leaves in the med33-23 mutant had senesced but the plant was still producing young, green leaves (Figure 4.5A).

If the Med33 genes play a role in preventing senescence, the double mutant might also be expected to senesce early when senescence is induced. To induce senescence, leaves were detached from three week-old wild-type, med33 double mutant, and semi-dominant ref4 mutant plants grown in long-day conditions, placed in the dark, and chlorophyll breakdown (as an early marker of senescence; (Guo and Crawford, 2005; Lim et al., 2007)) was monitored. Contrary to what was observed during growth in short-day conditions, detached leaves from the double mutant remained greener longer than wild-type and ref4 mutant leaves which showed signs of chlorosis (Figure 4.6).
Figure 4.5 Older leaves of the *med33* double mutants develop lesions and senesce early

**A. The double mutant shows signs of senescence at seven weeks.** Wild type (WT) and the *med33-14* and *med33-23* double mutants were germinated and grown in soil in short-day conditions (8 h light/16 h dark). Photographs were taken after 7, 10, and 12 weeks of growth. Bar, 2.5 cm.

**B. Lesions form on older leaves of the double mutants.** Close-up images of lesions from 10 week-old wild-type and double mutant leaves shown in panel A.
Figure 4.5

A.

7 weeks 10 weeks 12 weeks

WT

med33-14

WT

med33-23

B.

WT  med33-23  med33-14
Figure 4.6 Detached leaves from the med33 double mutants have delayed senescence

A series of leaves were detached from 3 week-old wild type (WT), double med33 mutants, and the ref4 semi-dominant mutants, placed on filter paper soaked in MS, incubated in the dark for 3 days, and photographed. Bar, 1 cm. One representative image of three replicates.
Figure 4.6

WT

med33-14

med33-23

ref4-1

ref4-3

0 days

3 days
**Nutrients delay the med33 double mutant senescence phenotype**

In nutrient-limiting conditions, plants mobilize elements including nitrogen, potassium, and phosphorus from older leaves to support new growth (Himelblau and Amasino, 2001). If the med33 double mutant cannot properly accumulate nutrients, senescence of older leaves might be required for element transfer to support and maintain new growth. If this is the case, the senescence phenotype might be delayed in plants grown in nutrient-rich conditions. To test this, wild type and the med33 double mutant were grown in short-day conditions and treated with distilled water or with nutrient solution. After nine weeks, the double mutant watered with distilled water was smaller than its wild-type control and the older leaves had developed lesions and were senescing (Figure 4.7). The double mutant watered with nutrient solution was similar in size to its wild-type control and was just beginning to show signs of lesions and chlorosis (Figure 4.7). These results demonstrate that nutrient solution delays, but does not prevent, the early senescence phenotype of the double mutant.

**The cbl10;cbl7 single or double mutants do not phenocopy the med33 double mutant**

While results from the yeast two-hybrid assay suggest that Med33a and Med33b interact with CBL10 and CBL7 *in vitro* (Figure 4.1C), the med33 double and cbl10 single mutants display different ion sensitivities to NaCl (Figure 4.3; (Quan, 2007)) and the cbl10 single mutant does not display an early senescence phenotype (data not shown). These phenotypic differences could indicate that the Med33 proteins and CBL10 do not function in the same pathway to regulate these processes, or that genetic redundancy
Figure 4.7 Addition of nutrients delays senescence in the med33 double mutant in short-day conditions

Wild type (WT) and the med33-14 double mutant were grown in short-day conditions (8 h light/16 h dark) and watered with distilled water or fertilized with nutrient solution once a week. Photographs were taken after nine weeks of growth. Bar, 2.5 cm.
Figure 4.7

Distilled Water

WT

med33-14

Nutrient Solution
masks the interactions. To determine if CBL10 and CBL7 function redundantly in response to growth in high concentrations of chloride or in short-days, a cbl10;cbl7 double mutant was generated. To screen for ion sensitivity, wild type and both double mutants were grown on 100 mM NaCl or KCl. Both the med33-14 and cbl10;cbl7 double mutants were sensitive to NaCl as expected; however, only the med33-14 double mutant was sensitive to KCl (Figure 4.8A). To monitor the timing of senescence, wild type and the cbl10;cbl7 and med33-14 double mutants were grown in short-day conditions. After 10 weeks, the med33-14 double mutant was smaller than wild type and the older leaves had senesced (Figure 4.8B). However, growth of the cbl10;cbl7 double mutant was similar to that of wild type (Figure 4.8B). These results indicate that CBL10 and CBL7 do not function with Med33 during growth on high chloride or to regulate the timing of senescence in short-day conditions.
Figure 4.8 The cbl10;cbl7 double mutant does not phenocopy the med33 double mutant

A. The cbl10;cbl7 double mutant is insensitive to chloride. Seeds from wild-type (WT), cbl10;cbl7, and med33-14 double mutant were germinated on solid MS for five days and transferred to solid MS without or with 100 mM NaCl or KCl. Photographs were taken 19 days after transfer. Image of one replicate.

B. The cbl10;cbl7 double mutant does not have an early senescence phenotype. Wild type (WT), cbl10;cbl7, and med33-14 double mutants were germinated and grown in soil in short-day conditions (8 h light/16 h dark). Photographs of 10 week-old plants are shown. Bar, 2.5 cm.
Figure 4.8

A.

WT  cbl10;cbl7  med33-14

MS

100 mM NaCl

100 mM KCl

B.

WT  WT

cbl10;cbl7  med33-14
Discussion

**Med33a and Med33b are unlikely to interact with the CBL proteins *in planta***

In an *in silico* search for proteins that interact with the CBL family of calcium-binding proteins, Med33a and Med33b were identified. Using a yeast two-hybrid assay, the Med33 peptides containing a SOS2-like CBL10-interaction domain (FISL motif) were shown to interact with CBL10 and CBL7 (Figure 4.1). While these findings suggest that these proteins interact to regulate plant growth and development, the following results suggest they are unlikely to interact *in planta*. 1) While both CBL10 and the Med33 proteins are required for prolonged growth in the presence of NaCl, they appear to function in different aspects of tolerance; CBL10 to keep cytosolic sodium levels low (Kim et al., 2007; Quan, 2007) and Med33 to prevent the toxic effect of chloride. 2) While Med33 interacted with both CBL10 and CBL7 in a yeast-two hybrid assay, the *cbl10;cbl7* double mutant does not phenocopy either the *med33* double mutant chloride sensitivity or the early senescence phenotype in short-day conditions indicating that genetic redundancy is not masking the CBL10-Med33 interaction (Figure 4.8). 3) Localization of the proteins within the cell appears to be different. CBL10 is reported to localize to the vacuolar and plasma membranes and within the cytoplasm (Kim et al., 2007; Quan, 2007). Med33a and Med33b, as components of the Mediator complex, would be expected to localize to the nucleus and initial experiments using transient expression of protein fusions to green fluorescent protein (GFP) in tobacco confirm this (H. Renault, unpublished data).
Med33a and Med33b may play a role in DNA protection and/or repair

There has been significant research focused on the toxic effects of sodium, but very little is known about the effect of chloride on plant growth. Measurements of chloride in plant cells indicate that cytosolic levels of this ion increase when plants are grown in the presence of NaCl or KCl and there is a greater accumulation of chloride in leaves versus roots (Diedhiou and Golldack, 2006; Lorenzen et al., 2004). This higher leaf accumulation of chloride could explain the observation that sensitivity to chloride in the med33 double mutant is manifested in leaves.

One recently reported effect of chloride is increased genotoxic stress. Wild-type Arabidopsis grown in the presence of NaCl, KCl, and MgCl₂ but not Na₂SO₄ or MgSO₄ had higher levels of double stranded breaks in DNA and higher rates of recombination (Boyko et al., 2010). In addition, plants that were treated with high levels of chloride had elevated transcript levels of AtRad51, an enzyme involved in the homologous recombination pathway which functions to repair breaks in DNA (Boyko et al., 2010). These observations were also seen in the progeny of salt-treated plants which had elevated levels of DNA recombination and higher expression of enzymes involved in DNA repair compared to progeny from plants grown in control conditions (Boyko et al., 2010).

One model to explain the increased sensitivity of the med33 double mutant to chloride is that the Med33 proteins, as components of Mediator, play a role in co-activating the transcription of genes involved in DNA repair. If the Med33 Mediator has
a general role in the transcriptional regulation of DNA repair genes, then the double mutant would likely be sensitive to multiple DNA damaging agents. To test this model, the *med33* double mutant is being analyzed for sensitivity to other genotoxic agents including ultraviolet (UV) light and the chemicals, methyl methane sulfonate (MMS) and hydroxyurea. Preliminary results indicate that the double mutant is hypersensitive to all of these agents, suggesting that the Med33 proteins are involved in DNA damage repair (Dr. Yan Guo, China Agricultural University). To determine if the Med33 Mediator activates DNA repair responses, the expression of genes involved in DNA repair pathways could be compared in wild type and the double mutant using quantitative RT-PCR (qRT-PCR) and the ability of protein extracts from the mutant and wild type to repair DNA could be tested in an *in vitro* DNA repair assay (Boyko et al., 2010). If Med33 is involved in DNA repair, transcript levels of DNA repair genes would be lower in the double mutant and protein extracts would be unable to repair damaged DNA in the *in vitro* assay when compared to wild type.

While the mechanism underlying chloride’s role in genotoxic stress is unknown, one possibility is that chloride causes DNA damage through the production of ROS (Balestrazzi et al., 2010). If chloride effects are mediated by ROS, a wild-type response to excess chloride accumulation would require the expression of genes encoding enzymes that scavenge ROS to prevent protein, lipid, and DNA damage (Van Breusegem and Dat, 2006). If this is the case, another possible role for the Med33 Mediator might be to reduce ROS levels through the transcriptional regulation of the genes encoding
these ROS scavenging enzymes. If Med33 is involved in the removal of ROS, the double mutant might be sensitive to other inducers of oxidative stress that lead to increased ROS production. To test this, seedlings were exposed to CuSO$_4$ which has been shown to induce ROS accumulation (Keilig and Ludwig-Muller, 2009; Maksymiec et al., 2005). Sensitivity in the roots of the double mutant was observed; however, the phenotype was not reproducible suggesting that Med33 does not play a role in response to oxidative stress caused by copper (Appendix Figure 4.3). A more direct method to determine if chloride generates higher levels of ROS in the med33 double mutant would be to visualize the levels of hydrogen peroxide in the double mutant and wild type grown without and with NaCl using 3,3’-diaminobenzidine (Guo and Crawford, 2005).

**Med33a and Med33b may be involved in maintaining growth in nutrient-limiting environments**

Leaf senescence involves the break down and transport of cellular materials and nutrients from a mature leaf to a sink such as a developing leaf or fruit (Guiboileau et al., 2010; Himelblau and Amasino, 2001; Lim et al., 2007). Relative to senescence, leaf development appears to progress through three distinct phases (Guiboileau et al., 2010). During phase one, young, developing leaves do not senesce. In the second phase, leaves can senesce early in response to hormonal or environmental cues, potentially enabling plants to respond to changing conditions (Guiboileau et al., 2010; Himelblau and Amasino, 2001; Lim et al., 2007). Endogenous signals that trigger leaf senescence include the plant hormones abscisic acid (ABA), JA, and SA (Guiboileau et al., 2010; Lim
et al., 2007). Environmental signals triggering senescence include drought, nutrient limitation, extreme temperatures, oxidative stress by UV light, pathogen attack, and shading by other plants (Lim et al., 2007). In phase three, developmental senescence is initiated in an age-dependent manner and occurs even in optimal growing conditions with no signs of environmental stress.

Regardless of the photoperiodic conditions during growth, med33 double mutant leaves accumulated anthocyanins, developed lesions, and senesced early compared to wild-type leaves. The senescence phenotype is first seen in leaves that are likely in phase two of developmental senescence, suggesting that an environmental signal could be responsible for inducing this response (Figure 4.5). Because the early senescence phenotype of the double mutant is delayed with the addition of nutrients (Figure 4.7), nutrient limitation might be the signal inducing this senescence. Perception of nutrient limitation would trigger mobilization of nutrients from older leaves to support and maintain new growth (Himelblau and Amasino, 2001). Addition of nutrients might delay some of the early signs of nutrient-starvation by providing additional nutrients for the plant. Nutrients added during growth also delay early senescence in the autoinhibited Calcium-ATPase aca4;aca11 double mutant (Boursiac et al., 2010). This double mutant has a pattern of senescence similar to that of the med33 double mutant; lesions first appear as spots and then spread throughout the leaf. The early senescence phenotype of the aca double mutant does not appear to be in response to a specific nutrient
because addition of different salts, including \( \text{NH}_4\text{NO}_3 \), KCl, or \( \text{KH}_2\text{PO}_4 \), delayed senescence.

A process underlying nutrient mobilization is autophagy, the degradation of cytoplasmic constituents that have been encased in vesicles (Guiboileau et al., 2010). Mutations in the Arabidopsis *Autophagy-Related Genes (ATG)* disrupt autophagy, but plants are still able to germinate, flower, and reproduce in nutrient-rich conditions. However, these mutants show signs of early senescence during growth in short-day conditions and are hypersensitive to growth in nutrient-limiting conditions suggesting that maintenance of leaf development in wild-type plants is due to the combined contributions of nutrient uptake, assimilation, and recycling of cellular components (Doelling et al., 2002; Yoshimoto et al., 2009). Based on similarities in the phenotypes of the *med33* double and *atg* mutants, the early senescence phenotype of the *med33* double mutant could be due to a disruption in autophagy. To determine if the Med33 proteins play a role in the regulation of genes involved in this process, growth of the double mutant could be monitored in nutrient-limiting conditions (e.g., seedling growth on nitrogen-depleted media; (Yoshimoto et al., 2009)) where seedlings would be expected to be hypersensitive relative to the growth of wild type. The role of Med33 proteins in the co-activation of autophagy genes could be determined by comparing transcription levels of specific genes (e.g., *ATG* genes) in wild-type and the *med33* double mutant during growth in short-day conditions using qRT-PCR or through transcriptional profiling with microarray analysis.
SA signaling might link Med33a and Med33b to senescence through autophagy

In addition to its role in nutrient cycling, autophagy has also been shown to regulate levels of SA (Yoshimoto et al., 2009). Experiments with *atg2*, *atg5*, and the *aca4;aca11* double mutants demonstrated that the early senescence phenotypes were dependent on SA (Boursiac et al., 2010; Yoshimoto et al., 2009). A model, based on these experiments, suggests that SA triggers autophagy which then initiates a negative feedback regulatory loop to modulate SA signaling (Yoshimoto et al., 2009). Med33 could participate in this pathway by regulating transcription of autophagy genes, thereby preventing the over-accumulation of SA through the activation of autophagic activities. Without Med33, autophagy would not be activated and SA levels would increase leading to the development of lesions and the early senescence phenotype. If this is the case, the phenotype of the *med33* double mutant would be suppressed with the removal of SA, which could be tested by over-expression of the *Pseudomonas* SA hydroxylase, NahG, or through disruption of SA biosynthesis by mutating *SALICYLIC ACID INDUCTION-DEFICIENT2* (*SID2*), a SA biosynthetic gene. To directly link Mediator to SA-mediated activation of autophagy, the SA agonist, benzo (1, 2, and 3) thiadiazole-7carbothioic acid (BTH), could be used to induce autophagy in wild type and the *med33* double mutant. In wild-type plants treated with BTH, the formation of vesicles representing autophagosome-related structures can be observed using the autophagosome marker GFP-ATG8a (Yoshimoto et al., 2009); if Mediator is important for this process, the
absence of or a reduced number of vesicles would be expected in the med33 double mutant.

**Med33a and Med33b may have multiple roles during senescence**

Relative to the growth of wild type, the med33 double mutant has an early senescence phenotype when grown in both long and short-days (Figure 4.4 and Figure 4.5), but if senescence is induced by incubating detached leaves in the dark, the double mutant displays delayed senescence (Figure 4.6). Studies of the atg2 and atg5 mutants offer insight into how Med33 could have opposite phenotypes in senescence-related processes. Both atg2 and atg5 have an early senescence phenotype in standard growth conditions, during seedling growth on nitrogen-limited media, and during dark-induced senescence in seedlings and detached leaves. However, when SA biosynthesis or signaling is disrupted, the senescence phenotype of the mutants is suppressed in standard growth conditions but not during starvation or dark-induction. This suggests that there are at least two senescence processes; one is SA-dependent (during standard growth) while the other is SA-independent (during starvation and dark-induction). In the med33 double mutant, there is early senescence in conditions that might correlate with the SA-dependent phenotype (during standard growth), but delayed senescence for those conditions that correlate with the SA-independent phenotype (during dark-induction). Mediator’s proposed function as both an activator or repressor of transcription (Casamassimi and Napoli, 2007) might explain the mechanism underlying these opposite phenotypes in the double mutant in the two senescence processes. For
example, without Med33, the double mutant might senesce early when grown in short-day conditions (SA-dependent) because Med33 is not present to activate genes that prevent senescence or maintain growth. To initiate senescence in response to starvation or dark induction (SA-independent), Mediator might interact with CDK8 or a CDK8-like complex (Bjorklund and Gustafsson, 2005) to prevent Pol II from transcribing genes involved in repressing senescence or maintaining growth. In the absence of Med33, genes that are involved in these processes would be expressed allowing detached leaves of the med33 double mutant to remain green even when senescence is induced.

Large scale approaches will be needed to understand the Med33 Mediator complex

My characterizations of the med33 double mutant have demonstrated that the Med33 proteins are important for plant growth in high levels of chloride and to prevent premature senescence (Figure 4.3 and Figure 4.5). The information included in the sections above provides examples of potential mechanisms that might underlie Med33 function and outlines experiments to link mechanism to function. Additional approaches that will contribute to our understanding of the functions of a Mediator complex containing Med33a and Med33b, will be to identify both the transcriptional activators with which the complex interacts and genes whose expression is regulated by the Med33 Mediator (complex targets). The Med33 proteins are plant specific suggesting that they might interact with transcriptional activators rather than binding to Pol II (Backstrom et al., 2007). A yeast two-hybrid assay could be used to screen a
transcriptional activator library to identify Med33-interacting transcription factors.

Several groups have demonstrated protein interaction with specific Mediator subunits in yeast two-hybrid assays, suggesting that it is possible to use one component of Mediator to identify interacting proteins (de la Cera et al., 2002; Dhawan et al., 2009; Leonard et al., 2006; Stumpf et al., 2006). To identify Med33 Mediator targets, a microarray comparing gene transcript levels in wild type and the double mutant could be performed on plants that have been exposed to high levels of chloride or grown in short-day conditions.
**Appendix Figure 4.1 Med33 proteins interact with some of the CBL proteins**

In the upper panels, *Med33* cDNAs encoding the protein sequence surrounding the putative SOS2-like CBL10-interaction domain (FISL motif, indicated in Figure 4.1B) were cloned into the pGAD vector and fused to the GAL4 activation domain (Med33aFISL-AD and Med33bFISL-AD). All ten full-length *CBL* cDNAs were cloned into the pGBK vector and fused to the GAL4 DNA binding domain (CBL-BD). Serial dilutions of diploid yeast harboring both constructs were spotted onto synthetic complete medium minus leucine, tryptophan, adenine, and histidine (SD-LWAH) to determine strength of interaction. The CALMODULIN4 (CaM4) cDNA was cloned into the pGBK vector (CaM4-BD) and, along with empty vector (BD), was used as a negative control. Also included as controls for this experiment (lower panels) was a fragment of SOS2/CIPK24 cDNA containing the FISL domain (SOS2FISL-AD) cloned into the pGAD and the pGBK vectors paired with SOS3/CBL4 cDNA cloned into the pGBK and the pGAD vectors, respectively. All yeast were grown for 6 days. One representative image of two replicates.
### Appendix Figure 4.1

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The images show the results of experiments with different constructs under conditions SD-LWAH.
Appendix Figure 4.2  The *med33* single mutants have wild-type like growth on NaCl

Seeds from wild type (WT), *cbl10*, and the *med33* single mutants were germinated on solid MS media for five days and transferred to solid MS without or with 100 mM NaCl. Photographs were taken 10 days after transfer. One representative image of two replicates.
Appendix Figure 4.2

WT  med33a-1  med33b-4  cbl10

MS

100 mM NaCl
Appendix Figure 4.3 The *med33* double mutants are sensitive to growth on Cu$_2$SO$_4$

Seeds from wild type (WT) and the *med33* double mutant were germinated on solid MS media for five days and transferred to solid MS without or with 75 μM CuSO$_4$. Photographs were taken 10 days after transfer. Two technical reps are shown. This phenotype was only seen in one experiment of three.
Appendix Figure 4.3

WT    med33-14    med33-23

MS

75 μM CuSO₄

75 μM CuSO₄
CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Calcium is a component of signal transduction pathways that allow plants to respond to numerous endogenous and environmental signals during growth and development. Calcium-binding proteins perceive changes in cytosolic calcium levels and transduce those changes into specific cellular responses. One method for achieving specificity during calcium signaling is through regulation of these proteins. Calcium-binding proteins can be regulated through differences in expression in response to stimuli, localization within the cell or plant, affinity for calcium, and interaction with downstream proteins; all of which can result in specific cellular responses (Reddy and Reddy, 2004).

The goal of my research has been to understand the mechanisms underlying calcium-mediated plant responses to salinity through characterization of the CALCINEURIN B-LIKE10 (CBL10) calcium-binding protein. I have shown that CBL10 is involved in responses to salinity in leaves and flowers and that post-transcriptional regulation of the protein is used to modulate signaling in leaves. In order to more fully understand how specificity is achieved during calcium-mediated responses to growth in saline environments, I would: 1) compare calcium-binding proteins with similar but organ-specific roles during plant growth in saline conditions and 2) characterize a
calcium-binding protein in a salt-tolerant plant to determine its contribution to the maintenance of plant growth in salt-affected soils.

**Compare calcium-binding proteins with similar but organ-specific roles during plant growth in saline conditions**

In Arabidopsis, *SALT-OVERLY-SENSITIVE3 (SOS3)/CBL4* and *CBL10/ SOS3-LIKE CALCIUM BINDING PROTEIN8 (SCaBP8)*, two members of the CBL family of calcium-binding proteins, have roles in plant growth in saline conditions. Genetic analyses have demonstrated that *SOS3* primarily plays a role in roots while *CBL10* plays a role in leaves and flowers. During growth in salt, the *sos3* mutant displays a strong root phenotype and a mild leaf phenotype compared to wild type and *cbl10* (Halfter et al., 2000; Liu and Zhu, 1998; Quan, 2007), while the *cbl10* mutant displays a strong leaf phenotype and a mild branch root phenotype compared to wild type and *sos3* (Quan, 2007). Analysis of transcript levels in leaves and roots supports this model; *SOS3* is expressed in roots while *CBL10* is predominantly expressed in leaves (Quan, 2007). The double mutant (*cbl10; sos3*) has an additive phenotype, both leaf and root growth are severely affected compared to the two single mutants (Quan, 2007), further confirming that the two proteins have unique, non-overlapping roles. Both proteins have been shown to interact with and activate the SOS2 serine/threonine protein kinase to regulate the activity of the SOS1 Na⁺/H⁺ exchanger (Halfter et al., 2000; Qiu et al., 2002; Quan, 2007).
To understand how these related calcium-binding proteins achieve their organ-specific pathway functions, I would determine the organs in which they function, characterize any structural differences between the two proteins, and identify their pathway interactions.

**In which organs do SOS3 and CBL10 function?**

While the model for SOS3 and CBL10 function based on genetic analyses and gene expression data suggests that SOS3 regulates SOS1 in the root and CBL10 regulates SOS1 in leaves, initial experiments demonstrating SOS3 regulation of Na\(^+\)/H\(^+\) exchange were performed with plasma membrane vesicles isolated from leaves. In these studies, analysis of Na\(^+\)/H\(^+\) exchange activity in sos1 and sos3 indicated that both mutants had a similar reduction in Na\(^+\)/H\(^+\) exchange compared to wild type (Qiu et al., 2002). These results suggest that SOS3 non-redundantly regulates SOS1 activity in leaves. However, analysis of Na\(^+\)/H\(^+\) exchange in plasma membrane vesicles isolated from leaves of the cbl10 mutant revealed that SOS1 activity is also decreased compared to activity in wild-type suggesting that CBL10 also regulates SOS1 activity in leaves. To better understand how SOS3 and CBL10 function in the regulation of SOS1, I would measure Na\(^+\)/H\(^+\) exchange activity in plasma membrane vesicles isolated from wild-type, sos1, cbl10, sos3, and cbl10;sos3 leaves and roots of plants grown in hydroponics. These studies would identify the tissues in which each protein functions.
To determine if *SOS3* and *CBL10* have similar functions during plant responses to growth in saline conditions and if only their patterns of expression differ, reciprocal complementation analyses were performed (Quan, 2007). In these studies, the *SOS3* gene was transformed into the *cbl10* mutant and the *CBL10* gene was transformed into the *sos3* mutant, both under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter to ensure expression throughout the plant. Since both proteins activate the SOS pathway, the expectation was that expression of either gene in the other mutant should activate the pathway and complement the salt-sensitive phenotype. *SOS3* was unable to complement the *cbl10* leaf phenotype while *CBL10* complemented the *sos3* leaf phenotype, but not the root phenotype. Both proteins were able to complement their respective mutants when expressed from the 35S promoter, which suggests that the lack of complementation in the reciprocal analysis was not due to problems with gene expression. Instead, lack of complementation was most likely due to unique properties of the proteins in either activation of the SOS pathway or of another pathway involved in salt tolerance. To determine if activation of the SOS pathway is general (either protein can activate the pathway if present) or organ specific (*CBL10* must be present in leaves and *SOS3* must be present in roots), I would monitor activation of *SOS1* by measuring Na⁺/H⁺ exchange in leaves and roots of *sos3; p35S:CBL10* and *cbl10; p35S:SOS3* transgenic plants, using wild type, *sos1*, *cbl10*, and *sos3* as controls. If the SOS pathway is activated with expression of either gene, then the *sos3* leaf phenotype is due to a lack of SOS pathway activation but the root phenotype is due to a
loss of a function that is specific for SOS3. Likewise, the cbl10 leaf phenotype is due to a loss of a function that is specific for CBL10. If the SOS pathway is not activated in either of the transgenic plants, then activation of the SOS pathway might occur in an organ-specific manner (SOS3 has a specific characteristic that is required for activation of the SOS pathway in roots and CBL10 has a specific characteristic that is required for activation of the pathway in leaves).

**What contributes specificity to the SOS3 and CBL10 calcium-binding proteins?**

Since neither SOS3 nor CBL10 can fully compensate for the absence of the other, there must be characteristics that specifically regulate protein function (e.g., affinity for calcium or post-translational modifications) or intrinsic properties of each protein that regulate their interaction with specific target proteins.

**Calcium affinity assays** - Calcium sensors like SOS3 and CBL10 function by binding calcium leading to a conformational change in the protein which facilitates interaction with target proteins. These proteins bind calcium through a helix-loop-helix motif called an EF-hand. (Tuteja and Mahajan, 2007). The affinity of the EF-hand for calcium depends on the amino acid residues within the loop (Tuteja and Mahajan, 2007). Changes in the amino acids present at a given position modulate binding affinity allowing calcium sensors to respond to different calcium signatures (Kolukisaoglu et al., 2004). CBL10 and SOS3 both have four motifs with similarity to EF-hands (Kolukisaoglu et al., 2004). In CBL10, the fourth EF-hand represents a canonical EF-hand with
conserved amino acids at each position in the loop (Kolukisaoglu et al., 2004). The second and third EF-hands have basic amino acid substitutions at key positions and the first EF-hand has non-oxygen containing amino acid substitutions which most likely decrease the affinity of the motif for calcium or may prevent binding (Kolukisaoglu et al., 2004). In contrast, the SOS3 protein does not have a canonical EF-hand. The second, third, and fourth EF-hands have basic amino acids at key positions and the first EF-hand has non-oxygen containing amino acid substitutions. This suggests that SOS3 might have a lower affinity for calcium than CBL10. To begin to understand how EF-hand structures might contribute to the specificity of the SOS3 and CBL10 proteins, the affinity of SOS3 and CBL10 for calcium could be compared using flow dialysis with labeled calcium (Kakalis et al., 1995). CBL10 might bind at low concentrations of calcium, with higher concentrations required for SOS3 binding. To link calcium binding to function, the EF-hands of SOS3 could be altered to more closely match those of CBL10. When comparisons of EF-hands are made between the two proteins, the first and third EF-hands are identical, there are three amino acid differences in the second EF-hands (none of the changes reside in key positions), and there are five amino acid differences in the fourth EF-hands (two of which are in key positions). Within the fourth EF-hand, one of the positions altered in the SOS3 sequence seems to determine whether the motif is canonical among all of the CBLs (Kolukisaoglu et al., 2004). This position could be changed in SOS3 to generate a canonical EF-hand and its effect on the ability of SOS3 to complement the sos3 and cbl10 mutants could be examined. Results from these
studies should help us understand if changing the affinity for calcium changes protein function (the mutant SOS3 protein can now complement the \textit{cbl10} mutant) and/or abolishes protein function (the mutant SOS3 protein cannot complement the \textit{sos3} mutant).

\textbf{Interaction screens} – An alternative explanation for the organ specific function of SOS3 and CBL10 during plant growth in salinity is that they interact with unique target proteins. As a result, identification of these interacting proteins will be an important complementary approach to understand how these related calcium-binding proteins achieve their specific functions. To verify interaction between CBL10 and SOS2, a bimolecular fluorescence complementation assay was used (Quan, 2007). Each gene was transcriptionally fused to a portion of the yellow fluorescent protein (YFP), transiently transformed into Arabidopsis protoplasts and detection of YFP fluorescence was used as an indicator of interaction. A similar assay might be used to identify novel SOS3 and CBL10 interacting proteins using a cDNA library enriched for genes that are up-regulated during growth in salt (Ohad et al., 2007). A significant benefit of this approach is that protein interaction can be detected at the plasma membrane, the site at which SOS3 and CBL10 localize.
Characterize a calcium-binding protein in a salt-tolerant plant and determine its contribution to the maintenance of plant growth in salt-affected soils

The many genetic resources available in Arabidopsis have greatly facilitated the identification and characterization of genes involved in plant responses to salinity including the SOS pathway genes. However, Arabidopsis is a salt-sensitive plant whose growth is severely reduced upon exposure to salt. Therefore, an understanding of the mechanisms operating in salt-tolerant plants will be important for understanding how some plants maintain growth in salt-affected soils. Thellungiella halophila (Thellungiella, also called Eutrema salsugineum) is a salt-tolerant relative of Arabidopsis that is able to maintain growth in soils watered with NaCl as high as 300 mM (J. Barrero-Gil, unpublished). SOS1 has been identified in Thellungiella, shown to be critical for its salt-tolerance (Oh et al., 2007), and to be the functional ortholog of the Arabidopsis SOS1 gene (D.E. Jarvis, unpublished). In Arabidopsis, CBL10 regulates the activity of the SOS1 plasma membrane Na⁺/H⁺ exchanger through activation of the SOS2 protein kinase. Because CBL10 responds to changes in intracellular calcium when plants are grown in saline conditions, it likely initiates and regulates the SOS pathway. To determine if CBL10 plays a similar role in Thellungiella’s salt-tolerance, I would focus future studies on identifying the CBL10 gene in Thellungiella, functionally determining its importance in salt-tolerance, and characterizing its regulation.
Two CBL10-like genes are present in Thellungiella

In Thellungiella, two CBL10-like genes have been identified. To verify that AtCBL10 is the most closely related CBL in Arabidopsis, the sequences of the two Thellungiella genes were compared with all sequences in the Arabidopsis genome - AtCBL10 had the highest homology. The two ThCBL10 genes show 86% and 81% identity with AtCBL10 and share 85% identity with each other (S. M. Monihan, D. E. Jarvis, unpublished).

Gene duplication is thought to be one of the driving forces for adaptive evolution (Flagel and Wendel, 2009) and there are several potential fates for duplicated genes in plants. Both genes could have completely redundant functions or, through differences in regulatory sequences or accumulation of amino acid changes, one gene might develop a novel function or become non-functional. Duplication of CBL10 in Thellungiella leads to a number of important questions; for example, 1) Do both genes have a role in Thellungiella’s salt tolerance? 2) Do the two genes have overlapping functions? 3) Do both genes have functions similar to CBL10 in Arabidopsis? 4) Does either have a novel role outside the SOS pathway? 5) Do the two genes function in different organs of the plant? Using the approaches and tools available in Arabidopsis and those being developed for Thellungiella, questions like these can be addressed functionally.

What are the functions of the Thellungiella CBL10 genes?
**Genetic analyses** – To learn more about the role of the two *CBL10* genes in Thellungiella, I would focus on understanding if both genes are involved in salt tolerance, if they have overlapping or unique functions, and if their functions are similar to that of *AtCBL10*. To determine if both genes are involved in salt tolerance, the expression of each gene separately and in combination would be reduced using RNA interference (RNAi) technology. If the single mutants are more sensitive to salt during growth than wild-type Thellungiella, this would indicate that both genes are involved in salt tolerance (Question 1) and could have unique functions. If a salt-sensitive phenotype is only seen in the double mutant, the duplication resulted in two genes with overlapping functions (Question 2). If only one of the single mutants is salt-sensitive, it is possible that one gene has a function similar to *AtCBL10* while the other has developed a novel role outside salt-tolerance or is non-functional (Question 3). If the genes are involved in Thellungiella’s salt tolerance, they would be transformed singly or in combination into the Arabidopsis *cbl10* mutant to determine if the genes have the same function as *AtCBL10* (Questions 2 and 4). Transformation of the Thellungiella genes singly and together into the Arabidopsis *cbl10* mutant would also allow me to determine if the Thellungiella gene(s) improves the salt tolerance of Arabidopsis.

**Expression analyses** – Gene duplication can result in genes whose expression is regulated by different cis-regulatory elements. To determine if the two *ThCBL10* genes are differentially expressed relative to each other and to *AtCBL10*, reverse transcriptase polymerase chain reaction and protein fusions to GFP would be used to monitor
localization within the plant and cell (Question 5). Initial experiments looking at the promoter activity of AtSOS1 in Arabidopsis and ThSOS1 in both Thellungiella and Arabidopsis roots suggest that expression of ThSOS1 might be found in more cell layers than just the stele (where AtSOS1 promoter activity is seen in Arabidopsis) (D. E. Jarvis and J. Barrero-Gil, unpublished).

Are signaling mechanisms conserved in Arabidopsis and Thellungiella?

In Arabidopsis, alternative splicing of CBL10 produces two proteins with antagonistic functions; one activates the SOS pathway while the other inhibits pathway activity. The relative ratio of the two proteins could determine the level of SOS pathway activity. One potential benefit of this signaling mechanism might be that cells are kept in a state where they are poised to respond to increases in cytosolic sodium. A signaling mechanism like this would likely be very important for plants that need to respond to high levels of salt quickly or frequently. To determine if this same mechanism is present in a salt-tolerant plant, the two Thellungiella CBL10-like genes could be amplified from cDNA to determine if alternatively spliced transcripts are present - initial EST data from the Thellungiella genome project suggests that both genes are alternatively spliced. To determine the dynamics of alternative splicing, the presence of the variants could be examined in plants grown in the absence and presence of salt to determine if the variants are differentially regulated during growth in saline conditions.
REFERENCES


