LEE, SANG YOON. The Involvement of ER Calcium in Abiotic Stress Tolerance. (Under the direction of Dr. Dominique Robertson.)

Calcium application is known to reduce the deleterious effects of NaCl in many plant species. Ca\(^{2+}\) supplementation is thought to act by inhibiting ion channels that allow Na\(^+\) influx and by blocking Na\(^+\)-induced K\(^+\) efflux. I generated transgenic Arabidopsis lines that constitutively express a low affinity, high capacity Calcium Binding Peptide (CBP) localized to the endoplasmic reticulum. Four independent transformed lines, two that also contained GFP and two that lacked it, were analyzed and contained up to 10% more total calcium than GFP control and wild-type plants. Each of these lines also showed increased K\(^+\) that was balanced by a decrease in Na\(^+\) accumulation. There were no significant changes in the relative amounts of other ions.

ER-CBP transgenic plants exhibited better salt and osmotic tolerance, increased survival in soil under intermittent drought conditions, longer root growth, higher chlorophyll content, and higher total seed production compared to GFP transgenic plants and control plants. One member of the CIPK (CBL-Interacting Protein Kinase) family, CIPK6, showed higher transcript levels in ER-CBP lines along with other drought-associated genes, such as DREB1a and rd29a, even under non-stress conditions. However, DREB2a transcript level was not affected by ER-CBP.

CIPK6 interacts with a Calcineurin B-Like Protein(s) (CBL) and was recently shown to interact with the C-terminus of the Arabidopsis Potassium Transporter1 (AKT1) protein.
Using *cipk6* null mutants, I showed that expression of both ER-CBP and CIPK6 was needed to achieve salt tolerance.

I used two methods, confocal ratio imaging of Indo-1 and cytoplasmic expression of aequorin, to detect transient changes in \([\text{Ca}^{2+}]_{\text{cyt}}\) in response to a salt stimulus. There were no significant differences in \([\text{Ca}^{2+}]_{\text{cyt}}\) measured by confocal ratio imaging between ER-CBP transgenic plants and control Arabidopsis plants in response to a short term salt treatment (~ 20 min). However, after three days incubation on 100 mM NaCl, ER-CBP lines had a higher steady state level of \([\text{Ca}^{2+}]_{\text{cyt}}\) than wild type plants. Similarly, ER-CBP transgenic plants expressing aequorin in the cytoplasm did not show significant differences in \(\text{Ca}^{2+}\) spikes in response to 150 mM or 300 mM NaCl. After seedlings were grown on \(\text{Ca}^{2+}\) depleted media for 5 days, ER-CBP transgenic plants maintained \(\text{Ca}^{2+}\) peak heights similar to that seen before the low-calcium treatment, but control plants showed a decrease in \(\text{Ca}^{2+}\) spikes. This suggests that ER-CBP transgenic lines utilized extra ER \(\text{Ca}^{2+}\) stores under continual stress to maintain optimal \(\text{Ca}^{2+}\) levels in the cytoplasm.

Trehalose has been shown to play an important role in drought tolerance and is one of the best studied osmoprotectants in plants. ER-CBP transgenic lines exhibited increased *Trehalose 6-phosphate synthase* (*TPS*) and *Trehalose-6-phosphate phosphatase* (*TPP*) expression. Accordingly, the trehalose content of ER-CBP lines was significantly higher compared to control plants. These results strongly implicate ER calcium as being critical for sustained tolerance to drought and salt in Arabidopsis.
The Involvement of ER Calcium in Abiotic Stress Tolerance

by
Sang Yoon Lee

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Biology

Raleigh, North Carolina

2009

APPROVED BY:

Dr. Dominique Robertson
Chair of Advisory Committee

Dr. Nina Strömgren Allen
Advisory Committee Member

Dr. George Allen
Advisory Committee Member

Dr. Heike Winter-Sederoff
Advisory Committee Member
BIOGRAPHY

Sang Yoon Lee was born in Seoul, South Korea in 1975. He grew up in an outskirt of Seoul where nature and wildlife were nearby. He developed an interest in science to help find a way to protect the environment from pollution by human activity and to find answers in science to solve world poverty. He did an outstanding award-winning scientific research project about environmental water pollution in high school and it encouraged him to pursue a biology degree in college. He excelled in math and science in high school and represented his high school for a national competition. He enrolled for biology studies at Yonsei University and developed his interest in Plant Biology under the direction of Dr. Kim Woo Taek. After serving in the US army for two years earning multiple awards and recognition by the brigade general for his outstanding service, he went back to college and finished his B.S degree in 2002.

He was awarded the KEF International Scholarship in 2003 to study at North Carolina State University. He was intrigued by the dynamic and complex roles that calcium plays in cell biology and wanted to study calcium in plants. He took over a calcium project on the Calreticulin C-domain from the previous lab member, Dr. Pei-Lan Tsou. His research goal was to understand the role of Calreticulin in *Arabidopsis thaliana* and the effect of increased ER calcium in plant stimuli and responses under the direction of Dr. Dominique Robertson.
ACKNOWLEDGEMENTS

I wish to thank Dr. Dominique Robertson for her guidance as my advisor, committee chair, research advisor, teaching advisor and being a person who I can trust. She has been a great source of encouragement and optimism. She helped me to think scientifically through numerous discussions we had together. I also wish to thank Dr. Nina S. Allen, Dr. George Allen and Dr. Heike Winter-Sederoff for their great advice and precious time.

I would like to thank CMIF (Cell and Molecular Imaging Facility) members, Dr. Nina Strömgren Allen and Dr. Eva Johannes. Dr. Eva Johnannes provided constant help throughout my Indo-1 experiments and taught me how to use a confocal microscope and to prepare samples for my experiments. Thanks and appreciations are also extended to Dr. Pamela Abit for helping me with drought tolerance assays of CBP transgenic plants. I would like to thank Dr. Imara Perera for providing the aequorin transgenic plants and dispensing useful advice for my experiments.

I would like to thank Dr. Chad Jordan, Steve Bernacki and Rich Tuttle for helping out and working together to keep things running smoothly in the lab. I would like to thank Steve Bernacki for being one of the nicest friends I have known and a person I can rely on whenever I have personal issues.

Finally, I wish to express gratitude to the members of the Plant Biology Department for their support and encouragement. I would like to acknowledge Mrs. Sue Vitello, Mrs. Carol Apperson, Mrs. Vicki Lemaster, and Ms. Christine Brownfield for their kind help.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>viii</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ix</td>
</tr>
</tbody>
</table>

## Chapter 1. Introduction: Calcium Stores, Calcium Binding Peptide, and Calcium Signaling

Summary ................................................................. 2

Introduction: Calcium as a Second Messenger .......................... 3

Methods of Measuring \([\text{Ca}^{2+}]_i\) ........................................ 4

Calcium Stores and Calcium Levels in Plants .......................... 10

Endoplasmic Reticulum ................................................. 17

Characterization of CRT ................................................ 19

CRT Regulation of Calcium Homeostasis and Binding in ER ............ 22

Plant CRT ....................................................................... 23

CIPK6 (Calcineurin B Like Protein Interacting Protein Kinase 6) ............ 29

References ........................................................................ 31

## Chapter 2. Transgenic Arabidopsis expressing an ER Targeted calcium binding peptide (CBP) show increased drought and salt tolerance

Abstract ......................................................................... 52

Introduction ..................................................................... 53

Results ............................................................................. 56

Expression of GFP-CBP ..................................................... 56
The GFP-CBP protein binds Ca$^{2+}$ .......................................................... 66

CBP Expression Increases Drought and Salt Tolerance ...................... 67

GFP-CBP and CBP increase CIPK6 Expression .................................. 75

CIPK6 is required for the salt tolerance of CBP transgenic plants ........ 76

Discussion .................................................................................................. 82

Material and Methods ............................................................................. 87

Plasmid Constructs .................................................................................. 87

Bombardment of Onion Epidermal Peels .............................................. 88

Plant Transformation and Selection .................................................. 88

Analysis of GFP Expression ................................................................. 90

Protein Extraction and Immunoblot Analysis .................................... 90

Assessment of Calcium Binding .......................................................... 91

Assessment of relative root growth on salt medium ......................... 91

Assessment of $CIPK6$ expression by semi-quantitative RT-PCR and
Real Time RT-PCR .................................................................................. 92

Combined cycling drought assay (CD) and yield analysis from BASF, the
Chemical Company ............................................................................... 93

Chlorophyll measurements ................................................................... 93

Calcium, Potassium and Sodium Measurements in CBP transgenic
Plants and control plants ...................................................................... 94

Acknowledgements .................................................................................. 94

References ............................................................................................... 95
Chapter 3. ER-CBP transgenic Plants maintain $\text{Ca}^{2+}$ signaling after a long term salt stress and $\text{Ca}^{2+}$ depletion

Abstract ............................................................................................................. 105

Introduction ........................................................................................................... 105

Results .................................................................................................................. 108

$[\text{Ca}^{2+}]_{\text{cyt}}$ measurements using confocal ratio analysis ......................... 108

ER-CBP transgenic plants do not exhibit difference in $[\text{Ca}^{2+}]_{\text{cyt}}$
comparied to wild type control Col-0 under a short term NaCl treatment .................. 111

ER-CBP transgenic plants exhibit a significant difference in $[\text{Ca}^{2+}]_{\text{cyt}}$
comparied to wild type control Col-0 under a long term salt stress ........... 111

$[\text{Ca}^{2+}]_{\text{cyt}}$ measurements using Aequorin transgenic plants ................. 112

ER-CBP transgenic plants (GFP-CBP and CBP), GFP, and
wild type plants show no significant difference in initial cytoplasmic calcium spikes ................................................................. 113

ER-CBP transgenic plants (GFP-CBP and CBP) exhibit
maintenance in initial rapid $\text{Ca}^{2+}$ spikes while control plants exhibit reduced initial $\text{Ca}^{2+}$ spikes after
grown in $\text{Ca}^{2+}$ depleted media .............................................................. 113

Discussion ......................................................................................................... 119

Materials and Methods ....................................................................................... 121

RNA isolation and semi-quantitative RT-PCR ............................................. 121

Indo-1 Dye Loading ........................................................................................ 122

Standard curves for ratio imaging ................................................................. 122

Indo-1 Confocal Ratio Imaging Measurement ........................................... 122
Chapter 4. ER-CBP transgenic plants exhibit changes in ion content and increased production of trehalose

Abstract ................................................................. 130
Introduction ............................................................. 131
Results ................................................................. 133

ER-CBP transgenic plants show reduced Na$^+$ accumulation under NaCl stress ........................................ 133
Increased expression of TPS and TPP in ER CBP transgenic plants ........ 135
Trehalose production is increased in ER-CBP transgenic plants .......... 136
Recovery of CBP transgenic plants from drought stress ................. 137
Discussion .............................................................. 137
Materials and Methods ............................................... 139
References ............................................................. 141
LIST OF TABLES

Chapter 2

Table 1. Transgenic lines, parents and plasmid constructs used to generate GFP-CBP and CBP transgenic plants ......................................................... 61

Appendix

Table 1. Confocal ratio analysis of $[\text{Ca}^{2+}]_{\text{cyt}}$ (nM) after 100 mM NaCl treatment for 30 seconds and 20 minutes ......................... 146

Table 2. Confocal ration analysis of $[\text{Ca}^{2+}]_{\text{cyt}}$ (nM) after 3 days in MS media only and MS media + 100 mM NaCl ....................... 147

Table 3. Summary of aequorin luminescence converted into $[\text{Ca}^{2+}]_{\text{cyt}}$ peak after 150 mM NaCl and 300 mM NaCl treatment ...................... 148

Table 4. Summary of aequorin luminescence converted into $[\text{Ca}^{2+}]_{\text{cyt}}$ peak to 150 mM NaCl and 300 mM NaCl treatment after 5 day Ca$^{2+}$ depletion ................................................................. 149
LIST OF FIGURES

Chapter 1

Figure 1. A schematic plant cell image .................................................. 10

Figure 2. The NMR structure of the rat calreticulin P-domain of CRT, residues from 189 to 288 (Ellgaard et al. 2001) ............................... 21

Chapter 2

Figure 1. Expression of different CBP constructs in Arabidopsis ...................... 59

Figure 2. Expression of the GFP-CBP fusion protein in different transformants .... 63

Figure 3. Increased seed production and higher chlorophyll content in CBP transgenic plants ................................................................. 65

Figure 4. Calcium-dependent electrophoretic mobility of ER:GFP-CBP ................. 67

Figure 5. Drought and salt tolerance is increased in ER-GFP-CBP transgenic plants ................................................................. 69

Figure 6. qPCR of drought associated genes in 5 day old GFP-CBP transgenic plant leave ................................................................. 72

Figure 7. GFP-CBP transgenic plants maintain root growth under osmotic stress ................................................................. 74

Figure 8. Salt tolerance of GFP-CBP transgenic plants .............................. 75

Figure 9. CIPK6 is specifically upregulated in transgenic plants ...................... 78

Figure 10. Expression of CIPK6 is required for the increased salt tolerance of CBP plants ................................................................. 80
Chapter 3

Figure 1. A representative image of a growth environment for Arabidopsis seedlings used in confocal ratio analysis ........................................ 109

Figure 2. Confocal ratio analysis of Indo-1 fluorescence ............................ 110

A. A representative image of confocal microscope fluorescence image of a Col-0 Arabidopsis root hair ................................................................. 110

B. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 30 seconds at 10 second intervals after 100 mM NaCl treatment ................................................................. 110

C. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 20 minutes at 5 minute intervals after 100 mM NaCl treatment ................................................................. 110

D. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 20 minutes at 5 minute intervals after growing them on MS media ................................................................. 112

E. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 20 minutes at 5 minute intervals after growing them on MS media + 100 mM NaCl ........................................ 112

Figure 3. Aequorin Luminescence Analysis .................................................. 114

A. Semi-quantitative verification of aequorin gene expression in transgenic plants ................................................................. 115

B. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement using Aequorin luminescence in CBP transgenic plants and control plants after 150 mM NaCl ......................... 115

C. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement using Aequorin luminescence in CBP transgenic plants and control plants after 300 mM NaCl ......................... 115

Figure 4. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement using Aequorin luminescence after 5 days of calcium depletion .................................................................117

A. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement using Aequorin luminescence in CBP transgenic plants and control plants after 150 mM NaCl .........................118
B. \([\text{Ca}^{2+}]_{\text{cyt}}\) measurement using Aequorin luminescence in CBP transgenic plants and control plants after 300 mM NaCl ............. 118

**Chapter 4**

Figure 1. ER-CBP transgenic seedlings show reduced accumulation of Na\(^+\) under NaCl stress ........................................................... 134

Figure 2. Semi-quantitative RT-PCR of trehalose 6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) ............................... 135

Figure 3. Trehalose measurement in wild type Col-0, CBP transgenic plants, cipk6 mutant and CBP X cipk6 mutant grown on normal MS media ......................................................... 136

Figure 4. Recovery of ER-CBP transgenic plants after an intermittent drought stress ................................................................. 137

**Appendix**

Figure 1. GFP fluorescence from NT1 cells transformed with 35S-ER-GFP-CBP construct ................................................................. 139
Chapter 1

Introduction: Calcium Stores, Calcium Binding Peptide and Calcium Signaling

Key Words: Calreticulin, calcium-binding peptide, calcium storage, endoplasmic reticulum, Arabidopsis, salt tolerance, drought tolerance

Abbreviations: Ca^{2+}, calcium; CBL, Calcineurin B Like; CRT, calreticulin; ER, endoplasmic reticulum; GFP, green fluorescent protein; HS, heat shock; BiP, immunoglobulin binding protein; ATP, adenosine triphosphate; EGTA, Ethylenebis(oxyethylene nitrilo) tetraacetic acid; CBP, calcium binding peptide; IP3, Inositol (1,4,5) trisphosphate; NT1 cells, Nicotiana tabacum suspension cells; WT, wild type
Summary

Calcium is an important plant nutrient that is necessary for the integrity of plant cell walls and membranes (Burstrom et al., 1968). Inside the cell, calcium acts as a cation to counter inorganic and organic anions in the vacuole to maintain ionic equilibrium, and as a secondary messenger in the cytoplasm to transmit information from numerous stimuli to their respective response pathways in plants (White et al., 2003). There are a multitude of calcium binding proteins that are either activated by calcium (such as calcium-dependent protein kinases, CDPK), store calcium (such as calreticulin, CRT), undergo a conformational change (calmodulin, CAM), or change in binding partner following an increase in calcium (Calcineurin B-Like, CBL) (Michalak et al., 1999; Luan et al., 2002). Calcium released into the cytosol by different stimuli generally constitutes a “signal” that could differ in amplitude, frequency and duration from that of other stimuli. Different stimuli generate specific calcium signals, which in turn generate the response. Calcium binding proteins such as CAM, CDPK and CBL are involved in sensing changes in cytosolic calcium content and decode these changes to a specific response by activating their targets such as protein kinases (Sanders et al. 2002, White et al. 2003).

The goal of this thesis is to ask whether increasing the intracellular calcium content in plants, by ectopic expression of a nonspecific calcium-binding protein localized in the endoplasmic reticulum (ER) could affect cytosol calcium signaling, and to determine if a particular class(es) of responses would be affected. In this introductory chapter, I will first briefly discuss the role of calcium as a second messenger, and the method of measuring
cellular calcium levels to detect calcium signaling will be reviewed. Second, the function of the ER and its role in calcium storage and in calcium signal generation will be analyzed. Third, different roles of calreticulin (CRT), the major calcium-binding protein in the ER, will be discussed. Lastly, drought and salt tolerance will be covered as examples of downstream responses that are affected in transgenic Arabidopsis plants expressing ER-CBP.

**Introduction: Calcium as a Second Messenger**

Sydney Ringer first identified one of calcium’s important regulatory roles in muscle contraction in 1882 (Ringer et al. 1882a, 1882b, 1883a, 1883b) but it took almost a century to understand that calcium acts primarily as a secondary messenger (Rasmussen et al. 1976, Prince et al. 1972, and Berridge et al. 1998). Calcium is a ubiquitous secondary messenger that transduces various cellular signals in animals, fungi and plants (Berridge et al. 2000, Sanders et al. 1999). Calcium is released from intracellular compartments upon reception of stimuli into the cytoplasm (Knight et al. 2000). Interestingly, this release of calcium exhibits certain patterns. In animal cells, it has been demonstrated that the amplitude, the duration, and the frequency of Ca$^{2+}$ signaling are important in inducing different responses (Li et al. 1998, Sanders et al. 1999). Variations in the amplitude and duration of Ca$^{2+}$ signaling in B lymphocytes, a type of white blood cell that functions in the immune system, have been correlated with differential activation of transcription factors (Dolmetsch et al. 1997). Moreover, the Ca$^{2+}$ oscillation frequency in the cytosol controls the specific pattern of transcriptional activation (Dolmetsch et al. 1998), the activation of Ca$^{2+}$- dependent enzymes (De Koninck et al. 1998) and neuronal migration direction and speed (Gomez et al. 1999).
In plants, it has been demonstrated that the amplitude of Ca$^{2+}$ signaling controls cytoplasmic streaming (Yamaguchi et al. 1981 and Takagi et al. 1983). For example, increasing [Ca$^{2+}$]i concentration of Vallisneria mesophyll cells to 1.0 μM decreased cytoplasmic streaming by 20% and [Ca$^{2+}$]i at 10 μM inhibited cytoplasmic streaming more than 80% (Williamson et al. 1975 and Tazawa et al. 1976). Cytosolic calcium signaling in plants has been linked to closure of the stomatal pore. Both the length of time between Ca$^{2+}$ oscillations and the duration of the oscillations were important in inducing stomatal closure in Arabidopsis (Allen et al., 2000 and 2001). Recently, it has been shown that dampened Ca$^{2+}$ signal amplitudes, in response to abiotic stress such as cold and salt in transgenic plants expressing type-1 inositol-5-phosphatase, correlated with increased transcription of the drought-associated transcription factor DREB2a (Perera et al. 2008). This is the first evidence suggesting that altering the amplitude of Ca$^{2+}$ signaling can result in differential activation of transcription factors in plants.

**Methods of Measuring [Ca$^{2+}$]i**

Measuring transient Ca$^{2+}$ increases in plant cells is not a trivial task. To study Ca$^{2+}$ signaling in both animals and plants it is important to accurately measure [Ca$^{2+}$]i of both whole cells and tissues, and of various cellular compartments. Ridgeway and Ashley performed the first reliable measurements of [Ca$^{2+}$]i using the photoprotein aequorin by injecting it into the giant muscle fiber of the barnacle (Ridgeway et al. 1967). In the 1980s, Roger Tsien and his colleagues produced a variety of chemical fluorescent dyes
(Gryniewicz et al. 1985, Minta et al. 1989, Takahashi et al. 1999) that reliably measured calcium concentrations. In plants, Ca$^{2+}$ sensitive microelectrodes, fluorescent dyes, the bioluminescent photoprotein aequorin, and the calcium ion probe cameleon have been adapted to measure intracellular calcium dynamics (Read et al. 1993, Allen et al. 1999).

I. Ca$^{2+}$-sensitive microelectrodes

This method inserts two microelectrodes, the Ca$^{2+}$ sensitive microelectrode and a reference electrode, or a double-barreled microelectrode into the cytoplasm of a single cell (Felle et al. 1989). The range of concentration of free Ca$^{2+}$ that this method can easily measure is between 10 nM and 1 mM. Spatial resolution is at the single-cell level, localized to one cellular region. The response time to changes in calcium concentration is slow (a few seconds) compared to fluorescent dyes and the recombinant aequorin method. Microelectrodes can be good for the direct measurement of cytosolic Ca$^{2+}$ concentration (Read et al. 1993) because they don’t require a light emitting probe and photo bleaching, dye cytotoxicity and radiation damage can be avoided. However, the procedure requires microinjection, which can damage the cell.

II. The Fluorescent dye

Chemical fluorescent probes (or dyes) are the Ca$^{2+}$ indicators most widely used because their signal to noise ratio is low compared to bioluminescent indicators such as aequorin (Takahashi et al. 1999). There are many fluorescent Ca$^{2+}$ dyes with different features such as excitation and emission spectra, quantum yields, affinities for free calcium, and response times (Read et al. 1993). It is essential to examine each feature of a fluorescent
dye before using it for biological applications.

The concentration of free calcium that can be easily measured by chemical fluorescent dyes is between 50 nM and 5 μM. The quantum yield of light-emitting probes is high and good spatial resolution can be achieved at subcellular levels. The response time to changes in calcium concentration is less than 100 msec (Read et al. 1993), which is much better than the response time for microelectrodes. As is the case with other methods, there are some disadvantages to using fluorescent dyes. Compared to the targetable photo-protein aequorin and GFP-based probes, fluorescent dyes are hard to target to subcellular compartments, except for rhod-2 which is largely retained within the mitochondrial matrix (Minta et al. 1989). Fluorescent dyes can cause intracellular buffering of calcium, depending on the affinity of the dye, and can be cytotoxic at high concentrations. Other common problems are the compartmentalization of fluorescent dyes to organelles, dye leakage, and non-specific binding to other ions and proteins.

Fluorescent dyes can be divided into two groups: single wavelength fluorescent dyes for non-ratiometric methods, and dual wavelength dyes for ratiometric methods. Dual wavelength fluorescent dyes, such as Fura-2 and Indo-1, exhibit shifts in their excitation or emission spectra upon binding free Ca²⁺. Ratiometric methods use these dual wavelength dyes and one can then calculate calcium concentration from the ratio between two fluorescence intensities. The advantage of ratiometric methods is that it can correct artifacts due to bleaching, changes in focus, and variations in laser intensity. However, acquisition and data manipulation is more complicated due to the use of fluorescence ratios. Only
specific microscopes are suitable for these measurements because the method requires changing the excitation and emission wavelengths at suitable rates. If the investigator is trying to measure a Ca\textsuperscript{2+} peak, the microscope must be capable of very rapid shifts in wavelengths and rapid recording methods. Moreover, many ratiometric indicators (Fura-2, Indo-1, and their low- affinity derivates) require the use of UV excitation, which may cause radiation damage and high background fluorescence. In addition, the microscope must be able to transmit UV and a UV source must be available. Non-ratiometric methods use dyes with a single wavelength. It is easier to accomplish data processing and calculation with these methods than with ratiometric methods. Single wavelength dyes cause less phototoxicity because they are excited mostly by visible wavelength. In addition, single wavelength dyes usually have better fluorescence response to calcium than dual ratiometric dyes. This suggests that a smaller concentration of dye can be used, which results in less cytotoxicity. However, fluorescence signals can be affected by factors other than calcium concentration. Cytosol thickness, dye concentration, the distribution of dye, dye photo-bleaching, optical photo length, pH, temperature, and acquisition conditions all affect the observed fluorescence intensities.

III. Bioluminescent Calcium Indicators

1.) Photoproteins

There are several Ca\textsuperscript{2+}-binding photo-proteins such aequorin, sobelin, mitrocomin, and clytin (Tsuji et al. 1995). The recombinant protein aequorin, derived from jellyfish, has
been widely used to measure [Ca\(^{2+}\)]\text{c}, in plants over other bioluminescent calcium indicators. Aequorin has been modified to measure the free Ca\(^{2+}\) in cell organelles by using targeting sequences to direct it to a specific subcellular compartment (Rizzuto et al. 1994). Aequorin has two components - a photo-protein apoaequorin, and a luminophore coelenterazine. It emits visible blue light at \(\sim 460\) nm by an intra-molecular reaction in the presence of Ca\(^{2+}\). The required instrumentation for photon measurements is relatively simple and photo bleaching, a problem with dyes, does not occur. The most common problems with these probes have been the methods required for loading the bioluminescent [Ca\(^{2+}\)]\text{c} indicator probes such as aequorin, and the detection and calibration of the bioluminescence. However, the problem of loading the aequorin can be partially overcome by transforming cells to express organelle-targeted aequorin (Gilory et al. 1993).

The number of photons emitted from a single cell (with the exception of large cells) can be low because each aequorin molecule emits only one photon when it binds to Ca\(^{2+}\). Because sufficient signal accumulation is necessary to rise above background noise, this method shows limited spatial resolution. However, this method can be very useful to monitor Ca\(^{2+}\) homeostasis and signaling at the whole-tissue level in intact plants because of its rapid response time.

2.) **Green Fluorescent Protein-Based Ca\(^{2+}\) Indicators**

Green fluorescent protein (GFP) is produced by the jellyfish *Aequorea*. GFP absorbs the blue luminescent emission of aequorin and emits green fluorescence. There are three
main types of GFP-based Ca\textsuperscript{2+} probes: the cameleon, the camgarross and the pericams (Miyawaki et al. 1997, Baird et al. 1999, Nagai et al. 2001). Cameleon consists of two different-colored mutants of GFP at the ends of the molecule, with calmodulin and the calmodulin target peptide M13 in the middle. The conformation of cameleon changes when calcium binds to this hybrid protein. This results in a decrease in the distance between the two GFP mutants and an increase in fluorescence resonance energy transfer (Miyawaki et al. 1997). Camgaroo probes consist of YFP and CaM. The conformational change in CaM occurs upon calcium binding and results in an increase in the fluorescence of YFP (Baird et al. 1999). Pericam probes consist of circularly permuted YFP, calmodulin and the CaM binding peptide M13. The presence of Ca\textsuperscript{2+} induces an interaction between CaM and the M13, which causes changes in YFP fluorescence (Nagai et al. 2001). GFP-based probes can be used in ratiometric measurement and, unlike chemical dyes, Ca\textsuperscript{2+} dependent fluorescence is reversible. Because of the relatively high fluorescence compared to that obtained with aequorin, GFP probes have better time resolution and can be measured by a simplified photon detection system. Unlike chemical fluorescent dyes, the sensitivity of Ca\textsuperscript{2+} binding is independent of the concentration of the GFP-based probes. Like aequorin, GFP-based probes can be targeted to specific intracellular compartments.

On the other hand, GFP-based indicators have a small dynamic range of fluorescence intensity and the signal intensity upon calcium binding is relatively low compared to chemical fluorescent dyes. GFP fluorescence is also pH sensitive (Miesenbock et al. 1998) and this can be a problem that must be controlled.
Calcium Stores and Calcium Levels in Plants

**Fig 1. A schematic plant cell image.** A plant cell has a large vacuole, chloroplasts and cell wall compared to an animal cell (http://www.biologycorner.com, the plant cell image was created by Shannan Muskopf).
- **Cell Wall**

Plant cells have several places that can store calcium, one of which is the cell wall. Ca\(^{2+}\) plays a crucial role in determining the structural rigidity of the cell wall (Burstrom et al. 1968). Calcium binding sites are mainly located on the extensive network of pectic polymers and the Ca\(^{2+}\) concentration in the cell wall is in the mM range (Trewavas et al. 1999).

- **Aploplast**

There is a big Ca\(^{2+}\) concentration difference across the plasma membrane in a plant cell. The apoplastic Ca\(^{2+}\) levels for land plants usually range from 100 μM to 1 mM, which is much higher than their cytosolic Ca\(^{2+}\) concentrations of 100 nM (Bush et al. 1995, Bjorkman et al. 1991 and Harker et al. 1991).

- **Cytosol**

The cytosol is the unstructured aqueous phase of the cytoplasm without organelles, membranes, and insoluble cytoskeletal components (Lodish et al. 1999). Ca\(^{2+}\) concentrations must be maintained within the cytosol in the 100 - 600 nM range because Ca\(^{2+}\) precipitates phosphate, which is an important component of ATP (adenosine triphosphate) (Sze et al. 2000, Buchanan et al. 2001). The cytosolic Ca\(^{2+}\) concentration in plant cells measured using various techniques is around 30 to 200 nM except during a Ca\(^{2+}\) signaling event (Bush et al. 1993). In plants, [Ca\(^{2+}\)]\(_{\text{cyt}}\) increases of up to 3 μM have been reported in response to various stimuli (Lecourieux et al. 2002).

The cytosolic calcium concentration of mung bean root protoplasts was measured to be 171 nM (Gilroy et al. 1986). The cytosolic calcium concentration of a root hair cell is
about 100-200 nM in *Arabidopsis thaliana* (Gilroy et al. 1993 and Wymer et al. 1997). There is a calcium gradient in growing root hair cells of *Sinapis alba* (Felle et al. 1997). Two different methods, microelectrodes and fluorescent dyes, showed that the cytosolic Ca$^{2+}$ concentration ranged from 158 to 251 nM for the basal area of *Sinapis alba* root hair cells while in the tip, elevated Ca$^{2+}$ concentrations of 446 to 707 nM were observed. Ca$^{2+}$ flux studies suggest that a Ca$^{2+}$ influx at the tip of root hair cells is necessary for generating the Ca$^{2+}$ gradient (Schiefelbein et al. 1992, Felle et al. 1997).

- **Mitochondria**

  The concentration of Ca$^{2+}$ varies among the plant cell organelles. The mitochondria and chloroplast have mM levels of total Ca$^{2+}$ and they are capable of generating their own calcium signals (Bush et al. 1995, Xiong et al. 2006). Mitochondria from *Arabidopsis thaliana* transformants that expressed mitochondrial-targeted aequorin showed free Ca$^{2+}$ measurements of around 200 nM (Logan et al. 2003). It was first shown in maize that plant mitochondria actively participate in the uptake of Ca$^{2+}$ and that energy is needed for this process (Hodges et al. 1965). Chen et al. showed that mitochondria isolated from 14 different plants and fungi are also able to absorb calcium in the presence of respiratory substrates (Chen et al. 1973). Studies from oat mitochondria suggested that the plant photoreceptor, phytochrome, regulates Ca$^{2+}$ flux in a photo-reversible manner (Roux et al. 1981). Roux et al. showed that red light irradiation reduced net Ca$^{2+}$ uptake in oat mitochondria, a phenomenon that was reversed following far-red irradiation. In the presence of ruthenium red, an inhibitor of active Ca$^{2+}$ uptake, red light caused Ca$^{2+}$ to be released through a
passive efflux mechanism. These results show that light regulates Ca\textsuperscript{2+} uptake by plant mitochondria and suggest that diurnal rhythms may impact mitochondrial function.

It has been demonstrated that not only is Ca\textsuperscript{2+} sequestered in plant mitochondria, it also controls NADH oxidation (Møller et al. 1981). Furthermore, mitochondria respond to relatively small changes in cytosolic calcium levels (Rizzuto et al., 1994, Carafoli et al., 2001). The relative contribution of ER calcium to mitochondrial function is not understood, but as much as 20\% of the mitochondrial envelope surface is in direct contact with the ER of HeLa cells (Rizzuto et al. 1998). This association implies that dynamic and highly regulated communication could occur between the ER and mitochondria.

- **Chloroplast**

  The chloroplast has a resting free calcium concentration of 150 nM, as measured in transgenic tobacco expressing chloroplast-targeted aequorin (Johnson et al. 1995). Chloroplasts from wheat (Muto et al. 1982) and spinach (Kreimer et al. 1985a and 1985b) exhibit a dramatic, light-dependent uptake of Ca\textsuperscript{2+}; a process that depends on photosynthetic electron transport (Miller et al. 1987). Miller and Sanders demonstrated that cytosolic Ca\textsuperscript{2+} levels were lower when plants were illuminated with strong light, suggesting that Ca\textsuperscript{2+} fluxes across the chloroplast envelope contribute to the regulation of cytosolic Ca\textsuperscript{2+}.

  Calcium plays important roles within the chloroplastidic stroma. Fru-1,6-bisphosphatase, a Calvin cycle enzyme, is activated by low concentrations and inhibited by high concentrations of Ca\textsuperscript{2+} in the stroma (Hertig et al. 1983, Kreimer et al. 1988). Ca\textsuperscript{2+} activates NAD kinase in the stroma, which is required to convert NAD to NADP (Muto et al.
1981, Jarrett et al. 1982). High Ca$^{2+}$ concentrations in the stroma have been linked to the inhibition of CO$_2$ fixation (Portis et al. 1976, Demmig et al. 1979).

- Nucleus

There does not seem to be much difference between the nucleus and cytosol in free Ca$^{2+}$ concentration (Gilroy et al. 1991). However, this assumes that calcium is uniformly distributed throughout the nucleus. Localized areas with increased or decreased calcium levels may persist, for example, in subdomains such as the nuclear speckles, which contain splicing factors. Nuclear speckles were first identified in mammalian cells (Lamond et al. 2003) and later in plant cells (Lorkovic et al. 2004).

The free calcium concentration of some cellular compartments such as chloroplast, mitochondria, and nuclei are similar to the free calcium concentration of cytosol. However, it has been suggested that calcium is regulated differently in distinct compartments of the plant cell and that the calcium oscillation patterns in response to a specific stimulus are different between the cytosol and other cellular compartments (Gilroy et al. 1991, Johnson et al. 1995, Logan et al. 2003, Xiong et al. 2006). Nuclei extracted from tobacco cells show a transient increase in [Ca$^{2+}$]$_{\text{nuc}}$ in response to mastoparan (Pauly et al. 2000), temperature changes, and mechanical stimulation (Xiong et al. 2004). Incubation of nuclei in a medium containing high concentrations of Ca$^{2+}$ has no effect on nucleoplasmic calcium levels in the absence of stimulation, which rules out the possibility of passive diffusion across the nuclear envelope. Furthermore, chelating external calcium by adding 5 mM EGTA had no effect on the intensity or kinetics of the [Ca$^{2+}$]$_{\text{nuc}}$ elicited by mechanical or thermal stimuli (Xiong et al.
2004) This suggests that nuclei can generate calcium signals that are independent from those of the cytosol and that the source of \( \text{Ca}^{2+} \) for transient \( [\text{Ca}^{2+}]_{\text{nuc}} \) signals may be the lumen of the nuclear membrane, which is contiguous with the ER.

- **Vacuole**

The vacuole is considered to be the main storage area of intracellular \( \text{Ca}^{2+} \) because of its large volume and the key role that it plays in the ionic homeostasis of the plant cell. The concentration of free \( \text{Ca}^{2+} \) in the vacuole is about 1 mM, which is 5-10 fold higher than the \( \text{Ca}^{2+} \) concentration of the cytoplasm (Felle et al. 1988). There are several \( \text{Ca}^{2+} \) transporters and \( \text{Ca}^{2+} \) release channels in the vacuolar membrane, confirming its role as a major stimulus-reservoir of \( \text{Ca}^{2+} \) for signaling (Sanders et al. 1999). \( \text{Ca}^{2+} \) buffering may be conferred by organic and inorganic ions in the vacuole. Because of the low pH (pH 3-6), proteins such as CRT would not be functional. A low-affinity, high-capacity \( \text{Ca}^{2+} \) binding protein was recently characterized in the radish vacuole that does not show sequence homology with CRT (Yuasa et al. 2000).

Transport of \( \text{Ca}^{2+} \) from the cytoplasm to intracellular organelles against an electrochemical gradient requires energy. \( \text{Ca}^{2+} \)-ATPases (ACA4) and \( \text{H}^+/\text{Ca}^{2+} \)-antiporters encoded by the CAX genes are present in the tonoplast (Axelsen et al. 2001, Hirschi et al. 2001). Over-expressing Arabidopsis CAX proteins increases total \( \text{Ca}^{2+} \) and confers new phenotypes (Hirschi et al. 1999, Park et al. 2004, 2005). Over-expression of the Arabidopsis \( CAX1 \) in tomato resulted in symptoms that resembled calcium deficiency, despite increased total \( \text{Ca}^{2+} \) content in the plant (Hirschi et al. 1999, Park et al. 2005), suggesting that the
increased calcium was confined to the vacuole and was not bio-available. However, over-expressing another family member, CAX4, increased the Ca$^{2+}$ content in tomatoes and prolonged shelf life without causing calcium deficiency.

- **Golgi apparatus (GA)**

  Golgi apparatus are stacks of membranous structures that function in the processing and sorting of proteins and lipids destined for other cellular compartments or for secretion in eukaryotic cells (Lodish et al. 1999). In animal cells, the Golgi apparatus sequesters or releases calcium when cytoplasmic Ca$^{2+}$ increases (Connor 1993, Zha et al. 1995). La$^{3+}$ releases Ca$^{2+}$ from the Golgi apparatus in LLC-PK$_1$ cells, measured via ion microscopy (Zha et al. 1995). The concentration of Ca$^{2+}$ in the Golgi apparatus in LLC-PK$_1$ cells is about 1 mM. Plant cells have several Golgi bodies while animal cells have only one. Active Ca$^{2+}$ accumulation in the plant cell Golgi apparatus was first demonstrated in the elongating zone of etiolated pea (Ordenes et al. 2002). Thapsigargin-sensitive calcium uptake was also detected in a cauliflower (Brassica oleracea) Golgi-enriched fraction, suggesting that other plants may also possess thapsigargin-sensitive Golgi calcium pumps (Ordenes et al. 2002). We do not know how much free calcium is present in plant Golgi stacks at this time.

- **Endoplasmic reticulum**

  The ER has also been recognized as an intracellular Ca$^{2+}$ store that plays a potentially important role in Ca$^{2+}$ signaling in plants. The lumen, or interior of the ER, is distinct from the cytoplasm and is known to contain higher levels of free Ca$^{2+}$; around 400 μM in HeLa cells (Chami et al. 2001) and about 3 - 4 μM in aleurone cells (Bush et al. 1989).
Endoplasmic Reticulum

The endoplasmic reticulum (ER) was first discovered in mammalian cells using transmission electron microscopy (TEM) by Keith R. Porter (Satir et al. 1997, Ostwald et al. 1974). By using osmium tetroxide, which stains lipids, to fix mammalian cells, Porter was able to describe the existence of an extensive network of membranes contiguous with the outer membrane of the nuclear envelope and spreading throughout the cytosol of the cell. The ER appeared to be a double-membrane structure with a consistent, small distance between the membranes, now known as the ER lumen. The use of more modern methods, such as fluorescence microscopy (Mathur et al. 2007) and 3-D reconstruction (Donohoe et al. 2006), showed that the ER is a single organelle that forms a reticulated network extending from the outer nuclear membrane to the plasmalemma and through plasmodesmata. Although the ER appears to be similar in both plant and animal cells, more research has used animal cells to understand ER function.

A TEM image first showed that more than one form of ER could be distinguished in cells. ER membranes with ribosomes on the cytosolic surface, which are needed for protein synthesis, are called “rough ER” while smooth ER was similar in structure but lacked ribosomes. Later studies showed that microtubules play an important role in the organization of the ER (Gupton et al. 2006) and that there are sub-domains with heterogeneous distributions of Ca$^{2+}$ binding proteins, Ca$^{2+}$ pumps and release channels within the ER (Papp et al. 2003). This heterogeneity may enable it to perform various complex functions (Papp et al. 2003). The ER can also show rapid increases in size in response to various stimuli.
(Rajasekaran et al. 1995), demonstrating the dynamic nature of this organelle.

- **Function of the ER**

  The ER is important for protein synthesis, protein sorting, protein folding, post-translational modification, lipid synthesis, calcium storage, and calcium release (Bauman et al. 2001, Arnaudeau et al. 2002). In animals, steroid synthesis and the storage and production of glycogen also occur in ER (Michalak et al. 1999). Plants are known to modulate the endoplasmic reticulum to cope with external stresses (Staehelin et al. 1997). One of the differences that separate the plant ER from that of other eukaryotes is its ability to form protein, oil, or rubber-containing subcellular structures known as ER bodies (Matsushima et al. 2003). ER bodies compartmentalize the synthesis of specific proteins and glycoproteins (Chrispeel et al. 2000). Moreover, ER bodies store lipids, oil, waxes, rubber, sterols, and isoprenoids, which are controlled by development, stress, and environmental conditions in plants (Herman et al. 2004).

- **Calreticulin**

  CRT was first discovered as a high affinity calcium binding protein in the mammalian sarcoplasmic reticulum (SR) (Ostwald et al. 1974). SR is the term used in muscle for the smooth ER and it was from studies of the SR that the importance of calcium uptake and release first became apparent. CRT was one of the five water-soluble, acidic proteins isolated from the SR. Among these five proteins, CRT is the only protein with high affinity for Ca$^{2+}$. CRT was subsequently shown to be a highly conserved protein that is found in almost all eukaryotic cell types except for yeast, which lacks a well-developed ER (Kraus et al. 1997,
Li et al. 2000). In plants, CRT was first purified and sequenced in tobacco (Denecke et al. 1993) and spinach (Menegazzi et al. 1993).

Since its discovery, CRT has been associated with numerous cellular functions in cell biology. In the ER, CRT is important for protein folding and Ca\(^{2+}\) homeostasis. In animals, CRT has also been suggested to play a role in apoptosis (Groenendyk et al. 2005). CRT on the surface of animal cells has been shown to be involved with the membrane-associated protein integrin, and with integrin-mediated calcium signaling and cell adhesion (Coppolino et al. 1997). Extracellular CRT has also been found in patches on the surface of cultured plant cells using TEM with immunogold labeling (Borisjuk et al. 1998).

**Characterization of CRT**

CRT is a multifunctional protein that consists of three domains that show sequence conservation across the plant and animal kingdoms. The domains are always found together, even though their function can be separated biochemically. The first domain of CRT is the N-domain, which is globular and highly conserved. The N-domain is followed by the P-domain. The P-domain is the central region of the protein and is rich in proline. The P-domain binds Ca\(^{2+}\) with high affinity and low capacity (Baksh et al. 1991 and Michalak et al. 1999). The last domain of CRT is the C-domain. It is the acidic, carboxy-terminal region of CRT. The C-domain binds Ca\(^{2+}\) with low affinity and high capacity and plays a role in Ca\(^{2+}\) storage in the ER (Arnaudeau et al. 2002, Nakamura et al. 2001).
1. N-Domain

The N-domain of the CRT proteins starts with an ER-targeting signal sequence that is necessary for translation on rough ER and secretion into the lumen. The N-domain consists of about 180 amino acids, depending on the organism. It is predicted that the N-domain forms a globular β-sheet structure based on the known 3-D structure of calnexin (CXN), a protein closely related to CRT (Schrag et al. 2001, and Camacho et al. 1995). The N-domain is highly conserved among all CRT genes and is more conserved than the P- and C-domains. Zn$^{2+}$ is known to bind to the N-domain of CRT in animal cells (Michalak et al. 1992), although the exact binding site(s) of Zn$^{2+}$ have not been identified. There is a region containing five histidine residues in the N-domain that could be involved in binding, based on research with other zinc-binding proteins (Baksh et al. 1995).

2. P-Domain

The P-domain is a proline-rich segment consisting of residues from amino acids 181 to 290 in the human CRT protein. It contains tandem repeat segments. One such repeat is P-x-x-I-x-D-P-D-A-x-K-P-E-D-W-D-E and the second repeat is G-x-W-x-P-P-x-I-N-P-x-Y-x and they are repeated 3 times, respectively, in mouse and human CRTs. The two repeats for plant CRTs are slightly different with the consensus repeating sequence being PXXIXDPXXKKPEXWDD and GXWXAXXIXNPXYK in Arabidopsis thaliana. These repeats are suggested to play a role in high affinity Ca$^{2+}$ binding. However, studies of the NMR structure of the rat CRT P-domain (Figure 2) have not revealed Ca$^{2+}$ binding sites (Ellgaard et al. 2001). The P-domain has a nuclear localization signal, P-P-K-K-I-K-D-P-D
in human *Crt1* and P-A-K-K-I-K-D-P in *Arabidopsis thaliana Crt1*. CRT has been suggested to play a role in nuclear export of the glucocorticoid receptor (Holaska *et al.* 2001), providing additional evidence that the nuclear localization signal may be functional.

**Figure 2. The NMR structure of the rat calreticulin P-domain of CRT, residues from 189 to 288 (Ellgaard *et al.* 2001).** It shows a hairpin fold that involves the entire polypeptide chain. It has the two chain ends in close spatial proximity, and does not fold back on itself. This globally extended structure is stabilized by three anti-parallel beta-sheets, with the beta-strands comprising the residues 189-192 and 276-279, 206-209 and 262-265, and 223-226 and 248-251.
3. C-Domain

The C-terminal domain of the human CRT protein consists of amino acid residues 291-400. This domain is not as conserved as other domains but the acidic nature of the amino acid residues is conserved. Thirty seven of the last 57 residues of the C-domain are acidic, consisting of aspartic and glutamic acid, which have the ability to bind to positively charged ions such as Ca$^{2+}$. Basic residues, especially those with lysine side chains, are interspersed with acidic residues in the last region of the C-domain. These are suggested to be important for the calcium storage function and may affect the structure for calcium binding (Breier et al. 1994). Ectopic expression of the C-domain of CRT has been reported to compensate for the loss of calcium storage capacity in CRT-deficient animal cells (Nakamura et al. 2000) demonstrating that this portion of the protein is functional. The C-domain of animal CRTs has an ER retrieval amino acid sequence KDEL (Lys-Asp-Glu-Leu)-COOH, which is one of the signals necessary for ER luminal retention of CRT (Pelham et al. 1989). In plants the sequence for ER luminal retention is HDEL and histidine replaces lysine.

**CRT Regulation of Calcium Homeostasis and Binding in ER**

In animals, it has been suggested that the increased sensitivity to apoptosis seen in CRT-overexpressing cells is the result of the modulation of Ca$^{2+}$ homeostasis by CRT (Nakamura et al. 2000). The function of the Ca$^{2+}$ release channel of the ER, the inositol triphosphate receptor (IP$_3$R), has been shown to be influenced by CRT. Overexpression of CRT results in suppression of inositol 1,4,5-triphosphate-induced Ca$^{2+}$ oscillations by inhibiting Ca$^{2+}$ uptake into the endoplasmic reticulum (John et al. 1998). SERCA is a
Ca\(^{2+}\) - ATPase that transfers Ca\(^{2+}\) from the cytosol of the cell to the lumen of the sarcoplasmic reticulum using ATP hydrolysis during muscle relaxation. The suggested mechanism is that CRT interacts with the COOH terminus of SERCA2b and glycosylates asparagine N1036 (John et al. 1998). The glycosylation of the COOH terminus of SERCA2b results in inhibition of Ca\(^{2+}\) uptake into the ER. This results in an increase in cytosolic calcium content and an inhibition of inositol 1,4,5-triphosphate-induced Ca\(^{2+}\) oscillations in order to prevent a further increase in the cytoplasmic Ca\(^{2+}\) content. CRT has been shown to co-localize with Ca\(^{2+}\) release channels such as SERCA and IP\(_3\)Rs in animal cells (John et al., 1998) suggesting that CRT in animal cells carefully maintains Ca\(^{2+}\) homeostasis.

**Plant CRT**

In plant cells, the endoplasmic reticulum provides a specialized environment promoting the folding and oligomeric assembly of proteins. The plant endoplasmic reticulum is equipped with several molecular chaperones and folding sensors largely similar to those in all eukaryotes. The endoplasmic reticulum is important for newly synthesized and properly folded proteins that go through the subsequent steps of the secretory pathways (Vitale et al. 1999). One of the conserved ER resident proteins in plants is CRT. CRT binds calcium, functions as a chaperone during protein folding, and is localized to plasmodesmata (Chen et al. 2005). Plasmodesmata are intercellular channels that contain plasma membrane and ER that are continuous with both cells. CRT ensures that newly synthesized proteins are properly folded and glycosylated before they traffic to the Golgi bodies for further modification. An important difference between animal and plant CRT is that only plant CRT
is glycosylated (Navazio et al. 1995, Pagny et al. 2000). The significance of the glycosylation is unclear. The true functions of CRT in plants are just now beginning to be understood and it is likely that CRT has other functions that are different from those of animals.

There are two different groups of CRTs in plants, CRT1 and CRT2, and CRT3 (Persson et al. 2003) that can be distinguished by their response to tunicamycin (Person et al. 2007), an inhibitor of glycosylation. CRT1 and CRT2 are up regulated in tunicamycin-treated tissues, which show a response characterized as the “unfolded protein response” (Fontes et al., 1991, Koizumi et al., 2001). These CRTs bind to Binding Protein (BiP), which is also induced by tunicamycin (Crofts et al. 1998). CRT3 does not participate in the unfolded response but does interact with the tobacco mosaic virus movement protein (Chen et al. 2005), which localizes to plasmodesmata (Baluska et al. 1999, Laporte et al. 2003). CRT3 is up regulated by viruses and may therefore play a role in viral cell-to-cell movement. Interestingly, over-expression of CRT3 disrupted TMV movement (Chen et al. 2005). T-DNA knockout mutants have been identified in all three Arabidopsis CRT genes (Christensen et al. 2008). Mutations in crt1 and crt2 showed that a lack of CRT renders plants sensitive to tunicamycin (Christensen et al. 2008). The crt2 mutant also showed stunted growth in Arabidopsis (Persson et al. unpublished data).

- CRT and Drought Tolerance

It has been reported that over-expression of TaCRT (Triticum aestivum L.; wheat) can modulate plant response to drought stress and is a potential strategy for transgenic...
improvement of plant water-stress (Jia et al. 2008). Subcellular localization of TaCRT in onion epidermal cells showed that it is localized in the cytoplasm and the nucleus which suggests that (i) TaCRT acts as a calcium buffer in the cytoplasm and the buffered Ca\textsuperscript{2+} homeostasis in the cytoplasm changes the gene expression pattern, or (ii) nuclear-localized CRT is either buffering Ca\textsuperscript{2+} or interacting with transcription factors to regulate gene expression. There is evidence in animal systems that CRT is involved in gene regulation (Burns et al. 1993, Dedhar 1994). However, it was suggested that the amino terminal portion of CRT, not the C terminal portion, is important in regulating gene expression in animal cells (Burns et al. 1994). It is possible that there are fundamental differences in CRT function between plant and animals cells. For example, it was not possible to generate transgenic lines in animal cells that express only the CRT C-domain (Camacho et al. 1995). Alternatively, CBP may act as a calcium buffer that changes a putative calcium signature in the nucleus.

- Function of CRT in the nucleus

It has been suggested that Ca\textsuperscript{2+} is responsible for the regulation of many nuclear events, thus regulation of nucleoplasmic Ca\textsuperscript{2+} is of high importance in animal cells (Badminton et al. 1998, Gerasimenko et al. 2004). The nuclear envelope lumen is continuous with the ER lumen (Figure 1). The nuclear envelope stores calcium and may regulate nuclear calcium (Badminton et al. 1998, Peterson et al. 1998). CRT localizes to the nuclear envelope in several animal cell types and to the nucleus in L6 cells (Holaska et al. 2001; Burns et al. 1993, Opas et al. 1991). CRT has been shown to interact with the rat ER membrane and nuclear proteins via the C-domain of CRT (Burns et al. 1993). As previously mentioned, the
wheat CRT localizes to both the cytoplasm and nucleus (Jia et al. 2008). It is possible that the C-domain of CRT of plants, like animals, also interacts with the nuclear envelope or nuclear proteins, but this has not been demonstrated.

- **The Role of Calreticulin in Ca$^{2+}$ Signaling in Plants**

  The interrelationships between CRT and Ca$^{2+}$ in the ER have been thoroughly investigated in animal cells. It is a widely accepted fact that CRT is involved in more than one part of a complex sensing and signaling network. CRT has a lectin-like chaperone activity, interacts with other ER chaperones, regulates [Ca$^{2+}$]$_{ER}$ and participates in the ER signaling network (Corbett et al. 2000). However, very little is known about the involvement of CRT in these roles in plant cells.

  Unlike animal cells, plant cells have a large vacuole that contains calcium and is responsible for maintaining turgor pressure in the plant cell through modulation of ion and solute concentrations (Apse et al. 2003). At least some of the roles for calcium storage, release, and signaling could theoretically be fulfilled by the vacuole instead of the ER. However, the conservation of the Ca$^{2+}$ binding properties of plant CRT suggests that it plays a role in the maintenance of intracellular Ca$^{2+}$ homeostasis, as in animal cells. Evidence for this comes from over-expression studies of the maize CRT in tobacco suspension cells, which increases the endoplasmic reticulum Ca$^{2+}$ pool (Persson et al. 2001). Ectopic expression of CRT also resulted in increased ATP-dependent Ca$^{2+}$ accumulation in isolated microsomes, and in a higher Ca$^{2+}$ retention after ionophore treatment (Persson et al. 2001). Overexpression of CRT improved growth of tobacco cell suspensions in medium containing
high Ca\(^{2+}\) (Akesson \textit{et al.} 2005). It is not known whether this protective effect was exerted via modulation of the activity of the ER Ca\(^{2+}\) ATPases and/or agonist-triggered Ca\(^{2+}\) channels, as shown in animal cells (Camacho \textit{et al.} 1995 and John \textit{et al.} 1998).

Expression of both maize CRT and an ER-targeted maize CRT C-domain gene under the control of a heat shock promoters helped Arabidopsis seedlings to survive on calcium-depleted media (Persson \textit{et al.} 2001 and Wyatt \textit{et al.} 2002). The seedlings were grown on normal medium during the heat shock treatment and then switched to media with no calcium but containing EGTA. Because the C-domain can bind a 30-50 fold molar excess of calcium, it was hypothesized that over expressing the CRT C-domain resulted in an increased Ca\(^{2+}\) buffering ability, which conferred survival value to the seedlings. These experiments demonstrated that higher ER calcium stores could have an impact on plant growth, suggesting that it contributed to signaling responses or to help restore homeostasis in areas where growth processes were limited by low levels of intracellular calcium.

In conclusion, it is widely accepted that CRT is conserved and expressed in plant cells. However, the roles of CRT in Ca\(^{2+}\) signaling in plants have not clearly been demonstrated.

- \textbf{Characteristics of GFP-CBP Expression}

The remainder of this thesis concentrates on the analysis of ectopic expression of the C-domain of the maize CTR1a gene in Arabidopsis. This domain is now referred to as the calcium-binding domain (CBD) to distinguish it from CRT. The original work used a heat shock promoter to drive the expression of an ER-targeted GFP-CBP fusion protein to avoid
potentially deleterious effects of altering cellular calcium levels. When the heat-shock version of GFP-CBP did well, we decided to test constitutive expression. Over 20 different 35S:GFP-CBP transgenic lines were created by Dr. Pei-Lan Tsou as part of her PhD thesis research. Although most of these lines were carried to the F2 and F3 generations to obtain homozygous lines (Tsou, 2001), there was not enough time to characterize individual lines for their response to stress. Western blots showed large variations in expression and the presence of breakdown products in some lines, but the impact of expression level and response to NaCl was not known.

In collaboration with Shantha Sumanasinghe and Dr. Nina Strömgren Allen, thirteen independent 35S:GFP-CBP lines were analyzed by microscopy for GFP expression. Figure 3 shows two lines that were selected for further use and a third line that initially showed high levels of expression but then became silenced. Jenny Bradfield and Dr. Niki Robertson characterized some of these lines on NaCl and showed that lines with high expression were more susceptible than wild type to NaCl, the opposite of what was expected (Figure 4). When I began to work on this project, two lines with low, but stable GFP expression were chosen for further investigation. I also constructed and selected two independent 35S-CBP lines lacking GFP to be used for measuring cytosolic calcium levels. The lines expressing GFP-CBP fusion proteins are referred to as ER-GFP-CBP1 and ER-GFP-CBP2 and lines without GFP as ER-CBP3 and ER-CBP4 (identical but lacking GFP).

I determined that 150 mM NaCl was the highest level that 35S ER-GFP-CBP seedlings could tolerate, in contrast to Tsou (2001), who showed that they survived on
200 mM NaCl. The line used by Tsou became silenced and was no longer available for testing. I also found that root growth was higher in ER-GFP-CBP plants on NaCl-containing media and that even on MS + 1% sucrose, ER-GFP-CBP roots continued to grow for a longer period of time than ER-GFP controls and wild type seedlings. I determined the molecular basis for drought tolerance in ER-GFP-CBP transgenic Arabidopsis, which is discussed in Chapter 2.

I wanted to determine whether ER-CBP alters the cytosolic calcium concentrations and/or induces altered calcium signaling in response to salt stress (final concentration of 150 mM and 300 mM) compared to control plants without ER-CBP. Altered calcium signaling in plant cells might explain how ectopic ER-CBP expression could generate different transcription levels of genes compared to controls. Using both indole-1 and 35S-aquorin_cyt plants, I measured the effect of salt treatments on cytosolic calcium. These results are discussed in Chapter 3.

**CIPK6 (Calcineurin B-like Interacting Protein Kinase 6)**

CIPK is a serine/threonine protein kinase in the SNF1/AMPK family that physically interacts with and is activated by CBLs. The CIPK gene family in Arabidopsis has 25 members that are differentially regulated (Luan *et al.* 2002, Batistic *et al.* 2004). Calcineurin B-Like proteins (CBL) have an EF hand calcium-binding site and function as a calcium sensor. They have sequence similarities with neuronal calcium sensors in animals and the B component of calcineurin in yeast (Shi *et al.* 1999).

We observed that CBP-transgenic plants with more total calcium express higher
levels of CIPK6 even in the absence of stress. Preliminary microarray results (Bradford, Robertson, Kimbrough and Sederoff) showed that CIPK6 was the only member of the 25-member CIPK family to be significantly affected in the CBP plants. Genevestigator showed that CIPK6 was induced by salt and drought, but not by cold. SOS2, later classified as CIPK24, is required for salt tolerance and Na$^+$ and K$^+$ homeostasis in plants (Zhu et al. 2000). CIPK24 is activated by the calcium sensor, SOS3, a CBL member (Zhu et al. 2000).

It has been reported that CIPK6, like CIPK24, interacts with SOS3 (Kim et al. 2000), the calcium sensor CAM1 (Ponsecu et al. 2007), and the potassium channel AKT1 (Lee et al. 2007). However, a relationship between CIPK6 and SOS3 in salt tolerance is not suggested because the Arabidopsis cipk6 mutant is not more salt sensitive than WT control plants (Chapter 2). In the subsequent chapters, I will discuss the effect of ER-CBP expression on the stress response, stress associated genes that show altered expression in the presence of ER-CBP, and the effect of induced expression of CIPK6 in CBP transgenic plants (Chapter 2). [Ca$^{2+}$]$_{cyt}$ measurement of salt stressed ER-CBP transgenic plants using confocal ratio analysis and aequorin$_{cyt}$ luminescence will be discussed in Chapter 3. In Chapter 4, the increased production of the compatible solute trehalose in ER-CBP transgenic plants will be discussed. Finally, the Appendix presents evidence for nuclear-localization of the GFP-CBP recombinant protein, which could explain induction of CIPK6.
References:


42


Ringer, S. (1882b) Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. J Physiol. 3:380-393.

Ringer, S. (1883a) A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. J Physiol. 4:29-42.

Ringer, S. (1883b) A third contribution regarding the influence of the inorganic constituents of the blood on the ventricular contraction. J Physiol 4:222-225.


46


Chapter 2

Transgenic Arabidopsis expressing an ER targeted calcium binding peptide (ER-CBP) show increased drought and salt tolerance

**Key Words:** Calreticulin, calcium-binding peptide, calcium storage, endoplasmic reticulum, Arabidopsis, salt tolerance, drought tolerance

Abbreviations: Ca$^{2+}$, calcium; CBL, Calcineurin B Like; CRT, calreticulin; ER, endoplasmic reticulum; GFP, green fluorescent protein; HS, heat shock; BiP, immunoglobulin binding protein; ATP, adenosine triphosphate; EGTA, Ethylenebis (oxyethylene nitrilo) tetraacetic acid; CBP, calcium binding peptide
Abstract

Calcium is an essential second messenger that generates a specific response to a stimulus. Calcium has to be tightly transported and stored to efficiently act as a second messenger. The endoplasmic reticulum is one of the main calcium storage compartments in plants. We tested whether increasing \( \text{Ca}^{2+} \) stores in the ER would affect salt and drought stress responses. To do this, we generated transgenic lines of *Arabidopsis thaliana* Col-0 plants that constitutively expressed either an ER-targeted green fluorescent protein-calcium binding peptide fusion protein (GFP-CBP) or an ER-targeted CBP protein without GFP. The CBP was derived from the maize calreticulin (*CRTI*) C-domain, and shows high capacity, low affinity calcium binding in the ER. GFP-CBP was previously shown to increase total calcium levels when transiently expressed from a heat shock-inducible promoter in Arabidopsis. The 35S:GFP-CBP plants showed higher expression than HSP:GFP-CBP but, in several lines, breakdown products and gene silencing were problems. We chose two independent transgenic lines with low, but stable GFP-CBP protein accumulation. The ER looked normal, as determined by GFP fluorescence, and full-length GFP-CBP fusion protein could be isolated that bound \( \text{Ca}^{2+} \) in vitro. These two lines and two other independently transformed 35S-CBP lines lacking GFP were used for further analyses. All four CBP transgenic lines had more chlorophyll, increased seed production and delayed senescence compared to 35S:GFP and wild type controls. CBP-transgenic seedlings exhibited better survival on NaCl and sorbitol-containing media and showed increased root growth compared to wild type or GFP control plants. GFP-CBP plants grown in soil had a 30% higher survival
rate than GFP control plants after 8 weeks of intermittent drought, and also had increased root mass. Microarray and qPCR analyses demonstrated that CIPK6, a CBL (calcineurin B-like protein) interacting protein kinase, was specifically up-regulated in the GFP-CBP plants under non-stress conditions while NaCl and sorbitol stress induced CIPK6 expression in the wild type and GFP control plants. Although growth of a cipk6 null mutant was similar to wild type plants on NaCl containing media, cipk6/CBP progeny did not have the increased growth conferred by expression of CBP on NaCl-containing media. Therefore, CIPK6 induction by CBP is what was necessary for the enhanced salt tolerance conferred by CBP. Homozygous cipk6/CBP progeny did present increased tolerance to low calcium levels compared to ciplk6 mutants and wild type plants. We conclude that ectopic expression of the ER-targeted CBP increases Ca$^{2+}$ in the ER, which is responsible for inducing CIPK6 expression. CBP expression confers an increased tolerance to salt and osmotic stress, largely through the action of CIPK6.

**Introduction**

Reduced and unpredictable rainfall and increased salinity in irrigated land has resulted in serious drought and salt stress to crop plants around the globe. It is estimated that around 50% of irrigated land is affected by salinization (Ghassemi et al. 1995). Calcium is an essential nutrient and a secondary messenger that participates in responses to external stimuli and is implicated in many cellular functions and developmental processes (Hirschi et al. 2004, Groenendyk et al. 2004, and White et al. 2003). Cytoplasmic Ca$^{2+}$ concentrations are tightly regulated at 100-200 nM, but higher levels of free calcium are found in sub-cellular
organelles. In both plants and animals, free Ca\(^{2+}\) is found at 3-4 \(\mu\)M in the endoplasmic reticulum (ER) (Groenendyk et al. 2004). In plants, Ca\(^{2+}\) is also stored in vacuoles, chloroplasts and cell walls. Ca\(^{2+}\) is involved in cellular responses to salt stress as well as other external and internal stimuli (Liu et al. 1997), and external Ca\(^{2+}\) can enhance salt tolerance when used as a supplement in plants (Epstein et al. 1998 and Liu et al. 1998).

Salt tolerance appears to be related to the plant’s ability to retain Ca\(^{2+}\) under salt stress. Maize and squash, which are salt tolerant, have higher Ca\(^{2+}\) accumulations in shoots compared to salt sensitive reed canary grass and cucumber (Unno et al. 2002). Liu et al. (1997) showed that a mutation in SOS3 (Salt Overly Sensitive 3 or AtCBL4), encoding one of 10 Arabidopsis calcineurin B-like (CBL) proteins involved in Ca\(^{2+}\) sensing, resulted in lower potassium/sodium ratios than wild type control plants and that SOS3 is needed to suppress the activity of High Affinity Potassium Transporter1 (HKT1), which also controls sodium transport into plant roots. Supplemental Ca\(^{2+}\), however, alleviated both mutant phenotypes (Liu et al. 1997, Rus et al. 2004). Higher external Ca\(^{2+}\) resulted in a higher ratio of potassium/sodium in salt stressed plants (Liu et al. 1997). This suggests that one mechanism whereby Ca\(^{2+}\) mitigates the effect of salt stress is the altering of potassium/sodium ratios in plant cells through the activation of potassium pumps (Liu et al. 1997).

In animal cells, the ER is a major source of cellular Ca\(^{2+}\) (Groenendyk et al. 2004, Navazio et al. 2001, Mailhot et al. 2000). Animal cells use ER calcium for IP\(_3\)-mediated signaling, but although the IP\(_3\) biosynthetic pathway is conserved (Berdy et al. 2001), there is no evidence yet for an IP\(_3\) receptor in plants.
Plants have additional compartments including the cell wall, vacuole, and chloroplasts, that also have high levels of Ca$^{2+}$ (Hirschi et al. 1999), but it is not known if, when, or how calcium from these compartments play a role in signaling and/or regulation of metabolism. Moreover, there are a multitude of calcium-dependent protein kinases (CDPK) and calcium sensors (CBL and calmodulins) in the cytoplasm (Sanders et al. 2002 and Roberts et al. 1992) but it is not known if their cellular location is regulated or even relevant to signaling. The vacuole is the most likely source of Ca$^{2+}$ because it contains the highest calcium levels, but maintenance of vacuolar osmotic potential is also crucial to the integrity of the cell. The ER is an attractive candidate for Ca$^{2+}$-mediated signaling because it is located along the cell wall (cortical ER), in plasmodesmata, and is contiguous with the nuclear envelope.

The plant ER contains a variety of Ca$^{2+}$-binding proteins including the molecular chaperone Binding Protein (BiP), calnexin (CXN), and calreticulin (CRT). Among the ER proteins, CRT is primarily associated with sequestering Ca$^{2+}$ (Persson et al. 2001; Michalak et al. 1999). CRT is a multifunctional protein that is highly conserved in eukaryotic cells (Michalak et al. 1999). The C-domain of CRT has low affinity, high-capacity calcium binding activity in the ER and has been expressed independently of the N- and P-domains in Arabidopsis (Wyatt et al. 2002). Overexpression of CRT in both plants (Persson et al. 2001, Wyatt et al. 2002) and animals (Mery et al. 1996, Bastianutto et al. 1995, Opas et al. 1996) increases total ER Ca$^{2+}$ stores. Over-expression of the C-domain alone in the ER also increases Ca$^{2+}$ stores, creating a reserve that appears to enhance plant survival in low Ca$^{2+}$

We tested plants expressing an ER-targeted fusion gene containing an N terminal
green fluorescent protein (GFP) and a C terminal gene fragment encoding 126 amino acids
from the maize CRT1 C-domain, which served as a Ca\(^{2+}\) binding peptide (CBP) gene (Wyatt
et al. 2002). Previous work showed that heat shock inducible expression of the GFP-CBP
fusion protein increased total calcium by 10-15\% and decreased chlorophyll loss on media
lacking external calcium (Wyatt et al. 2002). Here, we test plants transformed with the same
GFP-CBP construct, or CBP alone, under the control of the strong, constitutive promoter, the
Cauliflower Mosaic Virus 35S promoter (CaMV 35S). We find that transgenic plants
constitutively expressing low amounts of the GFP-CBP fusion protein or the CBP protein by
itself were more tolerant to osmotic stress than GFP or wild type control plants. One of the
genes up-regulated in the CBP transgenic lines is CIPK6 (CBL-interacting protein kinase 6).
CIPK6 belongs to the Calcineurin B-like Interacting Protein Kinase family that has
previously been shown to have a role in salt tolerance in Arabidopsis (Shi et al. 1999). Here
we suggest that the increased stress tolerance of CBP-transgenic plants is conferred by an
increased store of ER Ca\(^{2+}\) and by the subsequent constitutive induction of CIPK6.

Results

Expression of GFP- CBP

Our previous work suggested that intracellular Ca\(^{2+}\) levels could be manipulated in
Arabidopsis by heat shock induction of an ER-targeted GFP-CBP transgene (Wyatt et al.
2002). When HS:GFP-CBP plants were induced on calcium-containing medium, the plants

56
accumulated more Ca$^{2+}$ and survived longer than GFP control plants after transfer to calcium-depleted medium (Wyatt et al. 2002). This work suggested that the ER capacity for Ca$^{2+}$ could be directly related to a physiological response, early senescence in the absence of Ca$^{2+}$.

To further examine physiological differences in these plants, and to avoid the complications of heat shock induction, we transformed Arabidopsis with both the same GFP-CBP construct, and a CBP only construct, under the control of the constitutive CaMV 35S promoter (Fig. 1A).

Fig. 1A shows plants with three plasmid constructs that were cloned into a pUCAP vector (Grebenok et al. 1997) with a CaMV 35S promoter and octopine synthase terminator. The mGFP5 sequence with a 5’ signal sequence derived from chitinase and a HDEL ER retention sequence (Haseloff et al. 1997) was inserted between CaMV 35S promoter and octopine synthase to generate pPLT1011. pPLT1311 was made by inserting 377 bp of the maize CrtI C-domain into pPLT1011. The HDEL from the mGFP5 gene was removed in pPLT1311, leaving only the native CRT HDEL sequence at the carboxy-terminus of the fusion protein. pSYL1301 was generated by removing the GFP gene from pPLT1311.

Table 1 shows the transgenic lines, parents and constructs used to generate transgenic plants used for further analysis. Twenty independently transformed lines were screened for GFP-CBP expression by visual inspection of GFP fluorescence and by immunoblots of isolated protein using the GFP antibody. Fourteen independent transformants carrying only CBP were screened by kanamycin selection and immunoblots of isolated protein. We selected two independent GFP-CBP transgenic lines (GFP-CBP-1 and GFP-CBP-2), one GFP vector
control line and two independent CBP transgenic lines (CBP-3 and CBP-4) for further characterization.

**Fig. 1B** shows the expression pattern of GFP-CBP in a high (1311-10) and low-expressing line (1311-8) and a GFP control line (1011), containing the same construct but lacking CBP. GFP fluorescence in different transformed lines varied with respect to intensity and pattern of expression and some of the lines showed high plant-to-plant variability in expression, suggesting that their transgene was being silenced. GFP-CBP1 and GFP-CBP2 showed low expression compared to a transgenic plant expressing high CBP and the GFP control and GFP was visible only in roots (**Fig. 1B**). To ascertain whether increased CBP expression affected the morphology of the ER, the same constructs used for transformation were bombarded into onion cells (Scott et al. 1999). Similar fluorescence patterns of reticulate ER were seen (**Fig. 1C**), demonstrating that GFP-CBP was uniformly expressed in the ER.

To determine whether there was stable expression of the GFP-CBP transgene, we analyzed mRNA levels in shoots and roots. Quantitative RT-PCR of the GFP-CBP transcription levels in lines GFP-CBP1 and GFP-CBP2 showed that transgene expression was similar in roots and shoots of the same plant (**Fig. 1D**). This indicates that GFP fluorescence in the shoot (**Fig. 1B**) was masked by chlorophyll auto-fluorescence. The GFP-CBP-High line showed similar levels of transgene expression compared to the GFP vector control but also showed degraded protein and unstable expression such that no GFP fluorescence could be detected when the plants were older than 3 weeks. In contrast, lines
Figure 1. Expression of different CBP constructs in Arabidopsis. A. Constructs used in this chapter. GFP-CBP construct (pPLT1011), GFP-only construct (pPLT1311), and CBP-only construct (pSYL1301). All constructs used the CaMV 35S promoter and octopine synthase terminator. pPLT1011 contains the mGFP5 sequence, with a 5’ signal sequence derived from chitinase and an HDEL ER retention sequence. pPLT1311 is identical to pPLT1011 except that it contains a GFP 3’ translational fusion with 377 bp of the maize CRT C-domain. The HDEL from the mGFP5 gene was removed in pPLT1311, leaving only the native CRT HDEL sequence at the carboxy-terminus of the fusion protein. pSYL1301 is identical to pPLT1311 except that it does not have GFP. B. Under control of the 35S promoter, GFP expression was visible in both roots and shoots of GFP (lower left) and GFP-CBP-HIGH (upper left) and roots of GFP-CBP-low (upper right, longer exposure) as compared to a wild type seedling (lower right). Chlorophyll autofluorescence (red) masked GFP signal in hypocotyls and leaves of GFP-CBP-low. The scale bar = 1 mm. C. Fluorescence images of onion epidermal cells bombarded with the GFP-only construct (left) or GFP-CBP constructs (right). Both proteins localized to the ER and showed similar fluorescence patterns. The scale bar = 25 μm. D. Quantitative RT-PCR of transgene expression in leaf and root tissues of 5-day-old seedlings from GFP-CBP-High, GFP-CBP-1 and GFP-CBP-2, and GFP lines. GFP-CBP-High, and GFP are from the same lines shown in Fig. 1B and GFP-CBP-1 is referred to as GFP-CBP low in Fig. 1B. Error bars are standard deviation of three independent experiments.
Table 1. Transgenic lines, parents and plasmid constructs used to generate GFP-CBP and CBP transgenic plants.

<table>
<thead>
<tr>
<th>Transgenic Arabidopsis Line</th>
<th>Parent</th>
<th>Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>1011</td>
<td>pPLT1011 (35S-ER-GFP5)</td>
</tr>
<tr>
<td>GFP-CBP-1</td>
<td>1311-8</td>
<td>pPLT1311 (35S-ER-GFP-CBP)</td>
</tr>
<tr>
<td>GFP-CBP-2</td>
<td>1311-11</td>
<td>pPLT1311 (35S-ER-GFP-CBP)</td>
</tr>
<tr>
<td>CBP 3</td>
<td>1301-3</td>
<td>pSYL1301 (35S-ER-CBP)</td>
</tr>
<tr>
<td>CBP 4</td>
<td>1301-4</td>
<td>pSYL1301 (35S-ER-CBP)</td>
</tr>
</tbody>
</table>

GFP-CBP-1 and GFP-CBP-2 did not show degradation (Fig. 1B) and exhibited stable levels of expression throughout the plant life cycle. Two lines, GFP-CBP-1 and GFP-CBP-2, with low transgene expression were used for further testing.

The expression levels of GFP-CBP varied in different transformants. Figure 2 shows total protein from seedlings of 5 independent GFP-CBP transgenic lines and the GFP control. Most of the 35S-CaMV GFP-CBP plants showed higher expression than the same construct driven by a heat shock promoter (Fig. 2 lane 1, data not shown). Unlike heat shock-inducible GFP-CBP expression (Wyatt et al. 2002) there was evidence of protein degradation in lines showing high expression (Fig. 2A). The uniform size of the breakdown product (approximately 27 kDa) and its ability to cross-react with the anti-GFP antiserum suggested that the carboxy-terminal CBP (C-domain) was degraded, as previously described (Persson et al. 2002). We suspected that the presence of high amounts of GFP-CBP in the ER may have triggered an unfolded protein stress response. If this is the case, the expression of the BiP
chaperone should increase (Cascardo et al. 2000, Urade 2007). The amount of BiP was constant in different transformants and showed no direct correlation with the amount of GFP-CBP fusion or the presence of breakdown product expressed in these plants (Fig. 2). Proteolytic cleavage of the CBP portion of the GFP-CBP fusion protein would remove the KDEL ER retention sequence located in CBP and the truncated fusion protein was not expected to remain in the ER. If it were secreted into the apoplast, it would be unlikely to fluoresce (Scott et al. 1999). Therefore the fluorescence shown in Fig. 1C, although proteolysis occurred, is still likely to represent mostly intact fusion protein.

We used a crt1 null mutant to provide an additional comparison for physiological processes of the CBP transgenic plants. RT-PCR of the crt1 showed that no message accumulated, consistent with an insertion of the T-DNA into an exon of CRT1. Under normal growing conditions, there were no significant differences in rosette leaves, inflorescence height, architecture, or time to flower between wild type plants, the GFP control, and two different GFP-CBP lines grown in soil (data not shown). Senescence is a genetically programmed process, accelerated by environmental stress such as drought, heat and nitrogen deficiency. The primary expression of leaf senescence is the breakdown of chlorophyll and the subsequent collapse of photosynthesis. Chlorophyll measurement has been widely used as an indicator of plant responses to stresses such as water deficit (Cornic et al. 1996), high and low temperatures (Kristjansdotirr et al. 1993) and salinity (Smillie et al. 1982). It has been used as a screening method for identifying drought tolerant durum and bread wheat (Flagella et al. 1994 and 1995). Lines GFP-CBP-1 and 2 had significantly higher concentrations of leaf
Figure 2. Expression of the GFP-CBP fusion protein in different transformants. A. Total protein was extracted from seedlings, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was probed with polyclonal antibodies against GFP and detected by chemiluminescence. The western blot analysis indicated the appropriate size shift (13 kDa) for the GFP-CBP fusion protein, lane 3 to 8 as compared to lane 2, the Arabidopsis expressing mGFP5 under the 35S promoter (GFP control). Lane 1 plants express the GFP-CBP fusion protein under the control of a heat shock inducible promoter. The high expression lines shown in lane 3 and lane 6 have breakdown products whereas lower expression lines (lane 4, 5, 7 and 8) show no detectable breakdown. Lane 5 shows GFP-CBP-1 and Lane 7 shows GFP-CBP-2. B. The same membrane shown in A was also probed with BiP antibodies. There was no direct correlation with the expression level of BiP and the expression of GFP-CBP in different transformants. C. Total proteins were visualized with Gelcode Blue (Pierce, Rockford, IL). Lanes are as indicated in A.
chlorophyll, showed delayed senescence, and produced an increased number of seeds under normal growth conditions (Fig. 3). This difference increased when GFP-CBP was compared to *crt1* (Fig. 3A). There was a statistically significant increase in seed weight of GFP-CBP compared to GFP, Col-0 and *crt1* (P < 0.001). CBP also showed a statistically significant increase in seed weight compared to GFP, Col-0 and *crt1* (P < 0.001). There were no significant differences in seed weight among GFP-CBP transgenic plants and CBP transgenic plants. Expression of ER-targeted CBP increases chlorophyll content in leaves of soil-grown plants (Fig. 3B). There was statistically a significant increase in chlorophyll contents of GFP-CBP transgenic plants compared to wild type Col-0 and GFP control (P < 0.001). CBP (without GFP) also showed a significant increase in chlorophyll content compared to wild type Col-0 and GFP control (P < 0.001). Lines with high expression of the GFP-CBP gene showed non-uniform expression and evidence of gene silencing in subsequent generations while the GFP control, which also showed high expression, did not. As a result, the phenotype of GFP-CBP high-expressing lines varied. Thus, two GFP-CBP lines referred to as GFP-CBP-1 and GFP-CBP-2, which showed low but uniform expression of GFP (Fig. 2) and lacked breakdown products, were selected for further phenotypic and genetic analyses. The GFP control was selected for several generations for stable expression and a high-expressing line was chosen for use as a control.
Figure 3. Increased seed production and higher chlorophyll content in CBP transgenic plants. A. Total seed production of 5 plants per line was measured. SEM is represented as error bars. B. Chlorophyll content of GFP-CBP, CBP transgenic plants, wild type Col-0, GFP vector control plants. Total chlorophyll was measured in leaves of two independent lines of 5 day old GFP-CBP and CBP transgenic plants, wild type (Col-0), and 35S:GFP control plants. 50 mg fresh tissue was used to extract chlorophyll for each line. The experiment was repeated 3 times. Bars are SEM.
**The GFP-CBP Fusion Protein Binds Ca$^{2+}$**

Although the CRT C-domain has been demonstrated to have high capacity, low affinity Ca$^{2+}$ binding activity in the ER (Corbett et al. 2000), we needed to verify that expression of the maize CRT C-domain as a GFP-CBP fusion protein expressed could retain Ca$^{2+}$ binding activity in Arabidopsis. Total leaf protein from different GFP-CBP transgenic lines and a GFP control plant was separated by electrophoresis on two separate SDS/PAGE gels in the presence of either 1 mM EGTA or 1 mM CaCl$_2$. GFP-CBP fusion proteins and GFP were visualized by immunoblotting using polyclonal antibodies made against recombinant GFP (Clontech, Palo Alto, CA). Identical molecular weight standards were used to align the two gels (**Fig. 4**). In each case, the GFP-CBP fusion proteins migrated more slowly in the presence of EGTA (**Fig. 4**, lane 3-8) than in the presence of Ca$^{2+}$ (**Fig. 4**, lane 11-16), whereas the GFP control (**Fig. 4**, lanes 2 and 10) and GFP-cross-reacting breakdown products (**Fig. 4**, lanes 3, 7, 11 and 15) showed no difference. The apparent molecular mass of the GFP-CBP fusion protein shifted from 43 kDa to about 48 kDa. This result is consistent with the Ca$^{2+}$- dependent electrophoretic mobility shift shown by other calcium binding proteins (Teahan et al. 1992, Hooker 1999, Burgess et al. 1980), including full length CRT (Hooker et al. 1999, Li et al. 2000). We conclude that the recombinant GFP-CBP fusion protein, but not GFP, binds calcium in vitro.
**Figure 4. Calcium-dependent electrophoretic mobility of ER-GFP-CBP.**
SDS-PAGE of protein extracts from independent transformants of At1311 are shown in lane 3-8 and 11-16. Protein extracts from At1011-1 is loaded in lane 2 and 10. The GFP-CBP fusion protein and GFP were visualized by immunoblotting with anti-GFP serum. The migration of calcium-loaded or calcium-depleted GFP-CBP (lane 3-8 and 11-16) was compared using molecular-mass standards (Kaleidoscope prestained standards), lane 1 and 9, and with GFP alone (lane 2 and 10). **A.** A gel containing 1 mM EGTA. **B.** A gel containing 1 mM CaCl₂.

**CBP Expression Increases Drought and Salt Tolerance**
Calcium plays a role in stress response signaling, and adding calcium to growth media has been shown to help alleviate salt stress in plants (Grattan et al. 1999). When calcium was supplied to salt-stressed plants, root and shoot elongation was restored and the total amount of Na⁺ content in the plant was reduced (Cramer et al. 1989, Zidan et al.)
1991). We conducted several experiments to see whether the increased internal calcium content in GFP-CBP transgenic plants could mitigate abiotic stresses such as drought, salt, and increased osmoticum. We followed a protocol from BASF (Bryan McKersie, personal communication) for assessing intermittent drought tolerance in plants grown in soil to test drought tolerance. GFP-CBP-1, GFP-CBP-2, GFP, and Col-0 were grown in pots with equal amounts of soil. Pots were placed in a randomized pot design in a growth chamber and rotated every other day. GFP-CBP plants showed enhanced drought tolerance compared to wild type and GFP control plants (Fig. 5A). Root growth was much greater in the GFP-CBP plants compared to wild type plants. To further determine if continued intermittent drought had an impact on survival, plants were kept under similar drought conditions for 5 weeks, watered normally, and given two weeks to recover. GFP-CBP plants showed a higher survival rate (~60%) after the intermittent drought than wild type or GFP controls (Fig. 5B). When two week-old seedlings were transferred to individual pots and subjected to drought, they showed more growth than the one week-old seedlings and developed more flowers and fruits. However they were still significantly more robust compared to control plants (Fig. 5C). One of the striking phenotypes of GFP-CBP transgenic plants when drought stressed (watered once a week) was that the root growth of GFP-CBP transgenic plants was significantly higher compared to control plants (Col-0 and GFP) under drought stress (Fig. 5D).

There are many genes regulated in response to drought stress at the transcriptional
Figure 5. Drought tolerance of CBP transgenic plants. A. Four-week old seedlings after 3 weeks of intermittent drought stress. Plants were watered only once a week. All GFP-CBP-1 plants are green while other plants showed high anthocyanin levels. B. Survival rate of GFP-CBP transgenic plants, wild type (WT) and GFP control plants. One week after transfer from media to soil, 20 seedlings from each line were subjected to drought stress for 5 weeks and normal watering for 2 weeks. C. Phenotype of older seedlings subjected to drought stress. From right to left: 35S GFP-CBP1 and 35S GFP-CBP2 Seven-week-old seedlings after 5 weeks of intermittent drought by watering once a week. The two Col-0 wild type plants on the left have wilted compared to 35S-GFP-CBP transgenic plants. D. 35S GFP-CBP (left) and wild type Col-0 (right) plants photographed after 4 weeks of intermittent drought stress (watering once a week). 35S GFP-CBP has more root growth than wild type under drought stress. Increased root mass was observed in GFP-CBP transgenic plants, however, shoot mass was variable.
level, and several genes important in drought tolerance (Shinozaki et al. 2000). To determine whether the transcriptional levels of drought-associated genes were altered in GFP-CBP transgenic plants, we measured the expression levels of several previously-identified drought induced genes. RT-PCR was performed on stressed and unstressed plants for the RD29a and DREB1a genes (Behnam et al. 2007). DREB1a and RD29a were from 2 to 4 fold up-regulated in GFP-CBP transgenic plants in their leaves (Fig. 6A). CBP transgenic plants exhibit a high transcript level of CIPK6 in a normal growth condition (Fig. 9B and 9C). We investigated whether the increased transcription of CIPK6 is required in the induction of DREB1a and RD29a in CBP transgenic plants compared to GFP vector control plants and Col-0 plants. Real time PCR analysis of CBP x cipk6 mutant showed that CIPK6 is needed to exhibit higher transcription levels of DREB1a and RD29a in CBP transgenic plants compared to control plants (Fig. 6A). In order to determine whether GFP-CBP transgenic plant’s drought tolerance was due to their ability to reduce stomatal conductance, which results in the elevated water use efficiency (Jakab et al. 2005), we monitored stomatal conductance for 6 hour period during the day. GFP-CBP transgenic plants exhibited lower stomatal conductance compared to WT and GFP controls (Fig. 6B). Initial measurement early in the day did not demonstrate significant difference in stomatal conductance among four lines tested. However, it showed a significant difference between GFP-CBP lines and control plants during the mid-day but the difference started to narrow as it headed toward late afternoon.
Figure 6. A. qPCR of drought associated genes in 5 day old GFP-CBP transgenic plant leaves. Transcript levels of drought associated genes DREB1a and RD29a were increased compared to Col-0 and GFP control. Error bars are standard deviation. B. Measurement of stomatal conductance. Stomatal conductance was measured for 5 leaves in 5 plants per line and repeated 3 times. Error bars mean SEM.
We have tested GFP-CBP transgenic plants under osmotic stress to determine whether they can tolerate osmotic stress because drought stress is closely related to osmotic stress. We added 150 mM sorbitol to MS media with 1% sucrose and transferred 10 day-old seedlings. They were grown for 2 weeks and photographed (Fig. 7A). Root growth is a convenient and accurate indicator of Arabidopsis seedling growth (Wu et al. 1996). The primary root was longer in 35S-GFP-CBP transgenic plants than control GFP vector control plants and wild type plants. Root lengths were measured at 20 days after transferring seedlings to MS medium with 150 mM sorbitol (Fig. 7B). Thirty seedlings were measured for each line. GFP-CBP transgenic plants showed osmotic stress tolerance and maintained their root growth compared to control plants.

We tested GFP-CBP transgenic plants as well as control plants in salt stress to see whether expressing of GFP-CBP could also make transgenic Arabidopsis salt tolerant since they were osmotic stress tolerant. When GFP-CBP and control plants were exposed to salt stress, by growing 10-day-old seedlings on MS media plus 100 mM NaCl, the GFP-CBP plants had delayed stress symptoms, showed longer primary root growth and had less chlorophyll loss (Fig. 8A). Wild type seedlings showed a steady decline in root growth with increasing salt concentrations, the GFP-CBP lines appeared to tolerate NaCl concentrations of up to 100 mM before showing a steep decline in root growth (Fig. 8B). When seeds were germinated directly on the medium with high salt (100 mM or higher) most of the seeds did not germinate. Therefore, seeds were germinated on normal MS medium and then after 5 days transferred onto medium containing different concentrations of NaCl. GFP-CBP
Figure 7. GFP-CBP transgenic plants maintain root growth under osmotic stress. 

A. 10 day-old seedlings were transferred to MS + 0.15 M Sorbitol + 1% sucrose and photographed after two weeks. The primary root is longer in 35S-GFP-CBP transgenic plants compared to WT plants. 

B. Roots measured at 20 days after transferring seedlings to MS medium with 150 mM sorbitol. Thirty seedlings were measured for each line. Error bars are standard deviation.

genic plants showed salt tolerance and exhibited better primary root growth under salt stress compared to control plants (Fig. 8B). Wild type seedlings showed a steady decline in root growth with increasing salt concentrations, the GFP-CBP lines appeared to tolerate NaCl concentrations of up to 100 mM before showing a steep decline in root growth (Fig. 8B). When seeds were germinate directly on the medium with high salt (100 mM or higher) most of the seeds did not germinate. Therefore, seeds were germinated on normal MS medium and then after 5 days transferred onto medium containing different concentration of NaCl. GFP-CBP transgenic plants showed salt tolerance and showed better primary root growth under salt stress compared to control plants (Fig. 8B). Although GFP-CBP transgenic plants were able to tolerate salt stress and maintain their root growth in MS media with salt concentration from 50 mM to 150 mM NaCl, they could not tolerate salt concentrations above 200 mM.
CIPK6 mutant Arabidopsis seedlings did not exhibit any more salt sensitivity than wild type control seedlings.

**GFP-CBP and CBP increase CIPK6 Expression**

CIPK6 is a serine/threonine protein kinase that interacts with calcium sensors such as CBL, calmodulin, and AKT1 (Popescue et al. 2007, Lee et al. 2007). CIPK6 appears to be up regulated by ABA, osmotic, and salt stress according to data obtained from the Geninvestigator microarray database (https://www.genevestigator.ethz.ch/) (Fig. 9A). Transcription of CIPK6 was higher in the High-GFP-CBP line (Table 1) when compared to the GFP control.
in preliminary microarray experiment (data not shown). We performed qPCR and confirmed that the expression of CIPK6 also increases in both GFP-CBP1 and 2 and the CBP1 and 2 transgenic lines compared to controls (Fig. 9B and 9C). One of the CIPK family members, CIPK24 (Salt Overly Sensitive 2, SOS2), was previously identified in a screen for mutants that were overly sensitive to salt. SOS2 functions in salt tolerance by regulating Na\(^+\) channels in Arabidopsis. It has also been reported that CIPK6 interacts with the C terminal domain of AKT1 (Arabidopsis K\(^+\) transporter 1) (Lee et al. 2007) but it is not known whether CIPK6 increases the potassium content of Arabidopsis. Inductively Coupled Plasma (ICP) analysis for P, Ca\(^{2+}\), Mg\(^{2+}\), K\(^+\) and Na\(^+\) showed that GFP-CBP transgenic plants with constitutively increased CIPK6 transcriptional level had an increased in total Ca\(^{2+}\) as well as in total K\(^+\) compared to control plants, but also had decreased Na\(^+\) content (Fig. 9D). These results raise the possibility that, like CIPK24, potassium channels are activated by CIPK6 in GFP-CBP transgenic plants, which leads to increased K\(^+\) content.

CIPK6 is required for the salt tolerance of CBP transgenic plants

To test whether CIPK6 is needed for salt tolerance in CBP transgenic plants, we crossed a cipk6 homozygous mutant to GFP-CBP and CBP transgenic plants. Progenies of cipk6 mutant lines that also expressed CBP were verified by RT-PCR (Fig. 10A). These CBP/cipk6 lines were subjected to salt stress by growing them on a MS media with 100 mM NaCl. We found that CBP transgenic plants maintained their root growth and were able to delay their stress symptoms while the CBP/cipk6 lines and Col-0 control plants were susceptible to salt stress (Fig. 10B). Root growth of wild type, two independent CBP
transgenic plant lines and homozygous cipk6 progeny from two independent CBP X cipk6 lines were measured for 20 plants, respectively, and compared to those grown on MS media without NaCl (Fig. 10C). CBP transgenic plants showed better primary root growth compared to both wild type Col-0 and the CBP X cipk6 progeny on different concentration of NaCl. CBP/cipk6 was not salt tolerant and did not show significant differences from the control plants. This suggests that CIPK6 expression is necessary for the CBP transgenic plants to be salt tolerant.
Figure 9. CIPK6 is specifically upregulated in CBP transgenic plants. A. The CIPK6 microarray data compiled at Geninvestigator (https://www.genevestigator.ethz.ch/) shows that it is expressed in all tissues but is significantly up-regulated by abscisic acid, osmotic stress, and salt stress. B. Semi-quantitative RT-PCR for CIPK6 expression in wild type, the GFP vector control, and two independent CBP transgenic lines. C. Quantitative RT-PCR for CIPK6 in GFP-CBP, CBP, GFP control and WT plants. Seedlings were grown on MS Medium without sucrose for 5 days after germination and then harvested for qRT PCR. RT-PCR showed that there was an increase in the expression of CIPK6 in both GFP-CBP and CBP transgenic plants. D. Increased potassium and decreased sodium in CBP transgenic plants. Two grams of Arabidopsis seedlings grown on MS media for 2 weeks were ground and analyzed at the NCSU Soil Science Analytical Lab using Inductively Coupled Plasma. Experiments were repeated 3 times and graphs show the average of the 3 replicates. GFP-CBP and CBP plants showed a statistically significant difference in both Ca^{2+} and K^{+} in CBP transgenic plants compared to that of control plants (P < 0.001).
A. Fold Change in Log2

- Hormone: ABA,3 (+)
- Stress: cold,2
- Hormone: ethylene (+)
- Stress: osmotic_roots_early
- Nutrient: K (+)
- Hormone: ABA,7 (+)
- Nutrient: P, long-term
- Light intensity: high light,2
- Nutrient: glucose,2-4-6h
- Chemical: 2,4-Dichlorophenoxyacetic acid (+1)
- Stress: osmotic_green,early
- Chemical: ozone,1
- Nutrient: nitrate,low
- Nutrient: nitrate(0) sucrose(90mM)
- Hormone: ABA,6 (+)
- Biotic: P. infestans (+)
- Stress: salt,roots,late
- Biotic: P. syringae,4 (avrRpm1)
- Stress: salt,green,late
- Chemical: isoxaben (+)
- Stress: osmotic_roots,late
- Hormone: ABA,1 (+)
- Stress: osmotic_green,late
- Hormone: BL / H8803 (+1)

B. Semi-Quantitative RT-PCR

WT  WT  GFP  CBP1  CBP2

CIPK6

Actin8

C. qPCR CIPK6

D. Nutrient Content (Whole Seedling)

WT  CBP  GFP-CBP

Figure 10. Expression of CIPK6 is required for the increased salt tolerance of CBP plants. A. RT-PCR verification of lack of CIPK6 expression in the mutant and in selected CBP X cipk6 progeny. B. Salt stress assay demonstrates that CIPK6 is necessary for the increased salt tolerance of CBP plants. Seedlings were germinated on MS medium and transferred to MS + 150 mM NaCl after 4 days. The picture was taken after 4 days in MS +150 mM NaCl. C. CBP transgenic plants grown on different MS+NaCl media with control plants. Root growth was measured for 5 lines on MS media, MS+25 mM NaCl, MS+50 mM NaCl, MS+75mM NaCl, MS+100 mM NaCl, MS+125 mM NaCl, MS+150 mM NaCl, and MS+200 mM NaCl. Seedlings were grown for 4 days on vertical agar plates containing MS media and then transferred to vertical agar plates containing MS media supplemented with NaCl. Root growth was measured 6 days after the transfer. Growth on MS media was considered as 100%. Values are the averages of 20 seedlings. Error bars are SEM.
Discussion

Transgenic plants were created that express CBP in the ER to test whether increased ER Ca\(^{2+}\) would be available for modulating stress responses following exposure to salt and drought. When the 35S promoter was used, only plants with low CBP expression levels showed stable, beneficial responses. The total calcium content of transgenic plants increased by up to 10\% (Wyatt et al. 2001 and Fig. 9D). The ability of CBP to bind Ca\(^{2+}\) was experimentally verified by comparing the mobility of the GFP-CBP fusion protein with GFP in calcium or EDTA-containing SDS PAGE (Fig. 4). The retarded mobility of GFP-CBP compared to GFP in the presence of Ca\(^{2+}\) is consistent with the protein acquiring a more positive net charge. In the absence of Ca\(^{2+}\), the relative mobility of GFP-CBP increased as it was not bound by Ca\(^{2+}\).

The increased ER calcium conferred by CBP resulted in enhanced drought tolerance and salt tolerance. Drought-associated genes, which include *rd29a* and *DREB2a* were up-regulated in CBP transgenic plants (Fig. 6A). Trehalose levels were also higher in CBP-expressing plants (data not shown) and increased trehalose has been shown to play a role in drought tolerance in rice (Garg et al. 2002). We speculated that too much CBP expression could cause an ER stress response. However, the fact that the ER stress-associated protein, BiP, was not up-regulated (Fig. 2) suggests that CBP expression did not induce the unfolded protein response (Cascardo et al. 2000, Urade 2007).

A striking phenotype of CBP-transgenic plants under drought stress was the alteration of development (Fig. 5D). CBP plants exhibited longer and enhanced root growth
compared to controls, suggesting that CBP expression not only alters gene expression but also can have an effect on root development.

CBP-expressing plants are salt tolerant (Fig. 8A and Fig. 8B) and can maintain root growth under salt stress compared to control plants. One of the genes upregulated by CBP in the transgenic plants is CIPK6. CIPK6 is a protein kinase that interacts with CBL and has also shown to interact with CaM (Camodulin)1 in vivo (Popescu et al. 2007). We initially speculated that CIPK6 was needed for salt tolerance. However, the cipk6 mutant is not more sensitive to salt than wild type plants. We crossed the cipk6 mutant to CBP to see whether cipk6 was needed for the enhanced salt tolerance in CBP transgenic plants. Progeny from this cross subjected to salt stress showed that CIPK6 was needed; plants that were homozygous for cipk6 and CBP were not more salt tolerant than wild type plants (Fig. 10B and Fig. 10C). CIPK6 was up-regulated in CBP transgenic plants even before they were stressed (Fig. 9B and Fig. 9C). These results demonstrate that CIPK6 is needed for CBP to confer salt tolerance, but unless CIPK6 is over-expressed, it does not confer salt tolerance.

CBP transgenic plants with increased Ca\(^{2+}\) also exhibited increased K\(^{+}\) content, but show a decreased Na\(^{+}\) content (Fig. 9D). This suggests that CIPK6 is important for maintaining the homeostasis of other ions in CBP transgenic plants. It has been reported that CIPK6 is one of the serine/threonine protein kinases that interacts with the C-terminus of AKT1 (Arabidopsis K\(^{+}\) Transpoter 1) (Lee et al. 2007). Thus, constitutive expression of CIPK6 by CBP activates potassium channels, leading to an increase in total potassium. The
salt tolerance of CBP transgenic plants can be explained by increased K\(^+\) and decreased Na\(^+\). Having lower intracellular Na\(^+\) suggests that CBP-expressing plants are able to counteract Na\(^+\) influx; thereby increasing the amount of external Na\(^+\) required to reach cell toxicity.

Additional important phenotypes of CBP transgenic plants include increased chlorophyll content, increased seed mass, and delayed senescence (Fig. 3).

Studies in mammalian systems have shown that up-regulation of CRT expression resulted in increased accumulation of cellular Ca\(^{2+}\) (Mery et al. 1996, Bastianutto et al. 1995). Recent studies in plants also have shown that CRT levels were positively correlated with Ca\(^{2+}\) accumulation, which required ATP (Persson et al. 2001). The increased Ca\(^{2+}\) accumulation observed in the ER of NT1 tobacco suspension cells appeared to result from buffering by CRT (Persson et al. 2001, Akesson et al. 2005). In addition, CRT-transgenic Arabidopsis plants showed delayed chlorosis on Ca\(^{2+}\)-depleted medium (Persson et al. 2001). Support for the calcium-buffering property of CRT as a critical mediator of the response comes from experiments following heat shock induction of the GFP-CBP fusion protein, which includes the CRT C domain (Wyatt et al. 2002).

It has been proposed that plants use different Ca\(^{2+}\) stores for signaling patterns in response to different signals (Sanders et al. 1999, Knight et al. 1996, Knight et al. 1997, Trewavas et al. 1999). Although the ER contains lower total Ca\(^{2+}\) than the vacuole (Bush et al. 1989), it was shown that the ER Ca\(^{2+}\) pool is important in plant tolerance for calcium stress (Wyatt et al. 2002). Unlike Ca\(^{2+}\) stored in the vacuole, ER Ca\(^{2+}\) was available to plants when they were subjected to stress (Wyatt et al. 2002). The location of the Ca\(^{2+}\) store appears
to be important for whether calcium stores are available for the plants when they are under stress.

Both the vacuole and the endoplasmic reticulum have been suggested to serve as Ca$^{2+}$ reservoirs for cytoplasmic signaling. There is also evidence that Ca$^{2+}$ transport into the vacuole by Ca$^{2+}$ pumps and Ca$^{2+}$/H$^+$ antiporters dampens cytoplasmic Ca$^{2+}$ signals (Catala et al. 2003). In plants, Ca$^{2+}$ can be released from the ER by cADP-ribose, a NaD$^+$ metabolite (Navazio et al. 2001). The cell’s sensitivity and response to various stresses is affected by its ability to sequester and use Ca$^{2+}$ from internal Ca$^{2+}$-signaling stores (Lemtiri-Chlieh et al. 2003, Catala et al. 2003).

Our data suggest that the ER Ca$^{2+}$ pool is important for salt and drought stress. A large amount of information has accumulated on the role of Ca$^{2+}$ in salt tolerance (Rengel et al. 1992, LaHaye et al. 1969, Liu et al. 1998, Epstein et al. 1998). It is thought that the toxic levels of sodium may displace Ca$^{2+}$ from the membrane of root cells (Lynch et al. 1989, Cramer et al. 1985, Cramer et al. 1987, Lynch et al. 1985), leading to increased membrane leakage. The GFP-CBP expressing plants were able to store more total calcium and these plants were more salt tolerant when transplanted from normal medium onto a high salt medium. Our data suggest that the ER calcium store is also important in relieving Na$^+$ toxicity by changing K$^+$/Na$^+$ in the plant (Fig. 9D). The increased K$^+$/Na$^+$ content in plants may be due to the constitutive expression of CIPK6 in GFP-CBP transgenic plants. Recently, it has been reported that CIPK6 interacts with the C-terminus region of Arabidopsis Potassium Transporter1 (AKT1) (Lee et al. 2007). We speculate that CIPK6 interacts with
calcium sensors such as AtCBL4 (SOS3) (Kim et al. 2000) or CaM1 (Popsecu et al. 2007), leading to activation of ion channels such as the potassium transporter (AKT1) to control ion homeostasis in CBP transgenic plants. The mechanism for how activation of ion channels by CIPK6 is contributing to salt tolerance in GFP-CBP transgenic plants is not clear but CIPK6 is required for CBP transgenic plants to have stress tolerant phenotypes. The phenotypes of these plants were normal under non-stress conditions. Transgenic expression of individual genes has also been shown to increase salt and drought tolerance without a detrimental phenotypic effect. The complexity of the signaling response is illustrated by results with the rice CDPK7 gene, which confers salt and drought tolerance when over-expressed as a transgene. Immunoblots demonstrated that accumulation of the CDPK7 protein did not change with exposure to stress or in the transgenic plants (Martín et al. 2001). This suggests that post-translational regulation of the protein may have affected steady state levels of CDPK7.

The discovery of SOS (salt overly sensitive) genes in Arabidopsis provides us an indication of how the pathway for plant salt tolerance is regulated. The SOS3 gene has been proposed to be involved in mediating the beneficial effects of Ca\(^{2+}\) (Zhu et al. 1998, Zhu et al. 2000). A mutation of SOS3 gene increases the level of extracellular Ca\(^{2+}\) required to relieve salt stress (Liu et al. 1997). SOS3 encodes a putative Ca\(^{2+}\) binding protein with 3 predicted EF-hands (Liu et al. 1998) and is most similar to the B-subunit of calcineurin (Mendoza et al. 1994) and animal neuronal Ca\(^{2+}\) sensors (NCS) (Schaad et al. 1996). In yeast, calcineurin regulates K\(^{+}\), Na\(^{+}\), Ca\(^{2+}\) homeostasis and pheromone responses (Mendoza
induction of genes coding for a Na\(^+\)-ATPase, Ca\(^{2+}\)-ATPase, and a cell wall β-1, 3 glucan
synthase (Stathopoulos et al. 1997). This study also showed that SOS3 is capable of binding
Ca\(^{2+}\). The novel Ca\(^{2+}\)-binding properties of SOS3 are likely to be important in determining
the specificity of Ca\(^{2+}\) signaling under Na\(^+\) stress.

Our earlier data suggested that the CRT C-domain not only has the ability to store
Ca\(^{2+}\) but that it can act as a “capacitor” to store calcium until needed (Wyatt et al. 2002).
This model of cellular Ca\(^{2+}\) control suggests that Ca\(^{2+}\) stores when depleted in the ER are re-
filled from channels in the plasmalemma upon depletion, and that the ER is key to sensing
cellular Ca\(^{2+}\) homeostasis (Thomas et al. 1996). CRT levels may be a limiting factor for Ca\(^{2+}\)
storage in the ER and over-expressing the C-domain increases the calcium “capacity” of the
ER. Clearly, the expression of the GFP-CBP fusion protein has a beneficial effect on
modulation of plant responses to drought and salt stress. Besides salt stress, calcium is also a
key player in other stress responses. We are currently testing transgenic expression of GFP-
CBP for protecting plants from other environmental stresses.

**Material and Methods**

**Plasmid Constructs**

The AtHSP-mgfp5-CRT-C domain-ocs construct (pBIN2311) contains mGFP5
modified to remove the HDEL and replaced with a 400 bp sequence from the maize CRT
gene (accession # AF190454) and was previously described (Wyatt et al. 2002). The heat
shock promoter of clone pPLT2311 was replaced by a HindIII/BamHI fragment containing
the CaMV 35S promoter from pBINmGFP5 (Haseloff et al. 1997) to make pPLT1311. An Ascl/ PacI DNA fragment of pPLT1311 consisting of the CaMV35S-GFP-CBP-ocs sequence was ligated into the binary plasmid pBINPLUS (van Engelen et al. 1995), and the resulting plasmid was labeled pBIN1311. To serve as a control, a 35S-mGFP5er construct (pPLT1011) was similarly constructed from pWy2011 (Wyatt et al. 2002) to make pBIN1011. Removing the GFP sequence from pPLT1011 using BamHI and replaced it with the maize CRT C-domain sequence (CBP). The 400-bp CBP fragment was generated by PCR using primers with embedded BamHI and PacI restriction sites. The final products of each clone were verified by sequencing.

**Bombardment of Onion Epidermal Peels**

Before the constructs were inserted into binary vectors, the pUC based constructs pPLT1011 and pPLT1311 were bombarded into onion epidermal cells as described (Scott et al. 1999). Images were taken using a Zeiss Axiophot® microscope equipped with 25X and 40X water, and 100X oil immersion lenses (Carl Zeiss, Thornwood, NY, USA). Images were captured using a Pentamax cooled CCD Camera (Model TE/ CCD-K1317; Princeton, NJ, USA) and analyzed using Image One™ software (Universal Imaging, West Chester, PA, USA). Images were acquired and processed using Adobe Photoshop software. GFP was excited using a super-high pressure mercury lamp placed behind a 425/40 nm filter and visualized using a 540/50 nm emission filter.
Plant Transformation and Selection

Binary constructs pBIN1311, and pBIN1011 were electroporated into Agrobacterium tumefaciens, strain GV3101, using a Bio-Rad electroporator according to the manufacture’s instructions (Bio-Rad, Hercules, CA). Wild type A. thaliana Col-0 plants (generation T₀) were then transformed by vacuum infiltration as described (Bechtold et al. 1993 and 1998, Love et al. 2000). Seeds from these plants, labeled generation T₁, were sterilized for 30 min with 50% (v/v) bleach, 0.02% (w/v) Triton, then plated onto AT medium [4.3 g/l Murashige and Skoog salts (Gibco BRL, Bethesda, MD), 1X B5 vitamins, 2% sucrose, 0.05% MES pH 5.8, 1% Phytagar (Gibco BRL, Bethesda, MD)] containing 30 mg/l kanamycin. Seedlings were grown for 2 weeks at 21°C in constant light. Kanamycin-resistant seedlings were transferred to soil and cultivated at 21°C, under an 8-h light/16-h dark photoperiod. Plants were then transferred to a 16-h light/8-h dark photoperiod, allowed to self fertilize, and the resulting seed collected (T₂ generation). Progeny were selected from each T₁ line that showed a 3:1 ratio of kanamycin resistance consistent with single locus insertion of the transgene. Seedlings from the T₂ generation were analyzed by fluorescence microscopy and leaf samples were taken from all plants, weighed, frozen in liquid N₂, and stored at -80°C for analysis of protein expression.

We screened seed from 35 independent transgenic T₁ lines containing the 35S GFP-CBP and 20 independent transgenic T₁ lines containing the 35S CBP only construct and chose 14 lines, respectively, that gave a 3:1 ratio of kanamycin resistance. Kanamycin-resistant T₂ plants were then grown, selfed, and screened for GFP expression. Homozygous
lines were identified by testing T3. Transgenic lines of Arabidopsis showing high fluorescence (At1311-10-21), or low fluorescence (At1311-13 or GFP-CBP 1, At1311-11 or GFP-CBP 2), were selected as lines that express the GFP-CBP fusion protein, and At1011-1 for the ER-targeted GFP control, based on GFP expression.

**Analysis of GFP Expression**

The T2 generation of plants transformed with pBIN1011 and pBIN1311 were screened for GFP fluorescence. Fluorescence images were acquired 10 days after germination as described (Scott et al. 1999, Love et al. 2000) using a Leica MZ12 fluorescence dissecting microscope (1.6X) (Leica, Deerfield, IL, USA) and a Hamamatsu chilled 3CCD color camera.

**Protein Extraction and Immunoblot Analysis**

Plants were also analyzed for GFP expression by western blot analysis to ensure stability of transgene expression. Approximately 20-50 mg samples of young leaves were collected from each plant, weighed, and frozen in liquid nitrogen. Leaves were ground in 2x sample buffer (Ausubel et al. 1992) using 1 µl of buffer to 1 mg of plant material. Equal amounts of plant tissue extract were loaded into each lane of a 10% SDS polyacrylamide gel, and proteins were separated by electrophoresis. Separated proteins were either stained with Gelcode Blue (Pierce Scientific, Rockford, IL) or transferred to a nitrocellulose membrane (Bio-Rad, Bethesda, MD) using a Bio-Rad Mini Trans-Blot Assembly at 16 h at 30V. After transfer, the membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline with 0.2% (v/v) Tween 20 for 2 h at 22°C. Proteins were detected using polyclonal
antibodies against recombinant GFP (Clontech, Palo Alto, CA) diluted 1:2000, or an antiserum against BiP from maize (gift from Rebecca Boston) diluted 1:5000 and horseradish peroxidase-conjugated secondary antibodies (Pierce Scientific, Rockford, IL) using a chemiluminescent substrate, Supersignal West Pico blotting protocol (Pierce Scientific, Rockford, IL) according to the manufacturer’s instructions.

Assessment of Calcium Binding

To test for calcium-dependent electrophoretic mobility, protein extracts from individually transformed lines of 1311 and 1011 were analyzed by SDS/PAGE in the presence of either 1 mM EGTA (first gel) or 1 mM CaCl₂ (second gel) according to the methods of Li et al., 2000. The gels were then transferred to nitrocellulose membranes (Bio-Rad, Bethesda, MD) and proteins detected by immunostaining, as described above. The migration of calcium-loaded or calcium-depleted GFP-CBP (At1311) was compared in the two gels using molecular-mass standards (Kaleidoscope pre-stained standards from Bio-Rad, Bethesda, MD) and the GFP-alone control (At1011-1). The GFP breakdown product from high-expressing lines served as an internal standard.

Assessment of Relative Root Growth on Salt Medium

Wild-type and CBP transgenic lines (T₃) as controls and two independent F2 progenies from a CBP X cipk6 cross that lacked CIPK6 expression were grown on vertical MS agar plates at 22°C for four days. They were then transferred to MS with various concentrations of NaCl. Root growth was measured 6 days after the transfer and growth on MS medium without NaCl was considered as 100% for both controls and crosses. Values are
the averages of 20 seedlings per treatment. The following primers have been used for RT-PCR of CIPK6, CIPK6F: AATCACAACCACCGGAAGAGG and CIPK6R: ACGTCG-CGAATCTCATCTCTCT. After RT-PCR identified 5 lines that did not express CIPK6 but express CBP was verified using following primers, CBPF: GCATGCCCTATGGTGAC-AACC and CBPR: GCATGCGATCTAGAGCTCGTC. Segregation analysis on four lines showed 3:1 segregation. Two lines were chosen for analysis.

**Assessment of CIPK6 expression by Semi-Quantitative RT-PCR and Quantitative**

**Real Time RT-PCR**

Total RNA was extracted from Arabidopsis seedlings using the QIAshredder and RNeasy plant mini kits (Qiagen, CA/USA). DNA was removed by performing an on-column digestion using a DNase kit (Qiagen). cDNA was synthesized according to the ImProm-II™ Reverse Transcription System RT kit protocol on 300 ng of RNA with dT16-oligonucleotide as primer. Following synthesis, cDNA samples were diluted to 1,000 pg/μL with DNase/RNase-free water. PCR primers for CIPK6, CIPK6F: AATCACAACCACCGGA AGAGG and CIPK6R: ACGTCGCGAATCTCATCTCTCT were designed using Primer3 (http://frodo.wi.mit.edu/cgi women/ primer3/primer3 www.cgi) to create amplicons of 150 to 200 bp. Primers used had the following characteristics: melting temperature between 55°C to 60°C, 3' G/C clamp, and 40% to 60% G/C content overall. No-reverse transcriptase enzyme (RT) controls were used on each 96-well plate to ensure that no genomic DNA was carried over. To ensure consistent tissue collection, RNA extraction, and RT activity, primers
for an internal control (the Actin8 gene, accession number AT1G49240) were designed to amplify the 3' end of the cDNA (5'-CTTTCGTTACGCTTTT-3' and 5'-GAAACGCGGATTAGTGCCT-3').

**Combined cycling drought assay (CD) and yield analysis from BASF, the Chemical Company**

Each pot was filled with thoroughly mixed 50 g of 1/3 Turface and 2/3 Metro Mix 360 and then pots were placed in trays without holes (Model F1020NH, Hummer Cat#11-3050). The fertilizer solution was added approximately 2 liters to four trays containing 16 pots (fertilizer mix 0.4 g/l of miracle grow). Pots were soaked with fertilizer overnight and excess fertilizer solution was discarded from the tray. Seeds were refrigerated for several days in dH2O and then used a 1 ml pipet to transfer seeds to the soil surface in each pot. The tray was then covered with a standard transparent dome (Model CW221, Hummer Cat#14-2568-1). At day 7, only one seedling was left per pot and domes were placed back on the trays until day 10. Trays were placed in a Percival growth chamber with the following conditions: Photoperiod (16 h light/ 8 h dark), temperature 22°C, relative humidity 55-60% using fluorescent bulbs only (white) approximately 120 μmol m⁻² s⁻¹ at rosette level. One liter of water was directly poured into the tray Monday, Wednesday and Friday and the other group of plants (1/2) was given one liter of water once a week.

**Chlorophyll measurements**

50 mg of fresh plant leaves in the form of a disk punctured out from the leaves using the cap of an Eppendorff tube was immediately put into 8 ml of 100% methanol immediately
and incubated at 30°C for 24 h. The chlorophyll extract was subjected to spectrophotometric measurements at 603, 647, and 664 nm. Specific chlorophyll content was calculated according to Moran (1982) and normalized to the total fresh weight of tissue in each sample.

*Calcium, Potassium and Sodium Measurements in CBP transgenic plants and control plants*

Approximately 100 seedlings grown vertically on MS media were harvested and gently washed with nano-pure water. Tissue were dehydrated in a 50°C oven for at least 3 days and ground and weighed. 0.1g of each sample was submitted to NCSU Analytical Service Laboratory for analysis. The samples were analyzed by ICP (Inductively coupled plasma mass spectrometry).

**Acknowledgments**

Dr. Pei-Lan Tsou did the experiments shown in Figures 1B, 1C, 2 and 4. Sang Yoon Lee performed all of the other experiments. We thank Dr. Rebecca Boston for the maize CRT clone and BiP antibodies, Wayne Robarge for analytical ICP readings, and Shantha Sumanasinghe for GFP imaging. We also thank Bill Hoffman for help with stomatal conductance. GFP fluorescence was imaged in the NCSU Cell and Molecular Imaging Facility, and research was supported by NASA grant # NAGW-4984 and NC ARS.
References


Chapter 3

Extra Calcium Stores in CBP Transgenic Plants Contribute to Maintaining the Transient Cytosolic Ca\(^{2+}\) Increase after Long Term Salt Stress and Ca\(^{2+}\) Depletion

**Key Words:** Calreticulin, calcium-binding peptide, calcium storage, endoplasmic reticulum, Arabidopsis, salt tolerance, drought tolerance, salt tolerance

**Abbreviations:** Ca\(^{2+}\), calcium; [Ca\(^{2+}\)]\(_{cys}\) cytosolic calcium; CBL, Calcineurin B Like; CRT, calreticulin; ER, endoplasmic reticulum; GFP, green fluorescent protein; immunoglobulin binding protein; ATP, adenosine triphosphate; CBP, calcium binding peptide
Abstract

Previous studies showed that plants with a constitutively expressed, ER-localized Calcium Binding Peptide (CBP) have increased total calcium and survive over longer periods of drought and salt stress. To understand the calcium-based mechanism for increased stress tolerance in these plants, cytosolic calcium concentrations under normal and stress conditions were measured. There were no significant differences in resting cytosolic calcium levels between CBP (Calcium Binding Peptide) and control seedlings when measured by confocal ratio analysis of Indo-1 fluorescence or by aequorin-based luminescence. To determine how ER calcium participates in stress responses, plants were grown on normal media for 8 days and then transferred to calcium-depleted media until they doubled in size. Cytosolic calcium concentrations were reduced in control plants compared to CBP transgenic lines, which maintained normal levels of cytosolic Ca\(^{2+}\). These experiments strongly suggest that the plant ER plays an important role in cellular calcium homeostasis and that increased stores of ER calcium help to buffer cytoplasmic calcium levels over long periods of stress.

Introduction

Calcium plays an important role in the signaling and transduction of salt stress in plants. A rapid cytosolic calcium spike occurs within a few seconds when a plant is salt stressed (Knight et al. 1997, Kiegle et al. 2000, Knight et al. 2000, Moore et al. 2002). This increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) is thought to be sensed by one of several calcium sensors, such as the Arabidopsis Calcineurin B-Like (CBL) protein 4 (AtCBL4/SOS3), which interacts with Arabidopsis CBL-Interacting Protein Kinase (AtCIPK24/SOS2) to induce necessary salt
stress responses (Halfter et al. 2000). It is proposed that CIPK24/SOS2 enables the phosphorylation and Ca$^{2+}$-dependent activation of SOS1, a Na$^+$/H$^+$ anti-porter bound to the membrane (Qui et al. 2002; Quintero et al. 2002). This process increases Na$^+$ efflux to maintain Na$^+$/K$^+$ homeostasis in the cell.

It has been demonstrated that a mild salt stress inhibits uptake of K$^+$ and Ca$^{2+}$ in Arabidopsis (Attia et al. 2008) and homeostasis of three macro-elements: K$^+$, Ca$^{2+}$, and N in plants is known to be important for salt tolerance (Grattan et al. 1999). Many studies have demonstrated that adding extra Ca$^{2+}$ to the plant growth media alleviates salt stress in plants (Maathuis et al. 1999). Externally added Ca$^{2+}$ restored root elongation and shoot growth in salt-stressed plants, and reduced total Na$^+$ content (Cramer et al. 1996 and Zidan et al. 1991). Noninvasive ion flux measuring and patch-clamp techniques demonstrated that addition of Ca$^{2+}$ to the growth media alleviates salt stress by reducing Na$^+$ influx through non-selective cation channels and inhibiting Na$^+$-induced K$^+$ efflux through outwardly directed, K$^+$-permeable channels (Shabala et al. 2006).

The recombinant bioluminescent reporter of [Ca$^{2+}$]$_{cyt}$ aequorin, is a non-invasive method that can be compared to confocal ratio image analysis in studies of Ca$^{2+}$-mediated NaCl signaling in Arabidopsis. Aequorin studies have identified plant responses to NaCl treatments that include Ca$^{2+}$ influx across the plasma membrane and inositol (1,4,5) trisphosphate [Ins(1,4,5)P3]-mediated Ca$^{2+}$ release from the vacuole in Arabidopsis (Knight et al. 1997). More cell-specific NaCl-induced [Ca$^{2+}$]$_{cyt}$ increases have been demonstrated by root cell expression of aequorin in Arabidopsis (Kiegle et al. 2000). It has also been
demonstrated that NaCl treatment causes the increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ in plants (Knight et al. 1997, Kiegle et al. 2000), but the source of the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase and how this increase is translated to a specific response remains elusive (Reddy et al. 2004).

We increased both ER calcium and the overall plant calcium content of *Arabidopsis thaliana* by expressing the C-domain of calreticulin as an ER-localized CBP (ER-CBP) (Chapter 2). Our previous studies showed that CBP transgenic plants have up to 10% more calcium than control plants and exhibited increased salt tolerance (Chapter 2). It has been demonstrated that salinity stress inhibits uptake of calcium (Lynch et al. 1985, 1988, and 1989, Wolf et al. 1990). Furthermore, the symplastic $\text{Ca}^{2+}$ transport is more inhibited than the apoplastic transport in salinized roots (Halperin et al. 1997). We hypothesized that having extra calcium in CBP transgenic plants alleviates salt stress by using additional calcium in them when $\text{Ca}^{2+}$ uptake and transport are inhibited under salt stress.

Here, we measured the cytosolic calcium content in Arabidopsis root hairs using confocal-mediated fluorescence ratio imaging analysis. The cytoplasmic calcium content of Arabidopsis seedlings was also measured using recombinant aequorin-based methods. We found no significant differences in $[\text{Ca}^{2+}]_{\text{cyt}}$ in root hairs during the 20-min interval following exposure to 100 mM NaCl. However, prolonged exposure to salt stress showed that CBP transgenic plants maintain a $[\text{Ca}^{2+}]_{\text{cyt}}$ similar to that during the initial salt stress, while wild type controls showed reduced $[\text{Ca}^{2+}]_{\text{cyt}}$ increases after 3 days on media with 100 mM NaCl, as measured by confocal ratio analysis. Moreover, when plants were grown on media depleted of $\text{Ca}^{2+}$, CBP transgenic plants maintained $[\text{Ca}^{2+}]_{\text{cyt}}$ while wild type controls showed
reduced $[\text{Ca}^{2+}]_{\text{cyt}}$. We conclude that CBP transgenic plants maintain $[\text{Ca}^{2+}]_{\text{cyt}}$ levels under prolonged NaCl stress by using extra calcium stored in the ER.

Results

$[\text{Ca}^{2+}]_{\text{cyt}}$ measurements using confocal ratio analysis

To determine whether the salt tolerance characteristic of CBP transgenic plants was due to stronger $[\text{Ca}^{2+}]_{\text{cyt}}$ spikes following a salt stress stimulus or to sustained signaling for longer periods of time compared to control plants, we measured $[\text{Ca}^{2+}]_{\text{cyt}}$ concentrations during the administration of a high salt stimulus. We first used a confocal ratio imaging method to detect $[\text{Ca}^{2+}]_{\text{cyt}}$ by acid loading the fluorescent dye Indo-1 into Arabidopsis root hairs. Arabidopsis seedlings were embedded in 8% agarose gel to immobilize roots and promote root hair growth toward the cover slip placed under a microscope slide drilled with a 1.5” hole (Fig. 1). We could not use GFP-CBP transgenic lines for this experiment because GFP fluorescence interfered with Indo-1 fluorescence when excited by UV. A 35S-ER-CBP construct identical to the GFP-CBP gene was made that retained the ER-signal sequence from chitinase fused to the C-terminal 133 amino acids of the maize CRT1 gene containing an HDEL ER-retention sequence. Two independent lines of CBP transgenic plants, lines 3 and 4, were selected that showed Mendelian segregation of the transgene. Both lines showed similarities with the GFP-CBP transgenic plants with respect to salt and drought tolerance, seed mass, and chlorophyll content (see Chapter 2 for the characterization of these traits).

Arabidopsis seedlings on the microscope slide were grown for 2 days in a growth
Figure 1. A representative image of the growth environment for Arabidopsis seedlings used for confocal ratio analysis. Two Arabidopsis seedlings, one wild type control (left) and one CBP transgenic Arabidopsis (right) were embedded in 0.8% agarose on a slanted slide in a Magenta box to maintain humidity. Indo-1 was added to the hole in the microscope slide where seedlings were embedded.

chamber and the fluorescent dye Indo-1 was added to the hole in the microscope slide where seedlings were embedded in 0.8% agarose. We measured the ratio of the emission spectra at 400 - 445 nm and 460 - 500 nm in root hairs of Arabidopsis at a distance of 20 µm from the apex of root hair. If there was a difference in the [Ca\(^{2+}\)]\(_{cyt}\), it would be easily detected in this area because the [Ca\(^{2+}\)]\(_{cyt}\) is kept near the resting state (100 - 200 nM) in this region (Halperin et al. 2003). Moreover, calcium measurements in the vicinity of the root hair apex can be strongly affected by external Ca\(^{2+}\) influx and efflux.
Figure 2. A. Confocal microscope fluorescence image of a Col-0 Arabidopsis root hair acid-loaded with Indo-1 fluorescent dye. The first image to the left represents Indo-1 fluorescent dye emission at 440 - 445 nm. The image in the middle is emission at 460 - 500 nm and the image to the right is a Differential Interference Contrast (DIC) image of the Arabidopsis root hair. B. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 30 seconds at 10 second interval after 100 mM NaCl treatment. $[\text{Ca}^{2+}]_{\text{cyt}}$ at each time point represents an average measurement of 10 seedlings for 5 plants. A ratio of two emission spectra was used to calculate $[\text{Ca}^{2+}]_{\text{cyt}}$ from a standard curve. No significant difference was found among CBP transgenic plants and wild type control plants. C. $[\text{Ca}^{2+}]_{\text{cyt}}$ measurements for 20 minutes at 5 minute interval after 100 mM NaCl treatment. For each time point, we averaged $[\text{Ca}^{2+}]_{\text{cyt}}$ of 10 seedlings for 5 plants per line. A ratio of two emission spectra was used to calculate $[\text{Ca}^{2+}]_{\text{cyt}}$ from a calibration curve. No significant difference was found among CBP transgenic plants and wild type control plants.
ER-CBP transgenic plants do not exhibit differences in $[Ca^{2+}]_{cyt}$ compared to wild type control Col-0 under a short term NaCl treatment.

There was no significant difference in fluorescence ratios between CBP transgenic plants and wild type Arabidopsis in $[Ca^{2+}]_{cyt}$ measured in a 5 X 5 μm region 20 μm from the apex of the root hair after 30 seconds of 100 mM NaCl (Fig. 2B). The exposure time was extended to 20 minutes at 5-minute intervals but there was still no significant difference in the concentration of $[Ca^{2+}]_{cyt}$ between CBP transgenic plants and control plants under salt stress (Fig. 2C).

ER-CBP transgenic plants exhibit significant differences in $[Ca^{2+}]_{cyt}$ compared to wild type control Col-0 after long-term salt stress.

To see whether prolonged growth on NaCl-containing media would affect $[Ca^{2+}]_{cyt}$, seedlings were treated as above but then placed on MS media with or without 100 mM NaCl. As before, there was no significant difference in $[Ca^{2+}]_{cyt}$ of CBP transgenic and wild type plants in MS medium after 3 days (Fig. 2D). However, CBP-transgenic plants had higher levels of $[Ca^{2+}]_{cyt}$ than wild type plants grown on MS media + 100 mM NaCl (Fig. 2E). Both CBP transgenic lines on NaCl had $[Ca^{2+}]_{cyt}$ levels that were similar to plants grown without NaCl, whereas wild type plants showed significantly reduced calcium concentrations (Fig. 2E). This demonstrated that although the initial response to NaCl treatment was similar between CBP transgenic plants and wild type controls, a longer period of NaCl stress (3 days) did result in changes in the $[Ca^{2+}]_{cyt}$. 

111
**Fig 2.** D. [\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) measurement of Arabidopsis root hair after 3 day growth in MS only media. No significant difference was detected. An average of 10 individual hairs for 5 plants per experiment. Error bars are SEM. Experiments were repeated twice. E. [\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) of CBP transgenic Arabidopsis and Col-0 Arabidopsis ecotype after incubation in MS media + 100 mM NaCl for 3 days. [\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) was measured for 20 minutes for at five minute interval. Wild type Arabidopsis showed significantly decreased [\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) compared to CBP transgenic plants (p < 0.001) Each point represents measurements of 10 root hairs from 5 plants. Error bars are SEM. The experiments were repeated twice with similar results.

[\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) measurements using Aequorin transgenic plants

To verify that the maintenance of [\(\text{Ca}^{2+}\)]\(_{\text{cyt}}\) levels under long-term salt stress of CBP transgenic plants was significant, and not an artifact of spurious autofluorescence, we used a second method to determine cytoplasmic calcium levels. Transgenic Arabidopsis lines expressing aequorin in the cytoplasm have been used to accurately measure calcium (Kiegle et al. 2000). We crossed 35S- CBP transgenic plants to 35S-Aequorin\(_{\text{cyt}}\) Arabidopsis plants and generated both CBP-Aequorin\(_{\text{cyt}}\) and GFP-CBP- Aequorin\(_{\text{cyt}}\) expressing transgenic lines. We verified the transgene expression level of aequorin in each line and selected ones that
expressed aequorin stably.

**ER-CBP transgenic plants (GFP-CBP and CBP), GFP, and wild type plants show no significant difference in cytoplasmic calcium spikes.**

We used two different concentrations of salt solution to test the hypothesis that CBP transgenic plants would have different $[\text{Ca}^{2+}]_{\text{cyt}}$ following salt stress. However, there was no significant difference in response to 150 mM or 300 mM NaCl (final concentration) (Fig. 3B and C). The cytoplasmic calcium spike was similar in all four lines for both 150 mM NaCl and 300 mM NaCl treatments. The $[\text{Ca}^{2+}]_{\text{cyt}}$ fluctuated in several lines after the first spike, but this occurred in both CBP transgenic and control plants. Although the $[\text{Ca}^{2+}]_{\text{cyt}}$ peaks were higher following 300 mM NaCl than for 150 mM NaCl, there was no significant difference between CBP and control plants. This suggests that the plant does modify the amplitude of cytoplasmic Ca in response to NaCl, and that the change is proportional to the size of the stimulus. However, the increased ER calcium did not affect the cytoplasmic calcium level.

**ER-CBP transgenic plants (GFP-CBP and CBP) showed higher $\text{Ca}^{2+}$ spikes than control plants after growth in $\text{Ca}^{2+}$ depleted media.**

To determine if calcium depletion would impact $[\text{Ca}^{2+}]_{\text{cyt}}$ signaling, we grew seedlings in MS medium lacking $\text{Ca}^{2+}$ for 5 days and then measured luminescence. Although the $[\text{Ca}^{2+}]_{\text{cyt}}$ peak elicited by 150 mM NaCl was reduced in both CBP transgenic plants and control plants, control plants showed significantly more of a decrease than CBP plants (Fig. 4A and Fig. 4B). The duration of the $[\text{Ca}^{2+}]_{\text{cyt}}$ spike was also longer in CBP transgenic plants.
Fig 3. A. RT-PCR verification of aequorin gene expression in transgenic plants. Actin was used as an internal control for RNA. Lane 1 - Aequorin- CBP#3, 2 - Aequorin- GFP-CBP#2, 3 - Aequorin- CBP#4, 4 - Aequorin- GFP-CBP#2, 5 - Aequorin- GFP. B. [Ca$^{2+}$]$_{cyt}$ measurement using Aequorin luminescence in CBP transgenic plants, wild type and GFP control plants. 150 mM NaCl solution is applied to seedlings at 15 seconds (Indicated by an arrow). There was no significant difference in calcium spikes seen after the salt treatment. 5 replicates per line were used for each of three independent experiments. C. [Ca$^{2+}$]$_{cyt}$ measurement using Aequorin luminescence in CBP transgenic plants, wild type and GFP control plants. 300 mM NaCl is applied to seedlings at 15 seconds (Indicated by an arrow). There was no significant difference in calcium spikes to salt treatment among the four lines, Wild type Col-0 Arabidopsis, CBP transgenic Arabidopsis, GFP-CBP Arabidopsis and GFP Arabidopsis. 5 replicates per line were used for each of three independent experiments.
A. 

<table>
<thead>
<tr>
<th>RT PCR</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aequorin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B. NaCl 150 mM

\[ [\text{Ca}^2+]_{\text{cyt}} (\text{nM}) \]

C. NaCl 300 mM

\[ [\text{Ca}^2+]_{\text{cyt}} (\text{nM}) \]
compared to control plants (Fig. 4A). We used a higher NaCl solution of 300 mM NaCl to determine whether these two differences, a longer [Ca\(^{2+}\)]\(_{\text{cyt}}\) peak duration and a higher [Ca\(^{2+}\)]\(_{\text{cyt}}\) peak, can be amplified in CBP transgenic plants. Contrary to our hypothesis, although the [Ca\(^{2+}\)]\(_{\text{cyt}}\) peak was significantly higher in CBP transgenic plants compared to control plants, the duration of the [Ca\(^{2+}\)]\(_{\text{cyt}}\) peak was shorter in both CBP transgenic plants and control plants when compared to the lower 150 mM NaCl treatment (Fig. 4B). It is likely that there is a limited amount of Ca\(^{2+}\) available because plants were grown in the Ca\(^{2+}\) depleted media.
Fig 4. [Ca\textsuperscript{2+}]\textsubscript{cyt} measurements using Aequorin luminescence after 150 mM and 300 mM NaCl salt treatment after 5 days of calcium depletion. A. [Ca\textsuperscript{2+}]\textsubscript{cyt} measurement using aequorin luminescence in CBP transgenic plants, wild type Arabidopsis Col-0 and GFP control plants. 150 mM NaCl solution was applied to seedlings grown in calcium-depleted medium at 15 seconds. There were significant differences in calcium spikes in response to salt treatment between CBP transgenic plants (CBP and GFP-CBP transgenic plants) and control plants (wild type Col-0 Arabidopsis and GFP transgenic plants). 5 replicates per line were used for each of three independent experiments. B. [Ca\textsuperscript{2+}]\textsubscript{cyt} measurement using aequorin luminescence in CBP transgenic plants, wild type and GFP control plants after growing them in calcium-depleted medium for 3 days. 300 mM NaCl was applied to seedlings at 15 seconds. There were significant differences in calcium spikes after the 300 mM salt treatment between CBP transgenic plants and control plants (GFP:CBP P < 0.01, GFP:WT P < 0.24, GFP-GFP-CBP P < 0.01, WT:GFP-CBP P<0.01, WT:CBP P< 0.01). GFP-CBP transgenic plants showed a higher calcium spike compared to control plants. 5 replicates per line were used for each of three independent experiments.
A. NaCl 150 mM

B. NaCl 300 mM
Discussion

Ca^{2+} is important for plant development, plant stress responses and signal transduction (Sanders et al. 2000). Exogenously added Ca^{2+} enhances both root hair growth, root growth and shoot growth in plants under salt stress (Cramer et al. 1996 and Zidan et al. 1991). To determine if increased intracellular stores of calcium could have a similar effect, we used transgenic CBP lines that have higher levels of intracellular calcium (Persson et al. 2001, Wyatt et al. 2002) and show increased salt tolerance (Chapter 2 and Chapter 4). To test whether the increased internal store of calcium in CBP plants altered [Ca^{2+}]_{cyt} in root hairs under salt stress, we acid-loaded Indo-1 into root hairs and measured [Ca^{2+}]_{cyt} using confocal microscopy ratio imaging. The [Ca^{2+}]_{cyt} level in root hairs was not significantly different between CBP transgenic plants and control plants during a 20-min exposure to 150 mM NaCl (Fig. 2B and 2C). To determine whether a longer period of salt stress would affect [Ca^{2+}]_{cyt} level, we extended the 100 mM NaCl treatments for 3 days and repeated confocal microscopy ratio imaging. We observed that CBP transgenic plants had a slight decrease in [Ca^{2+}]_{cyt} after the 100 mM NaCl treatment compared to untreated plants, but the wild type control plants exhibited a significantly higher reduction in [Ca^{2+}]_{cyt} level (Fig. 2D and Fig. 2E). This suggested that the salt-tolerant CBP transgenic plants used the extra stored calcium to maintain [Ca^{2+}]_{cyt}.

We used a different method for measuring changes in [Ca^{2+}]_{cyt} following NaCl treatment that used homozygous progeny from a CBP x aequorin_{cyt} plants. Arabidopsis seedlings expressing the calcium reporter protein aequorin have been used in numerous
studies to measure $[Ca^{2+}]_{\text{cyt}}$ after various stimuli (Mazars et al. 2002). We used four aequorin lines, GFP-CBP-aequorin$_{\text{cyt}}$, CBP-aequorin$_{\text{cyt}}$, GFP-aequorin$_{\text{cyt}}$, and Col-0-aequorin$_{\text{cyt}}$, and observed no significant differences in $[Ca^{2+}]_{\text{cyt}}$ spikes for any of them following either a 150 mM or 300 mM NaCl treatment (Fig. 3A and Fig. 3B). However, there was a difference in $[Ca^{2+}]_{\text{cyt}}$ after growing seedlings in MS media without Ca$^{2+}$ for 5 days. CBP-Aequorin$_{\text{cyt}}$ Arabidopsis lines retained cytoplasmic Ca$^{2+}$ spike levels while control plants had reduced $[Ca^{2+}]_{\text{cyt}}$ spikes. The increased Ca$^{2+}$ stores of CBP plants may have functioned to protect against the depleted extracellular Ca$^{2+}$ by contributing to the $[Ca^{2+}]_{\text{cyt}}$ signal.

Both the confocal ratio analysis and the aequorin luminescent measurements demonstrated that even with higher ER calcium, the cytoplasmic levels were similar in CBP and wild type plants following a brief exposure to stress. However, after 5 days in Ca$^{2+}$ depleted media, cytoplasmic calcium spikes were significantly reduced in wild type plants. In contrast, CBP-transgenic plants showed spikes with a higher magnitude, more similar to non-stressed plants, suggesting that they could access calcium stored in the ER to compensate for the Ca$^{2+}$ depletion.

In addition we found that the duration of the $[Ca^{2+}]_{\text{cyt}}$ spike was longer in the CBP-Aequorin$_{\text{cyt}}$ transgenic plants treated with 150 mM NaCl (Fig. 4A). However, when higher NaCl concentrations were used, the difference in spike duration was lost (Fig. 4B). This suggests that when calcium is depleted for a period of days, the cells respond by limiting Ca$^{2+}$ release into the cytosol. The extra ER calcium could either increase the duration of Ca$^{2+}$ signaling or the $[Ca^{2+}]_{\text{cyt}}$ peak, but not both because the extra calcium in CBP transgenic
plants is still limited.

**Materials and Methods**

**RNA Isolation and semi-quantitative RT-PCR**

RNA was isolated from harvested leaves using the plant RNeasy mini kit (Qiagen, Valencia, CA) with the on-column RNase free DNase I treatment. Total RNA was extracted from Arabidopsis seedlings using the QIAshredder and RNeasy plant mini kits (Qiagen, CA/USA). DNA was removed by performing an on-column digestion using a DNase kit (Qiagen). cDNA was synthesized according to the ImProm-II™ Reverse Transcription System RT kit protocol on 300 ng of RNA with dT16-oligonucleotide as primer. Following synthesis, cDNA samples were diluted to 1,000 pg/μL with DNase/RNase-free water. RT-PCR primers were designed using a software Primer3 (v. 0.4.0) http://frodo.wi.mit.edu/cgi-bin/primer3/primer3-www.cgi to create amplicons of 150 to 200 bp. Primers used had the following characteristics: melting temperature between 55°C to 60°C, 3' G/C clamp, and 40% to 60% G/C content overall. No-reverse transcriptase enzyme (RT) controls were used on each 96-well plate to ensure that no genomic DNA was carried over. To ensure consistent tissue collection, RNA extraction, and RT activity, primers for an internal control (the Actin8 gene, accession number AT1G49240) Primers for Actins are CTTTCCGTTACACGTTTG-3' and 5'-GAAACGGTATAGTGCCCT-3'.

**Indo-1 Dye Loading**

Seedlings were acid-loaded with Indo-1 (Teflabs Inc., Austin, TX) as described previously (Wymer et al., 1997). A 1 mM stock solution of 2'-7'-bis-(2-carboxyethyl)-5-(and-
6)-carboxyfluorescein-acetoxymethylester (Teflabs Inc., Austin TX) in dimethylsulphoxide (DMSO) was diluted to a final concentration of 2.5 μM in growth medium and was added to the roots and allowed to load for at least 15 min at room temperature.

**Standard curves for ratio imaging**

In vitro and in vivo calibration of Indo-1 was performed as previously described (Wymer et al., 1997, Bibikova et al., 1997 and 1998). The in vitro curve was used for converting ratio measurements into Ca$^{2+}$ concentration because it lacked the additional variation resulting from the difficulties of accurately buffering Ca$^{2+}$ inside a plant cell (as also described in Wymer et al., 1997).

**Indo-1 Confocal Ratio Imaging Measurement**

Arabidopsis roothairs loaded with indo-1 were imaged using a Leica TCS SP1 confocal microscope system and a 40x 1.2 NA oil immersion objective. The samples were excited with a UV laser and the fluorescence emission was collected from 400 - 445 nm (E1) and from 460 - 500 nm (E2). DIC images were collected simultaneously with a transmitted light detector. Average cytosolic Ca$^{2+}$ levels were measured in a 5x5 μm region 20 μm from the tip of the hair from confocal ratio images from at least 10 individual hairs from > 5 separate plants per treatment. Plants were treated with 0, 100 mM, and 150 mM NaCl.

**Transgenic Aequorin$c_{yt}$ ER-CBP Crosses**

Wild type plants transformed with 35S-aequorin$c_{yt}$ were obtained from Dr. I. Perera (Perera et al. 2008). They were crossed to CBP and GFP- CBP transgenic lines, a ctrl mutant (Salk_093215), and GFP transgenic control lines. Progeny were screened by PCR
because the selectable marker for both transgenes was kanamycin resistance. Primers for aequorin are 5’ TCAACTTCCTCGACGTAAC 3’ and 5’ TCATCTCATCGACATCGAGC 3’. The expression levels of aequorin in progeny from these crosses were stable for 4 generations. We used homozygous F₂ and F₃ plants for [Ca²⁺]cyt measurement.

**Aequorin Luminescence Measurements**

Seedlings from Aequorin<sub>cyt</sub> transgenic control, Aequorin<sub>cyt</sub>-CBP, Aequorin<sub>cyt</sub> GFP-CBP, and Aequorin<sub>cyt</sub> GFP-transgenic lines were incubated overnight in 2.5 µM coelenterazine (Nanolight Technology, Pinetop, AZ) in the dark with gentle shaking. Individual seedlings were placed in the bottom of a luminometer cuvette containing DI water and allowed to settle. Luminescence was counted at 0.2-sec intervals for 2 min. 1 ml of DI water, 300 mM or 600 mM NaCl solution was injected to final NaCl concentrations of 0, 150 mM and 300 mM at 15 seconds. For calibration, the remaining aequorin was discharged with 100 µl of 2M CaCl₂, 20% ethanol at 1.5 min. Luminescence counts were converted to Ca²⁺ concentrations according to Knight et al., 1993 and Knight et al., 1997.

**Statistical Analysis for Aequorin Luminescence**

This experiment had two factors. Plant line types and two different salt treatments. Plant line types are GFP-CBP transgenic plants, CBP transgenic plants, GFP transgenics plants, and Wild type Col-0. Two salt treatments used are 150 mM and 300 mM NaCl. A split-plot experimental design was used with the five replications serving as whole plot units, each receiving exactly one salt treatment and
had all GFP-CBP transgenic plants, CBP transgenic plants, GFP transgenic plants and WT measured. A mixed model appropriate to this design was used, with fixed effects for the salt treatment and line factors, and random effect for replication. The luminescence measurements exhibited variability that was increasing with the mean, so a log transformation was used. SAS/STAT® Software was used for analysis.

Acknowledgements

We thank Dr. Eva Johannes for help with indo-1 imaging, Dr. Imara Perara for providing cytoplasmic aequorin-expressing Arabidopsis, and Dr. Jason Osborn for help with statistical analyses.
References


Chapter 4

ER-CBP Transgenic Plants Exhibit Changes in Ion Contents and Increased Production of Trehalose

Key Words: Calreticulin, calcium-binding peptide, calcium storage, endoplasmic reticulum, Arabidopsis, salt tolerance, drought tolerance, trehalose.

Abbreviations: Ca$^{2+}$, calcium; [Ca$^{2+}$]_{cyt} cytosolic calcium; CBL, Calcineurin B Like; CRT, calreticulin; ER, endoplasmic reticulum; GFP, green fluorescent protein; immunoglobulin binding protein; ATP, adenosine triphosphate; CBP, calcium binding peptide
Abstract

Salinity is known to cause three major problems in plants: osmotic stress, nutrition imbalance and Na⁺ toxicity. Na⁺ toxicity is due to an excess accumulation of Na⁺ inside plant cells resulting in competition with K⁺ for many cellular processes. Supplementation with external Ca²⁺ alleviates salt stress and restores the optimal K⁺/Na⁺ ratio in plants by increasing selectivity for K⁺ transport and preventing K⁺ efflux out of the cell under salt stress. We have increased internal calcium stores by generating ER-localized, Calcium Binding Peptide (GFP-CBP) transgenic plants, which have better salt tolerance. To determine whether the salt tolerance of CBP transgenic plants was due to lower Na⁺ content while maintaining normal K⁺ content in the plant, we measure total ion content using Inductively Coupled Plasma (ICP) spectroscopy. Furthermore, we investigate how CBP transgenic plants adjust osmotic pressure to maintain growth under salinity stress. CBP transgenic plants showed an increase in K⁺ as well as Ca²⁺ but had decreased Na⁺ when compared to control plants. Trehalose, a sugar known to play an important role in osmotic adjustment, increased in CBP transgenic plants compared to control plants. Together, our experiments demonstrate that CBP transgenic plants have altered K⁺ flux and show an increase in drought-protective compounds such as trehalose.
Introduction

Potassium is important for many essential cellular processes and is the most abundant cation in plant cells. It is essential for plant cells to have optimal K⁺/Na⁺ in the cytoplasm (Marschner et al. 1995) because K⁺ and Na⁺ competitively bind to enzymes. If the Na⁺ content is too high, then essential cellular processes, such as protein synthesis, are disrupted. It has been reported that salt-tolerant crop lines often have more efficient mechanisms to exclude Na⁺ from the shoot than less tolerant crop lines (Tester et al. 2003). Extra Ca²⁺ supplementation to plant growth media or soil can alleviate salt stress and help plants to maintain growth (Cramer et al. 1987, Reid and Smith 2000, Shabala et al. 2003). Alleviation of salinity by extra Ca²⁺ supplementation is thought to occur by two possible mechanisms. One is by restricting Na⁺ uptake via the Non-Selective Cation Channel (NSCC), which has been reported as a main route for Na⁺ uptake under saline conditions (Apse and Blumwald 2007, Demidchik and Tester 2002, Tester and Davenport 2003). Another proposed mechanism is reducing Na⁺-induced K⁺ efflux via outwardly directed, K⁺-permeable channels (Shabala et al. 2006), which can be measured by a patch clamp method. Neither of the suggested mechanisms, however, address how extra Ca²⁺ supplementation helps plants to endure osmotic stress, which leads to a water deficit inside the plant cell.

There are two ways that supplemented Ca²⁺ is thought to mitigate osmotic stress. The first mechanism is in the production of compatible solutes such as trehalose (Serrano et al., 1999), which acts as an osmolyte or regulator to protect against oxidative and salinity stress (Hanson et al. 1982, Shabala et al. 2006a). For example, expression of the yeast TPS1 gene,
which encodes trehalose-6-phosphate synthase, in tobacco (*Nicotiana tabacum*) and rice plants (Garg et al. 2002) resulted in improvements in drought and salt tolerance (Romero et al., 1997; Serrano et al., 1999). However, the trehalose content in the tobacco transgenic plants was low (< 0.5 mM). Similarly, the level of trehalose in transgenic rice plants remained well below 1 mg/g fresh weight. These low concentrations could not osmotically balance the salt and suggested that trehalose may play a regulatory role in the stress response (Garg et al. 2002). Interestingly, Ca$^{2+}$, K$^+$ and Mg$^{2+}$ activated trehalose-6-phosphate synthase (SfTps1) in *Saccharomyces fibuligera* sdu, a high-trehalose-accumulating strain (Liang et al. 2006). The second mechanism by which extra Ca$^{2+}$ can alleviate osmotic stress is by increasing the uptake of inorganic ions. This mechanism has a low energetic cost because plants do not need to produce compatible solutes, such as proline, which require ATP. The difference in the ATP “cost” between active uptake and compartmentalization of inorganic ions and synthesis of compatible solutes is suggested to be approximately 10 fold (Raven et al. 1995). The production of compatible solutes may have physiological significance for long-term stress; however, uptake of inorganic ions is the faster way to execute osmotic adjustment.

We expressed ER-targeted CBP (Calcium Binding Peptide) to increase the ER calcium content in Arabidopsis and were able to increase total calcium by up to 10%. CBP transgenic plants also exhibited better tolerance to drought and salt stress (Chapter 2). When we performed ICP measurements for other ions, we noticed an increase in K$^+$ content in CBP expressing plants. CIPK6, which interacts with several calcium sensors (Kim et al. 2000) and
with the C-terminus of AKT1 (Lee et al. 2007) was necessary for the $K^+$ increase in CBP transgenic plants.

Importantly, CBP expressing plants exhibited an increased trehalose content, which has been reported to be important for drought and salt stress tolerance. We propose that increasing the intracellular $Ca^{2+}$ content in CBP expressing plants has several effects on ions, such as increasing $K^+$ and reducing $Na^+$ content. Furthermore, because of the low cytoplasmic concentration of trehalose, its role in osmotic stress remains unknown. Although higher than controls, the trehalose levels in CBP transgenic plants is still too low to act as an osmoprotectant. This suggests a regulatory role for trehalose in Arabidopsis, which appears to be associated with $Ca^{2+}$ levels in the cell.

Results

**ER-CBP transgenic plants show reduced $Na^+$ accumulation under NaCl stress**

We found that the concentration of three cations, which include $Ca^{2+}$, $K^+$ and $Na^+$, were different in CBP expressing plants compared to control plants grown under normal conditions (Chapter 2). $Ca^{2+}$ and $K^+$ levels were increased, and $Na^+$ levels were decreased in the CBP expressing plants. The increase in $K^+$ was equivalent to the decrease in $Na^+$, suggesting a balance mechanism. To further explore the relationship between $Na^+$ exclusion and NaCl tolerance, we grew CBP transgenic plants in different concentrations of NaCl and measured $Na^+$ content after one day. Fig. 1 shows that the percent $Na^+$ in dry weight was significantly lower in CBP plants compared to that of control plants. The percent $Na^+$ began to increase at 75 and 100 mM NaCl but was still lower than either the GFP-expressing or

133
wild-type plants. Fig. 1 also shows that CBP expressed in a mutant cipk6 background was not sufficient to confer an ability to exclude Na\(^+\). Together, these results demonstrate that CIPK6 is needed to achieve lower [Na\(^-\)] in the CBP transgenic plants (Fig. 1). We previously showed that constitutive expression of CIPK6 is needed for salt tolerance in CBP transgenic plants (Chapter 2). When CBP was expressed in a cipk6 mutant, Na\(^+\) levels remained the same instead of decreasing. This suggests that increased CIPK6 expression is responsible for excluding Na\(^+\) and for increasing salt tolerance.

**Fig 1. CBP transgenic seedlings show reduced accumulation of Na\(^+\) under NaCl stress.** Na\(^-\) content in CBP transgenic plants (CBP and GFP-CBP), GFP control plants, WT (wild-type), CBP x cipk6 transgenic mutant, and cipk6 mutant. The CBP transgenic plants had significantly lower amounts of Na\(^+\). Each point is the average of 50 seedlings. Error bars are SEM.
**Increased expression of TPS (Trehalose 6-phosphate synthase) and TPP (Trehalose-6-phosphate phosphatase (TPP) in CBP transgenic plants.**

It has been demonstrated that over-expressing yeast TPS and TPP confers tolerance to multiple and extreme abiotic stress conditions (e.g. salt and drought) in transgenic Arabidopsis thaliana (Miranda et al. 2007). We found that CBP expressing plants showed an increase in TPS and TPP expression when grown on MS media without any salt or sorbitol treatment (Fig. 2). CIPK6 is required for the CBP-expressing plants to increase the expression of the TPP and TPS genes.

![Image of RT-PCR results](image.png)

**Fig 2.** Semi-quantitative RT-PCR of trehalose 6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) in wild type Col-0, CBP transgenic plant, cipk6 mutant and CBP X cipk6 mutant. Lane 1. cipk6 mutant, 2, CBP X cipk6, 3. Wild type Col-0, 4. CBP
Trehalose content is increased in CBP transgenic plants

We measured both the trehalose and raffinose content in CBP-expressing plants to determine whether the salt and drought tolerance of CBP-transgenic plants was associated with trehalose content. We observed significant increase in the trehalose content in CBP expressing plants. Moreover, CIPK6 is required for the CBP-expressing plants to increase the trehalose production.

Recovery of ER-CBP transgenic plants from severe drought stress

CBP transgenic plants have up to 70% more trehalose compared to control plants. CBP transgenic plants were able to recover from the extreme drought stress (watered

Figure 3. Trehalose measurement in wild type Col-0, CBP transgenic plants, cipk6 mutant and CBP X cipk6 mutant grown on normal MS media. Transgenic plants expressing CBP exhibit increased trehalose content. Raffinose has been used as a control.
once a week for 4 weeks). CBP transgenic plants that appeared to be dead showed new growth when they were watered again regularly (Fig. 4).

**Figure 4. Recovery of CBP transgenic plants after intermittent drought stress.** 5-week old seedlings were watered only once a week in long-day conditions (16-hr day, 25°C / 8-hr night, 22°C) for 4 weeks. CBP transgenic plants recovered from drought stress and were able to form new rosettes while wild type control plants died.

**Discussion**

CBP transgenic plants were drought tolerant and able to recover from a drought stress (Fig. 4) when wild type plants could not recover. This raised a possibility that there were compatible solutes that were differentially accumulating in CBP transgenic plants. We hypothesized that trehalose was one of them because our intial microarray data on CBP transgenic plants showed increased transcription of the TPP gene (data not shown). RT-PCR
confirmed that *TPP* and *TPS* transcription was increased in CBP transgenic plants (Fig. 2). Most organisms produce trehalose when they are stressed using these two key enzymes (Sazzad et al. 2007). In plants, increased trehalose provides protection against dessiccation due to its high capacity for water replacement and vitrification (Miranda et al. 2007, Suarez et al. 2008, Watanabe et al. 2003). Watanabe et al. demonstrated that an increase in internal ion concentration triggers trehalose synthesis in larvae of *Polypedilum vaderplaki*. This is consistent with increased production of trehalose in ER-CBP transgenic plants, but the mechanism behind it still remains elusive. Low (5 mM) concentrations of trehalose significantly reduce NaCl-induced K⁺ efflux and OH⁻-induced K⁺ efflux in Arabidopsis roots (Cuin et al. 2005, 2007a and 2007b). Thus, there is a possibility that increased trehalose is contributing to increased K⁺ in CBP transgenic plants. Potassium is important for many essential cellular processes and is the most abundant cation in plant cells. It is required for plant cells to have optimal K⁺/Na⁺ in the cytoplasm due to the fact that K⁺ and Na⁺ competitively bind to enzymes and if the Na⁺ content is too high, then essential cellular processes such as protein synthesis are disrupted. It has been reported that salt-tolerant crop lines tend to have a more efficient mechanism to exclude Na⁺ from the shoot than less tolerant crop plants (Tester et al. 2003). It is necessary for plants to regulate the homeostasis of cations and anions to maintain cellular process and osmotic pressure inside the cell. Thus, plant cells have mechanisms that enable them to sense and regulate homeostasis. When a plant has higher amounts of cations, what effect does it have on K⁺/Na⁺ homeostasis? Here we increased the calcium content in plants by expressing CBP targeted to the ER. We have
noticed about a 9-10% increase in calcium content in these plants, compared to GFP controls and wild-type plants (Chapter 2). We have also measured the K\(^+\) and Na\(^+\) content in CBP-transgenic plants and found that the potassium content is increased while sodium content is decreased (Chapter 2). Comparisons between Arabidopsis and its salt-tolerant relative *Thellungiella halophila* have shown that *Thellungiella* has increased mesophyll K\(^+\) content under saline conditions while salt-sensitive Arabidopsis shows a reduction in mesophyll K\(^+\) content (Volkov et al. 2003). This suggests that CBP transgenic plants achieve salt tolerance by altering ionic homeostasis to favor retention of K\(^+\) and exclusion of Na\(^+\). More studies need to be done to understand the relationship between Ca\(^{2+}\) and trehalose accumulation, but the activation of AKT1 by CIPK6 through expression of CBP is consistent with a role for intracellular Ca\(^{2+}\) in signaling to provide optimal responses to salt stress.

**Material and Methods**

*Combined cycling drought assay (CD) and yield analysis from BASF, the Chemical Company*

Each pot was filled with thoroughly mixed 50 g of 1/3 Turface and 2/3 Metro Mix 360 and then pots were placed in trays without holes (Model F1020NH, Hummer Cat#11-3050). The fertilizer solution was added approximately 2 liters to four trays containing 16 pots (fertilizer mix 0.4 g/l of miracle grow). Pots were soaked with fertilizer overnight and excess fertilizer solution was discarded from the tray. Seeds were refrigerated for several days in dH\(_2\)O and then a 1 ml pipet as used to transfer seeds to the soil surface in each pot. The tray was then covered with a standard transparent dome (Model CW221, Hummer
Cat#14-2568-1). At day 7, only one seedling was left per pot and domes were placed back on the trays until day 10. Trays were placed in a Percival growth chamber with the following conditions: Photoperiod (16 h light/ 8 h dark), temperature 22°C, relative humidity 55-60% using fluorescent bulbs only (white) approximately 120 μm/m²/s⁻¹ at rosette level. 1/2 liter of water was directly poured into the tray once a week for 4 weeks and then watered regularly every other day, Monday, Wednesday and Friday.

**Calcium, Potassium and Sodium Measurements in CBP transgenic and controls plants**

Approximately 50 seedlings from each line, grown vertically on MS media + 0, 25, 50, 75 and 100 mM NaCl, were harvested and gently washed with Nnano-pure® water. Tissue were dehydrated in a 50°C oven for 7 days and ground and weighed. 0.1g of each sample was submitted to the NCSU Analytical Service Laboratory for analysis. The samples were analyzed by ICP (Inductively coupled plasma mass spectrometry).

**Trehalose measurement**

1g of seedlings from each line were grown on MS media for 7 days at 22°C constant light after germination. Approximately 1 g of tissue from each line was harvested and was frozen in liquid nitrogen. Samples were ground in 60:40 Methanol:H₂O to yield a 200 mg/ml homogenate. For measurements, 100 μl (~20 mg fresh weight) was used for each sample. Extracts were derivatized with N-methyl-N-(trimethylsilyl) trifluoracetamide (TMS). A GC/MS scan method was used with Single Ion Monitoring to measure trehalose derivatives as described (Fiehn et al. 2000). All measurements were done at the NCSU Metabolomics & Proteomics Laboratory.
References


Watanabe, M, Kikawada, T and Okuda T (2003) Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of Polypedilum vanderplanki. The journal of Experimental Biology 206, 2281 - 2286
Appendix
Figure 1. GFP fluorescence from NT1 cells transformed with 35S-ER-GFP-CBP construct.
Table 1. Confocal ratio analysis of $[\text{Ca}^{2+}]_{\text{cyt}}$ (nM) after 100 mM NaCl treatment for 30 seconds and 20 minutes.

<table>
<thead>
<tr>
<th></th>
<th>30 seconds</th>
<th>0 sec</th>
<th>10 sec</th>
<th>20 sec</th>
<th>30 sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (wild type)</td>
<td>180.8 ± 1.81</td>
<td>179 ± 2.62</td>
<td>178.1 ± 2.2</td>
<td>177.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>ER - CBP</td>
<td>179.2 ± 1.76</td>
<td>177.9 ± 1.54</td>
<td>178.2 ± 2.7</td>
<td>176.2 ± 1.72</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>20 minutes</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (wild type)</td>
<td>182.4 ± 2.2</td>
<td>180.5 ± 1.71</td>
<td>178.8 ± 1.9</td>
<td>176.4 ± 2.3</td>
<td>177.4 ± 1.52</td>
<td></td>
</tr>
<tr>
<td>ER - CBP</td>
<td>180.6 ± 1.9</td>
<td>182.2 ± 1.66</td>
<td>179.2 ± 1.7</td>
<td>178.2 ± 2.5</td>
<td>178.6 ± 1.74</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Confocal ration analysis of $[\text{Ca}^{2+}]_{\text{cyt}}$ (nM) after 3 days in MS media only and MS media + 100 mM NaCl.

<table>
<thead>
<tr>
<th>MS Media</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (wild type)</td>
<td>178.2 ± 2.3</td>
<td>181 ± 1.71</td>
<td>184.6 ± 1.9</td>
<td>178.3 ± 2.3</td>
<td>178.6 ± 1.52</td>
</tr>
<tr>
<td>ER - CBP</td>
<td>180.4 ± 1.9</td>
<td>183.5 ± 1.66</td>
<td>182.8 ± 1.7</td>
<td>183.2 ± 2.5</td>
<td>180.4 ± 1.74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>100 mM NaCl*</th>
<th>0 min</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
<th>20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 (wild type)</td>
<td>164.8 ± 2.2</td>
<td>165.8 ± 1.71</td>
<td>166.4 ± 1.9</td>
<td>164.2 ± 2.3</td>
<td>163.2 ± 1.52</td>
</tr>
<tr>
<td>ER - CBP</td>
<td>173 ± 1.9</td>
<td>175.5 ± 1.66</td>
<td>173.8 ± 1.7</td>
<td>172.2 ± 2.5</td>
<td>171.4 ± 1.74</td>
</tr>
</tbody>
</table>

* There is a significant difference between Col-0 and ER-CBP after 100 mM NaCl treatment for three days. ($P < 0.001$)
Table 3. Summary of aequorin luminescence converted into \([\text{Ca}^{2+}]_{\text{cyt}}\) peak after 150 mM NaCl and 300 mM NaCl treatment.

A. Peak \([\text{Ca}^{2+}]_{\text{cyt}}\) Concentration (\(\mu\text{M}\))

<table>
<thead>
<tr>
<th>Plant lines</th>
<th>150 mM NaCl</th>
<th>300 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (Col-0)</td>
<td>GFP</td>
</tr>
<tr>
<td>Peak ([\text{Ca}^{2+}]_{\text{cyt}}) Concentration ((\mu\text{M}))</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.81 ± 0.04</td>
<td>2.84 ± 0.03</td>
</tr>
</tbody>
</table>

B. P-Value

P- Value

<table>
<thead>
<tr>
<th>150 mM NaCl Treatment</th>
<th>GFP-CBP</th>
<th>CBP</th>
<th>GFP</th>
<th>WT</th>
<th>300 mM NaCl Treatment</th>
<th>GFP-CBP</th>
<th>CBP</th>
<th>GFP</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>P &lt; 0.18</td>
<td>P &lt; 0.29</td>
<td>P &lt; 0.24</td>
<td>-</td>
<td>WT</td>
<td>P &lt; 0.25</td>
<td>P &lt; 0.35</td>
<td>P &lt; 0.23</td>
<td>-</td>
</tr>
<tr>
<td>GFP</td>
<td>P &lt; 0.39</td>
<td>P &lt; 0.34</td>
<td>-</td>
<td>P &lt; 0.35</td>
<td>GFP</td>
<td>P &lt; 0.34</td>
<td>P &lt; 0.18</td>
<td>P &lt; 0.34</td>
<td>P &lt; 0.23</td>
</tr>
<tr>
<td>GFP-CBP</td>
<td>-</td>
<td>P &lt; 0.35</td>
<td>P &lt; 0.39</td>
<td>P &lt; 0.18</td>
<td>GFP-CBP</td>
<td>-</td>
<td>P &lt; 0.42</td>
<td>P &lt; 0.34</td>
<td>P &lt; 0.25</td>
</tr>
<tr>
<td>CBP</td>
<td>P &lt; 0.35</td>
<td>-</td>
<td>P &lt; 0.18</td>
<td>P &lt; 0.35</td>
<td>CBP</td>
<td>P &lt; 0.42</td>
<td>-</td>
<td>P &lt; 0.18</td>
<td>P &lt; 0.35</td>
</tr>
</tbody>
</table>
Table 4. Summary of aequorin luminescence converted into [Ca$^{2+}$]$_{cyt}$ peak to 150 mM NaCl and 300 mM NaCl treatment after 5 day Ca$^{2+}$ depletion.

A. Peak [Ca$^{2+}$]$_{cyt}$ Concentration (μM)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>5 day Ca$^{2+}$ depletion + 150 mM NaCl</th>
<th>5 day Ca$^{2+}$ depletion + 300 mM NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (Col-0)</td>
<td>GFP</td>
</tr>
<tr>
<td>Peak Ca$^{2+}$ Concentration (μM)</td>
<td>1.97 ± 0.05</td>
<td>1.94 ± 0.02</td>
</tr>
</tbody>
</table>

B. P-Value

<table>
<thead>
<tr>
<th></th>
<th>GFP-CBP</th>
<th>CBP</th>
<th>GFP</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 day Ca$^{2+}$ depletion + 150 mM NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.14</td>
<td>-</td>
</tr>
<tr>
<td>GFP</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>-</td>
<td>P &lt; 0.14</td>
</tr>
<tr>
<td>GFP-CBP</td>
<td>-</td>
<td>P &lt; 0.07</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>CBP</td>
<td>P &lt; 0.07</td>
<td>-</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GFP-CBP</th>
<th>CBP</th>
<th>GFP</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 day Ca$^{2+}$ depletion + 300 mM NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.23</td>
<td>-</td>
</tr>
<tr>
<td>GFP</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>-</td>
<td>P &lt; 0.23</td>
</tr>
<tr>
<td>GFP-CBP</td>
<td>-</td>
<td>P &lt; 0.15</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>CBP</td>
<td>P &lt; 0.15</td>
<td>-</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>