CHARACTERIZATION OF PLANT CATION/H$^+$ ANTIPORTERS AND HOW THEY

CAN IMPACT HUMAN NUTRITION

A Dissertation

by

JAMES LARRY MORRIS, JR.

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

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ABSTRACT

Characterization of Plant Cation/H^+ Antiporters and

How They Can Impact Human Nutrition. (December 2007)

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M.S., Texas Tech University

Chair of Advisory Committee: Dr. Kendal Hirschi

Calcium transporters regulate calcium fluxes within cells. Plants, like all organisms, contain channels, pumps and exchangers to carefully modulate intracellular calcium levels. Numerous Arabidopsis proteins have been characterized which can transport calcium.   However, there are numerous genes whose products have not been fully characterized. One method I used to infer function was to analyze various promoter lengths of 17 putative cation transporters fused to reporter and to observe changes in the reporter expression in response to various stimuli. Using a more in depth approach I set out to characterize the function of *At*CCX3. Here I show Arabidopsis *At*CCX3 can suppress yeast mutants defective in vacuolar Na^+ and K^+ transport. *AtCCX3*-expressing yeast cells conferred Mn^{2+} sensitivity when highly expressed. Functional epitope tagged *At*CCX3 fusion proteins were localized to endomembranes in plants and yeast. Expression of *AtCCX3* increased in plants treated with NaCl, KCl and $MnCl_2$ and caused increased Na^+ accumulation and increased K^+ transport. Ectopic expression of *AtCCX3* in tobacco produced lesions in the leaves, stunted growth, resulted in the accumulation of higher levels of numerous cations and increased protein

oxidation preceded alterations in leaf growth. These characteristics define *At*CCX3 as an endomembrane localized transporter with biochemical properties distinct from previously characterized plant transporters.

In the remaining studies I used the mouse model to determine how molecular changes to plants can improve the nutritional quality of the food. Previously, the *cod5 M. truncatula* mutant was identified which contains identical calcium concentrations to wild-type, but contains no oxalate crystals. Mice fed intrinsically labeled *cod5* plants had 22.87% (p<0.001) calcium absorption compared to wild type plants. In a second study, using mice I examined if increased expression of a calcium transporter which increases calcium concentration 2-fold, alters bioavailable calcium.

In mice feeding regimes (n = 120), I measured ^{45}Ca-incorporation into bones, and determined that mice required twice the serving size of control carrots to obtain the calcium found in *sCAX1*-carrots. Together, these two studies demonstrate how the potential utility of removing calcium absorption inhibitors and fortifying vegetables can improve calcium bioavailability.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

CHAPTER I

INTRODUCTION: REVIEW OF LITERATURE

Calcium (Ca^{2+}) has various important roles in both plants and animals. Minute changes in cytosolic Ca^{2+} regulate biological responses. While humans need Ca^{2+} to build strong bones, plants use Ca^{2+} to provide stress protection and to strengthen cell walls. Thus Ca^{2+} serves a dual purpose as both a nutrient and a signal. To help modulate Ca^{2+} levels, plants have Ca^{2+} transporters with specific kinetic properties located on membranes of various organelles. These Ca^{2+} transporters can be characterized into three major types: pumps, which require energy to move Ca^{2+} across the membrane, exchangers, which are driven by proton gradients, and channels, which allow large concentrations of Ca^{2+} to traverse membranes (Pittman and Hirschi, 2003). Understanding the unique roles these transporters have in Ca^{2+} homeostasis is an important area of plant biology.

Factors that cause cytosolic Ca^{2+} spikes range from temperature stress, hormones, to mechanical or tactile stimuli (Bothwell and Ng, 2005; Braam, 2005). These spikes are complex and are judiciously regulated. Coupled with the amplitude of the Ca^{2+} spike, the duration and location are hypothesized to be crucial to the specificity of the subsequent signal (Sanders et al., 2002; Scrase-Field and Knight, 2003; Plieth 2005). Calcium can increase up to 3μM in concentration in the cytosol during a spike (signaling event), which is up to a 1,000 to 1,0000 fold increase from resting cytosolic Ca^{2+} concentrations (Reddy and Reddy, 2004; Medvedev, 2005).   To facilitate the

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This dissertation follows the style of The Plant Cell.

localization and duration of these spikes, various types of Ca^{2+} transporters are located on membranes throughout the cell. Arabidopsis has Ca^{2+} channels on the plasma (PM), vacuole (VM), and endoplasmic reticulum (ER) membranes. Ca^{2+} pumps can be found on the PM, VM, ER, Golgi, small vacuole (sVM) and chloroplast membranes (CM). Finally, Ca^{2+} exchangers can be found predominantly on the VM in Arabidopsis plants (Figure 1.1; Reddy and Reddy, 2004). There are other calcium transporters present at mitochondrial, plastid and nuclear membranes for Ca^{2+} partitioning in and out of these organelles (Chigri et al., 2006; Oldroyd and Downie, 2006; Xiong et al., 2006). Activation and regulation of these transporters are thought to shape the dynamics of a Ca^{2+} spike and insure the specificity of the stimulus dependant responses (Scrase-Field and Knight, 2003; Harper et al., 2004; Bothwell and Ng, 2005; Medvedev, 2005). At one level, these Ca^{2+} signaling events appear simple: cells at rest have a low level of cytosolic Ca^{2+} that rises during a signal transduction event (Hirschi, 2004). Signal transductions related to Ca^{2+} oscillations can be thought of as a four-part process: (1) Ca^{2+} is mobilized by response triggers to become active, followed by the movement of Ca^{2+} into the cytosol; (2) this increase activates Ca^{2+}-regulated proteins, which induce the response; (3) Ca^{2+} then functions as a messenger to activate Ca^{2+} sensitive processes, which are mediated by proteins such as CDPKs (calcium-dependant protein kinases) and calmodulin; (4) binding proteins and transporters remove the Ca^{2+} from the cytoplasm, essentially turning "off" the Ca^{2+} mediated signaling event (Berridge et al., 2000). These signaling events, which transiently alter cytosolic Ca^{2+} concentrations, must be regulated with a high degree of fidelity.
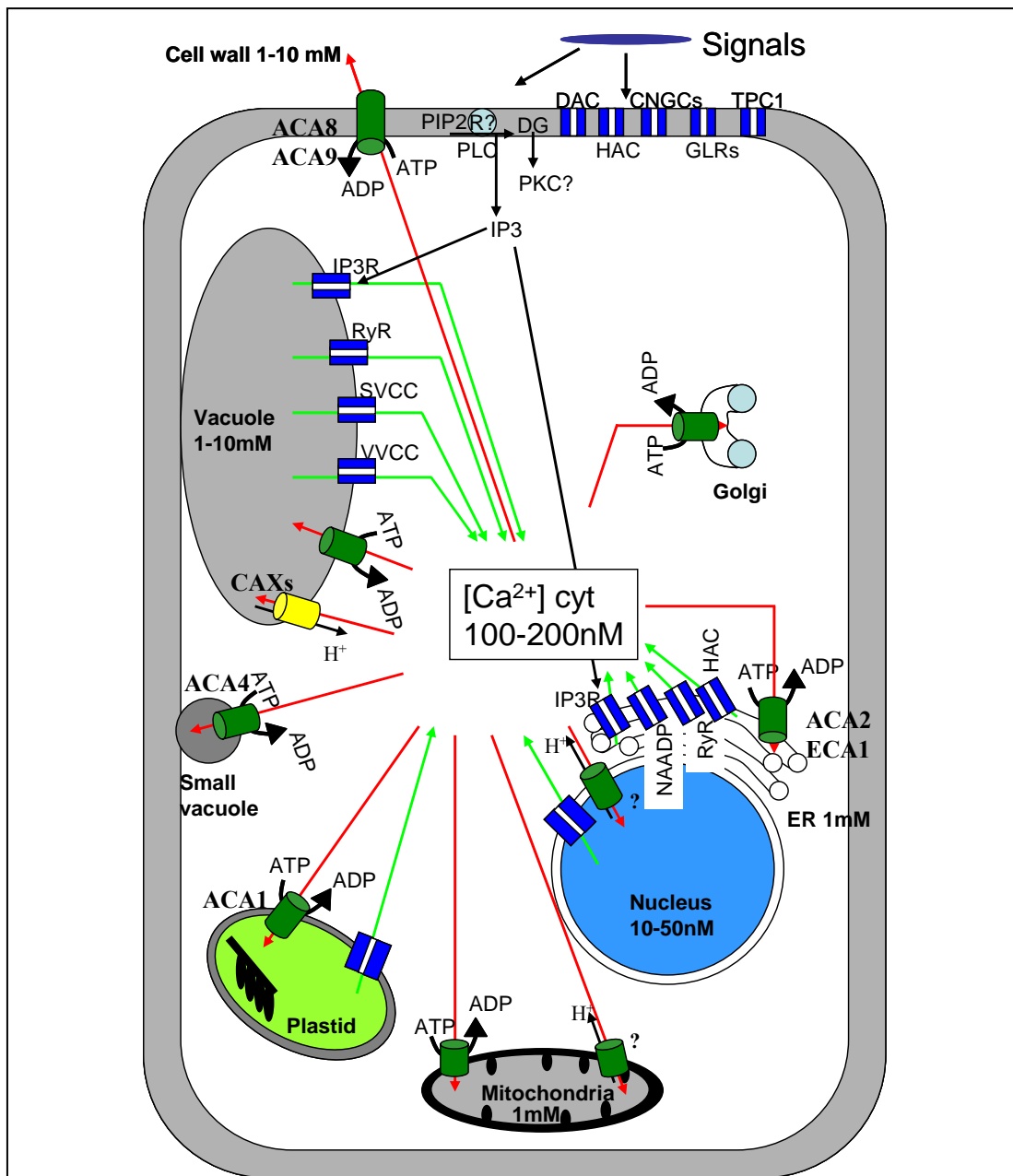
Figure 1.1 Diagram of Ca^{2+} in a plant cell.

Representation of Ca^{2+}-permable channels, pumps and transporters on various membranes of a plant cell thought to be in involved in the initiation and termination of Ca^{2+} specific signals. Ca^{2+} channels (blue/white boxes) allow Ca^{2+} entry from high concentration sources into the cytosol. Maintenance is done by Ca^{2+}-ATPase (green cylinders) and Ca^{2+} exchangers (yellow cylinders). Green and red arrows indicate influx and efflux sequestration of the Ca^{2+} stream. PLC, phospholipase C; ER, endoplasmic reticulum (Adapted from Reddy and Reddy 2004).

Several model systems have been used to characterize the biology of Ca^{2+} movement and transporters which help modulate these oscillations. Heterologous expression of plant transporters in the budding yeast *Saccharomyces cerevisiae* is a well developed and widely used technique (Ton and Rao, 2004). Yeast offers advantages such as simple and inexpensive growth conditions, tractable genetics and a conservation of basic cellular machinery and signal transduction pathways with higher eukaryotes (Ton and Rao, 2004). In plants, Ca^{2+} signals can be studied in highly coordinated events such as stomatal closure and pollen tube formation (Ng et al., 2001; Golovkin and Reddy 2003; Lemtiri-Chlich and Berkowitz, 2004; Levchenko et al., 2005; Shang et al., 2005). These processes involve highly specific Ca^{2+} spikes to induce directional growth in pollen tube formation (Wang et al., 2004) and the opening/closing of guard cells (Schiott et al., 2004). The directional growth of the pollen tube is important to ensure delivery of male gametophytic DNA to the female ovules. Pollen granules and guard cells are both single cell plant tissues and thus make ideal model systems to study Ca^{2+} transporters. These systems allow us to better understand the biology behind the highly organized system of transporters that ensures elaborate and effective regulation of the free Ca^{2+} level in cellular compartments (Yang and Pooviah, 2003; Medvedev, 2005).

To understand the function of any specific transporter among the collection of plant proteins is difficult. For instance, the Arabidopsis genome contains 855 open reading frames which encode for putative transporters (Sze et al., 2004; Shigaki and Hirschi, 2006). To study any transporter one must take a fundamental approach, which includes localization, expression and gene disruption studies. The determination of tissue

and cellular localization is often done by the use of reporter constructs, mainly green fluorescent protein (GFP) and β-glucuronidase (GUS). Often, specific transport studies are initially conducted in a heterologous host such as yeast and then function further clarified *in planta* (Kamiya et al., 2005; Ali et al., 2006). Another greatly utilized resource is gene knockout plants and subsequent forward/reverse genetics. Screening for phenotypes in mutant plants or identifying a phenotype in a known gene disruption can be beneficial in characterizing the function of a gene product. Along with biochemical studies in yeast, the changes in plant biochemistry related to specific gene knockouts help elucidate the function of individual gene products. However, many of the transporters are members of multigene families (Maser et al., 2001) and functional redundancy can mask the changes seen by disruption of a specific gene. The use of chameleon (ratiometric fluorescent proteins) Ca^{2+} indicators is also useful to measure local changes in Ca^{2+} concentrations in specific tissues due to their ability to compare the different Ca^{2+} oscillation patterns between the wild-type and the mutated gene product (Scrase-Field and Knight, 2003; Thuleau et al., 2003). These changes in Ca^{2+} concentrations, subtle or substantial, can be useful in trying to determine the biological function of a specific transporter. Experimental approaches using Ca^{2+} reporters to measure these types of changes have not yet been reported.

To date, numerous articles and reviews have been published on Ca^{2+} transporters and their specific functions in plants (Scrase-Field and Knight, 2003; Harper et al., 2004; Reddy and Reddy, 2004; Bothwell and Ng, 2005; Braam, 2005; Hepler, 2005; Medvedev, 2005). Here we will review the most current research, which shows the role

Ca^{2+} transporters have in controlling and maintaining Ca^{2+} movement within the cell as well as in various tissues. First, we will briefly discuss the use of yeast as a model system used to study plant Ca^{2+} transporters. Then, we will discuss the biology of Ca^{2+} channels and their related roles in Ca^{2+} signaling. The next section will detail the role Ca^{2+}-ATPases have in Ca^{2+} transport, followed by a section discussing Ca^{2+} exchangers. We conclude with a section dealing with how manipulating Ca^{2+} transporters can alter Ca^{2+} content and be used to improve plant production and benefit human nutrition.

Yeast as a Model

Ca^{2+} has been shown to be involved in numerous physiological processes in yeast, including adaptation to environmental stress, cell cycle control, mating response and processing of proteins in the secretory pathway (Kellermayer et al., 2003). A review by Ton and Rao (2004) described the advances in heterologous expression of proteins in yeast and their applications in the study of Ca^{2+} homeostasis. The greatest benefit to using yeast is the conservation of the essential components of cellular Ca^{2+} machinery, termed the "calciome" (Ton and Rao, 2004). The calciome includes Ca^{2+} channels and transporters, Ca^{2+} sensors and signal transducers (Figure 1.2). In yeast, Ca^{2+} enters the cytosol via the PM channel complex Cch1p/Mid1p or the vacuolar transient receptor-like channel Yvc1p (Ton and Rao, 2004). This increase in Ca^{2+} is in response to diverse environmental cues such as endoplasmic reticulum (ER) stress, osmotic shock or mating pheromone. The P-type ATPases Pmr1p (located in the Golgi) and Pmc1p (located in the vacuole) are primarily responsible for removing Ca^{2+} from the cytosol

Figure 1.2 Ca^{2+} signaling and transport pathways in *Saccharomyces cerevisiae*.

There are calcium transporters on all membranes; red circles indicate Ca^{2+} ions (Adapted from Ton and Rao, 2004).
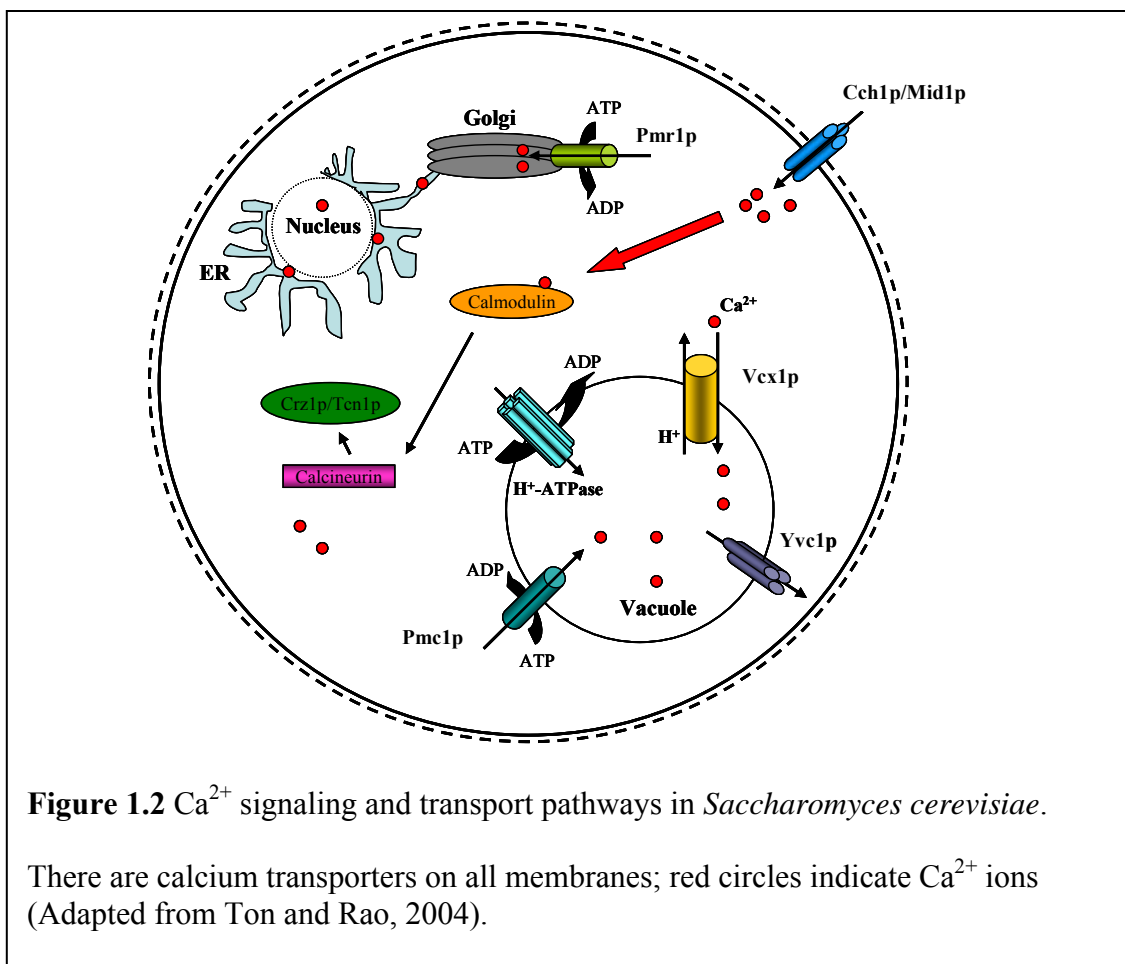
(Kellermayer et al 2003; Aiello et al., 2004; Vanoevelen et al., 2005). Along with these pumps, the vacuolar Ca^{2+}/H^+ exchanger Vcx1p transports Ca^{2+} into the vacuole by the use of a proton gradient (Miseta et al., 1999). It has been suggested that the ER P-type ATPase Cod1p (or Spf1p) is also involved in Ca^{2+} transport (Cronin et al., 2002). The yeast Ca^{2+} signaling and transport pathway is similar to that of plants and makes yeast a simple experimental model system to study the function of plant Ca^{2+} transporters.

Yeast lacks the redundancy of multiple isoforms and different splice variants found in mammalian cells. This allows for the deletion of individual or multiple genes with predictable effects (Ton and Rao, 2004). Conversely, plants have calcium transporter families consisting of numerous genes (Maser et al., 2001; Cai and Lyton, 2004, Shigaki et al., 2006). Designer yeast strains allow for the study of heterologous expression of plant genes without interference from endogenous host genes (Ton and Rao, 2004). For instance, a mutant strain lacking Vcx1p, the vacuolar Ca^{2+} pump Pmc1p and the regulatory subunit of the protein calcineurin (cnb1) is sensitive to high calcium and has been used to study the putative Ca^{2+} transport properties of plant proteins (Hirschi et al., 1996; Shigaki et al., 2001; Cheng et al., 2002). Rice Ca^{2+}/H^+ exchangers were also screened for the ability to suppress both the Ca^{2+} and Mn^{2+} sensitivity of a yeast strain devoid of Pmc1p and Vcx1p (Kamiya et al., 2005). The deletion of both vacuolar Ca^{2+} transporters disrupts the yeast cell's ability to remove Ca^{2+} from the cytosol.

Numerous yeast mutants have been used to study the function of plant transporters. A mutant lacking both Ca^{2+}-ATPases (Pmr1p & Pmc1p) along with *cnb1* can be used to study the biochemistry of yeast plasmids encoding Ca^{2+} pumps (Ton and Rao, 2004) and plant Ca^{2+} pumps (Wu et al., 2002; Bonza et al., 2004; Schiott et al., 2004; Schiott and Palmgren, 2005; Baekgaard et al., 2006) Additionally, yeast strains defective in Pmr1p and Pmc1p have perturbed cellular secretion (Kellermayer et al., 2003) and can be used to study plant genes thought to be involved in cellular secretion. Similarly, the yeast strains lacking a functional Cch1p PM channel have been used to

study the biochemistry of plant Ca^{2+} channels (Kurusu et al., 2004; Peiter et al., 2005). Calcium channels from Arabidopsis, rice and wheat have all been studied in the yeast *cch1* mutant background (Kurusu et al., 2004; Peiter et al., 2005; Wang et al., 2005). In sum, these yeast mutants are a powerful and simplistic tool used in studying plant Ca^{2+} transporters.

Yeast also has other advantages in the study of plant transporters. The first advantage is the ability to use yeast mutants to screen multiple variations in a specific plant protein which might have an effect on the function (Shigaki et al., 2001, Ton and Rao, 2004; Baekgaard et al., 2006). This allows rapid analysis of multiple variants of the plant protein to determine structure/function relationships by assessing their ability to complement the mutant phenotypes. These types of screens are markedly more difficult to do *in planta* due to the inherent growth difficulties and endogenous background activities (Ton and Rao, 2004). An example of the effectiveness of this approach was the screening of multiple chimeric constructs of Ca^{2+}/H^+ exchangers to identify functional domains (Shigaki et al., 2001). Along with the ability to study structure/function relations, one can also precisely regulate the expression level of the plant proteins in yeast cells. The yeast plasmid "tool kit" contains various promoters which allow for the control of gene expression levels. Some strong promoters have been shown to have high-level expression reaching up to 10% of the PM proteins (Ton and Rao, 2004). These promoters have been used to study the function of many heterologously expressed plant genes (Nakamura et al., 2001; Shigaki et al., 2001; Cheng et al., 2002). This ability to control expression is useful because it may be

necessary to express abundant amounts of the protein to delineate function (Ton and Rao, 2004). These methodologies, combined with the ability to quickly and efficiently transform yeast, make it a useful tool in studying the structure/function relationships of plant transporters.

One versatile tool to use with yeast to study Ca^{2+} transport function is the aequorin reporter system. The aequorin reporter protein system is a calcium receptor coupled to the aequorin luminescent protein. This protein has the ability to bioluminesce in the presence of Ca^{2+}. As more Ca^{2+} binds, the light intensity given off by the aequorin reporter protein increases (Allen et al., 1977), much like the cameleon reporter system used in plants (Allen et al., 1999) and animals (Thuleau et al., 2003). This calcium reporter system was used to show that Vcx1p plays an important role in the maintenance of resting cytosolic Ca^{2+} levels in a yeast strain deficient in Pmr1p and Pmc1p function (Kellermayer et al., 2003). The ability of this reporter system to detect subtle changes in Ca^{2+} is a major benefit which when coupled with genetic analysis creates a valuable tool to dissect the role of transporters in the modulation of cellular Ca^{2+} levels.

Despite all the previously mentioned benefits, sometimes using yeast to study the function of plant transporters can be problematic. Plant genes expressed in yeast encounter several problems such as localization to the proper membrane, mis-folding of the protein and a heterologous host which may contain foreign proteins that alter function (Ton and Rao, 2004). For example, a plant Na^{+} transporter expressed in yeast resides on the vacuole whereas the native yeast transport, that may have similar function, is localized on the prevacuole. Only after the plant transporter was targeted to the

prevacuole could functionality be obtained (Darley et al., 2000). Another illuminating example is the expression of barley Na^+ (or K^+) uniporter or Na^+, K^+ symporter, HvHKT1 (Haro et al., 2005). Only the uniport function was observed in the barley roots and it is suspected that symport function resulted from a different translation of *HKT1* when expressed in yeast. Another speculation is that K^+ inhibition of Na^+ uptake processes in roots cannot be reproduced in a heterologous yeast system (Haro et al., 2005). Expression in yeast can also be a problem for plant plasma membrane proteins where there may be difficulty targeting to the yeast PM. Exogenous proteins can also be retained in the ER due to delays or problems in the correct folding of the protein (Kauffman et al., 2002). Despite these issues, which we will discuss throughout the review, yeast is still a simple and valuable tool used to study the biology of plant transporters.

Ca^{2+} Channels

One component in the generation and duration of Ca^{2+} signals in the plant cell is a functional system of Ca^{2+} channels. Channels are activated by either a change in membrane polarization or binding of a ligand (hormone or second messenger). These channels account for low affinity transport of Ca^{2+} across the membrane and are an important portion of the generation and propagation of the Ca^{2+} signal (Medvedev, 2005). These channels are also thought to maintain the spatial and temporal increase in cytosolic Ca^{2+} as well as the duration and frequency of the Ca^{2+} spike (Nayyar 2003; Ng and McAinsh 2003; Miedema et al., 2003; Reddy and Reddy, 2004). In some plant

tissues, these Ca^{2+} channels create Ca^{2+} gradients in the tissues to create polar or

directional growth of the tissue (Demidchik et al., 2003; Mori and Schroeder 2004;

Wang et al., 2004; Prokic et al., 2005). For example, Ca^{2+} gradients may be involved in

the polarized growth of developing pollen tubes (Samaj et al., 2006; Qu et al., 2007).

Hyper-polarization of Ca^{2+} permeable channels have been reported in growing pollen

tubes from Arabidopsis (Schiott et al., 2004, Wang et al., 2004) and were first seen in the

growing apex from wheat roots (White et al., 2000). Calcium permeable channels play

an important role in stomatal closure in response to abscisic acid (ABA), a plant

hormone (Prokic et al., 2005). At pH 7 or higher, higher concentrations of Ca^{2+} are

needed to induce stomatal closure compared to an acidic pH (Prokic et al., 2005),

suggesting that cytosolic pH concentrations are important in regulation of Ca^{2+} channel

activity. Arabidopsis contains 51 potential channels, which imply a complex role of

channels in the regulation of cytosolic Ca^{2+} levels. This high number of channels is part

of the complex coordinated system of events maintaining the spatial temporal

relationship of Ca^{2+} signaling. To accomplish this, these channels are located in the

plasma and tonoplast membranes along with the ER, chloroplasts and the nuclear

envelope (Medvedev, 2005). This allows for Ca^{2+} entry from these endomembrane stores

and from extra cellular spaces through numerous channels to maintain the specificity of

the signal (Harper et al., 2004; Hetherington and Brownlee, 2004; Bothwell and Ng,

2005). Ca^{2+} permeable channels, cyclic nucleotide gated channels (CNGCs) and

glutamine receptors (GLRs) are located on the PM. Various other channels from

different endomembranes also play a role in the spatial temporal increase in cytosolic

Ca^{2+} (Reddy and Reddy, 2004). As we will discuss below, these channels may play an important role in the initiation and termination steps of a calcium spike.

TPC1 is a 2-pore channel

A recently characterized two-pore channel from Arabidopsis, *At*TPC1 was shown to play an important role in Ca^{2+} signaling (Furuichi et al., 2001; Peiter et al., 2005). *At*TPC1 is the only annotated cation channel in Arabidopsis and was first characterized by heterologous expression in yeast (Furuichi et al., 2001). This protein consists of 12 predicted transmembrane (TM) domains, with two EF hand Ca^{2+} motifs, and two pores, which allows for the transport of Ca^{2+} (Figure 1.3A). *At*TPC1 contains two Shaker domains with a basic region in the forth TM of each Shaker domain. Shaker domains are named after K^+ channels characterized in *Drosophila melonogaster* (Tanouye et al., 1981). These Shaker domains are structures which form the pore of the transporter and contain one region with numerous basic residues. This protein conformation is similar to yeast and mammalian two pore Ca^{2+} channels.

Expression of *AtTPC1* in a mutant yeast strain, deficient in the Ca^{2+} channel CCH1p, enhances the growth rate by suppressing mating-induced cell death and low basal Ca^{2+} uptake phenotypes (Hetherington and Brownlee, 2004; Peiter et al., 2005) suggesting a similar function to the yeast Ca^{2+} permeable channel. A GFP fusion of *At*TPC1 shows the protein localizes to the vacuolar membrane of Arabidopsis mesophyll protoplasts. Vacuolar proteomics and western blots reveal that AtTPC1 is the only Ca^{2+} permeable channel located on the plant vacuole membrane (Peiter et al., 2005). Whole plant analysis shows expression of *At*TPC1 in leaves, stems, root tissue, and green and

Figure 1.3 Topology Models of Putative PM proteins involved in Ca^{2+} influx into the Arabidopsis cytosol.

A) TPC1 is a 2-pore channel with 2 EF calcium binding hands, the pore loop (P) located between the 5th and 6th TM domains. The 4th TM domain is enriched in basic residues.
B) CNGC structure contains a P loop, and overlapping calmodulin and cyclic nucleotide binding domains
C) GLRs have 2 glutamate binding domains (GlnH) located on the outside of the membrane with 4 TM domains (Adapted from Sanders et al., 2002).

developing seed pods (Medvedev, 2005). This suggests *At*TPC1 plays an important role in Ca^{2+} transport from the plant vacuole.

When mesophyll vacuolar membranes from *At*TPC1 mutants were analyzed by patch clamp electrophysiology, complete absence of the slow voltage currents occurred. This suggests that *TPC1* encodes a slow-vacuolar (SV) Ca^{2+} channel (Peiter et al., 2005). This lack of current in the presence of Ca^{2+} was restored in the mutants by transformation with the wild-type gene (Peiter et al., 2005). Suppression of the phenotype from the mutant mesophyll vacuolar membranes by the wild-type *At*TPC1 confirms it is a Ca^{2+} permeable channel on the tonoplast. Wild type and *AtTPC1* over-expressers show a reduction in stomatal aperture when treated with external Ca^{2+} but the mutant stomata remain unresponsive (Peiter, et al., 2005). The reason for this difference in sensitivity is not known. It is possible that the transgenic plants already had more *At*TPC1 proteins and absorb the Ca^{2+} in guard cells, thus making the stomatal closing less sensitive to external Ca^{2+} concentration. These findings suggest that *At*TPC1 plays an essential role in voltage gated Ca^{2+} transport in stomatal closure. Taken together, these findings suggest that *At*TPC1 is an important slow voltage Ca^{2+} channel ubiquitously expressed with roles in stomatal closure and Ca^{2+} homeostasis.

*At*TPC1 is the first characterized voltage gated Ca^{2+} channel from plants and more detailed analysis will lead to a better understanding of this type of channel in plant cell signaling. Though some of the phenotypes observed *in planta* might be related to general cation homeostasis and turgor regulation (Peiter et al., 2005). This could be due to *At*TPC1 being relatively non-selective among the mono and divalent cations. In

order to determine the specific function of *At*TPC1, many factors need to be addressed.

Changes in pH, as well as difference in Ca^{2+} concentrations, need to be studied to see

how they affect the transport properties of *At*TPC1. Over-expression of *AtTPC1* leads to

ABA hypersensitivity in germinating seeds in Arabidopsis but no differences to

exogenous Ca^{2+} have yet to be observed in germinating seeds. The effect of Ca^{2+} on

ABA sensitivity of over-expressing *AtTPC1* lines seeds needs to be studied given that

ABA is involved in hyper-polarization of guard cells and pollen tubes (Wang et al.,

2004; Prokic et al., 2005).

Another recently characterized voltage gated Ca^{2+} channel, rice *Os*TPC1,

functions as a PM Ca^{2+} permeable channel (Kurusu et al., 2004; Kurusu et al., 2005).

*Os*TPC1 is the only TPC to be PM localized which is intriguing because it may suggest

some interesting differences in Ca^{2+} signaling in rice. This observation begs the

question as to what encodes SV channel activity in rice. Yeast has been used to

characterize other SV channels from plants. To determine *Os*TPC1 function,

complementation of the yeast *cch1* mutant was partially restored by expression of

OsTPC1 under Ca^{2+} limited conditions (Kurusu et al., 2004). The suppression of the

yeast phenotype is similar to *At*TPC1, suggesting that *Os*TPC1 functions as a Ca^{2+}

channel in rice. However, over-expression of *OsTPC1* in rice showed hypersensitivity

to excess Ca^{2+} but in limiting Ca^{2+} conditions plants had a higher growth rate. This

result suggests that *Os*TPC1 has a different function than *At*TPC1 *in planta*. This also

suggests that when Ca^{2+} is abundant, over-expression of *Os*TPC1 leads to Ca^{2+} toxicity,

where *At*TPC1 does not cause any Ca^{2+} toxicity. Prolonged increases in Ca^{2+} are

harmful because it can lead to activation of apoptosis (Kass and Orrenius, 1999). Conversely, cultured cells with an insertional knockout of *OsTPC1* displayed less sensitivity to extra-cellular free Ca^{2+} (Kurusu et al., 2004), suggesting that *Os*TPC1 has a role in Ca^{2+} uptake into the cell.   Similar to *At*TPC1, *Os*TPC1 functions as a Ca^{2+}-permeable channel with a possible role in the transduction of stimuli from either the cytoplasm or the external matrix (Medvedev, 2005).

This initial research indicates the presence of Ca^{2+}-permeable channels in plants. Nonetheless, there are several questions that need to be answered to better understand the biological function of *Os*TPC1. The cellular localization is one important aspect that needs to be determined. The Arabidopsis homolog localizes to the vacuole, however, it appears that *Os*TPC1 could localize to the PM due to toxicity of plant over-expressing *OsTPC1*. Another future area of inquiry is to address the role of *Os*TPC1 in stomatal closure in response to external Ca^{2+}. *AtTPC1* over-expression in Arabidopsis reduces stomatal aperture, suggesting that *At*TPC1 has a role in stomatal closure in plants (Peiter et al., 2005). The use of a chameleon reporter system in lines altered in *AtTPC1* expression will allow us to observe the effects *At*TPC1 has in regulating calcium levels in the stomates of Arabidopsis.

CNGCs

The plant CNGCs (Cyclic Nucleotide Gated Channel) are permeable to mono and divalent cations and were first isolated from barley aleurone cells (Medvedev, 2004). Plant CNGCs are inwardly rectified, ligand gated, non-selective cation channels with different selectivity profiles. In all cases published to date their conductance is

increased is the presence of cyclic nucleotides (this is unique to CNGCs; Leng et al.,

2002; Chan et al., 2003; Hua et al., 2003). CNGCs have both cyclic nucleotide and

calmodulin domains at the C-terminus, a TM core and a pore domain similar to the

Shaker family (Figure 1.3B; Ali et al., 2006). These channels can bind cyclic

nucleotides (cAMP, cGMP) and CaM, which allows for the integration of signals that

arrive from different signaling event pathways (Arazi et al., 2000). These inwardly

conducting channels located on the PM are activated by cAMP (Lemtiri-Chlieh and

Berkowitz, 2004; Ali et al., 2006). The Arabidopsis genome contains about 20 genes

encoding CNGCs (Ali et al., 2006) and they are capable of forming heterotetramers

(Kaupp and Seifert, 2002).

Recently, *CNGC1* was expressed in a mutant yeast strain to try and elucidate the

function of this protein. The Ca^{2+}-uptake yeast mutant *mid1/cch1* was used to

functionally characterize CNGC1 and CNGC1 mutants with domain deletions which

cause these transporters to localize to different membranes (Ali et al., 2006). A GFP

fusion of CNGC1 shows the protein localizes to the PM in yeast cells. Conversely,

mutants of CNGC1 predominantly localize to the vacuolar membrane, with some

fluorescent at the PM (Ali et al., 2006). This suggests that deletions in the CNGC1

protein can affect the localization of the protein and can have an affect on function. This

deletion could have a positive effect on Ca^{2+} transport by altering the pore selectivity

filter (Ali et al., 2006).

These results have shown that yeast can be an efficient model for studying the

function of plant *CNGCs*. As mentioned previously, it appears that there may be factors

in the yeast cell which also affect the efficacy of the assay system and need to be taken into account when heterologously expressing plant *CNGCs*. Endogenous yeast CaM and perhaps cyclic nucleotides in the yeast cytosol could affect the activity of CNGCs. Given the success of using yeast to characterize other plant transporters, this biochemical assay has the potential to be used in determining biochemical functions of other CNGCs.

CNGCs are thought to play a role in programmed cell death and tissue senescence in response to pathogen related signals (Balague et al., 2003). When *CNGC2* is mutated spontaneous cell death occurs resulting in constitutively active plant defenses systems. *CNGC2*::GUS expression is constitutive at rather high levels and is repressed upon infection suggesting that *CNGC2* is repressed in response to pathogenic infection. In contrast, when *CNGC4* is mutated, plants display necrotic lesions and a constitutive expression upon activation after inoculation by a pathogen (Balague et al., 2003). These lesions of clustered dead cells show an accumulation of β-glucoronidase (GUS) expression in the surrounding cells. *CNGC2* expression is higher in *cngc4* plants. An Arabidopsis mutant with a 3KB deletion (produced from a cross of two different ecotypes) fuses *CNGC11* and *CNGC12*. This chimeric *CNGC11/CNGC12* induces pathogen resistance through multiple signaling pathways, whereas the individual genes are a component of those pathways (Yoshioka et al., 2006). Together these mutants reveal that CNGCs have a role in plant stress recognition and their coordinated regulation could be related to the heterotetrameric nature of the proteins in the CNCGs family (Balague et al., 2003).

CNGC4 may also be involved in pathogen interactions but we know little regarding its biological function. *CNGC4* expression increases in response to plant pathogens at the site of inoculation (Balague et al., 2003). However, *CNGC4* expression does not increase in response to Ca^{2+} (Balague et al., 2003). To further determine the biological function of CNGC4, this transporter was expressed in Xenopus oocytes. *CNGC4* expression in oocytes shows more efficient activation by cGMP and is permeable by both K^+ and Na^+ but not Ca^{2+}. This lack of Ca^{2+} transport could be related to other endogenous proteins transporting Ca^{2+}, therefore masking any Ca^{2+} transport change resulting from expression of *CNGC4* (Balague et al., 2003). These findings suggest CNGC4 is not activated by Ca^{2+} but might be a component of the plant hypersensitive response (HR).

These results show that some CNGCs function as Ca^{2+} channels but there are problems heterologously expressing these proteins. One aspect to study further is the idea that CNGCs function as heterotetramers. *CNGC* mutations have been shown to induce or alter pathogen resistance and increase the HR (Yoshioka et al., 2006). Also, CNGC2 was recently shown to be involved in the pathogen response signal leading to nitric oxide production (Ali et al., 2007). The low level expression of some *CNGC* makes the determination of their biological function difficult but using the sensitive Ca^{2+} reporters could lead to a better understanding of their role in Ca^{2+} transport.

GLRs

There is little known about the physiological functions of GLRs (GLutamate Receptors) but they have been implicated in several plant processes from light signaling

to water balance (Davenport, 2002; Meyerhoff et al., 2005). The Ca^{2+} antagonist La^{3+} can inhibit the increase of root Ca^{2+} by GLRs, suggesting that the pore of GLRs has unique properties and can be related to the unusual pore sequences (Davenport, 2002; Hetherington and Brownlee, 2004). This pore sequence may act as a conserved filter throughout the GLR family. The conservation of the two-ligand binding domains implies a role in Ca^{2+} signaling in response to elevation in extra cellular glutamate levels (Davenport, 2002). The N-terminal domain has homology to mammalian GLRs and could be a sight for allosteric modulation by Ca^{2+} or metabolites (Davenport, 2002). In Arabidopsis, approximately 20 *GLRs* were identified with homology to animal GLRs (Lacombe et al., 2001). The general structure of GLR contains two glutamate binding domains and a PM signal sequence (Figure 1.3 panel C). There is emerging evidence that *At*GLRs may transport a variety of cations in plants but there have only been a few GLRs that have been characterized (Davenport, 2002; Meyerhoff et al., 2005; Li et al., 2006).

The Arabidopsis GLR family appears to have developed by multiple local gene duplications events (Chiu et al., 2002). It is possible that some of these genes have no function (Davenport, 2002). The large number of similar sequences makes the naming of GLR unique. There are 3 main groups of GLRs: *GLR1*, *GLR2* and *GLR3*. Within each of these groups different loci are given different names. For example, previous names like *GLR2* and *GLR6* are now termed *AtGLR3.1* and *AtGLR3.5* (Lacombe et al., 2001). This nomenclature suggests that *AtGLR3.1* and *AtGLR3.5* are almost identical in sequence but are located in different parts of the chromosome. In the case of *AtGLR3.1a*

and *AtGLR3.1b*, they are classified differently because they encode different RNAs (Lacombe et al., 2001). The differences among the *AtGLRs* RNAs could indicate specific functions for the different variants.

GLRs from Arabidopsis are predicted to target to the secretory pathways on the basis of their hydrophobic N-terminal sequences. The secretory pathway involves numerous organelles suggesting GLRs could be localized to numerous plant membranes. Attempts to visualize cellular localization with C-terminal GFP tags has been unsuccessful; however, GFP fusions within the N-terminus regions of GLRs did localize to the ER, revealing the possibility of internal C-terminus targeting sequences (Davenport 2002).

Interestingly the GUS expression data demonstrates that *AtGLR3.2* is expressed in vasculature tissue of roots and shoots and could play a role in unloading Ca^{2+} into the xylem (Davenport, 2002). Localization of other *AtGLR3s* was determined by RT-PCR. *AtGLR3.4* and *AtGLR3.5* are expressed in root and shoot tissue in 2-week old Arabidopsis plants. *AtGLR3.7* transcripts were also present in both leaf and root tissue (Davenport, 2002). Similarly, *AtGLR1.1* is expressed in all aerial tissue and *AtGLR2.1* is expressed in rosette leaves of Arabidopsis plants (Davenport, 2002), suggesting GLR proteins are expressed throughout the plant. Expression of *AtGLR3.7* in Xenopus oocytes demonstrates that this GLR is a Ca^{2+} permeable non-selective cation channel. Over-expression of *AtGLR3.2* in plants produces a Ca^{2+} deficiency phenotype in roots and the application of exogenous Ca^{2+} alleviates these symptoms (Davenport, 2002). This agrees with the oocyte data that GLR can transport Ca^{2+} into the cell.

At present, a better understanding of tissue and membrane localization, ion selectivity and the related sensitivities to different ligands is needed to define the role GLRs play in Ca^{2+} signaling/homeostasis in plants. Ectopic expression of *GLRs* could cause the proteins to mis-target or cause mis-folding within the membranes, thus confounding any experimental results. Some GLR subunits could function in Ca^{2+} signaling and nutrient uptake at the PM. Another function may be related to amino acid regulation due to specific distribution of glutamate within the cell from synthesis in the cytosol, plastids, mitochondria, and possibly in peroxisomes in response to the photorespiratory pathway (Medvedev, 2005). Transgenic plants and mutant analysis will help to characterize both gene function and the physiological role of GLRs. Given the numerous genes and the high occurrence of gene duplications, using multiple mutants could become too cumbersome an avenue in further characterizing GLRs.

Other channels

Calcium entry into the cytosol can occur from either the PM or from intracellular stores. In addition to the previously mentioned channels, there are several other types of Ca^{2+} channels, such as voltage-dependant and voltage-independent Ca^{2+} selective channels on the PM and ER, inositol 3 phosphate (IP_3), cyclic ADP ribose (cADPR) and nicotinic acid adenine dinuclotide phosphate (NAADP) gated channels (Becker et al., 2004; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Medvedev, 2005). So far none of these channels have been cloned from Arabidopsis (Hetherington and Brownlee, 2004). The only plant NAADP characterized so far is from red beet. This channel functions in Ca^{2+} release from the ER, even though the gene has not yet been

cloned (Navazio et al., 2000). Despite the lack of NAADP channels, other channels have been identified in Arabidopsis. Isolated vacuoles and tonoplast vesicles from plants treated with IP_3 induce Ca^{2+} efflux (Medvedev, 2005). This is compelling evidence for an IP_3 mediated channel on the tonoplast. There also appears to be a slow voltage (SV) channel in guard cells. This channel is involved in Ca^{2+} release from the vacuole by CaM activation and increases activity with increases in pH (White and Broadley, 2003). Further understanding of these channels will be a challenge and much remains to be discovered with regards to the regulation of these channels.

Annexins

Annexins have been suggested to be Ca^{2+} binding proteins which play a role in Ca^{2+} transport. They may function in either a direct manner by forming multimeric aggregation Ca^{2+} channels or in an indirect manner by activation of Ca^{2+} channels (White and Broadley, 2003; Dabitz et al., 2005). Multiple Arabidopsis annexin genes have been identified, but they remain poorly characterized. There is some physiological evidence of annexins playing a role in Ca^{2+} activity (Medvedev, 2005; Hetherington and Brownlee, 2004; Gorecka et al., 2005). In terms of their biological function, annexin 1 (*AnnAt1*) from Arabidopsis is involved in plant response to reactive oxygen species (ROS; Gorecka et al., 2005) and subsequent activation of Ca^{2+} channels (Mori and Schroeder, 2004; Rentel and Knight, 2004). ROS are part of the mutli-step pathogen response pathway in plants (Gorecka et al., 2005; Ali et al., 2007). Characterization of the redox sensing domain and identification of substrates involved in annexins response to oxidative stress has not yet been defined. Although annexins appear to have a role in

plant pathogen responses, like CNGCs, their specific role is unclear and it will be interesting to determine if CNGCs and annexins are coordinately regulated.

Ca^{2+} Pumps

Plant Ca^{2+} pumps are responsible for regulation of Ca^{2+} concentrations in endomembrane compartments as well as the cytoplasm. Pumps function by actively moving Ca^{2+} across membranes using ATP hydrolysis as the energy to drive transport against a Ca^{2+} concentration gradient (Marschner, 1995). This active transport in coordination with Ca^{2+} channels is used to create the specific spatiotemporal coding of a Ca^{2+} signal (Medvedev, 2005). As well as being involved in plant signal transduction pathways, Ca^{2+} pumps are involved in adaptation to stress conditions. After a Ca^{2+} spike, the removal of calcium requires active transport into endomembranes as well as efflux from the cell through the PM (Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Medvedev, 2005). Plant calcium pumps are expressed in all plant tissues from the vasculature to the guard cells (Wu et al., 2002; Bonza et al., 2004; Schiott et al., 2004; Schiott and Palmgren, 2005; Baekgaard et al., 2006). These pumps are involved in stomatal closure in response to cold stress (Schiott and Palmgren, 2005), pollen tube growth and fertilization (Schiott et al., 2004), acclimation to salt stress (Geisler et al., 2000b) and adaptation to variable nutrient conditions (Wu et al., 2002). To accomplish these processes, the pumps hydrolyze ATP to transport Ca^{2+} which causes a change in protein conformation that exposes the Ca^{2+} binding domain (high affinity E1) to the cytosol. Once bound, a conformational change E2 (low affinity) exposes the Ca^{2+} to the

opposite side of the membrane and allows the Ca^{2+} to dissociate from the transporter (Figure 1.4A; Xu et al., 2002; Sorensen et al., 2004). It is also likely that a proton is exchanged for the Ca^{2+} ion (Luoni et al., 2000).

Plants have two types of active Ca^{2+} pumps to accomplish the transport of Ca^{2+} from the cytosol, both of which are members of the P-type ATPase superfamily.   The ER-type Ca^{2+}-ATPase (ECA) and the autoinhibited Ca^{2+}-ATPase (ACA) make up the two classes of calcium pumps in the Arabidopsis genome (Geisler et al., 2000). Based on the structural similarities to the animal pumps, plant Ca^{2+}-ATPases are classified into 2 groups, type IIA (ECAs) and IIB (ACAs). The current nomenclature for the P-type ATPase subfamilies are P_{1B} (metal ATPases), P_{2A} (ECAs), P_{2B} (ACAs), and P_{2A} (H-ATPases) (Baxter et al., 2003). Type IIA pumps (ECAs) have approximately 50% protein sequence homology with animal SERCA (sacroplasmic/endoplasmic reticulum Ca^{2+}-ATPases) and are not activated by calmodulin. Type IIB pumps (ACAs) have approximately 50% protein to homology with animal PMCA (PM located Ca^{2+}-ATPases). ACAs are activated by calmodulin but the calmodulin and autoinhibitory domains are located on the N-terminal region instead of the C-terminus, as seen in mammalian PMCAs (Geisler et al., 2000). Both of the pumps have a similar general structure (Figure 1.4B). Only 20% of the protein is inside the membrane and they have between 8-10 TM domains. The largest part of the protein is exposed to the cytoplasm and contains two loops with the phosphorylation and ATP binding sites (Sorensen et al., 2004). Only about 10% of the protein faces the non-cytoplasmic side of the membrane. Animals have three Ca^{2+}-ATPase types: SERCA, PMCA and a third distinct class SPCA

Figure 1.4 Protein conformations and topology of a plant calcium pump.

A) conformation change of a calcium pump from E1 to E2 forms after activation by ATP hydrolysis (adapted from Sorensen et al., 2004).
B) Topology of the Ca2+-ATPases, the CaM/autoinhibitory domain is only present on type IIB Ca2+-ATPases (Adapted from Sanders et al., 2002).
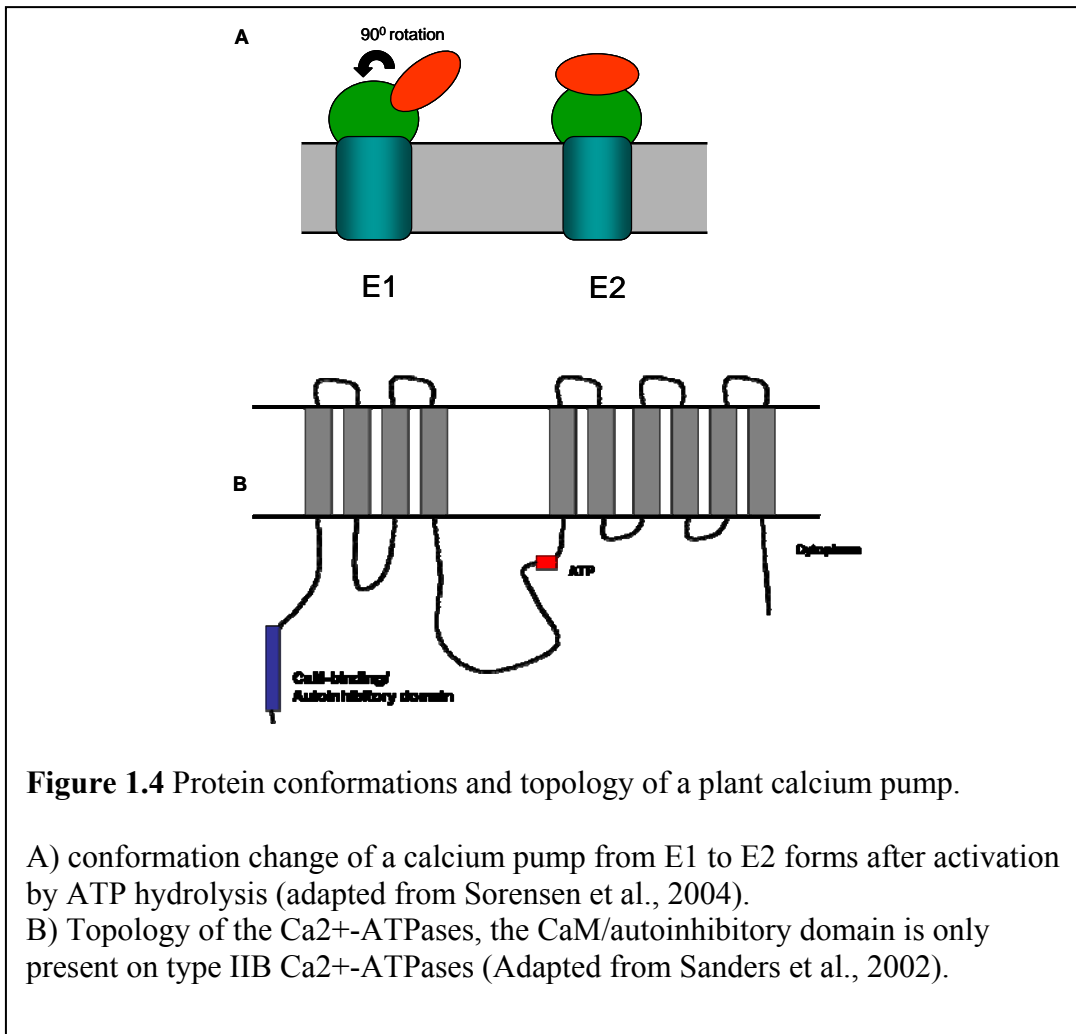
(secretory pathway Ca-ATPases) which are Golgi localized and found in yeast (PMR1)

and animals but not plants. The ECAs have some SPCA characteristics such as the

ability to transport other cations including Mn^{2+} (Dode et al., 2005).

ECAs

ECA1 (ER Ca^{2+}-ATPase) appears to be the most abundantly expressed of the

four Arabidopsis *ECAs* (Liang and Sze, 1998; Axelsen and Palmgren, 2001; Wu et al.,

2002).   ECA1 has been shown to suppress a yeast mutant defective in Ca^{2+}-ATPase activity by alleviating the sensitivity to Ca^{2+} in the growth medium (Liang and Sze, 1998). More recently, ECA1 was shown to increase tolerance of a yeast mutant to toxic levels of Mn^{2+} (1mM) and Zn^{2+} (3mM; Wu et al., 2002). This result suggests that ECA1 can transport multiple cations from the cytoplasm into the ER to facilitate growth of a yeast mutant strain.

The function of ECA1 was determined using standard reverse genetic analysis. The T-DNA was inserted into the last TM domain but it did not alter growth of *eca1-1* compared to wild type plants under normal growth conditions (Wu et al., 2002). Disruption of *ECA1* does not affect normal plant growth, possibly due to functional compensation by other Ca^{2+} transporters (Wu et al., 2002). When *eca1-1* lines are grown in media containing high Mn^{2+} (0.5mM) these mutants have a severely reduced growth rate (66% reduction; Wu et al., 2002). These lines also have a 22% reduction in vesicular Ca^{2+} pump activity. However, the plants display more tolerance to severe Ca^{2+} deficiency conditions when compared to controls (Wu et al., 2002). Root hair formation in the *eca1-1* plants is disrupted in media supplemented with 0.5mM Mn^{2+}. Disruption of *ECA1* expression alters the root hair formation by slowing hair growth but not root tip growth (Wu et al., 2002). Potentially, the plants could be slowing root hair growth to try and decrease the uptake of Mn^{2+} to lessen the toxicity created in the aerial tissues. The expression of the wild type *ECA1* gene in the *eca1-1* background was sufficient to rescue the mutant. Thus the sensitivity to Mn^{2+} stress was due to a disruption in *ECA1* expression.

Little is known about the other three *ECAs* from Arabidopsis. This might be related to abundant expression of *ECA1* in Arabidopsis or functional redundancy. In the case of *ECA4*, which is almost 97% identical to *ECA1*, there is speculation that it could be a pseudo gene (Kabala and Klobus, 2005). Numerous other *ECAs* have been cloned and/or characterized from other plants. Type IIA pumps have been cloned from tomato (*LCA1*, Wimmers et al., 1992) and rice (*OCA1*, Chen et al., 1997). Ectopic expression of Arabidopsis *ECAs* in these other plant species could provide insight about their function due to their divergent genetic background. Another way to possibly characterize the functions of these other Arabidopsis *ECAs* is to make double mutants of these transporters and look for changes in calcium transport or the location of calcium pools using the chameleon reporter system. Another ECA-like pump CAP1 from maize has also been identified to contain a calmodulin binding site at the C-terminus (Subbaiah and Sachs, 2000). This is interesting because little is known about regulation of plant ECAs, which is in contrast to the well understood regulation of animal SERCA pumps.

ACAs

The primary function of autoinhibited Ca^{2+}-ATPases (ACAs) are to remove Ca^{2+} from the cytosol into endomembrane compartments or into the extra cellular space (Bonza, et al., 2004; Schiott et al., 2004; Medvedev, 2005). *ACAs* main distinction from *ECAs* is their activation by Ca^{2+}-dependant protein calmodulin and Ca^{2+}-dependant protein kinases (CDPKs) (Hwang et al., 2000; Medvedev, 2005).   The first ACA that was shown to be stimulated by calmodulin was the vacuolar localized Ca^{2+}-ATPase BCA1 from cauliflower (Malmstrom et al., 1997). Unlike the C-terminus calmodulin-

binding domains of animal Ca^{2+}-ATPase, the BCA1 binding domain is localized to the N-terminus region (Malmstrom et al., 1997).

In Arabidopsis, ACAs function in pollen tube growth (Schiott et al., 2004) and in guard cell responses to cold stress (Schiott and Palmgren, 2005) among other physiological functions. There are 10 *ACAs* in Arabidopsis that are divided into 4 groups: Group 1 contains *ACA1,2,7*; Group 2 contains *ACA4, 11*; Group 3 contains *ACA12,13* and Group 4 contains *ACA8,9,10* (Baxter et al., 2003). Many group 3 *ACAs* contain intronless genes, where as group 4 genes can have as many as 33 introns (Baxter et al., 2003). *ACA2* was cloned and characterized by functional expression in a yeast Ca^{2+}-ATPase mutant (Harper et al., 1998). Truncation of the N-terminal domain of ACA2 increases transport activity 4 to 10 fold more than ACA2. This protein was also unresponsive to further stimulation by calmodulin (Hwang et al., 2000). This demonstrates that ACA2 contained a regulatory domain that is activated when Ca^{2+} induces calmodulin to bind to the N-terminal domain, thus suspending the inhibition. Since the characterization in yeast of ACA2, other plant Ca^{2+} pumps have subsequently been shown in yeast to have an N-terminus autoinhibitory domain, e.g. ACA4, ACA8, ACA9 and soybean SCA1 (Chung et al., 2000; Bonza et al., 2004; Geisler et al., 2004). These results show evidence that numerous ACAs contain an N-terminus autoinhibitory domain.

Within a plant cell, the ACAs are located on different membranes, (Sze et al., 2000; Schiott et al 2004; Bonza et al., 2004) unlike the animal PMCAs that are only PM pumps. This allows ACAs to tightly regulate Ca^{2+} at both the PM and other

endomembranes. Ca^{2+} cytosolic fluctuation requires tight regulation of both plasma and

endomembrane bound transporters. Two of the ACAs, ACA8 and ACA9, localize to the

PM (Bonza et al., 2000; Schiott et al., 2004). These enzymes function to remove Ca^{2+}

from the cell to help control the duration and intensity of the Ca^{2+} signal. Another

family member, ACA2, localizes to the ER and may have a unique role in modulating

the Ca^{2+} efflux into endomembrane compartments (Hwang et al., 2000).   It has been

previously reported that ACA4 localizes to small vacuole membranes (Geisler et al.,

2000) and ACA1 to the inner plastid envelope (Huang et al., 1993). Despite the

unknown cellular localization of other ACA family members (ACA7, 10,11,12 and 13)

the differing localizations implicate a diverse role of the ACAs in maintaining the spatial

and temporal relationship of cellular Ca^{2+} signals.

Along with the diverse cellular localization, the ACA family members also have

unique tissue localization. For instance, the PM localized *ACA9* expression is primarily

in pollen tubes (Schiott et al., 2004). Calcium efflux by ACA9 recycles calcium ions

entering through the PM channels. Gene disruption of *ACA9* results in a semi-sterile

phenotype in Arabidopsis plants. Three independent lines display reduction in pollen

tubes and the tubes that do reach ovulation have a high frequency of aborted fertilization

(Schiott et al., 2004). Transformation of these mutants with *ACA9* suppresses the male

sterile phenotype. Complementation of these mutants is also done by expressing *ACA8*

which suggests that *ACA8* functions downstream of *ACA9* in pollen tube formation.

Tissue specific localization of *ACA9* was shown by GUS promoter fusions. Expression

of *ACA9* promoter GUS expression was in flower anthers. Conformation of *ACA9*

expression by RT-PCR shows a greater than 500 fold increase in expression in stamen tissues (Schiott et al., 2004). This demonstrates that ACA9 function is critical for pollen tube formation and subsequent fertilization of the ovule.

Although *ACA9* is specifically expressed in pollen, other *ACAs* are expressed in different plant tissues (Schiott and Palmgren, 2005). For closely related *ACAs*, *ACA8* and *ACA10*, expression is found in Arabidopsis guard cells. Expression of a promoter::GUS fusion of *ACA8* is restricted to guard cells and vasculature tissue of both roots and shoots while *ACA10* promoter::GUS expression was more ubiquitous (Schiott and Palmgren, 2005). Both of these tissue expression patterns were confirmed by RT-PCR.

Even though *ACA8* and *10* are expressed in similar tissues they appear to have differing functions in response to cold stress. RT-PCR analysis of plant samples taken at 2, 8, 24 and 48 hrs treatment of 5 ^{0}C demonstrates that *ACA8* and *10* expression is altered by this treatment (Schiott and Palmgren, 2005). After 2 hrs, *ACA8* expression drops to almost zero, and then has a five fold increase at 8 hrs only to return to normal levels after 48 hrs. In contrast, *ACA10* expression decreases steadily all they way to a three fold decrease at 48 hrs (Schiott and Palmgren, 2005). Interestingly, mutations in either *ACA8* or *ACA10* do not cause a cold sensitive phenotype. These alterations in response to cold stress display that ACA8 and ACA10 may have a role in efflux of Ca^{2+} from the cytosol after a cold induced signal.

ACAs are located in several tissues and numerous membranes. Initial findings suggest ACAs have roles in cold accumulation and pollen tube growth. Throughout this

review it has been mentioned that genetic and functional redundancy creates difficulties

using standard genetic approaches. Heterologous expression systems help to alleviate

some of these limitations. As I discussed in the yeast section, valuable insights in *S.*

cerevisiae Ca^{2+} signaling were made when both Ca^{2+}-ATPase and Ca^{2+}/H^+ exchangers

were mutated. Potentially these types of mutations *in planta* will be just as beneficial in

further delineating biological functions of Ca^{2+} pumps. Specifically, it will be

interesting to determine if alterations in pollen tube growth or guard cell movement

occurs in these mutants.

Ca^{2+} Exchangers

The vacuole is an important storage organelle for many ions, including Ca^{2+}.

The vacuole allows for large concentrations of ions inside the luminal membrane. This

helps to maintain a low concentration of Ca^{2+} in the cytosol. To accomplish this,

Ca^{2+}/H^+ exchangers use a proton gradient, created by differences in pH between the

cytosol (pH ~7.5) and the vacuolar lumen (pH 3 – 6). This is physiologically different

from the previously mentioned Ca^{2+} pumps. The vacuolar Ca^{2+}-ATPase has a high

affinity (Km = 0.1-2 uM) and low capacity for Ca^{2+} (Sze et al., 2000), while Ca^{2+}/H^+

exchangers have a low affinity (Km=10-15uM) and a high capacity for Ca^{2+} (Schumaker

and Sze, 1986; Hirschi et al., 1996). This high capacity is thought to play a role in

removing Ca^{2+} from the cytosol after a signaling event, while the Ca^{2+}-ATPases may be

involved in fine tuning a Ca^{2+} oscillation (Pittman and Hirschi, 2003). The Ca^{2+}/H^+

exchangers may function as a component of the "off" mechanism of plant signaling events.

The Ca^{2+}/H^+ exchangers were identified by using a yeast suppression screen and are termed CAX1 and CAX2 (CAtion eXchangers; Hirschi et al., 1996). The membrane topology model for CAXs show 11 TM with an acidic motif between TM6 and TM7 (Figure 1.5A; Shigaki and Hirschi, 2006). Also indicated on the model are two distinct cation domains and an N-terminal auotregulatory domain (Pittman and Hirschi, 2001; Shigaki et al., 2001). The calcium domain was identified using chimeric constructs of CAX1 and CAX3 (Shigaki et al., 2001). This 9 amino acid domain from CAX1, when inserted into CAX3, allows CAX3 to now confer Ca^{2+} tolerance to a mutant yeast strain sensitive to high Ca^{2+} (Shigaki et al., 2001). This chimeric construct strategy was also useful in identifying other cation domains. CAX2 can also transport numerous other cations besides Ca^{2+}. Chimeric constructs were made between CAX1 and CAX2 to identify the Mn^{2+} domain critical for Mn^{2+} transport by CAX2 (Shigaki et al., 2003). Also, similar experiments using mutagenesis of CAXs in rice (OsCAXs) have identified other domains involved in substrate specificity (Kamiya and Meashima, 2004). Numerous reviews have mentioned how the first CAXs were characterized, I will focus this review on recent research of the CAX family of transporters (Scrase-Field and Knight, 2003; Harper et al., 2004; Reddy and Reddy, 2004; Bothwell and Ng, 2005; Hepler, 2005; Medvedev, 2005; Shigaki and Hirschi, 2006).

Phylogeny of CAX family

The plant CAX family has numerous members. Phylogenetic analysis of this family reveals an interesting relationship of the members. *CAX1-6* appear to be closely related to each other while *CAX7-11* are closely related to each other (Shigaki et al., 2006), simply based on sequence homology. Recently, *CAX7-11* were reclassified as *CCX1-5* due to higher homology to mammalian K^+ dependant Na^+/Ca^{2+} antiporters (Shigaki et al., 2006). These phylogenetic relationships suggest that *CCXs* have a different function compared to the previously characterized *CAXs*.

One difference that might exist between CAXs and CCXs is the existence of the N-terminal autoinhibitory domain (Figure 1.5A and 1.5B). The first functional clones, later named sCAX1 and sCAX2 (short-CAX; Hirschi et al., 1996; Pittman and Hirschi, 2001) were truncated cDNAs without the first 36 and 42 amino acids, respectively. The full length genes are unable to suppress the calcium sensitive phenotype of a yeast mutant when grown on high Ca^{2+} medium (Pittman and Hirschi, 2001; Pittman et al., 2004a). When N-terminal truncations were made in other CAXs in this CAX1-6 subfamily, these "short" forms were able to suppress the calcium sensitive yeast phenotype. In CAX1, specific residues within this N-terminal domain have been implicated in this regulation, including a putative phosphorylated serine residue and other residues that may be involved in protein interaction with another region of the CAX1 protein (Pittman et al., 2002). Although this N-terminal regulatory domain is seen with the ACA Ca^{2+}-ATPases, this CAX N-terminal domain does not have any sequence similarity with the ACA autoinhibitory domains and does not appear to bind

Figure 1.5. Topology of plant cations exchangers.

A) The calcium and manganese domain are indicted in blue and red, respectively. The N-terminal autoinhibitory domain is indicated in green (Adapted from Sanders et al., 2002).
B) Topology of the newly classified CCXs. This model is based on the TMHMM algorithm (Sonnhammer et al., 1998).
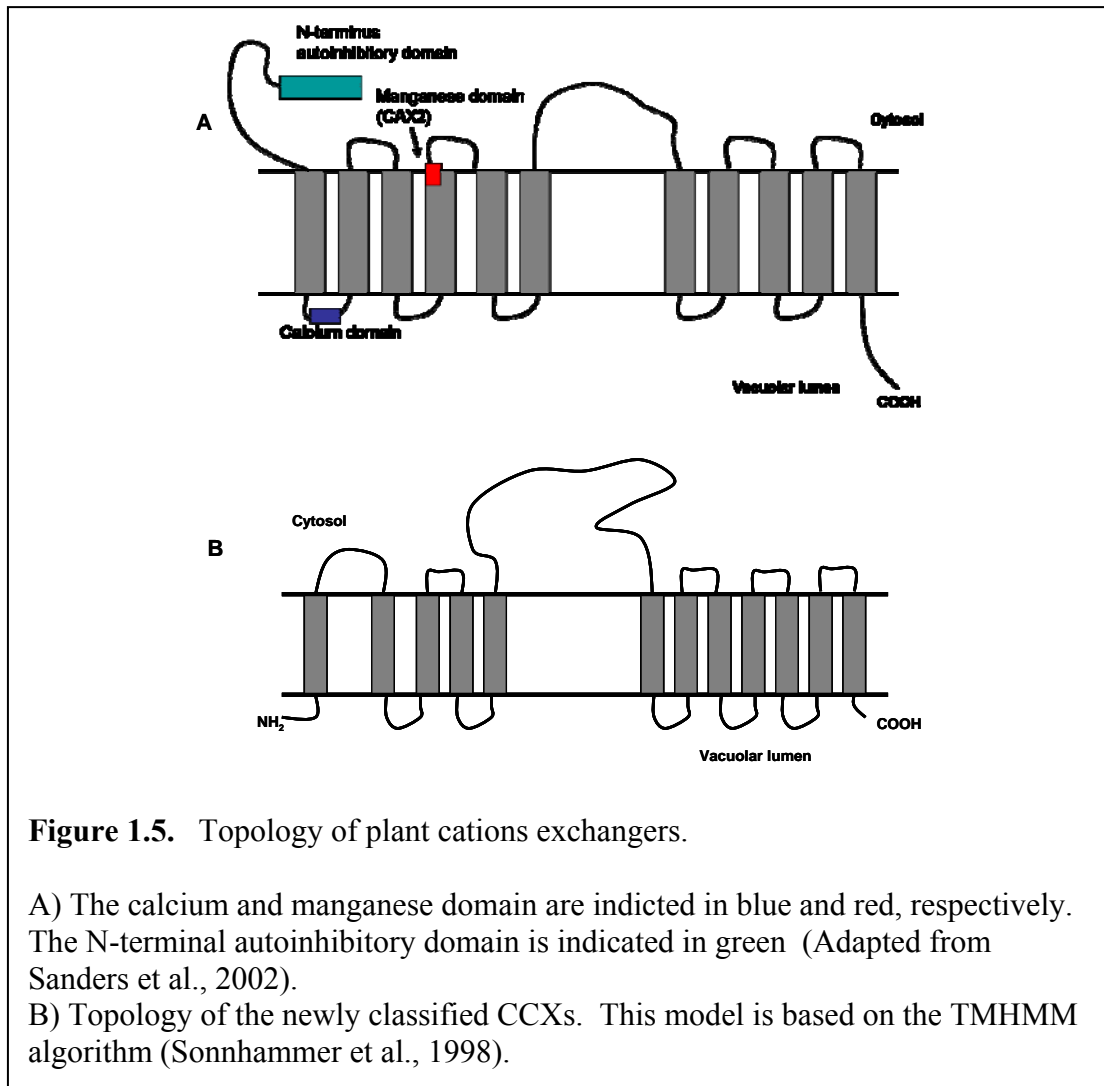
CaM (Pittman and Hirschi, 2001), but can interact with other proteins (Cheng and

Hirschi, 2003; Cheng et al., 2004a; 2004b). CCX1-5 have very little sequence homology

in the N-terminus region with CAX1-6 (Figure 1.5B). Rather, their N-terminal domains

are highly homologous to the N–terminal domain of mammalian NCKXs (Shigaki et al.,

2006). Potentially CCX transporters have different biochemical and regulatory properties than CAXs.

Physiological function of CAXs

As stated throughout this review, yeast is a useful tool to study plant Ca^{2+} transporters. However, *in planta* gene function is still critical. Heterologous yeast expression data combined with gene transcript expression data from microarray analysis, northern analysis and promoter-reporter fusions can be used to reveal the role CAXs have in plants. Phylogeny suggests that the CAXs have two closely related groups. The first group consists of *CAX1, 3,* and *4* and the second group contains *CAX2, 5* and *6* (Shigaki et al., 2006). To characterize the *CAXs* in these two groups, *CAX* promoter::GUS constructs have been used to discern their expression patterns in different Arabidopsis tissues. *CAX1* is highly expressed in shoots but only modestly expressed in the roots (Cheng et al., 2005). Conversely, *CAX3* expression is seen in roots and modestly in the leaves (Cheng et al., 2005). RT-PCR analysis of *CAX4* shows expression throughout the plant with the highest expression in root tissues (Cheng et al., 2002). *CAX2* promoter::GUS shows expression in the vasculature tissue of 10 day and 30 day old plants (Pittman et al., 2004a). Also *CAX2*::GUS expression is present in floral tissues and hydathodes. Many of the *CAX* genes are also expressed highly in inflorescence tissues (Cheng et al., 2005). *CAXs* are found in numerous other plants, yet only a few have been characterized. RT-PCR analysis of *OsCAXs*, from rice, shows expression in root, shoot, leaf and floral tissues (Kamiya et al., 2005).

Gene expression in response to various stimuli can be useful in determining biological function. *CAX1* and *CAX3* transcripts have been shown to be strongly induced by exogenous Ca^{2+}. However, *CAX4* is increased by Ni^{2+} and Mn^{2+} and not Ca^{2+} (Cheng et al., 2002). Induction by Na^+ has been seen with *CAX1, 3* and *4* and implicates a role for these *CAXs* in salt stress response (Hirschi, 1999; Shigaki and Hirschi, 2000; Cheng et al., 2004). Along with induction by different cations, transcripts of *CAX1* accumulate in leaves after 24 hrs cold treatment at 4 ^0C (Catala et al., 2003). *CAX* genes from other plant species, such as rice, show induction by stress conditions. Ca^{2+} and Mn^{2+} can induce expression of *OsCAXs* (Kamiya et al., 2005). These results suggest that *CAXs* play a role in numerous plant physiological processes from salt stress to acclimation to cold.

To better understand the functions of CAXs in plant, analyses of specific CAX mutants were conducted. For instance, deletion of *CAX1* causes a 40% reduction in vacuolar H^+-ATPase (V-ATPase) activity, along with a 50% reduction in vacuolar Ca^{2+}/H^+ activity, despite increased expression of *CAX3* and *CAX4* (Cheng et al., 2003). This shows that CAX1 is a major Ca^{2+} exchanger on the vacuole membrane. These plants also displayed a 36% increase in vacuolar Ca^{2+}-ATPase activity, revealing some mode of compensation by the plants to attenuate the decrease in vacuolar Ca^{2+} transport (Cheng et al., 2003). Similar reductions in V-ATPase were shown in *cax3* and *cax2* mutants (Cheng et al., 2005; Pittman et al., 2004b). This reduction in V-ATPase activity could mean that the deletion of CAX could disrupt the normal synthesis or assembly of the V-ATPase (Shigaki and Hirschi, 2006) or simply mean that H^+ pump turnover is

switched down as less H^+ are being transported out into the cytosol. The exact nature of this alteration has yet to be understood.

Mutations of *CAX* transporters have revealed some interesting phenotypes. Mutations of *cax1* produce a gain-of-function tolerance to high Mg^{2+} concentrations (Cheng et al., 2003). Additionally this same phenotype was identified in a mutant screen for tolerance to serpentine soils (low Ca:Mg ratio; Bradshaw, 2005). When mutations in multiple *CAX* genes were made, dramatic effects on the plant phenotype were observed. A double mutation of *cax1/cax3* produces plants with severely stunted growth even though these plants still have some Ca^{2+}/H^+ transport activity (Cheng et al., 2005). Individually, the two mutations do not have any dramatic phenotype; however, the results from the double mutant alludes to a functional association of CAX transporters. This could be due to some sort of heterodimer formation which could confer different biological properties (Cheng et al., 2005).

In the future, it will be paramount to determine further functions of the remaining *CAX* genes. Using yeast has been beneficial in determining substrate specificities and regulatory domains of CAX1-4, however little is still known about *CAX5* and *CAX6*. Although single mutants in *CAX* genes show subtle phenotypes, double mutants of *cax1/cax3* show dramatic phenotypes (Cheng et al., 2005). These double mutants will be good genetic backgrounds in which to study alterations in Ca^{2+} partitioning within the cell using Ca^{2+} reporters. A comprehensive approach like micro array analysis can also be useful to identify more specific characteristics of *CAXs*. Although very little is known about the newly classified *CCXs*, recently published research on *CCX5* showed

increases in Ca^{2+}, Cd^{2+}, and Zn^{2+} transport when ectopically expressed in tobacco (Koren'kov et al., 2007). The use of ectopic expression has been useful in characterizing *CAXs* and appears to create an avenue in which to elucidate the functions of *CCXs*.

Impact on Nutrition

In this section we will briefly discuss some of the applied research that is taking place with Ca^{2+} transporters in plants. A judicious use of transgenic plants will be an important option to improve human nutrition. Osteoporosis is a disease caused by reduced bone density and affects females to a greater extent than males. Adult humans should consume 1000 to 1200 mg/d of calcium, however Bryant and others (1999) point out that many adults consume less than this recommended daily intake. One way to overcome this problem, which has $13.8 billion in health care related costs each year, is to increase calcium levels in foods. Weaver and Plawecki (1994) state that vegetables make up the second largest source of calcium in the diet behind dairy products. Vegetables tend to be low in calcium but consumption of a wide variety of vegetable can have an impact on dietary calcium levels, including calcium-rich spinach and kale (Weaver and Plawecki, 1994). By creating vegetables or other agronomically important crops that contain higher calcium, a richer source of calcium would exist for people to use in maintaining a healthy diet.

The creation of genetically modified plants with increased nutritional benefits is an expanding field. The term "nutritional genomics" has been used to describe various studies which implement some form of plant biochemistry, genomics or human nutrition.

Transgenic plants are frequently analyzed for changes in plant metabolism and this is often where the experimentation ends. Ideally, these genetically modified plants need to be labeled and used in controlled animal and human feeding studies to assess nutritional impacts. This added nutritional benefit is not without issue. This increased Ca^{2+} needs to be targeted to the edible portions of the plant to ensure that excess Ca^{2+} is not sequestered into nutritionally unimportant plant tissues. Another area that needs to be investigated is accumulation of toxic metals in these plant tissues. Toxic metals might not have deleterious effects on the plants but can be detrimental to human health. When plants are grown in soils which could possibly contain heavy metals, uptake of these metals could occur due to their similar ionic radii with Ca^{2+} (Shigaki and Hirschi, 2006). Accumulation of these metals in the plants tissue needs to be assessed to determine if these foods are safe for consumption. Bioavailability of this increased Ca^{2+} also needs to be determined. To date, this type of analysis to assess the nutrient value of transgenic foods is minimal.

Aside from nutritional benefits, the use of genetic engineering to increase Ca^{2+} levels could improve plant productivity and extend product shelf life. Calcium has long been used to combat many post harvest issues (Kabak et al., 2006). Apples are immersed in a calcium solution to maintain firmness in shipping and prolong shelf life. Application of calcium solutions to plums (Alcaraz-Lopez et al., 2003) and pear fruits is used to increase firmness (Klein and Fergusson, 1987). Calcium is also added to soil to reduce the incidences of pathogen attack on potato tubers (Kabak et al., 2006) and to combat heat stress (Kabak et al., 2006). All of these preventative measures require time

and man power to apply these calcium containing solutions to the soil or fruits.

Advances in plant biotechnology can provide novel solutions to meet the benefits

exogenous Ca^{2+} creates for plants. One example of an alternative to exogenous

applications of Ca^{2+} is the expression of sCAX1 in tomatoes to increase fruit firmness

and prolonged shelf life (Park et al., 2005a). Another interesting aspect to increased

calcium accumulation is in long term space flight. In space, providing continuous light

can increase plant productivity but calcium deficiencies in plants become an issue that

cannot be solved by foliar Ca^{2+} applications (Bugbee, 1999). Using genetic engineering

to increase calcium levels in a wide variety of plants could positively impact plant

productivity, while simultaneously decreasing labor costs.

Conclusions

Calcium transport impacts growth, development and stress responses. I focused

this overview on the different types of transporters and the methodologies used to

determine their biological functions. *In planta* functional redundancy has hindered

identifying specific roles to transporters. Future research promises to visualize

alterations in calcium levels in lines with altered transport function. I concluded this

review by detailing how the manipulation of calcium transporters can be used to alter

plant productivity and benefit human nutrition.

In the future, detailing the precise biological functions of plant calcium

transporters will be essential to plant biology. Using yeast as a tool to elucidate function

of plant transporters is practical but the use of other heterologous systems is required.

With the abundance of genomic tools available, further inferences regarding function of Ca^{2+} transporters can be made. Once working hypotheses are formulated, multiple experimental approaches will aid in defining biological functions. After we better understand the biological functions of these calcium transporters, we can apply this knowledge towards improving plant productivity and human nutrition.

CHAPTER II

USING THE GUS REPORTER SYSTEM TO INFER FUNCTION OF

CATION/H$^+$ EXCHANGERS FROM ARABIDOPSIS*

Introduction

Plants have numerous mechanisms to transport and maintain levels of ions (Fox

and Guerinot, 1998). The transport of ions from the soil and into tissues and cellular

compartments regulate the nutrient levels in plants. The Arabidopsis genome has

approximately 855 open reading frames (ORFs) coding for transporters (Sze et al., 2004;

Shigaki et al., 2006). The transporters are located on endomembranes throughout the

cell as well as the plasma membrane (Schumaker and Sze, 1985; Salt and Wagner, 1993;

Apse et al., 1999; Gonzalez et al., 1999; Shigaki and Hirschi, 2000). Characterization of

transporter expression can be beneficial in order to understand the function of any given

transporter.

There are numerous families of cation transporters in Arabidopsis (Maser et al.,

2001). Some of these transporters can transport Na$^+$, CaP^{2+}, K$^+$ and numerous other

metals (Apse et al., 1999; Hirschi, 1999; Sze et al., 2004; Haro et al., 2005). An

important step in characterizing a gene's functions is to determine what factors can

affect expression. Little is known about factors affecting expression of the many cation

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* Part of this chapter is reprinted with permission from "Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter" by **Pittman, J.K., Shigaki, T., Marshall, J.L., Morris, J.L., Cheng, N-H., and Hirschi, K.D.** (2004). Plant Mol. Biol. **56:** 959-971 © 2005 with kind permission from Springer Science and Business Media.

transporters.

To determine the possible function of cation transporters, expression analysis was performed. The promoters of 17 cation transporters (Table 1) from Arabidopsis were cloned and fused to the reporter β- glucuronidase (GUS). I tested the expression of each promoter constructs in 10 independent lines of Arabidopsis (not all constructs generated 10 lines, see Table 2.1) and analyzed their expression and how they were induced by various stimuli. These gene expression profiles were analyzed to determine how various cations, hormones, temperature stress and cis-acting promoter elements affected expression.

Materials and Methods

Creation of promoter::GUS transgenic lines

A DNA sequence 5' of ATG of the open reading frame was amplified from genomic DNA by using a battery of primer sets (see Table 1). Two promoter fragments (transcriptional and translational [100bp 3' of ATG was included]) for *CHX26* and *CHX27* each were amplified by PCR from "Col" genomic DNA (100 bp 3' of ATG that provide transcriptional and translational signals were included). The primers used were as follows (underlined sequence is a *SfiI* site): *CHX26* transcriptional forward 5' ATC CGG CCA AAT CGG CCT CCC TCC TGC TAG GTA TTT CCC CTA AAG 3'; reverse 5' ATC CGG CCA TAA GG CCC TTG TTT CTC TTT GGG AAT ATT TTT TC 3' and *CHX26* translational reverse 5' GGA TCC GGC CAT AAG GGCC GGA TGC CTC GCA TAC AAA TTC ATG 3'. *CHX27* transcriptional forward 5' ATC CGG

CCA AAT CGG CCC TTG TTT CTC TTT GGG AAT ATT TTT C 3'; reverse 5' ATC

CGG CC ATAA GGG CCT CCC TCC TGC TAG GTA TTT CCC CTA AAG 3' and

CHX27 translational reverse 5' GAT GAT GGCC ATAA GGGCC GGA TCC GGC

CCT TAT GGC CAA AGA GCC TGC ACC TAC CCA TGG CTC 3'. These fragments

were cloned into the Topo2.1 vector (Invitrogen, USA). Once the sequences were

verified, these fragments were cloned in a recombination vector and recombined as

previously described (Shigaki et al, 2005). Agrobacterium transformation of

Table 2.1. Arabidopsis promoter::GUS constructs

Gene	Promoter length	Lines	Selection
CAX1	2000	10	Kanamycin
CAX1	1500	8	Kanamycin
CAX1	1000	18	Kanamycin
CAX1	500	10	Kanamycin
CAX1	250	10	Kanamycin
CAX2	2018	10	Kanamycin
CAX3	2000	25	Kanamycin
CAX5	1016	10	Kanamycin
CAX6	800	10	Kanamycin
CCX1	1000	4	Basta
CCX2	1000	10	Basta
CCX3	642	5	Basta
CCX4	494	24	Kanamycin
CCX5	593	5	Kanamycin
CHX13	2000	10	Kanamycin
CHX14	768	10	Kanamycin
CHX26	769	10	Kanamycin
CHX26	869	10	Kanamycin
CHX27	769	10	Kanamycin
CHX27	869	10	Kanamycin
CHX28	1000	7	Kanamycin
CCC	705	10	Kanamycin
MHX1	1000	10	Kanamycin

Arabidopsis plants was performed by the floral dip method (Clough and Bent, 1998).

Plant treatment and histochemical GUS analysis

The T3 generations of each line were germinated in soil and analysis was done at

10 and 30 days of age (unless noted otherwise). The plants were removed from soil, the

roots rinsed with water and treated for five hrs at room temperature. The following

treatments were used: 0.5mM $ZnCl_2$, 10mM $CaCl_2$, 2mM $MnSO_4$, 160mM NaCl, 10mM

$MgCl_2$, 10% (w/v) PEG, 100µM ABA, and 1µM NAA. Histochemical assays for *GUS*

Figure 2.1. *CAX1*::GUS expression is affected by promoter length.

A-B) 250bp fragment of the *CAX1* promoter::*GUS* expression in 30 day old leaf tip
and in the stamen tissue.
C-D) 2000 bp fragment of the *CAX1* promoter::*GUS* expression is strong in 30 day
old leaves and in the petals, sepals, stamens and developing embryos.

Figure 2.2. Analysis of GUS expression in *CAX2*::GUS transgenic Arabidopsis.

A) Cotyledons of 10 day-old transgenic seedlings expressing GUS in the vascular and vegetative meristematic tissue.

B) GUS expression in root vascular tissue from 10 day-old transgenic seedlings.

C) GUS expression in 30 day-old petiole tissue.

D) GUS expression in floral tissues of transgenic Arabidopsis including the petals, stamens, anthers and stigma, and also in pollen grains (inset).

E) Leaf vascular expression of GUS in 30 day-old transgenic Arabidopsis, also showing GUS expression in the hydathodes (inset; Pittman et al, 2004a).

activity in T3 generation lines of Arabidopsis plants harboring the GUS reporter constructs were performed (Cheng et al., 2003). One exception is worth noting, staining of *CCX3* occurred for 5 days.

Results

A complete listing of all the GUS lines, treatments and pictures can be seen at www.hirschilab.org/gus/.

CAX1-6

Several different promoter lengths of *CAX1* were analyzed to determine what effect deletions in specific cis- elements had on reporter expression. Although *CAX1* is induced by calcium (Shigaki et al., 2001) we did not observe any GUS induction from treatment with 10mM $CaCl_2$. *CAX1*::GUS expression was also affected by promoter length (Figure 2.1). As the promoter length decreased from 2000bp to 250bp the level of GUS expression also decreased. This suggests that cis-acting elements controlling *CAX1* expression are present in the region from 250 to 2000bp 5' of the start codon.

CAX2::GUS expression was seen in both leaf and root vasculature tissue of 10 day old plants. In older plants (30 days) GUS expression was observed in root vasculature tissue, hydathodes, flowers and pollen (Figure 2.2). Although *CAX2* RNA expression is induced by numerous cations (Pittman et al., 2002), none of the treatments tested induced *CAX2*::GUS expression.

CAX3::GUS expression was only observed in 30 day old plants in the root vasculature tissue and the flowers (Figure 2.3). In the single promoter length tested, we

Figure 2.3. Analysis of GUS expression in *CAX3*::GUS transgenic Arabidopsis.

A-B) Roots from 30 day old Arabidopsis show expression in vasculature tissue

Figure 2.4. Analysis of GUS expression in *CAX5*::GUS transgenic Arabidopsis.

Vasculature tissue in the roots and leaves expressing GUS in 10 day old Arabidopsis plants. GUS expression was seen in the SAM.
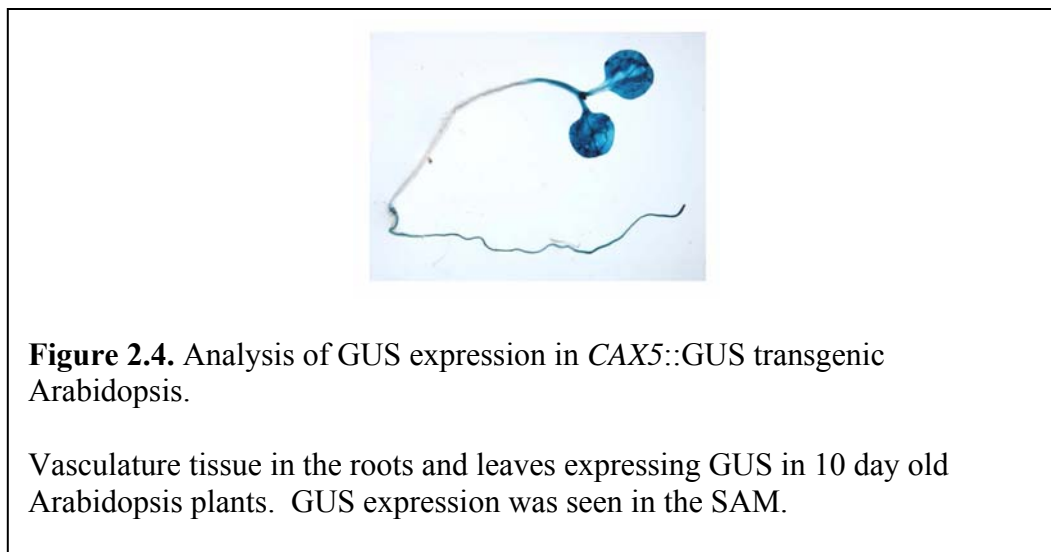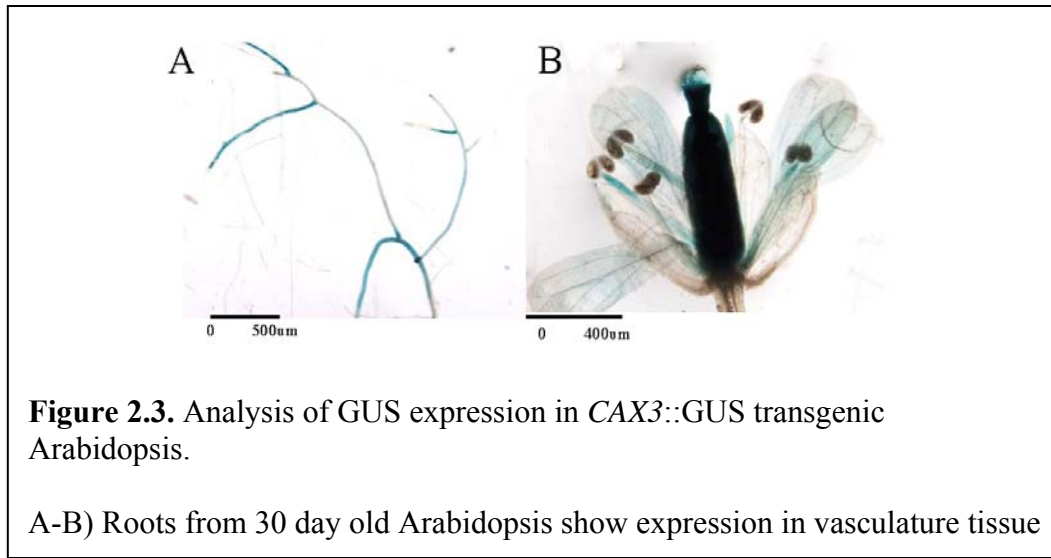
observed no activation by 10mM $CaCl_2$ stress, suggesting the Ca^{2+} cis elements may lie outside the promoter construct tested. Northern blot analysis shows induced expression of *CAX3* in response to 100mM $CaCl_2$ (Shigaki and Hirschi, 2000). No induction of *CAX3* was observed with other cations, hormones and cold stress. This expression is also noted by genevesitagator (https://www.genevestigator.ethz.ch/). Although the gene atlas feature shows *CAX3* expression in all 12 tissues, their data was compiled from RNA chip data which measures mRNA levels whereas GUS is a measure of active gene product. One difference that was observed is *CAX3* expression is much more abundant in roots compared to *CAX1* expression, which was more prominently expressed in the leaf tissue. This differential expression was only apparent when assaying the 2000bp promoter::GUS construct.

Two little studied genes within the *CAX* family are *CAX5* and *CAX6*. *CAX5*::GUS expression was only seen in 10 day old leaf and root tissue, as well as, in the shoot apical meristem (SAM; Figure 2.4). In contrast, *CAX6* expression was seen in both 10 and 30 day old samples. *CAX6*::GUS expression was also enhanced by treatment with cations and hormones (Figure 2.5). Both root tips and young leaf tissue showed higher expression when treated with Ca^{2+}, Mg^{2+}, PEG and ABA. The young leaves also had higher GUS expression when treated with Zn^{2+} and NAA. The expression profiles of *CAX5* and *CAX6* are somewhat similar with *CAX2* showing higher expression in most tissues (https://www.genevestigator.ethz.ch/). Although, we did not observe any expression in older plant tissues, *CAX5* and *CAX6* are expressed in the flowers of Arabidopsis plants.

Figure 2.5. *CAX6*::GUS expression increases in response to metal and osmotic stress and to ABA and NAA.

A-G) Water control of 10 day old plant shows no expression in SAM and trichomes. Induction of *CAX6*::GUS expression in SAM and trichomes in response to 50 uM $ZnCl_2$, 100mM $CaCl_2$, 10mM $MgCl_2$, 10%PEG, 100µM ABA, 1µM NAA.
H-L) *CAX6*::GUS expression in not apparent in 10day old root vasculature tissue but is expressed in response to 10mM $CaCl_2$, 10mM $MgCl_2$,10% PEG and 100µM ABA.

Figure 2.6. Analysis of GUS expression in *CCX1*::GUS and *CCX2*::GUS transgenic Arabidopsis.

A-B) Expression of *CCX1*::GUS in 30 day old leaves and root vasculature tissue. C) Expression of *CCX2*::GUS in 30 day old pollen grains.

CCX1-5

Recently, *CAX7-11* were renamed *CCX1-5* (Shigaki et al., 2006). This new nomenclature is based on *CCXs* having similar sequences to mammalian K$^+$-dependant Na$^+$/Ca^{2+} than to *CAXs* (Cai and Lyton, 2004; Shigaki et al., 2006). The *CCXs* are primarily expressed in pollen and flowers (Figures 2.6-2.8). *CCX1* and *CCX4* do show some expression in other plant tissues. *CCX2-CCX5* lines are all expressed in pollen and floral tissues with the exception of *CCX3* lines which are not expressed in floral tissue.

Figure 2.7. Analysis of GUS expression in *CCX3*::GUS and *CCX4*::GUS transgenic Arabidopsis.

A-B) Expression of *CCX3*::GUS in embryos and pollen.
C-G) Expression of *CCX4*::GUS in (water only) 10 day old plants increases in response to 10mM CaCl$_2$, 10mM MgCl$_2$, 2mM MnSO$_4$, and 100µM ABA.
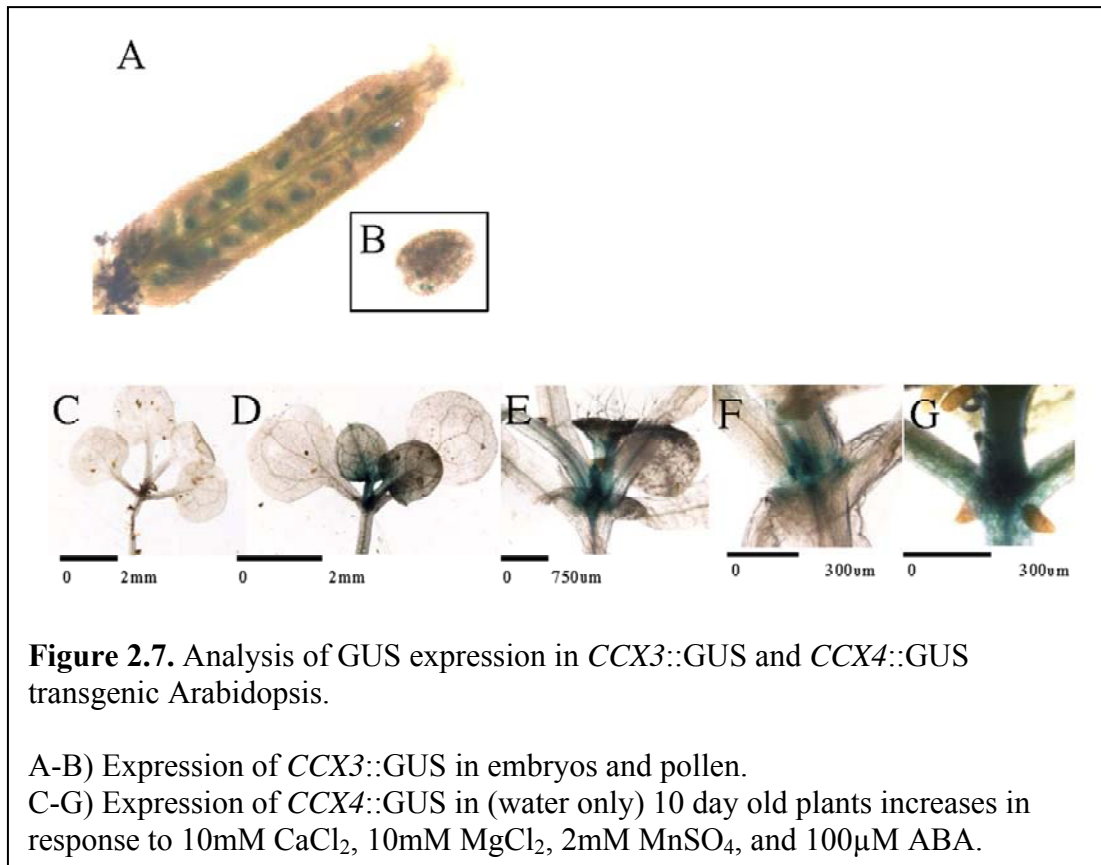
CCX3 expression was very low and it takes five days to observe GUS expression in these lines (Figure 2.7). Conversely, compared to *CAX1*::GUS lines, expression could be seen after 24 hrs and all of the lines analyzed showed expression after 48 hrs. Chip analysis of *CCX1* reveals expression in pollen. However, these lines were not stained for longer intervals like *CCX3* lines, therefore this could account for a lack of *GUS* expression in *CCX1*::GUS pollen. *CCX4*::GUS expression increased in lines treated with CaCl$_2$, MnSO$_4$, MgCl$_2$ and ABA; however, this was only
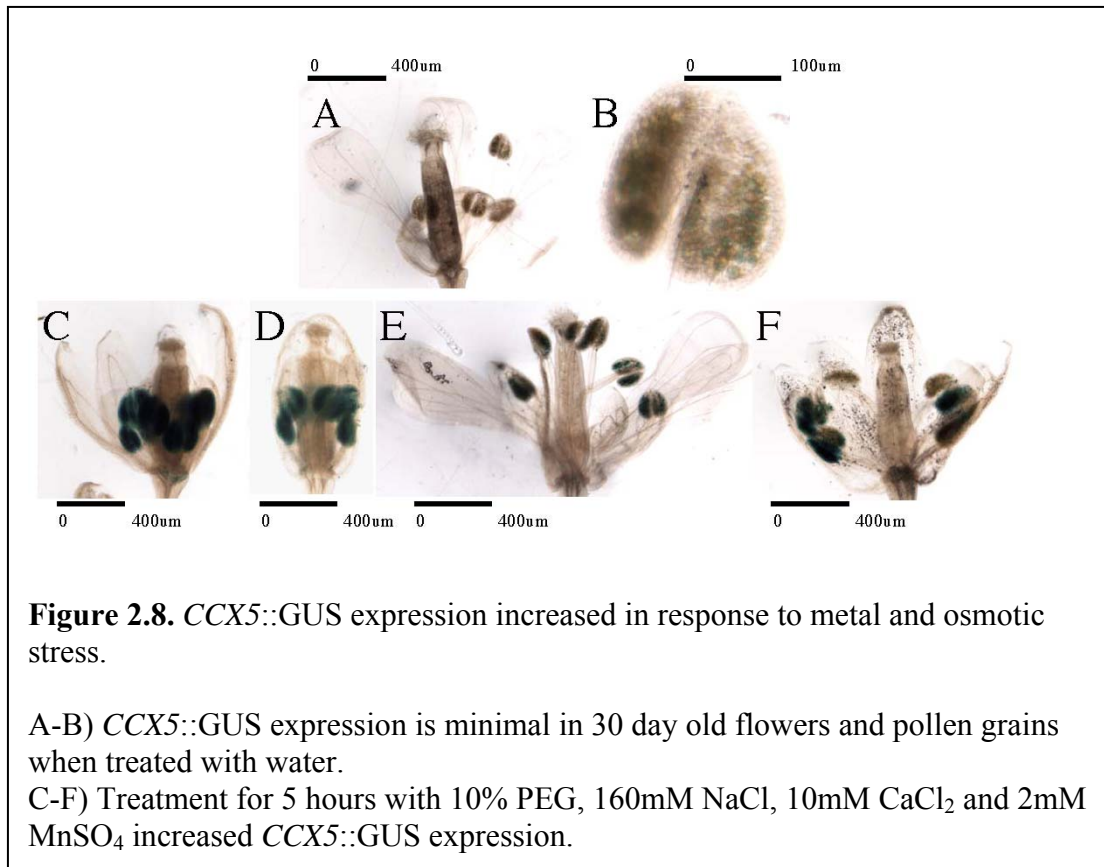
Figure 2.8. *CCX5*::GUS expression increased in response to metal and osmotic stress.

A-B) *CCX5*::GUS expression is minimal in 30 day old flowers and pollen grains when treated with water.
C-F) Treatment for 5 hours with 10% PEG, 160mM NaCl, 10mM $CaCl_2$ and 2mM $MnSO_4$ increased *CCX5*::GUS expression.
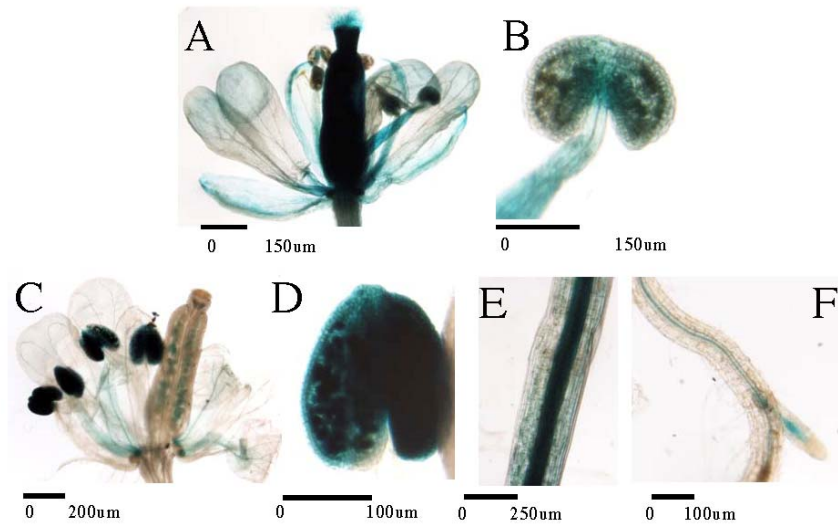
Figure 2.9. Analysis of GUS expression in *CHX13*::GUS and *CHX14*::GUS transgenic Arabidopsis.

A,B) Expression of *CHX13*::GUS in 30 day old flowers and pollen.
C-F) Expression of *CHX14*::GUS in 30 day old embryos, pollen, stem vasculature tissue and vasculature tissue of roots and lateral roots.

observed in 10 day plant tissue. *CCX5*::GUS (Figure 2.8) expression was also induced

by some cations, mainly $CaCl_2$, $MnSO_4$, NaCl, as well as osmotic stress when treated

with PEG. However, *CCX5*::GUS expression was seen in older floral tissue and the

above changes were not seen in young leaves and root tissue.   Comparison of *CCX4* and

CCX5 to their respective gene atlas profiles confirmed our GUS data

(https://www.genevestigator.ethz.ch/). These genes do not show induction by cations

and the hormones used in this study, although *CCX4* was highly induced by

brassionalides, however we did not analyze this compound. Promoter::reporter analysis

Figure 2.10. Analysis of GUS expression in *CHX26*::GUS and *CHX27*::GUS transgenic Arabidopsis.

A,B) A transcriptional fusion of *CHX26*::GUS (769 bp) displayed no expression in lateral roots; however, a translational fusion of *CHX26*::GUS (869bp) showed expression in 30 day old lateral root tips.
C,D) A transcriptional fusion of *CHX27*::GUS (769 bp) showed no expression in lateral roots; however, a translational fusion of *CHX27*::GUS (869bp) showed expression in 30 day old lateral root tips.

Figure 2.11. Analysis of GUS expression in *CCC*::GUS transgenic Arabidopsis.

A,B) Expression of GUS in the root tissue, petiole and SAM and trichomes in 10 day old plants.
C,D) GUS expression in pollen tissue of 30 day old flowers.

of this family of genes showed predominate expression in floral and pollen tissues.

Future studies of *CCX1* and *CCX4* will be interesting since they appear to be expressed

in other tissues within the plant suggesting a more diverse role in plant biology.

Other transporters

The genes in CHX family are expressed primarily in pollen grains of Arabidopsis

and are thought to play a role in pollen development (Sze et al., 2004; Bock et al., 2006).

Expression of GUS in *CHX13*::GUS, *CHX14*::GUS, *CHX26*::GUS and *CHX27*::GUS

lines was observed in pollen grains and stigmas (Figure 2.9). GUS expression was also

seen in the roots in *CHX14*::GUS, *CHX26*::GUS and *CHX27*::GUS lines (Figure 2.10).

In the *CHX26*::GUS and *CHX27*::GUS lines only the promoter fragment containing the

full length promoter plus 100bp of the first exon showed expression in lateral root tips.

This suggests either a regulatory or localization signal exists within this exon sequences.

It is worth noting that *CHX26* and *CHX27* share a common promoter sequence and their

100bp exon sequences are somewhat similar suggesting these genes could have similar

functions. Two other cation transporters were tested. *CCC* and *MHX1*::GUS

expressions were detected in pollen grains, like many other of the cation transporter

analyzed (Figures 2.11 and 2.12; Maser et al., 2001). We also observed GUS expression

in root and leaf vasculature tissue. However, we did not observe any changes in

expression with treatment of various cation, hormones or stress treatments.

Conclusions

Here we analyzed promoter::reporter fusions for a small subset of the 855 identified cation transporters from Arabidopsis (Sze et al, 2004; Shigaki and Hirschi, 2006). We used only limited 5' and, in a couple of cases, a small portion of the coding sequence to study gene expression patterns. Obviously this did not always represent or mimic native gene expression. For instance, we did not observe any induction of *CAX1*::GUS expression in response to Ca^{2+}. Given our limited set of promoter::reporter fusions and the inability to observe any post translation regulation we were not be able to qualitatively measure every factor which might influence expression. However, our survey still produced some interesting observations. For example, the induction of *CCX4* expression by numerous cation treatments suggests a general role in cation homeostasis. In the case of *CHX26* and *CHX27*, they appear to have a lateral root specific element within the first 100bp of exon one. In sum, this preliminary analysis suggests that observation of promoter::reporter expression can be utilized to infer function of cation transporters.

Figure 2.12. Analysis of GUS expression in *MHX1*::GUS transgenic Arabidopsis.

A,B) Expression of GUS in leaf vasculature tissue and pollen grains in 30 day old plants.
C,D) In 10 day old plants GUS was expressed in true leaves, hypocotyls, trichomes root vasculature tissue.
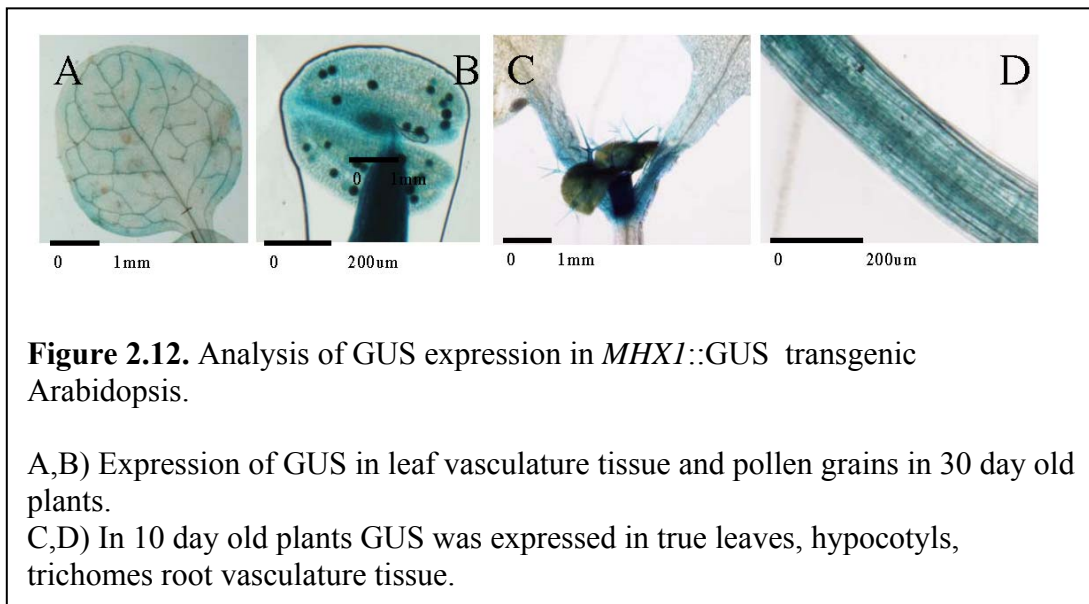
CHAPTER III

*At*CCX3, AN ARABIDOPSIS ENDOMEMBRANE TRANSPORTER, SIMILAR

TO K^+ DEPENDENT Na^+/Ca^{2+} EXCHANGERS, REGULATES VACUOLAR

CATION HOMEOSTASIS

Introduction

The plant vacuole and other endomembrane compartments play an important role

in the sequestration of various compounds (Marschner, 1995; Marty, 1999).

Concentration gradients of Na^+, Ca^{2+}, Cd^{2+}, NO_3^-, and Mn^{2+} are established across these

membranes by cation/H^+ exchange activities (Schumaker and Sze, 1985; Salt and

Wagner, 1993; Barkla and Pantoja, 1996; Gonzalez et al., 1999). Several genes

encoding these transport activities have been identified (Shigaki and Hirschi, 2006).

However, the biological function of many of the individual transporters remains for the

most part undefined.

CCXs (for Calcium Cation eXchangers) were previously identified as CAX (for

<u>CA</u>tion e<u>X</u>changers) homologs. Recently CAX7-11 were reclassified as CCX1-5 due to

higher homology to mammalian K^+ dependent Na^+/Ca^{2+} antiporters (Shigaki et al.,

2006). CAXs are Ca^{2+}/H^+ antiporters which show high-capacity, low-affinity transport

and have been characterized from a variety of plants (Blumwald and Poole, 1986; Kasai

and Muto, 1990; Ettinger et al., 1999; Cheng et al., 2002; Luo et al., 2005). CAXs are

energized by the pH gradient established by proton pumps such as H^+-ATPase or H^+-

pyrophosphatase (Kamiya and Maeshima, 2004). Several plant CAXs have been

characterized as vacuolar localized transporters, which function in H^+ coupled antiport of

Ca^{2+}, Mg^{2+}, and Mn^{2+}, resulting in accumulation of these cations in vacuoles (Hirschi, 1999; Pittman and Hirschi, 2001, Pittman et al., 2004a). CCXs have not been studied and it would be interesting to compare and contrast their activities to CAXs and the less closely related $Na^+(K^+)/H^+$ exchangers of the NHX family.

CAX proteins have N-terminal regulatory domains (Pittman and Hirschi, 2001), and *At*CAX1 and *At*CAX2 were originally cloned as functional N-terminal deletions (lacking the negative regulatory domain; Hirschi et al., 1996). We refer to these forms as sCAX1 and sCAX2 (Shigaki and Hirschi, 2006). Tobacco plants overexpressing *AtsCAX1* exhibit Ca^{2+} deficiency, leaf necrosis, tip burning and hypersensitivity to ion imbalance, as well as increased tonoplast Ca^{2+}/H^+ transport activity (Hirschi, 1999). The N-terminus of CCXs lacks homology with CAXs and it is not known if the N-terminus has a regulatory function.

One well characterized Arabidopsis transporter is the vacuolar localized Na^+/H^+ exchanger, *AtNHX1* (Apse et al., 1999; Apse et al., 2003). When highly expressed in Arabidopsis, *AtNHX1* confers tolerance to salt (Apse et al., 1999). Conversely, when *At*NHX1 is non-functional, the plants have diminished ability to transport cations and become sensitive to Na^+ and K^+ (Apse et al., 2003). In plants, *At*NHX1 functions as a vacuolar Na^+ or K^+ transporter but does not appear to be directly involved in the uptake of other cations, such as Ca^{2+}. To date, it is unclear how many other Arabidopsis transporters have functional properties similar to *At*NHX1.

Arabidopsis *CCX*s are related to mammalian plasma membrane Na^+/Ca^{2+} exchangers. The NCX are Na^+/Ca^{2+} exchangers are involved in Ca^{2+} movement which is

Na$^+$-dependent and NCKXs are defined by Ca^{2+} movement which is K$^+$-dependent (Cai and Lyton, 2004). Interestingly, both NCX and NCKX exchangers can operate in a forward (Ca^{2+} exit) or reverse (Ca^{2+} entry) mode which is mediated by the change in Na$^+$ gradients and the potential across the membrane (Cai and Lyton, 2004). However, the extent to which CCX transporters from Arabidopsis transport Na$^+$ or K$^+$ has not been addressed.

To investigate the function of CCX transporters, we cloned *AtCCX3* and the closely related *AtCCX4*. We expressed the transporters in various yeast strains in order to compare and contrast their function to *At*CAX1 and *At*NHX1 transporters. We monitored expression and localization of *At*CCX3 in yeast cells and *in planta*. Finally, we overexpressed *AtCCX3* in plants and examined ion uptake and plant growth. Collectively, these findings demonstrate *At*CCX3 is a newly identified endomembrane K$^+$ transporter.

Material and Methods

Cloning of AtCCX3 and AtCCX4 cDNAs

AtCCX3 and AtCCX4 genes do not have introns. Therefore, full length and N-terminal truncated version of *AtCCX3* (*At*3g14070) were amplified from Arabidopsis genomic DNA by using the polymerase chain reaction (PCR). The forward primer used for amplification of *AtCCX3* was 5'A GGA GG<u>G GCC AAA TCG GCC</u> ATG AGC GCG GTT AGT TTT CTT TAC AGC 3' and the reverse primer was 5' A GGA GG<u>G GCC ATA AGG GCC</u> TTA AGC AGC CCA TGG TAT GAA TCC CAT GGC 3'. The

underlined portion of the primer is the *SfiI* site used for directional cloning (Shigaki et al., 2006). The forward primer used to amplify the N-terminal truncation of *AtCCX3*, with a 38 amino acid deletion, is as follows: 5' A GGA GG<u>G GCC AAA TCG GCC</u> ATG GGA AAC CCT TTG TTA AGG AAC GCG 3'. Full length *AtCCX4* was amplified from a bacterial artificial chromosome (BAC; Assembly unit: 3600628, F15│1; Arabidopsis Biological Resource Center, Columbus, OH, USA) by PCR. The forward primer used to amplify *AtCCX4* (*At*1g54115) was: 5'A GGA GG<u>G GCC AAA TCG GCC </u>ATG AGA GCT GTG AAT TTT ATG TAT AGC 3'. The reverse primer was 5' A GGG GC<u>G GCC ATA AGG GCC</u> TCA AGA AGC CCA AGG TAT GAA TCC 3'. The 1.9 kb *AtCCX3* and 1.9 *AtCCX4* kb PCR products were cloned into the Topo 2.1 vector (Invitrogen, Carlsbad, CA, USA). These plasmids were then sequenced and contained no nucleotide changes and the encoded proteins contained no amino acid changes.

Plasmid DNA constructs

   AtCCX3, *At*s*CCX3*, and *AtCCX4* cDNAs were subcloned into the modified yeast expression vector piHGpd (Figure 3.1 A and 3.1B; Nathan et al., 1999) using *SfiI* restriction sites (Pittman and Hirschi, 2001; Pittman et al., 2002).

   The CAX2 plasmid was cloned previously (Shigaki et al. 2003). The Arabidopsis Na$^+$/H$^+$ exchanger *AtNHX1* was PCR cloned from template DNA derived from pDR196 plasmid containing *AtNHX1*. The following primers incorporated *SfiI* cut sites: forward 5' 5'A GGA GG<u>G GCC AAA TCG GCC</u> ATG CTA TCC AAG GTA TTG CTG AAT 3' and the reverse 5' A GGG GC<u>G GCC ATA AGG GCC</u> CTA GTG

GTT TTG GGA AGA GAA ATC TGC AGG 3'. The 1.9 *AtNHX1* PCR product was

cloned into the Topo 2.1 vector (Invitrogen, Carlsbad, CA, USA). *AtNHX1* fragments

Figure 3.1 Expression vectors modified to contain an *Sfi* linker cut site.

A-B) piHGpd with histidine selection and uracil selection.
C) pBIN19 with 35SCaMV promoter.

were then cloned into the yeast expression vector piHGpd (Nathan et al., 1999) using

SfiI restriction sites (Pittman and Hirschi, 2001; Pittman et al., 2002).

The *AtCCX3* and *At*s*CCX3* ORFs were cloned into modified pBIN19 (Figure

3.1C) by ligating the gene fragments using *SfiI* restrictions sites (Clontech, Palo Alto,

CA, USA).   The resulting constructs contained the cauliflower mosaic virus 35S-

promoter fragment driving expression of the transporters and the NOS terminator (Hull

et al., 2000).

The triple hemagglutinin (HA) epitope-tagged *AtCCX3* (HA:*AtCCX3*) was constructed as previously described (Shigaki et al., 2001). The primers used to amplify the HA tag were as follows: forward 5' A GGA GG<u>G GCC AAA TCG GCC</u> ATG GGC ATC TTT TAC 3'; reverse 5' GAA <u>TTC GAG</u> ACG GCA CTG AGC AGC GTA ATC TGG AAC GTC 3'. The *SfiI* and *BsmBI* sites are underlined. *AtCCX3* was engineered to contain a *BsmBI* site at the 5' and a *SfiI* cut site at the 3' end. The primers used were as follows: forward 5' GAA TTC <u>CGT CTC</u> CGT GAC ATG AGC GCG GTT AGT TTT CTT TAC 3'; reverse 5' A GGA GG<u>G GCC ATA AGG GCC</u> TTA AGC AGC CCA TGG TAT GAA TCC CAT GGC 3'. Each fragment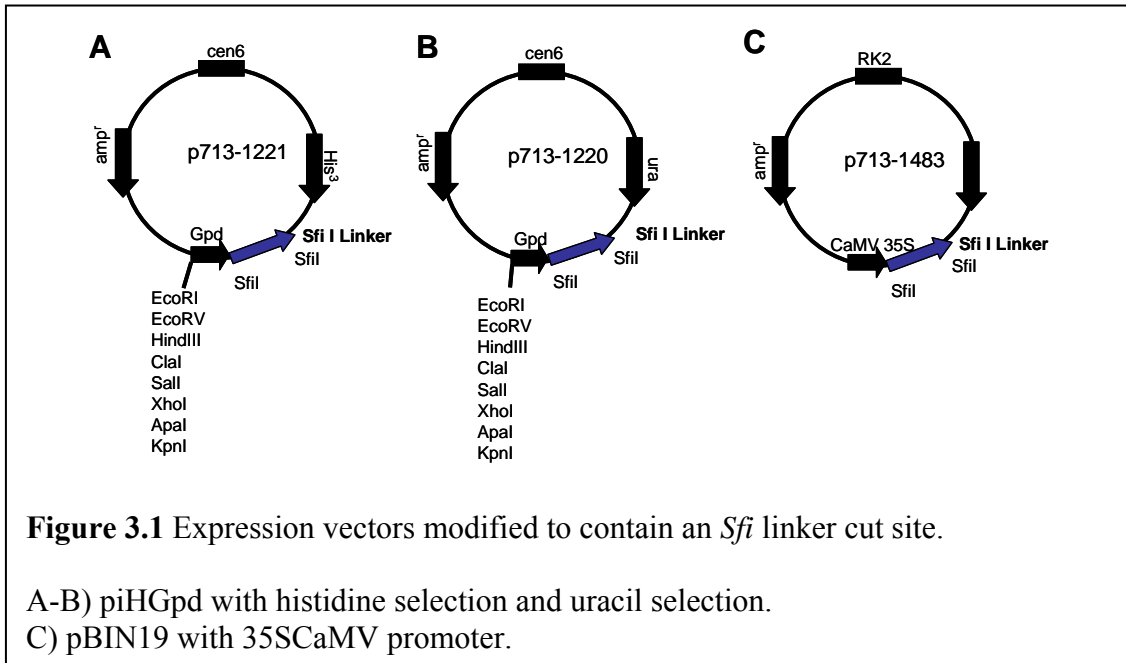 was cloned into the Topo 2.1 vector (Invitrogen, Carlsbad, CA, USA) and the sequences confirmed. The two fragments were ligated with the yeast expression vector piHGpd (Nathan et al., 1999) using the *SfiI* and the *BsmBI* restriction sites.

The C-terminal green fluorescent protein (GFP) tag *AtCCX3* (CCX3-GFP) was constructed as previously described (Cheng et al., 2004). The primers used to amplify *AtCCX3* and the GFP tag were as follows: *AtCCX3* forward 5'AGG AGG <u>GGC CAA ATC GGC C</u> ATG AGC GCG GTT AGT TTT CTT TAC AGC 3'; reverse 5' <u>GCG GCC GC</u>A AGC AGCC CAT GGT ATG AAT 3' with an *SfiI* site in the forward and a *NotI* site in the reverse primer. For GFP: forward 5' <u>GCG GCC GC</u>A TGG TGA GCA AGG GCG AGG AG 3'; reverse 5' GAG TTC <u>GGCC CTTAT GGCC</u> TTA CTT GTA CAG CTC GTC 3', with a *NotI* site in the forward and a *SfiI* in the reverse primer. Each fragment was cloned into the Topo 2.1 vector (Invitrogen, Carlsbad, CA, USA) and the sequences confirmed. The two fragments were ligated with the yeast expression vector

piHGpd (Nathan et al., 1999) in a three-way ligation using the *SfiI* and *NotI* restriction

sites. *AtCCX3*-GFP was also cloned in the plant transient expression vector pRTL using

KpnI and *XbaI* sites (Cheng et al., 2004). This was accomplished by amplifying the

CCX3-GFP cassette using the following primers: forward 5'AGG AGG GGT ACC

ATG AGC GCG GTT AGT TTT CTT TAC AGC 3' and reverse 5' GAG TCT AGA

TTA CTT GTA CAG CTC GTC 3'. This cassette was also cloned into pBIN19 by a

three way ligation using the *SfiI* and *NotI* sites (Clontech, Palo Alto, CA, USA; Hull et

al., 2000).

Yeast strains and growth

        The following *S. cerevisiae* yeast strains were used in this study: W303-1A

(*MATa* ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1; Wallis et al., 1989).

R100 (Δnhx1::URA3; Nass et al., 1997), which is isogenic to W303. The Mg^{2+} sensitive

yeast strain CM66 (*MATa* Δalr1: :HIS3, Δalr2: :TRP, his3-v200, ura3-52, leu2-v1, lys2-

v202 trp1-v63; Liu et al., 2002). The yeast strain K661 (*MATa* Δvcx1::URA3, ade2-1

can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1; Cunningham and Fink, 1996) and K667

(cnb1::LEU2 pmc1::TRP1 vcx1Δ; Cunningham and Fink, 1996) were also used in yeast

growth assays. Yeast metal-sensitive growth assays were performed as previously

described (Hirschi et al., 1996; Nass et al., 1997, Nass and Rao, 1998; Darley et al.,

2000; Shigaki et al., 2003; Padmanaban et al., 2007).

Yeast sample processing and ICP analysis

        Yeast culture conditions and sample processing was modified from a previous

study (Eide et al., 2005). Yeast cultures were inoculated in 5 ml YPD supplemented

with 100 mM Na^+ or 200 μM Mn^{2+}. The cells were grown at 30° C to middle log phase

and 2 ml of each culture was collected by vacuum filtration using isopore membrane

filters (1.2 μm pore size) (Fisher Scientific, Pittsburgh, PA, USA). Cells were washed

three times with 1ml of 1 μM EDTA, pH 8.0, followed by three washes of distilled

deionized water all under vacuum filtration. Filters were left to dry for 48 hr at 70° C

before reading by inductively coupled plasma atomic emission spectroscopy (ICP-AES)

analysis, performed as previously described (Lahner et al., 2003).

Membrane vesicle isolation and transport measurement

Yeast vacuolar membrane vesicles were prepared using a two-step sucrose

gradient as previously described (Nakanishi et al., 2001; Pittman et al., 2004b). Proton

coupled cation exchange was measured in yeast vacuole vesicles by recovery of

quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine

dihydrochloride) fluorescence. The vacuolar H^+-ATPase was activated using 250mM

BTP-ATP as previously described (Barkla et al., 1999). Changes in fluorescence

intensity over time were monitored using a fluorescence spectrometer (Perkin Elmer LS-

5, MA, USA), at excitation and emission wavelengths of 427nm and 495 nm,

respectively with a slit width of 5nm.

[86]Rb uptake assay in yeast

Yeast strains (*wx1*) expressing *At*CCX3, *At*NHX1 and vector were grown over

night in selection media. Dilutions of yeast were added to media containing 0.02 mM

and 20 mM K^+ at time points of 0, 10, 20, 40, 60, 100, 160, and 200 minutes, 1 ml of

culture was filtered and read in a gamma radiation counter as previously described (Fu and Luan, 1998).

Protein isolation and western analysis of epitope tagged AtCCX3 in yeast

Total protein was isolated from yeast expressing HA-*AtCCX3* using the glass bead method (Ausubel et al., 1998). Sucrose fractionation of membranes was done as previously described (Pittman et al., 2004b). Proteins (10μg) were separated out by SDS-PAGE on a 12% (w/v) precast gel (Bio Rad, CA, USA) and transferred to a polyvinylidene fluoride membrane (Amersham Bioscience, Piscataway, NJ, USA). Blots were blocked with a 5% w/v non-fat dried milk in phosphate-buffered saline with Tween 20 for 1 hr and then reacted with a 1:2000 dilution of anti-HA monoclonal antibody (Amersham Bioscience, Picataway, NJ, USA) for one hr at room temperature. The vacuolar marker ALP (Molecular Probes, Carlsbad, CA, USA) was used at a 1:2000 dilution and the plasma membrane marker, affinity purified polyclonal antibody, against the plasma membrane H^+-ATPase Pma1p at a 1:1000 dilution for one hr. The blots were washed with PBS-T (PBS-T; 10mM NaH_2PO_4/NaOH, ph 7.2) for one hr. Blots were then incubated in a 1:10,000 dilution of horseradish peroxidase coupled anti-mouse secondary antibody (Amersham Bioscience, Piscataway, NJ, USA) for one hr and then washed for one hr with PBS-T. The ECL Plus reagent kit (Amersham Bioscience, Piscataway, NJ, USA) was used to develop the blots which were then exposed to Hyperfilm photogenic film (Amersham Bioscience, Piscataway, NJ, USA). For the GFP tagged protein, total plant protein was isolated as previously described (Fitzpatrick and Keegstra, 2001).

Protein isolation and western analysis of epitope tagged AtCCX3 in plants

Microsomal membranes were prepared from *At*CCX3-GFP-expressing Arabidopsis leaf tissues as previously described (Cheng et al., 2003). Immunoblots were performed as previously described (Pittman and Hirschi, 2001). The GFP epitope and the membrane marker proteins were detected as described previously (Cheng et al., 2003).

Onion epidermis bombardment and visualization of the GFP subcellular localization

Single epidermis layers were removed from the white onion bulb and placed on the surface of a MS plate (Murashige and Skoog, 1962) containing 2% sucrose and 50μg/ ml ampicillin. The constructs 35S::CCX3-GFP and 35S::GFP were introduced into onion epidermal cells by particle bombardment. The process of particle preparation, coating and bombardment were done according to Sivitz et al., (2007). A PDS-1000/He Biolistic Particle Delivery System (Bio-RAD) was used for the experiment. 1100 psi rupture discs (Bio-Rad, CA, USA) and the Tungsten M-17 particles were used in bombardment under a vacuum of 26-28 Hg. GFP fluorescence was observed with a Nikon C1si™ laser scanning confocal microscope (Nikon, Japan) using a 488 laser line of an argon (Ar) laser, with the emission window set at 505-535nm. The GFP fluorescence acquisition was performed at Plan Apo 20×/ 0.75 NA objective. Images were recorded with picture size of 512 × 512 pixels. Individual sections along whole cells were captured in IDS format and then transferred into TIFF files.

Plant materials and growth

Arabidopsis 'Columbia' ecotype was used as wild-type. *Agrobacterium tumefaciens* GV3101 was transformed with recombinant plasmids of *AtCCX3*, *AtsCCX3* or vector controls (Sambrook et al., 1989). Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1999). Tobacco, *Nicotiana tabacum* L. cultivar KY14 (Koren'kov et al., 2006) transformation was done using the leaf disk method as previously described (Tarczynski et al., 1992). Transformants were selected on standard media containing 100 mg/mL kanamycin. Approximately 30 primary transformants harbouring the 35S::*AtCCX3* construct were placed in soil. T2 generation tobacco seeds were germinated on half strength MS $^+$ salts media containing the selection agent kanamycin. At 21 days, plants were transferred to soil. Plants were watered as needed and fertilized once every two weeks. At 40 days, one mature leaf (phenotype displayed throughout the leaf) and one young leaf (phenotype is present only at the leaf tip) was taken from the plant, dried at 75° C for three days, ground in a mortar and pestle and analyzed for mineral content. ICP was analysis was done as previously described (Franson, 1989).

^{86}Rb uptake in Arabidopsis

35S::*AtCCX3* lines 1, 2, 3 and vector control plants were grown hydroponically from seed in sterile flasks containing 2% sucrose and 1X B5 media (Caisson Labs, USA) at pH 5.0. After 14 days, seedlings were washed with K^+-free MS media and grown for an additional 48 hrs in 100ml K^+-free media. ^{86}Rb uptake was conducted in the K^+-free media, supplemented with 0.5uCi ^{86}Rb and either 0.02mM or 20mM KCl (Xiong et al., 2002; Philippar et al., 2004). Plants were then harvested (6-8 seedlings) at 10, 20, 30,

60, 80 and 120 minutes. The plants were washed 3 times with 10ml K$^+$-free media,

seedlings were blotted dry on filter paper and placed in a vial. Uptake of ^{86}Rb was

measure by gamma counting and measurements recorded as ^{86}Rb/g fresh wt.

RNA extraction and RT-PCR

RNA was isolated using the RNeasy Plant Kit (Qiagen USA), according to the

manufacturer's instructions. RNA samples were treated with DNase to minimize any

contamination of genomic DNA. RT-PCR was performed to detect mRNA transcript in

Arabidopsis of *AtCCX3* and *AtCCX4* knock out lines, Arabidopsis plants overexpressing

AtCCX3 and *AtsCCX3* and tobacco plants ectopically expressing *AtCCX3* and *At*s*CCX3*.

The first strand of cDNA was synthesized using 0.2 µg of total RNA as template in 20

µL of reaction mixture, which included 0.5 µg of oligo (dT) (12–18) primer and 200

units of Superscript II transcriptase (Invitrogen, Carlsbad, CA). One microliter of the

first strand cDNA was used to amplify an *AtCCX3* gene specific fragment and an *actin1*

fragment (Geisler et al., 2000). Forward and reverse primers for *AtCCX3*, *AtCCX4* and

actin1 were used at concentrations of 0.5µM each in amplification conditions identical

to those used to isolate *AtCCX3*. The primers used were as follows: *AtCCX3* forward 5'

A GGA GG<u>G GCC AAA TCG GCC</u> ATG GGA AAC CCT TTG TTA AGG AAC GCG

3' and reverse 5' A GGA GG<u>G GCC ATA AGG GCC</u> TTA AGC AGC CCA TGG TAT

GAA TCC CAT GGC 3' ; *AtCCX4* forward 5' A GGA GG<u>G GCC AAA TCG GCC</u>

ATG AGA GCT GTG AAT TTT ATG TAT AGC 3' and reverse 5' A GGG GC<u>G GCC</u>

<u>ATA AGG GCC</u> TCA AGA AGC CCA AGG TAT GAA TCC 3' ; *actin1* forward 5'

GTG CTC GAC TCT GGA GAT GGT GTG 3' and reverse 5' CGG CGA TTC CAG

GGA ACA TTG TGG 3'. PCR products were seperated on 2.5% (w/v) agarose gels and stained with ethidium bromide. The gels were photographed with a digital camera (Kodak, Rochester, NY), and the net intensity of individual PCR products was determined using Kodak ID 2.02 analysis software. The relative intensities in different lanes within each individual experiment were independent of the number of PCR cycles performed.

Isolation of homozygous T-DNA insertional lines

To isolate *ccx3* and *ccx4* null alleles, two T-DNA insertional lines were obtained from the SAIL T-DNA insertion collection (Sessions et al., 2002) for *AtCCX3* (SAIL_F-09, *ccx3-1*; SAIL_C30-05, *ccx3-2*) and two from the SALK T-DNA (SALK_113447, *ccx4-1*; SALK_040272, *ccx4-2*) insertion collection (Alonso et al., 2003) for *AtCCX4*. Homozygous plants from each T3 generation were obtained by PCR screening using *AtCCX3*-specific, *AtCCX4*-specific and T-DNA border primers. For *AtCCX3* the primers are as follows: reverse primer, 5' TTA AGC AGC CCA TGG TAT GAA TCC CAT GGC 3', and a T-DNA left border primer, 5' TAG CAT CTG AAT TTC ATA ACC AAT CTC GAT ACA 3' were used to screen for the *ccx3-1* and *ccx3-2* alleles (SAIL lines). For *AtCCX4*: reverse primer, 5' GAT GGC GGC CAA ATC GGC CAA ATT GGT TAG AGC GAG TAT TGA CGG GTT GAT 3' and a T-DNA right border primer, 5' TGG GAA AAC CTG GCG TTA CCC AAC TTA 3' were used to screen for the *ccx4-1* and *ccx4-2* alleles (SALK lines). To identify wild type copies of genes in heterozygote lines, gene fragments for *AtCCX3* were amplified by PCR using the primers listed in the plasmid DNA constructs section for *At*CCX3. For *AtCCX4* the

forward internal primer: 5' GAT GGC GGC CAA ATC GGC CAA ATT ATG TGG

ACC GAA GAT 3'; was used in conjunction with reverse primer listed in the DNA

constructs for *AtCCX4*.

Quantitative real time PCR analysis of AtCCX3

Arabidopsis plants were germinated on 1/2MS plates containing 0.5% sucrose.

At 14 days of age, 25 plants were transferred to liquid 1/2MS media containing standard

media or media supplemented with either 100mM KCl. 50mM NaCl or 1µM $MnCl_2$.

After 24 hrs, the plants were harvested and total plant RNA was extracted using an

RNeasy Plant Kit (Qiagen, USA). For floral RNA, 25 plants were transferred to soil and

watered weekly with either 50ml of 100mM KCl, 50mM NaCl or 1uM $MnCl_2$ solution.

After 3 weeks, flowers were harvested and total RNA was extracted using an RNeasy

Plant Kit (Qiagen, USA). Serial dilutions of RNA were made from 5µg to 5ng and first

strand cDNA was synthesized as previously described. The PCR amplification was

performed with 5µL of cDNA, 0.5µM of each primer and 1X SYBR Green PCR mix

(Invitrogen, USA). The following primers were used to amplify fragments from the

cDNA to use with the SYBR green kit (Invitrogen USA). *AtCCX3* forward 5' ATG

AGC GCG GTT AGT TTT CTT T 3' and reverse 5' TGA AAA CCC CTC GAA ATT

TGG 3'; *AtNHX1* forward 5' GAG CCT TCA GGG AAC CAC AA 3' and reverse 5'

CCG TGT CAA GAA GCC ACG TA 3'; 18S subunit forward 5' TGC AAC AAA CCC

CGA CTT ATG 3' and reverse 5' CCC GCG TCG ACC TTT TAT C 3'. Quantitative

PCR was done using the SYBR green probe, 1µg cDNA and the ABI 7900HT RT-PCR

system (Foster City, CA, USA). Real-time PCR amplification was performed and

calculations with ABI prism 7700 sequence detection system (Applied Biosystems, USA). Relative transcript abundance was determined using the comparative C_T method with SDS software version 2.2.2 (Applied Biosystems, USA). For a standard control, expression of the *18S* ribosomal subunit was used.

Protein oxidation analysis

Carbonyl assays for the analysis of oxidized proteins in plant cells were performed as previously described (Levine et al., 1994; Davletova et al., 2005; Cheng et al., 2006). Total protein was isolated from young and old leaves of six week old tobacco plants ectopically expressing *AtCCX3*, *At*s*CCX3* and vector only, as previously described (Cheng et al., 2006). The oxidized proteins were detected by protein gel blotting using anti-dinitrophenylhydrazone antibody (Davletova et al., 2005).

Pollen viability and tube growth

The viability of pollen from *ccx3-1*, *ccx3-2*, vector and 35S::*AtCCX3* tobacco plants was tested using an Alexander stain (Alexander, 1969). Pollen was harvested at 37 days of age and stage 12 maturation from Arabidopsis (Honys and Twell, 2003) and tobacco flowers. Pollen tube growth was assayed in these lines as previously described (Sze et al., 2004).

Results

AtCCXs are similar to mammalian NCKXs

A family of genes originally identified as members of plant *CAXs* (Mäser et al. 2001) were recently found to have high similarity to mammalian *NCKX* (Figure 3.2A).

Figure 3.2 *At*CCX3 and *At*CCX4 amino acid comparison to the CAX family of transporters and *Hs*NCKX6.

A) Phylogenetic tree showing the relative distance of predicted CAX to CCX proteins, including *Hs*NCKX6 (Prodist package in Phylip software; Felsenstein, 1989) Bar represents percent change in amino acids.
B) Protein sequence comparison of *At*CCX3 and *At*CCX4 to *At*CAX1 and *Hs*NCKX6. Alignment of deduced amino acid sequences were performed using ClustalW 1.8 program (Thompson et al., 1994). The autoregulatory domain of CAX1 is underlined in red. The yellow boxes mark the highly conserved α1 and α2 domains that are absent from *At*CAX1.
C) Predicted topology of *Hs*NCKX6, *At*CAX1, *At*CCX4 (*At*1g54115) and *At*CCX3 (*At*3g14070) analysis done using TMHMM algorithm (Sonnhammer et al., 1998; Krogh et al., 2001; http://www.cbs.dtu.dk/services/TMHMM /).

Thus, these transporters were reclassified as AtCCXs (Shigaki and Hirschi, 2006). We hypothesized that these putative transporters might function in cation homeostasis. We prepared cDNA clones for *At*CCX3 and *At*CCX4 from Arabidopsis (Columbia ecotype) genomic DNA since these genes were annotated not to contain introns. The cloned *AtCCX3* and *AtCCX4* open reading frames contained 1935 and 1938 nucleotides, which could encode 644 and 645 amino acids and produce putative proteins of 70.1 and 70.8-kD, respectively. *At*CCX3 and *At*CCX4 are 79.50 % identical and 86.02 % similar to one another. They share more identity (26.8 % identical; 46.9% similar) with *Hs*NCKX6 than with *At*CAX1 (12.1% identical; 25.1% similar). Both *At*CCX3 and *At*CCX4 contain short N-terminal hydrophilic domains, which are not related to the *At*CAX1 N-terminal autoinhibitory domain (Figure 3.2B). Comparisons of the AtCCX3, AtCCX4 and HsNKX6 proteins show a short (25 amino acids) N-terminal region, followed by five transmembrane (TM) domains separated from another seven TM domains by a long hydrophilic region (75 amino acids for *Hs*NCKX6 and 115 amino acids for *At*CCX4). By contrast, *At*CAX1 has a long N-terminal domain containing 65 amino acids and has two sets of four TM domains separated by a shorter hydrophilic domain (35 amino acids). Also, *At*CCX3, *At*CCX4 and *Hs*NCKX6, have very short (15 amino acids) C-terminal domains compared to *At*CAX1 which has longer C terminal region (25 amino acids; Figure 3.2C). Phylogenetic analysis of *At*CCX1-5, *At*CAXs and the human K^+-dependent Na^+/Ca^{2+} antiporter *Hs*NCKX6 clearly indicates CCXs are more homologous to the K^+-dependent Na^+/Ca^{2+} antiporter than to any of the *CAXs* (Figure 3.2A; Shigaki et al., 2006). Specifically, *At*CCX3 and *At*CCX4 share the
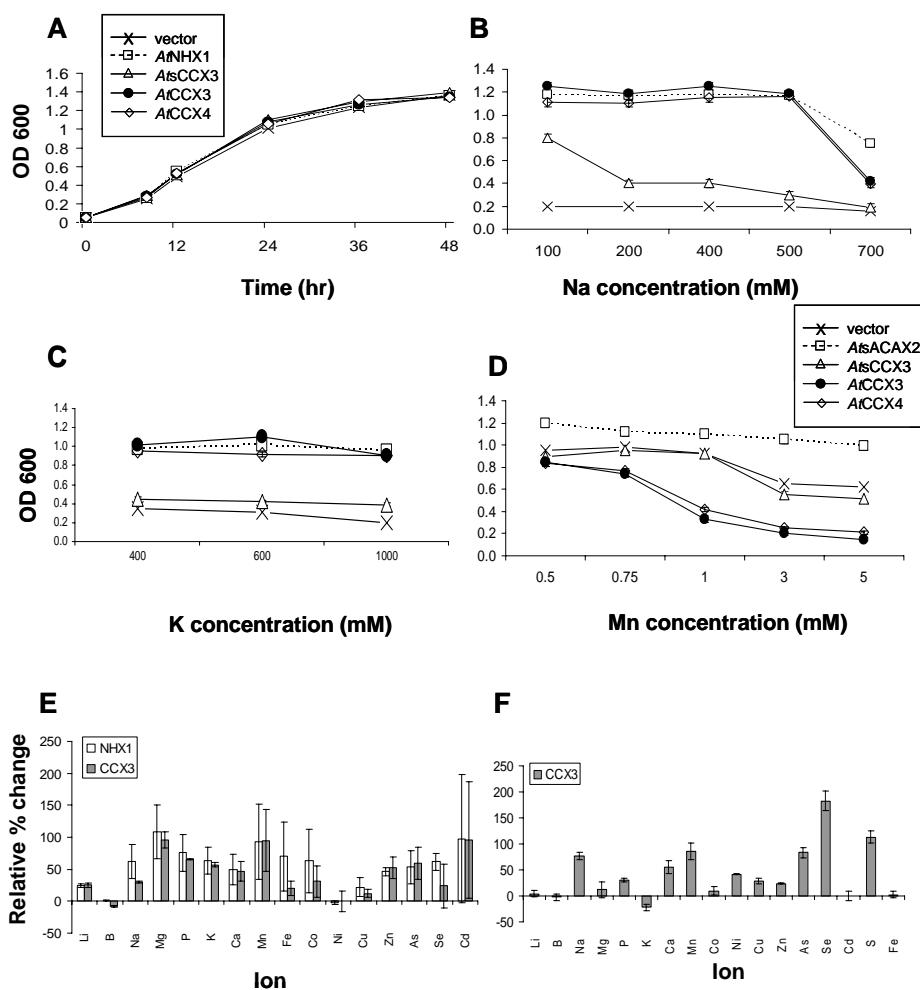
Figure 3.3 Growth of *AtCCX3* expressing yeast strains in YPD media with different cation concentrations.

A) Growth curve for yeast wx1 (*nhx1*) transformed with : vector (p2UGpd), *AtNHX1, AtCCX3, At*s*CCX3* and *AtCCX4* in YPD over various time points.
B-D) OD at 48 hrs for yeast strains grown in YPD with different concentrations of NaCl, KCl and MnCl$_2$. Yeast strains expressing the different plasmids were serial diluted and 10μl was placed into 190μL of liquid selection media containing NaCl, KCl or MnCl$_2$.
E) Relative percent change in cations between yeast wx1 vector control and wx1 transformed with *AtNHX1* or *AtCCX3* grown in media containing 100mM NaCl.
F) Relative percent change in cations between W303 yeast cells expressing controls and *AtCCX3* grown in media containing 200μM MnCl$_2$. Error bars represent standard error for the relative percent difference of the means (http://www.census.gov/acs/www/Downloads/ACS/PercChg.pdf).

characteristic α-repeats GNG(A/S)PD in α1 and (G/S)(N/D) SxGD in α2 with

HsNCKX6 (Figure 3.2B; Cai and Lytton 2004), these are highly conserved Ca^{2+} and Na^+

domains (Winkfein et al., 2003; Kang et al., 2005).

Expression of AtCCX3 and AtCCX4 in yeast

AtCCX3 and AtCCX4 were cloned into the piHGpd vector (Nathan et al., 1999)

for expression in yeast under control of the GPD promoter. To test the possibility of a

CCX N-terminal regulatory domain, we made a truncation in the AtCCX3 ORF

(AtsCCX3). These plasmids were then introduced into the yeast strain *wx1* (*nhx1* mutant)

with defects in vacuolar Na^+, K^+ transport (Nass et al., 1997). *AtCCX3* and *AtCCX4*

expression in *wx1* did not alter growth of the yeast in YPD (Figure 3.3A) compared to

vector controls. Also, expression of *AtsCCX3* or *AtNHX1* did not affect growth of *wx1*

(Figure 3.3A). AtCCX3, and AtCCX4 suppressed the Na^+ and K^+ sensitivity of this

yeast strain deficient in vacuolar Na^+/H^+ transport (Figure 3.3B and 3.3C). We also

tested both the HA-AtCCX3 and AtCCX3-GFP tagged proteins and they both showed

similar phenotypes to the native AtCCX3 (Figure 3.4A-C). Yeast cells expressing all of

the *AtCCX3* and *AtCCX4* variants were unable to suppress the Ca^{2+} sensitivity of yeast

strains deficient in vacuolar Ca^{2+} transport (data not shown). Yeast cells expressing

AtCAXs all show increased Ca^{2+} transport when truncated in their N-terminal domain

(Hirschi, 1999; Shigaki et al., 2001, Shigaki et al., 2003; Pittman et al., 2004a). A lack

of N-terminal regulation, the ability to suppress Na^+ sensitive phenotypes and the

inability to suppress Ca^{2+} sensitive phenotypes in yeast suggests functions for AtCCX3

and AtCCX4 that differ from CAXs.
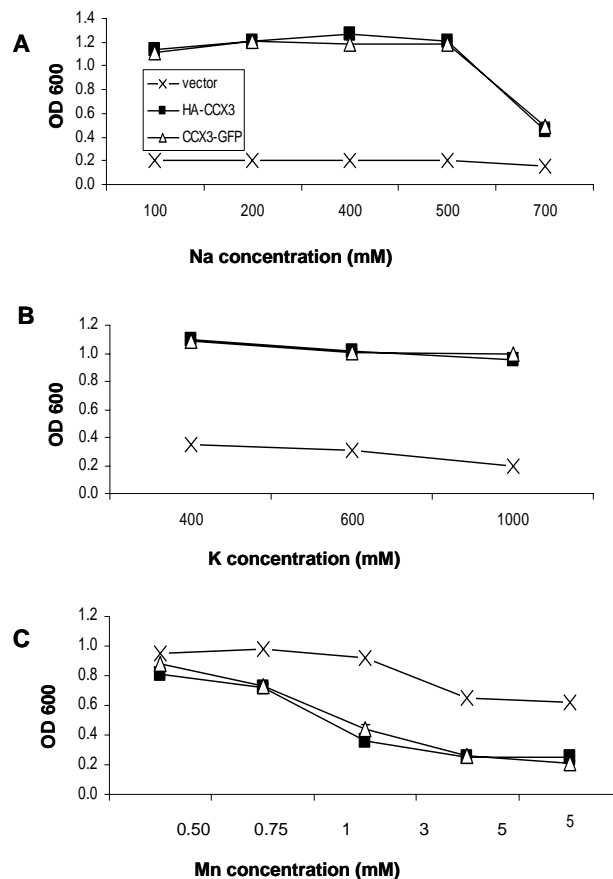
Figure 3.4 Growth of epitope tagged-*At*CCX3 in different media.

A) Growth curve of yeast wx1 (*nhx1*) strains expressing: vector (p2UGpd), *HA-AtCCX3* and *AtCCX3-GFP* in YPD supplemented with various concentrations of NaCl after 48 hrs.
B) OD at 48 hrs for yeast strains grown in YPD with different concentrations of KCl.
C) OD at 48 hrs of wild type W303-1A yeast grown in YPD with various concentrations of MnCl$_2$. Yeast strains expressing the different plasmids were serial diluted and 10µl was placed into 190µL of liquid selection media containing NaCl, KCl or MnCl$_2$. Yeast growth at 30°C is shown after 48 hr.

We also tested the possibility that *At*CCX3 and *At*CCX4 transport other metals,
thus making the host yeast cells tolerant or hypersensitive to these metals. Expression of
AtCCX3 and *AtCCX4* in an Mg^{2+} requiring strain (CM66) did not suppress the ion
sensitivity of these strains (MacDiarmid and Gardner, 1998). *AtCCX3* expressing cells
were also tested with a range of metals, such as Al^{3+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} in
various mutant yeast strains (K661; Cunningham and Fink, 1996). In each case,
AtCCX3-cells were indistinguishable from vector controls in our assay conditions.
However, wild type W303-1A yeast cells expressing *AtCCX3*, and *AtCCX4* were
hypersensitive to Mn^{2+} in the media compared to *At*s*CCX3* and vector expressing cells
(Figure 3.3D). Yeast cells expressing *At*s*CAX2* confer tolerance to Mn^{2+} (Figure 3.3D;
Pittman et al., 2004a). The lack of sensitivity in cells expressing *At*s*CCX3* and the lack
of Mn^{2+} tolerance similar to *At*s*CAX2* expressing cells, confirms that *At*CCX3 and
*At*CCX4 have distinct "non-CAX" functions.

Ionome measurements in yeast expressing AtCCX3 and AtNHX1

We tested the ionome changes in various yeast strains expressing *AtCCX3* and
AtNHX1. *At*CCX3 altereed the ionome in both the *wx1* and W303-1A yeast
backgrounds. Yeast cells expressing *AtCCX3* and *AtNHX1* in media containing elevated
levels of NaCl and $MnCl_2$ have very similar ion accumulation profiles (Figure 3.3E and
3.3F). *AtCCX3* and *AtNHX1* expressing cells accumulated 25% more Na^+ and 50% K^+
compared to vector expressing lines. Expression of both genes also increased the
accumulation of Mg^{2+}, P, Ca^{2+}, and Mn^{2+}, compared to the vector expressing lines.
Increases in micronutrients Fe^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mo^{2+} and Cd^{2+} were variable but

consistent.   In the wild type yeast strain, W303-1A, *AtCCX3* expressing cells also

altered the ionomes as evidenced by the 50% increase in Na^+ and Mn^{2+}, along with a

modest increase in P, Ni^{2+}, Ca^{2+}, Cu^{2+} and Zn^{2+}. *AtCCX3*-expressing yeast cells ability

to accumulate cations suggests *At*CCX3 plays a role in Na^+, K^+ and Mn^{2+} homeostasis.

Transport properties of AtCCX3 in yeast

In order to determine the K^+ - transport properties of *At*CCX3 we used direct

uptake assays of the radioactive tracer ^{86}Rb (Fu and Luan, 1998). In *wx1* yeast, only

cells expressing *AtCCX3* or *AtNHX1* showed higher ^{86}Rb uptake at high K^+

concentrations (20mM; Figure 3.5). At low external K^+ (0.02 mM) concentrations, ^{86}Rb

uptake by cells expressing *AtCCX3* and *AtNHX1* were similar to vector controls (Figure

3.5). This suggests that in yeast *AtC*CX3 can function as a low affinity K^+ transporter,

analogous to *At*NHX1.

Localization of AtCCX3 in yeast and plants

An N-terminal HA tagged *At*CCX3 construct conferred resistance to high Na^+

and K^+ stress in wx1 (Figure 3.4A and 3.4B). We utilized this construct to identify the

cellular location of *At*CCX3. As shown in Figure 3.6A, western-blot analysis of yeast

membranes fractionated on sucrose gradients showed that *At*CCX3 co-localized with

vacuolar membranes. The distribution of HA-*At*CCX3 corresponded with the yeast

vacuolar membrane marker alkaline phosphatase but not with the plasma membrane

marker Pma1p. As further confirmation of yeast endomembrane localization, the

*At*CCX3-GFP expressed in yeast also appeared to reside on the vacuolar membrane as

the signal overlapped with vacuolar marker protein Vac fused to RFP (Figure 3.6B;
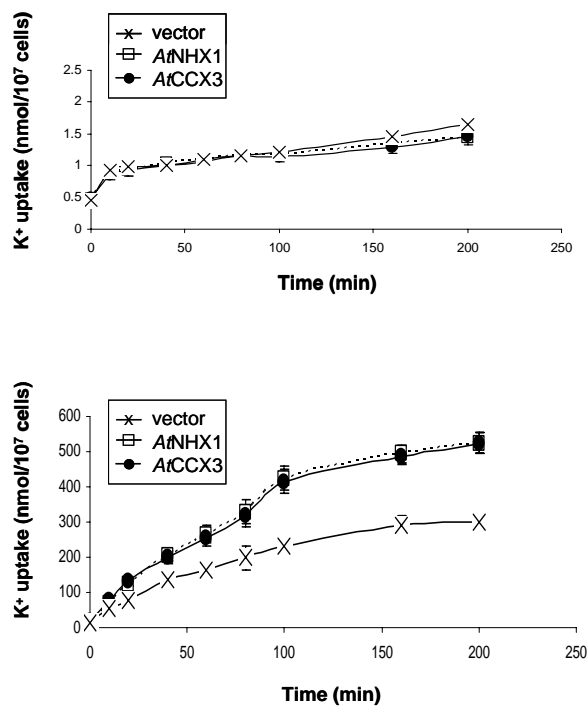
Figure 3.5 K⁺ uptake in yeast strains expressing *AtCCX3*.

⁸⁶Rb uptake into yeast was measured in whole cells at two different concentrations of external K⁺ (0.02 mM - top and 20 mM - bottom). Yeast cells expressing vector (filled triangle), *AtNHX1* (filled diamond) or *AtCCX3* (filled square).
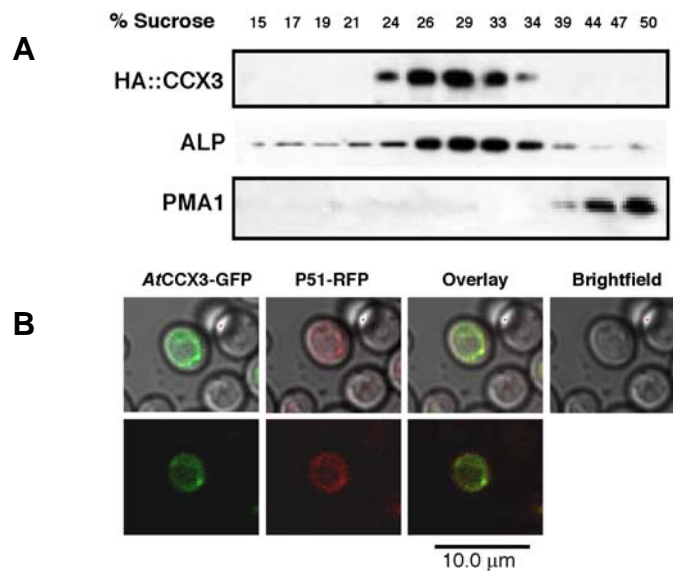
Figure 3.6 Subcellular localization of *At*CCX3 in yeast.

A) Subcellular localization of epitope-tagged HA-*At*CCX3 to the vacuole membrane. Yeast membranes were fractionated on 10-50% (w/w) sucrose gradients and equal amounts of protein (15µg) were separated by SDS-PAGE, blotted and subjected to western blot analysis using antibodies against HA, the vacuolar membrane marker alkaline phosphatase, ALP and the plasma membrane marker H^+-ATPase, PMA1. B) Subcellular localization of CCX3-GFP. GFP was fused to the C-terminus end of full-length *At*CCX3 and co expressed with the yeast vacuolar membrane protein P51. The transient expression and subcellular localization of the fusion protein and vacuolar marker were observed by confocal microscopy. The green channel (excitation at 488nm, emission at 522nm, and barrier filter at 522-535nm) and red channel (excitation at 480nm, emission at 530nm with a fluorescent long pass filter) images were captured with Fluoview software (Olympus America Inc., Center Valley, PA, USA).

Carter et al., 2004). These results suggest *At*CCX3 functions in yeast as an

endomembrane cation transporter.

To investigate the subcellular localization of *At*CAX3 in plants, microsomal

membranes from transgenic lines harbouring the *At*CCX3-GFP fusion protein were

fractionated. Centrifugation through a linear sucrose gradient was first used to compare

the distribution of the epitope-tagged transporter in transgenic Arabidopsis (Figure 3.7A)

to that of markers for the tonoplast, plasma membrane (PM), and endoplasmic reticulum

(ER) lumen. As shown in Figure 3.6A, when membrane fractions were screened for the

presence of *At*CCX3-GFP accumulation, the tagged proteins accumulated in fractions of

26% to 37% sucrose. The *At*CCX3-GFP protein accumulated in fractions enriched in

tonoplast, as indicated by the sedimentation profiles, which overlapped with a resident

protein (V-ATPase subunit B, Cheng et al., 2001) from this membrane but not with

marker proteins from the ER (BiP; Cheng et al., 2002) or the PM (AHA3; Pardo and

Serrano, 1989). To provide further confirmation for the localization in plants we

examined the transient expression of *At*CCX3-GFP in onion cells (Sivitz et al., 2007). In

onion cells expressing 35S::*At*CCX3-GFP, the green fluorescence protein (GFP) was

localized to endomembrane compartments (Figure 3.7B). In contrast, GFP fluorescence

associated with 35S::GFP was localized both to the cytosol and nucleus.

Expression of AtCCX3 and AtCCX4 in Arabidopsis

Analysis of publicly available Arabidopsis microarray data indicates that

AtCCX3 is expressed at very low levels and predominately in flowers and pollen grains

(Bock et al., 2006; https://www.genevestigator.ethz.ch; Figure 3.8A). This low level of

Figure 3.7 *At*CCX3 localization in plants.

A) Subcellular localization of epitope-tagged CCX3-GFP to the tonoplast. Arabidopsis microsomal membranes were fractionated on 10-50% (w/w) sucrose gradients. Aliquots were collected and equal amounts of protein (10μg) were separated by SDS-PAGE, blotted and subjected to western blot analysis using antibodies against GFP and plant membrane markers: the vacuolar membrane ATPase subunit B and the plasma membrane H$^+$-ATPase, AHA3 and the endoplasmic reticulum membrane protein, BiP (StressGen Biotechnologies, Victoria, British Columbia).
B) Confocal images of onion epidermis transiently expressing 35S::GFP (left) and 35S::CCX3-GFP (right). The 35S::GFP fluorescence signal was localized both in the cytosol and nucleus, whereas, 35S::CCX3-GFP, fluorescence was localized to the vacuolar membrane. Images are single optical sections.

Figure 3.8 Expression profiles of *At*CCX3 and *At*CCX4 from genevestigator.

A-B) *At*CCX3 (At3g14070) and *At*CCX4 (At1g54115).

expression made the use of Northern blot and GUS promoter::reporter analysis difficult (data not shown). In order to more precisely monitor expression, we conducted RT-PCR and detected expression of *AtCCX3* primarily in flowers, roots and stems of WT Arabidopsis (Figure 3.9A). *AtCCX4* appears to be expressed in pollen and throughout the plant and at levels substantially higher than *AtCCX3* (Bock et al. 2006; https://www.genevestigator.ethz.ch; Figure 3.8B). The ability of *At*CCX3 to suppress a yeast mutant sensitive to Na^+ and K^+ and the increased sensitivity of wild-type yeast to Mn^{2+}, prompted us to examine whether these or other cations could induce *AtCCX3* expression in Arabidopsis roots, leaves and flowers. Expression of *AtCCX3* increased two to three-fold in response to exogenous Na^+ and K^+, and by 0.5 fold to Mn^{2+}, in both roots and flowers (Figure 3.9B). We then directly compared the changes in *AtCCX3* expression to those of *AtNHX1* under salt stress conditions. An increase in *AtNHX1* in response to Na^+ has been reported previously (Yokoi et al., 2002). Although *AtCCX3* is expressed at much lower levels than *AtNHX1*, expression of both genes was induced by Na^+ treatment in roots, leaves and flowers (Figure 3.9C). However, the basal level of *AtNHX1* expression was much higher than *AtCCX3*.

Analysis of T-DNA insertional mutants of AtCCX3 and AtCCX4

To investigate the physiological function of *At*CCX3 and *At*CCX4 in Arabidopsis, we obtained two independent lines containing T-DNA insertions inside each open reading frame (Figure 3.10D). Homozygous lines were isolated by screening for the presence of the T-DNA insert and lack of native *AtCCX*. We isolated two different mutant alleles for *AtCCX3* termed *atccx3-1* and *atccx3-2*, and two alleles for

Figure 3.9 Expression of *AtCCX3* in Arabidopsis.

A) RT-PCR showing *AtCCX3* expression in Col WT tissues.
B) The relative expression levels of *At*CCX3 in roots, leaves and flowers in response to various cation treatments were normalized to expression of the *18S* subunit. The results were obtained from 2 independent RNA pools for each tissue and treatment combinations analyzed.
C) Comparison of *AtCCX3* expression to *AtNHX1* in roots, leaves and flowers in response to 50mM NaCl. Expression levels of *AtCCX3* and *AtNHX1* were normalized to expression of the *18S* subunit

Figure 3.10 Altered expression of *AtCCX3* in Arabidopsis.

A-C) RT-PCR showing *AtCCX3* expression in 35S::*AtCCX3* lines, *ccx3* and *ccx4*.
D) Diagram of T-DNA insertions for *ccx3-1*, *ccx3-2*, *ccx4-1* and *ccx4-2*. Arrows
indicate location of primers used to amplify from the T-DNA border and the
genomic ORF.

AtCCX4 termed *atccx4-1* and *atccx4-2*. RT-PCR analysis of the four different alleles showed no expression of *AtCCX3* or *AtCCX4* in the respective mutants (Figure 3.10B and 3.10C). Pollen viability, pollen tube growth, and seed set were not altered in these mutants (data not shown), nor were there any other discernable growth abnormalities.

Ectopic expression of AtCCX3

To further test the function of *At*CCX3 in Arabidopsis, *At*s*CCX3* and *AtCCX3* were expressed using the 35S promoter. Despite increased expression of *AtCCX3* RNA in the Arabidopsis lines, no visible changes in plant growth or development were detected (Figure 3.10A). However, lack of an *AtCCX3*-specific antibody prevented actual quantification of alterations of *At*CCX3 protein amounts.

35S::*AtCCX3* plants treated with exogenous NaCl accumulated 35% more Na^+ compared to controls (26,490 $\pm$ 370 μg/g for 35S::*AtCCX3*; 17,305 $\pm$ 2,145 μg/g for control). In contrast, when grown under normal conditions, there was only a modest Na^+ accumulation in 35S::*AtCCX3* lines (6,044 $\pm$ 55 μg/g for 35S::*At*CCX3; 5,772 $\pm$ 73 μg/g for controls). We also measured K^+ uptake in 35S::*AtCCX3* lines using [86]Rb. The 35S::*AtCCX3* lines were able to uptake K^+ at higher levels compared to controls (Figure 3.11). This increase in K^+ uptake was only observed at 20 mM external K^+, but not when K^+ concentration in the media was 0.02 mM (Figure 3.11B).

The Arabidopsis *AtCCX3* and *At*s*CCX3* constructs were also heterologously expressed in tobacco (KY14 variety). As shown in Figure 3.12C, *AtCCX3* RNA accumulated in all 35S::*AtCCX3* transgenic lines. The inability to detect transcript in the vector lines indicates the specificity of the primers used during the amplification process.

Figure 3.11 K$^+$ uptake in Arabidopsis expressing 35S::*AtCCX3*.

A-B) Plants were starved of K$^+$ for 48 hrs and then uptake of ^{86}Rb was determined at different time points in low K$^+$ media (top; 0.02 mM) and in high K$^+$ media (bottom; 20 mM).

Figure 3.12 Ectopic expression of *AtCCX3* in tobacco.

A) Phenotypes of tobacco plants expressing vector, *AtCCX3* or truncated *AtCCX3* (*AtsCCX3*). Only *AtCCX3*-expressing lines displayed stunted growth, yellowing of the leaves and necrosis of the leaf inter-venial leaf tissue.
B) *AtCCX3* expressing tobacco plants displayed yellow lesions at the leaf tips in young leaves and this became more apparent as the leaves aged (mature). These growth abnormalities were never seen in control lines regardless of age.
C) *AtCCX3* expression in the tobacco transgenic lines. Total RNA was isolated from 4-week-old leaf tissue and 5μg of RNA was used in the RT-PCR reaction.

Visible alterations in plant growth were readily apparent in the 35S::*AtCCX3*

expressing lines. After three weeks growth in sterile conditions, 100% (17 of 17) of the

primary transformants expressing 35S::*AtCCX3* formed leaves with small yellowing

necrotic lesions (Figure 3.12A). After being transferred to soil, the lines appeared to

partially recover for a period of three to five days. After a week, these phenotypes

reoccurred in all 17 of the primary transformants; however, the roots of these plants did

not show altered growth. As shown in Figure 3.12A and 3.12B, after three months all

the 35S::*AtCCX3* expressing plants were severely stunted. The 35S::*AtCCX3* transgenic

lines with the least dramatic growth changes always displayed low levels of

35S::*AtCCX3* expression (data not shown).   In contrast to these dramatic phenotypes,

the 12 35S::*At*s*CCX3*-expressing lines displayed phenotypes indistinguishable from the

vector expressing controls. Because the 35S::*At*CCX3 phenotypes were so dramatic, we

repeated the transformation process and obtained identical results in a replicate

experiment (data not shown).

35S::*AtCCX3* lines were selected for further study on the basis of their T1

phenotype and their ability to make seeds. All lines analyzed that displayed the altered

morphology were fertile (n=13). The same growth abnormalities revisited all of the

lines in the second generation.   When grown from seed in tissue culture, the T2 plants

appeared normal and unperturbed for the first two weeks, after which time the leaves

began to display altered growth phenotypes (data not shown).   T2 35S::*AtCCX3*-

transformed plants that were sown and grown in the greenhouse also displayed leaf

Figure 3.13 ICP analysis in young and mature leaves of *AtCCX3* expressing tobacco plants.

ICP analysis from tobacco plants expressing *AtCCX3*. The relative percent difference in the averages of 7 independent lines is shown in the graphs. A-B) ICP analysis of young leaves from 28-day old tobacco plants and mature leaves from 40-day old tobacco plants. Error bars represent standard error for the relative percent difference of the means (http://www.census.gov/acs/www/Downloads/ACS/PercChg.pdf).

symptoms after 3 weeks of growth (Figure 3.12B). Once again, none of the

35S::*At*s*CCX3* or vector control expressing plants displayed these phenotypes.

Effects of AtCCX3 on plant growth

The symptoms of the 35S::*AtCCX3*-expressing tobacco plants could not be

phenocopied in vector control lines under any growth conditions tested (excess and

depleted Na^+, Mg^{2+}, K^+, Mn^{2+} and Ca^{2+}; data not shown). Primary transformants and T2

35S::*AtCCX3* expressing plants displayed altered morphology regardless of the media

used for growth. Conceivably, constitutive *AtCCX3* expression may alter various

mineral levels simultaneously making suppression of the phenotypes dependent on the

addition or subtraction of multiple components.   To ascertain whether 35S::*AtCCX3*

expression altered ion content, we measure the total accumulation of ions in both mature

and young leaves. In 35S::*AtCCX3* expressing plants, young leaves accumulated at least

40% more of K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} (Figure 3.13A) compared to control lines

and maintained these increased levels for K^+ and Na^+, even as the leaves matured

(Figure 3.13B). One explanation for these growth defects is that the alterations in

cellular Mn^{2+} and other cations may produce excess reactive oxygen species (ROS). To

test this, we isolated total leaf protein from vector and 35S::*AtCCX3* expressing plants

and detected carbonyl content of the proteins.   As shown in Figure 3.14, the protein

carbonyl content was higher in 35S::*AtCCX3* expressing plants compared to vector

controls and *AtsCCX3* expressing lines.   In fact, these alterations were detectable before

the onset of morphological changes in leaf architecture. Although there are dramatic

changes in leaf architecture and phenotype there is not alteration in pollen viability in

Figure 3.14 Protein oxidation from tobacco plants expressing *AtCCX3*.

A) Plant protein carbonyl contents of vector control and *AtCCX3*, were analyzed by western blotting of total protein isolated from tobacco plants with anti-dinitrophenylhydrazone antibody (1:1000) in onset and old leaves.
B) Coomassie brilliant blue stained gel of protein to show loading.
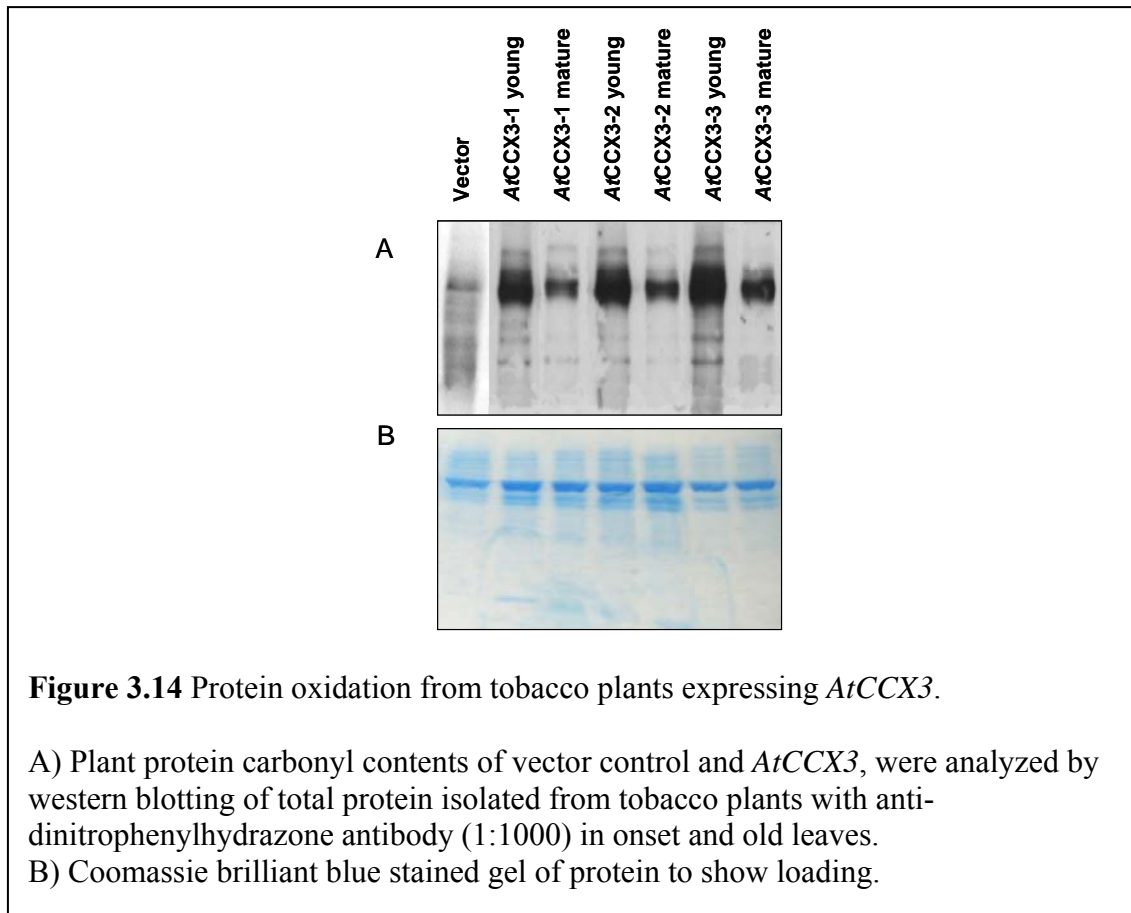
Arabidopsis (data not shown) and tobacco (Figure 3.15). Together these findings

suggest *AtCCX3* has a role in ion homeostasis in plants, and when *AtCCX3* expression is

heightened increases in protein oxidation occurs.

Figure 3.15 Pollen
viability is unaltered in
tobacco plants expressing
35S:*AtCCX3*.
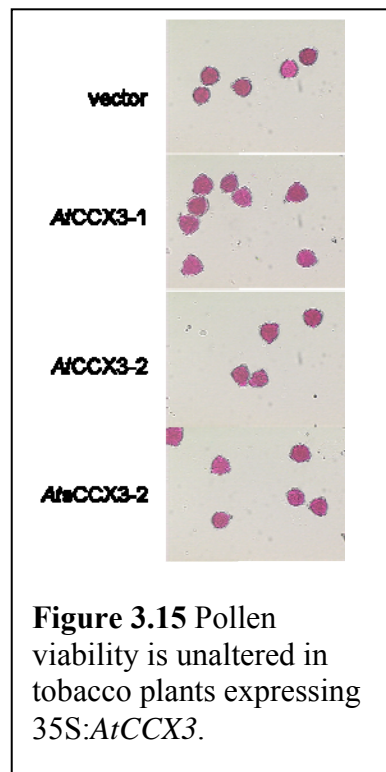
Discussion

The plant vacuole is a vital cation depot and numerous transporters have been

proposed to mediate influx and efflux from this end membrane compartment (Marty,

1999; Maeshima, 2000; Ratajczak, 2000; Hirschi, 2001). Here we show that yeast

suppression screens, *in planta* expression analysis, yeast and plant transport studies, and AtCCX-generated phenotypes in transgenic tobacco all suggest *AtCCX3* expression is judiciously regulated and is part of an ensemble of transporters regulating K^+, Na^+ and possibly Mn^{2+} levels within plant cells.

Sequence comparison of AtCCXs to HsNKCX6

Phylogenetic analysis has recently identified five Arabidopsis CCX transporters as being closely related to mammalian K^+-dependent Na^+/Ca^{2+} exchangers. (Figure 3.2A; Shigaki et al., 2006). The functions of *At*CCX1-5 in plants are unknown, though they may functionally resemble NCKXs. NCKX exchangers are involved in mammalian signaling (Blaustein and Lederer, 1999; Lee et al., 2002) by catalyzing the electrogenic counter transport of 4 Na^+ for 1 Ca^{2+} and 1 K^+ (Crevetto et al., 1989; Dong et al., 2001). Like the *Hs*NKCX6, *At*CCX3 and *At*CCX4 have similar conserved alpha repeats (Figure 3.2B) which play a critical role in maintaining a proper chemical microenvironment for ion binding (Cai and Lytton, 2004). *At*CCX3, *At*CCX4 and *Hs*NCKX6 also share similar membrane topology, which differ from that of *At*CAX1 (Figure 3.2C).

Function of AtCCX3 and AtCCX4 in yeast

Our data show *At*CCX3 and *At*CCX4 have functions distinct from *At*NHX1. Our yeast data support that of the phylogenetic analysis which indicates possible Na^+/K^+ transport properties for *At*CCX3 and *At*CCX4 (Figure 3.3; Shigaki et al., 2006), Like *AtNHX1*, *AtCCX3* and *AtCCX4*-expressing cells can suppress the Na^+ and K^+ sensitivities of mutant yeast strains defective in vacuolar Na^+ and K^+ transport (Figure 3.3B and 3.3C). Similarly, *At*CCX3-mediated K^+ (^{86}Rb) uptake in yeast cells is

indistinguishable from *AtNHX1*-expressing cells (Figure 3.5). Furthermore, *AtCCX3*-expressing yeast cells facilitated the uptake of various cations at concentrations comparable to that of *AtNHX1*-expressing cells (Figure 3.3E and 3.3F). Results support the idea that *At*CCX3 activity promotes K^+ or Na^+ accumulation into endomembrane compartments, such as the vacuole.

AtCCX3 and AtCCX4 expressing yeast cells did not completely phenocopy *AtNHX1* expressing yeast cells. *AtCCX3* and *AtCCX4* expressing cells could not suppress the hygromycin sensitivity of *nhx1* deficient cells (data not shown; Nass and Rao, 1998; Darley et al., 2000). Both *At*CCX3 and *At*NHX1 are endomembrane localized (see below), in contrast to the prevacuoler localization of yeast NHXp1 (Nass and Rao, 1998). This altered localization could explain the inability of *AtCCX3*-expressing cells to suppress this defect in the yeast secretory pathway. *AtCCX3* and *AtCCX4* expressing yeast cells were also hypersensitive to excess Mn^{2+} (Figure 3.3D). This phenotype resembles high-level expression of yeast vacuolar protein Mam3p (Yang et al., 2005). Interestingly, Mam3p does not appear to direct Mn^{2+} transport and is thought to confer Mn^{2+} tolerance indirectly (Yang et al., 2005). Possibly, the Mn^{2+} transport mediated by *At*CCX3, like Mam3p, may be indirect. The precise mechanisms of this *At*CCX3- mediated yeast Mn^{2+} sensitivity and the reversible nature of *At*CCX3 transport requires further investigation; however, the compelling observation remains that this Mn^{2+} sensitivity has not been seen with heterologous expression of Arabidopsis CAX or NHX transporters.

Our other yeast data further support *At*CCX3 and *At*CCX4 having biochemical functions distinct from CAX transporters. For example, N-terminal truncations of *At*CAX1 and *At*CAX2 suppress the Ca^{2+} sensitivity of yeast cells defective in vacuolar Ca^{2+} transport (Hirschi et al., 1996). In contrast, expression of both full-length and N-terminal truncations of *At*CCX3 and *At*CCX4 in yeast could not suppress these Ca^{2+} transport defects. Furthermore, the N-terminal domains of *At*CCX3 and *At*CCX4 were required for function. The requirement of the N-terminal domain in these yeast assays suggests that *At*CCX3 and *At*CCX4 do not contain a CAX-like N-terminal regulatory domain.

AtCCX3 is an endomembrane localized transporter in flowers

Functional epitope tags of *At*CCX3 demonstrated that *At*CCX3 localized to the endomembrane in both yeast and plants (Figure 3.6 and 3.7). Furthermore, *At*CCX3 appeared to function at the yeast vacuolar membrane as a cation transporter (Figure 3.3 and 3.5). Whether *At*CCX3 is localized exclusively on the plant vacuole or also on the trans-golgi network and prevacuolar compartment is unclear at this time. Additionally, RT-PCR showed expression of low levels of *At*CCX3 predominantly in roots and flowers and modestly increased expression in response to Na^+, K^+ and Mn^{2+} (Figure 3.9).

Although the precise role of *At*CCX3 is still unclear, these low *At*CCX3 expression levels suggest that *At*CCX3 might have a signaling role rather than be involved in bulk cation uptake. Furthermore, the localization of *At*CCX3 to the plant vacuole, and possibly other endomembrane compartments, combined with expression in

floral tissue (Figure 3.7A, 3.9B and 3.10A) suggests functions associated with the pollen

vacuole during tube elongation and polarized tip growth (Cheung et al., 2003;

Holdaway-Clarke et al., 2003).

AtCCX3 phenotypes in plants

*At*CCX3 and *At*CCX4 may function in concert with numerous other transporters

to regulate pollen growth.   The *atccx3* and *atccx4* lines displayed no altered pollen

phenotypes and general plant growth appeared robust in all our assays (Figure 3.8). This

lack of altered growth could be related to the expression of 2-3 other *At*CCX transporters

during vegetative and pollen development according to whole genome transcriptome

data (Sze et al., 2004, Bock et al., 2006). Many Arabidopsis T-DNA mutants lack any

morphological phenotype presumably due to functional redundancy (Krysan et al.,

1999). Our working hypothesis is that *At*CCX3 and *At*CCX4 have similar functions as

they arose from a gene duplication, and in future work it will be interesting to determine

if *atccx3/atccx4* double mutant lines display any alteration in growth, particularly floral

development.

Our tobacco results suggest *At*CCX3 activity must be carefully modulated.

Tobacco plants ectopically expressing 35S::*AtCCX3* were stunted in growth and

contained necrotic lesions in the leaf interveinal regions (Figure 3.12A). These tobacco

phenotypes differentiate *AtCCX3* from both *AtCAX1* and *AtNHX1* over-expressing

phenotypes. The *AtCCX3* phenotypes were not observed in plants expressing *At*s*CCX3*

(Figure 3.12A). In contrast, ectopic expression of *At*s*CAX1* in tobacco produces

dramatic phenotypes (Hirschi, 1999).   This further suggests that *AtCCX3* does not

contain an N-terminal regulatory domain. The fundamental cause of *At*CCX3 mediated tobacco phenotypes is less apparent than with the *At*s*CAX1* expressing lines. That is, *At*s*CAX1* expressing lines are Ca^{2+} deficient and application of exogenous Ca^{2+} can restore normal growth (Hirschi, 1999). In contrast, the *AtCCX3*-expressing lines could not be rescued by enhancing or reducing NaCl, KCl or $MnCl_2$ levels (data not shown). Possibly, the *AtCCX3*-expressing lines disrupt tonoplast V-type H^+-translocating ATPase activity causing a general disruption in pH homeostasis. In fact, altered expression of CAX transporters can produce alterations in vacuolar H^+-ATPase activity (Shigaki and Hirschi, 2006); however, these CAX phenotypes are not as severe at those documented here. Like *AtNHX1* expressing plants, *AtCCX3* lines were able to accumulate Na^+ (Figure 3.13A); however, unlike *AtNHX1* expressing lines, these plants did not appear to be Na^+ tolerant (data not shown; Apse et al., 1999, Apse et al., 2003)

Protein oxidation is increased in plants expressing AtCCX3

 *At*CCX3 function may be related to plant ROS signalling (Figure 3.14). Indeed, tobacco plants expressing 35S::*AtCCX3* showed much higher oxidation of proteins compared to controls (Figure 3.14). The drastic phenotypes observed in 35S::*AtCCX3* tobacco lines may be due to the perturbation of transient metal concentrations in *AtCCX3* expressing tobacco lines (Figure 3.13A). Regulation of metal concentrations is essential for plant antioxidant systems (Halliwell and Gutteridge, 2006). When in excess, these metals can produce highly toxic hydroxyl radicals through a Fenton reaction resulting in oxidative damage (Halliwell, 2006).

In summary, I have characterized the first CCX transporters from plants. Here I demonstrate that AtCCX3 resides on an endomembrane and functions as a K^+ transporter that may also transport Na^+ and Mn^{2+}.
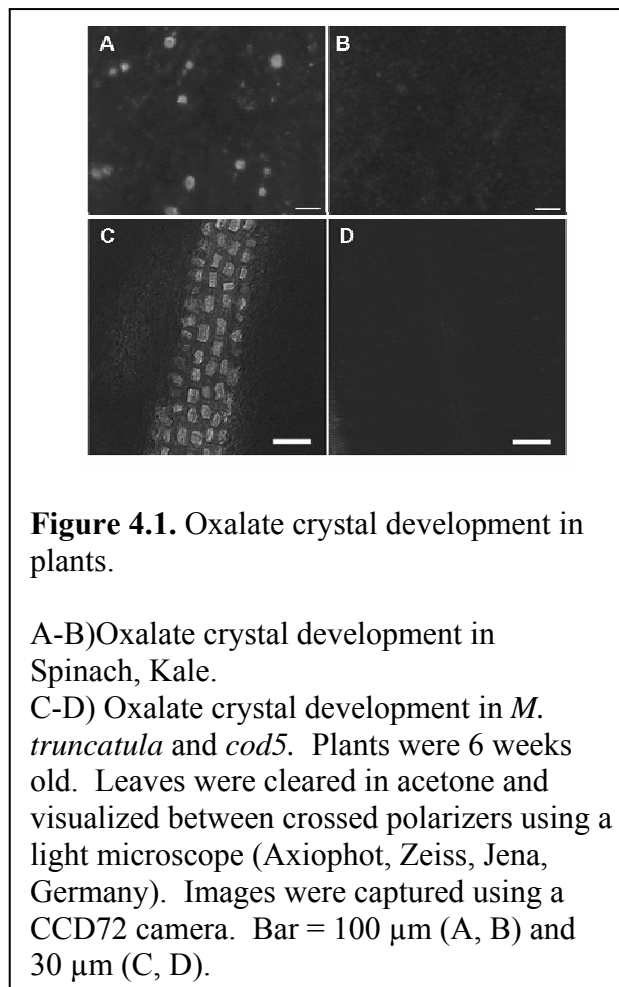
CHAPTER IV

INCREASED CALCIUM BOAVAILABILITY IN MICE FED GENETICALLY

ENGINEERED PLANTS LACKING CALCIUM OXALATE*

Introduction

Osteoporosis is a multifactorial disease in which calcium nutrition plays a role

(Ellis et al., 1972; Weaver et al., 1990; Bryant et al., 1990; Bachrach, 2001). Calcium

can be obtained from a variety of sources, most notably dairy products; however some

vegetables such as kale, broccoli and bok choy are also excellent sources of calcium

(Singh et al., 1972; Weaver et al., 1990). Unfortunately, many vegetables such as

spinach contain high levels of calcium but bioavailability is low. There is an abundance

of literature that infers that the plant content of oxalate and phytate interfere with

calcium absorption (Heaney et al., 1988; Weaver et al., 1990). For instance, spinach

contains 23.8 to 26.7 mg/g of calcium, while kale contains 26.3 to 27.6 mg/g of calcium

(Weaver et al., 1987). In these observational studies, calcium absorption appears to be

inversely proportional to the oxalic acid content in the food (Prenen et al., 1984; Weaver

et al., 1987; Heaney et al., 1988; Weaver et al., 1990) For example, kale is low in oxalate

(2.8mg/g) whereas spinach is high (105 mg/g; Figure 4.1; Weaver et al., 1987), although

a notable exception to this correlation is soybeans, where oxalate levels are high

(35mg/g; Massey et al., 2001) but bioavailable calcium is also high (total Ca 27.7 mg/g;

Massey et al., 2001). This association between bioavailable calcium content and plant

calcium partitioning (such as through oxalate formation) would be more convincingly

demonstrated by feeding studies utilizing plant sources containing a single genetic

change in a gene associated with calcium partitioning.

Figure 4.1. Oxalate crystal development in plants.

A-B)Oxalate crystal development in Spinach, Kale.
C-D) Oxalate crystal development in *M. truncatula* and *cod5*. Plants were 6 weeks old. Leaves were cleared in acetone and visualized between crossed polarizers using a light microscope (Axiophot, Zeiss, Jena, Germany). Images were captured using a CCD72 camera. Bar = 100 μm (A, B) and 30 μm (C, D).

Ideally, we would create spinach mutants which lack calcium oxalate and assay bioavailability as previously reported (Weaver et al., 1987); however, this is impractical when using agricultural crops like spinach as it requires a genetically tractable model system. Fortunately, the genetically tractable forage legume *Medicago truncatula* contains insoluble calcium oxalate crystals (Figure 4.1) in leaf tissue similar to crystals found in spinach. However, *M. truncatula* contains less that 1% of the total oxalate in a water soluble form whereas spinach varieties can contain anywhere from 20%-90% of the oxalate in the soluble form (Bohn et al., 2004). Thus, by focusing on *M. truncatula* studies we cannot address the impact of removing soluble oxalate but we can define the nutritional consequences of a single genetic change which removes insoluble calcium oxalate.
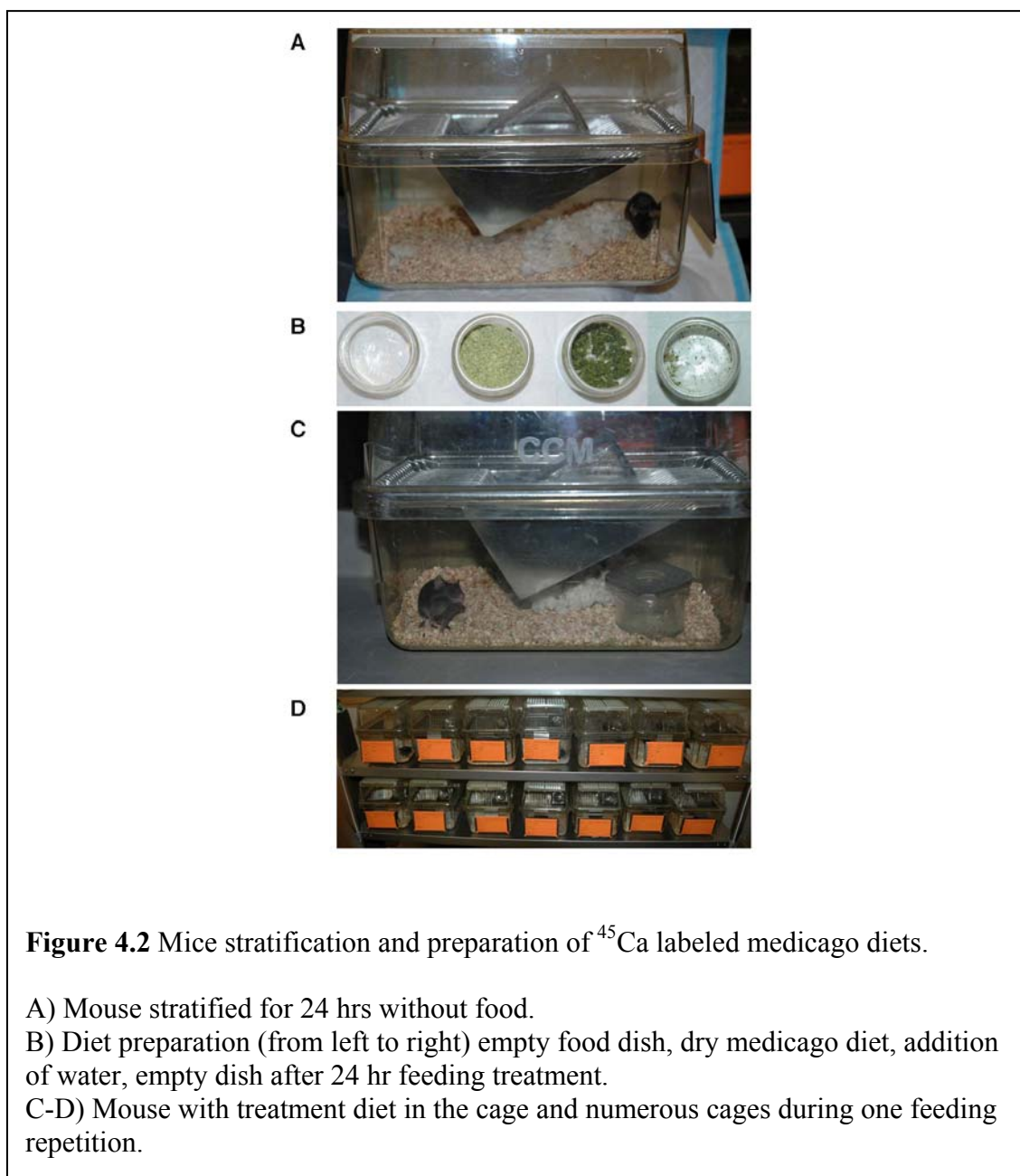
Previously, the *cod5 M. truncatula* mutant was identified which lacks oxalate crystals in the leaf tissue (Nakata and McConn, 2006). The mutation reduced the oxalate concentration from 20.9 +/- 1.13 mg/g to 1.5 +/- 0.14 mg/g. Microscopic observations and biochemical measurements confirmed that *cod5* leaves resemble kale in terms of the lack of oxalate crystals (Figure 4.1). Leaves from the mutant have similar calcium levels when compared to the wild-type, 12.3 +/- 2.0 mg/g compared to 17.6 +/- 3.5 mg/g, respectively. Additionally, lack of insoluble oxalate crystals does not alter *cod5* growth rates (Nakata and McConn, 2003). Using the *cod5* plants in controlled feeding studies should eliminate the variables introduced through the use of two different genetic varieties (e.g. spinach and kale) and allow direct assessment of a single genetic loci on calcium nutritional status within a plant.

Currently, plant biologists are manipulating the synthesis of micronutrients in order to improve crop nutritional quality (Foyer et al., 2006). The term "nutritional genomics" has been coined to describe work at the interface of plant biochemistry, genomics and human nutrition. However, the actual work done to assess the nutrient value of various novel plant foods to date is minimal. Using genetics to determine the precise role of oxalate crystals in calcium nutrition will provide important information for constructing "designer" foods to improve human nutrition. With this emerging goal in mind, utilizing a mice-feeding regime, this study demonstrates for the first time the nutritional impact of a single genetic lesion on calcium partitioning and bioavailability. These findings offer a general strategy to enhance bioavailable calcium levels in agriculturally important crops.

Materials and Methods

Mice feeding study

Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. C57-BL6 (Charles River Labs, Wilmington, MA) mice were housed in cages with ad libitum access to water and food (AIN93G diet; Reeves et al., 1993). We monitored food intake and calculated that the mice consumed approximately 3.5 to 4 grams of diet per day at six to seven weeks of age (n = 120) The mice were stratified and held without food for 24 hr separated into treatment groups using a randomized block design (Figure 4.2A). After 24 hr, 3.5g of each respective diet was placed in a glass food dish and placed into the cage (Figure 4.2C and 4.2D). The

Figure 4.2 Mice stratification and preparation of ^{45}Ca labeled medicago diets.

A) Mouse stratified for 24 hrs without food.
B) Diet preparation (from left to right) empty food dish, dry medicago diet, addition of water, empty dish after 24 hr feeding treatment.
C-D) Mouse with treatment diet in the cage and numerous cages during one feeding repetition.

amount of diet fed to each mouse represented 14.0 kcal, which is similar to the 14.4 to

16 kcal/d that control mice of identical age consume. This amount of diet also insured

that the mice ate the majority of the test over the allotted 24 hr. All mice were killed

with CO_2 after 24 hr of feeding and placed in a cooler at 4 C for 24 hr. The remaining

diet, if any, was placed in a bag and saved for analysis.

Preparation of labeled plants

Medicago truncatula, *cod5* and Spinach (*Spinacea oleracea* v 'Samish') plants

were germinated on paper and transferred to hydroponic growth containers (Grusak et

al., 1996; Grusak, 1997; Nakata and McConn, 2000). *M. truncatula* plants were grown

in hydroponics for 30 days and then the leaves were cut back. For intrinsically labeled

M. truncatula, the solution was supplemented with 1µCi ^{45}Ca/L after the removal of the

leaves and grown for 30 additional days. Spinach plants were grown in hydroponic

solution for two weeks prior to addition of 1µCi ^{45}Ca/L label and then grown in the

solution for 14 days.   For the extrinsically labeled spinach diets (the ^{45}Ca label will be

added during diet preparation), the plants were grown for 30 days in the hydroponic

solution.

The plant material was harvested and dried at 25 C. The *M. truncatula* plants

were harvested and freeze dried. Once dried, the material was frozen in liquid nitrogen

and ground to a powder with a mortar and pestle.

Oxalic acid determination

Oxalic acid concentration of the *M. truncatula* samples was determined by

methods outlined in Nakata and McConn (2000).

Diet preparation

Initially, we determined the nutritional composition of *M. truncatula* and *cod5* for the following constituents: fiber, total protein, calcium, potassium and magnesium (Sheldrick, 1986; Franson, 1989; Kmarek, 1993). Two diet mixes were then obtained from Research Diets (New Brunswick, NJ) and used for mice feeding. These mixes allowed for the incorporation of 0.5g of *M. truncatula* or *cod5* to be mixed with 3.0 g of prepared diet. This 3.5g diet was calorically and nutritionally equivalent (including 5.0mg/g calcium) to the standard AIN93G diet eaten by mice during a given 24 hr period (Reeves et al., 1993). Prior to feeding, diets were mixed to homogeneity with 2ml of dH$_2$O (Figure 4.2B). For the extrinsically labeled diet, the ^{45}Ca label was a component in the water. Spinach, CaCl$_2$, CaCO$_3$ and CaC$_2$O$_4$ labeled diets were prepared as previously described (Weaver et al., 1987).

Mice bioavailability analysis

The bones of the two hind limbs (femur, tibia, and fibula) were removed. The soft tissue was surgically removed with a scalpel and the bones were then ashed to remove all the organic matter (Figure 4.3). The ashing was performed in a muffle furnace (Thermolyne Furnace, Barnsted International, Dubuque, IW) at 700 C for 30 hr. The amount ^{45}Ca incorporated in the bones was done as previously described (Weaver et al., 1987). For determination of percent ^{45}Ca absorption the decay of the dose was adjusted for the intrinsically labeled plants. For the extrinsically labeled diets, a portion of the original standard label solution was used to determine the activity of the tracer at the time of the analysis of the bone samples.
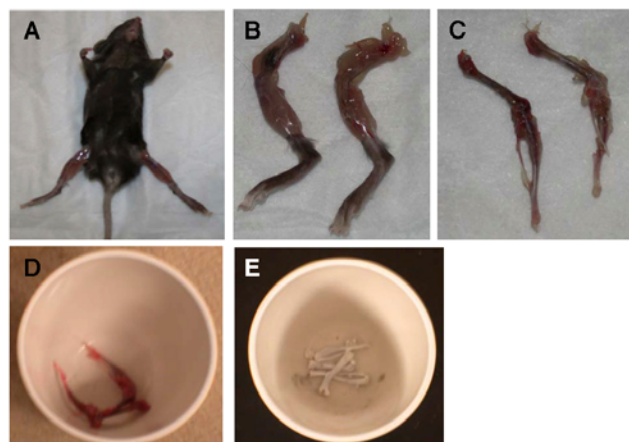
Figure 4.3 Bone tissue surgery and analysis.

A) Euthanized mouse with skin removed.
B) Lower limbs removed.
C) Soft tissue surgically removed with a scalpel.
D) Bones prior to ashing in the muffle furnace.
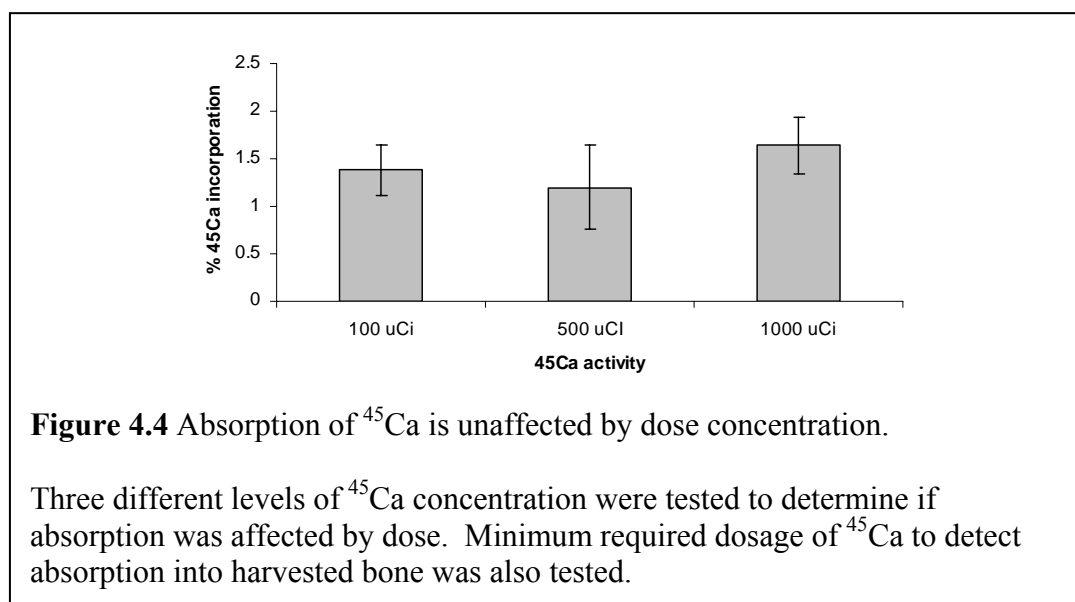E) Bones after ashing process which removes all organic matter.

Approximately 95% of the mice ate the entire labeled diet. If any diet was left uneaten, it was processed and analyzed as previously described. The ^{45}Ca activity in the uneaten diet was then subtracted from the total amount of ^{45}Ca label in the diets.

Percent dose of ^{45}Ca in hind limbs was calculated using Excel (Microsoft, Redmond, WA) and statistical differences calculated using ANOVA in SPSS (Chicago, IL).

Results

M. truncatula is a widely used model plant species, but is not commonly consumed by humans. Thus, feeding studies utilizing this plant must be tested in an animal model. As a first step, we demonstrated the validity of using tracer methods previously applied in rats (Weaver et al., 1987) for estimating calcium bioavailability in mice. Absorption of the tracer was determined by measuring ^{45}Ca concentrations in the legs of the dosed animals one day after consumption of extrinsically labeled test meals. Initially, we tested the sensitivity and linearity of the assay by using serial dilutions of ^{45}Ca in the food to determine that measurable amounts of ^{45}Ca could be obtained from animals fed as little as 100nCi ^{45}Ca. All three activities of ^{45}Ca (100, 500 and 1000 nCi ^{45}Ca) fed to mice demonstrated similar incorporation rates (1.38% +/- 0.27, 1.20% +/- 0.44 and 1.64 %+/- 0.30; Figure 4.4) into the hind limbs when the calcium concentration was kept constant (5 mg/g). This finding demonstrates that labeled foods having as little as 100 nCi ^{45}Ca could be fed to the mice and be accurately measured. To validate our mouse model, we then tested if the calcium source could impact calcium bioavailability

as had been reported using other experimental animals. As in previous studies using rats, the calcium absorption rate from calcium oxalate was approximately 6% of that from CaCl$_2$ and CaCO$_3$ fed mice (Table 4.1; Weaver et al., 1987). These levels of incorporation were lower than our *Medicago* test diets simply because the total calcium was approximately 58% lower than the plant based diets.

Figure 4.4 Absorption of ^{45}Ca is unaffected by dose concentration.

Three different levels of ^{45}Ca concentration were tested to determine if absorption was affected by dose. Minimum required dosage of ^{45}Ca to detect absorption into harvested bone was also tested.

In order to perform intrinsic labeling experiments, we labeled the plants with ^{45}Ca. Calcium uptake of 29.58% and 19.82% of the administered dose of ^{45}Ca were accumulated by edible parts of spinach and *M. truncatula* while the *cod5* lines incorporated 17.83% of the label. This efficiency of labeling assured that we had

adequate label for intrinsic diet preparation. In the feeding studies, both intrinsically and extrinsically labeled spinach diets (Table 4.1), displayed a marked reduction in the absorption of calcium in mice compared to the ^{45}CaCl$_2$ control diet fed to mice (Table 4.1). This reduction was not seen in the mice fed extrinsically (1.73 +/- 0.06 %) or

Table 4.1. Comparison of calcium bioavailability in rodents fed different calcium sources[1]

Diet	Rat[5] % ^{45}Ca dose in femur	Mice % ^{45}Ca dose in hind limbs[2]
CaCl$_2$ [3]	1.97 +/- 0.12	1.26 +/- 0.11[a]
CaCO$_3$ [3]	1.76 +/- 0.23	1.17 +/- 0.10[a]
CaC$_2$O$_4$ [3]	0.196 +/- 0.02	0.07 +/- 0.01[c]
Spinach intrinsically labeled [4]	0.17 +/- 0.02	0.246 +/- 0.029[b]
Spinach extrinsically labeled [4]	0.422 +/- 0.067	0.441 +/- 0.02[b]

[1] Mean +/- sem
[2] different lowercase superscripts indicates significant differences among mean (p < 0.001), LS means test.
[3] n=7 mice per treatment
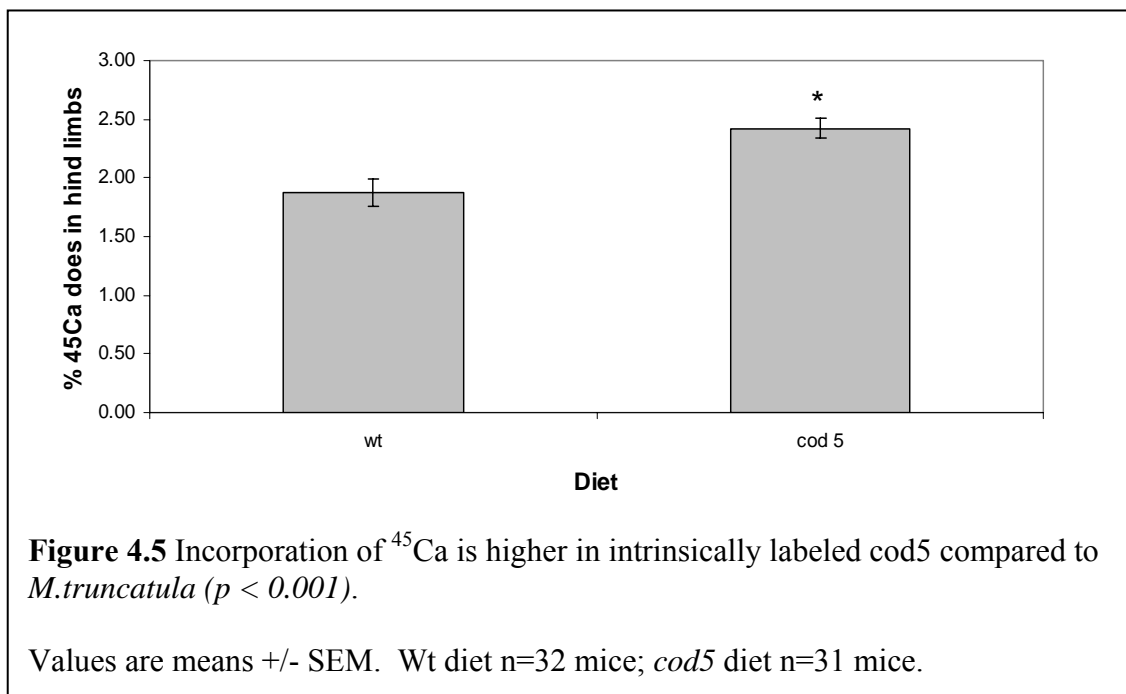[4] n=10 mice per treatment
[5] Rat ^{45}Ca dose numbers are included to allow comparisons and were taken from previous studies (Weaver et al., 1987).

intrinsically (1.81 +/- 0.06) labeled wild-type *M. truncatula.* Importantly, the

intrinsically labeled diets from the *cod5* plants had 22.87% more bioavailable calcium

than *M. truncatula* controls (Figure 4.5). This indicates that a genetic mutation that

disrupts oxalate crystal formation increases the bioavailability of calcium.

Discussion

Previous studies have suggested that calcium when sequestered in the form of

calcium oxalate is unavailable for nutritional absorption (Heaney et al., 1988; Hanes et

al., 1999a; Hanes et al., 1999b; Weaver et al., 1990). Such studies compared absorption

among a variety of plants foods; but some exceptions to this correlation have been

found. These previous studies lacked precision because the genetic mechanisms

underlying the inherent differences in the plants were too numerous to accurately

determine the components involved with oxalate formation. In order to clarify these

studies, I compared calcium absorption from isogenic lines of a plant that differed in a

single gene, which mediates oxalate crystal content. Using genetic analysis, we

convincingly demonstrate that plants lacking calcium oxalate crystals are better sources

of bioavailable calcium.

Here I have modified protocols used in rat feeding studies for analyzing calcium

bioavailability in mice (Weaver et al., 1987). Our data on calcium absorption with the

$CaCl_2$, CaC_2O_4 and spinach (both extrinsic and intrinsic) compare favorably to similar

studies in rats and thus establish the equivalence between the two in vivo models. For

example, the published rat studies have absorption values around 1.8% for both $CaCl_2$

Figure 4.5 Incorporation of ^{45}Ca is higher in intrinsically labeled cod5 compared to *M.truncatula (p < 0.001)*.

Values are means +/- SEM. Wt diet n=32 mice; *cod5* diet n=31 mice.

and CaCO$_3$ diets and only 0.2% from CaC$_2$O$_4$. In our work, we showed absorption of ^{45}Ca from CaCl$_2$ and CaCO$_3$ diets at 1.21% and 1.17%, compared to only 0.07% from CaC$_2$O$_4$ (Table 4.1). Using spinach as the calcium source for rats gave absorption values of 0.17% for intrinsically labeled diets and 0.42% in the extrinsic labeling. Meanwhile, mice fed the spinach had absorption values around 0.24% for intrinsically and 0.44% for extrinsically labeled diets. While the precise absorption values are not identical to the rat studies (Weaver et al., 1987), the findings from both studies suggest that oxalic acid binds calcium, rendering it unavailable for absorption.

In order to directly test the effects of oxalate crystal formation on bioavailable calcium, it was necessary to intrinsically label the near-isogenic *M. truncatula* and *cod 5* lines. Calcium uptake by plants in not generally as efficient as with other minerals (Kirby and Pilbeam, 1982). My ability to label spinach plants with 29.58% percent of the administered dose compared favorably with previous studies. Weaver et al. (1987) reported a similar percentage (24%) for their spinach studies. Our labeling of *M. truncatula* was 19.8% and 17.8% for the *cod5* lines. These numbers are less than reported for kale at 57% (Weaver et al., 1987) and may be due to *M. truncatula* having substantially less leaf area than kale. In leaves, calcium exists predominately in exchangeable forms in cell walls and on the exterior surface as free Ca^{2+} absorbed onto carboxylic, phosphorylic or phenolic hydroxy residues unless it is precipitated by the presence of oxalates, carbonates or phosphataes (Webb, 1999). Interestingly, we did not see a difference in labeling between *M. truncatula* and *cod5* plants, suggesting that the absence of crystals has little impact on labeling efficiency.

The low calcium content of common plant sources makes it difficult for most humans to meet their requirements exclusively from these foods (Weaver et al., 1990). Spinach is a particularly poor source of bioavailable calcium while kale is an excellent source of calcium. Here, I have used *M. truncatula* as a model for foods like spinach, which contain high levels of calcium oxalate crystals (Figure 4.1). However, unlike spinach, both intrinsic and extrinsically labeled *M. truncatula* diets showed reasonable calcium absorption (Figure 4.5). This may be due to the fact hat *M. truncatula* has low levels of soluble calcium oxalate compared to spinach (Heaney et al., 1988; Nakata and

McConn, 2000). It is worth noting that the operational pathways involved in oxalate production and in crystal formation may not be the same (Franceschi and Nakata, 2005). The *cod5* lines model kale, and indeed we can document a 22.87% (p< 0.001) increase in bioavailable calcium in the intrinsic feeding lines (Figure 4.5). We postulate from our findings that during the formation of crystals that calcium is bound in a form that is not bioavailable. Currently, work is undergoing to determine the genetic lesion in the *cod5* lines. Once the COD5 gene is cloned and characterized, work can be undertaken to modify the COD5 homologous in spinach and other vegetables as a means to increase bioavailable calcium levels.

In this study, I have developed a mouse model for measuring calcium bioavailability in foods. This single meal assay and the burgeoning fields of plant and mice molecular genetics provide a wealth of resources in the analysis of nutrient-gene interactions. Here I have used genetics to conclusively demonstrate for the first time that a single gene can significantly affect calcium bioavailability through the formation of calcium oxalate crystals. My results clearly demonstrate that removing calcium oxalate in plants improved bioavailable calcium levels. These findings offer a general strategy to engineer bioavailable calcium levels in an assortment of agriculturally important crops, either for humans or for animals.

CHAPTER V

INCREASED CALCIUM BIOAVAILABILITY IN MICE FED CARROTS

MODIFIED IN CALCIUM TRANSPORT ACTIVITY

Introduction

Lack of calcium in the diet can negatively impact health, as diseases like

osteoporosis are related to poor calcium nutrition (Gennari, 2001; New, 2001; Cashman,

2002; Nieves, 2003). However, one way to diminish this enormous health care issue

(Bryant et al, 1999; Bachrach, 2001) is to increase calcium consumption. Carrots are

among one of the most popular vegetables in the United States and contain high levels of

beta carotene (the precursor to Vitamin A) and other vitamins and minerals; however,

like most vegetables they are not great sources of dietary calcium (Weaver and

Plawecki, 1994). By engineering vegetables to contain increased calcium levels, we

may boost calcium uptake and reduce the incidence of calcium deficiencies (Park et al.,

2004).

Previously, we have demonstrated that the Ca levels in plants can be engineered

through high level expression of a deregulated Arabidopsis Ca transporter. An

Arabidopsis vacuolar Ca antiporter, termed Cation exchanger 1 (CAX1) contains an N-

terminal autoinhibitory domain (Pittman and Hirschi, 2001). Expression of N-terminal

truncations of *CAX1* (*sCAX*1) in plants such as potatoes, tomatoes and carrots increases

the calcium content in the edible portion of these foods (Shigaki and Hirschi, 2006).

Presumably these *sCAX1* expressing plants have heightened sequestration of calcium

into the large central plant vacuoles. However, these engineered foods have not been tested to determine if these altered calcium levels translate into increased bioavailable calcium.

Recently, we have developed a mouse model for measuring calcium bioavailability in foods (Morris et al., 2007). The bioavailability data from various diets using this mice protocol compares favorably with previous data obtained using diets fed to rats (Weaver et al., 1987). Insights obtained pertaining to calcium bioavailability in animal feeding studies eventually need to be recapitulated in human feeding studies; however, special concerns are present when evaluating mineral requirements and bioavailability in humans (Abrams, 2003). Stable isotopes provide a safe means of examining the relationship between a food and a particular nutrient's metabolic effect (Abrams, 2003; Labayen et al., 2004). To date, this established technology has not been utilized extensively in connection with genetically engineered foods.
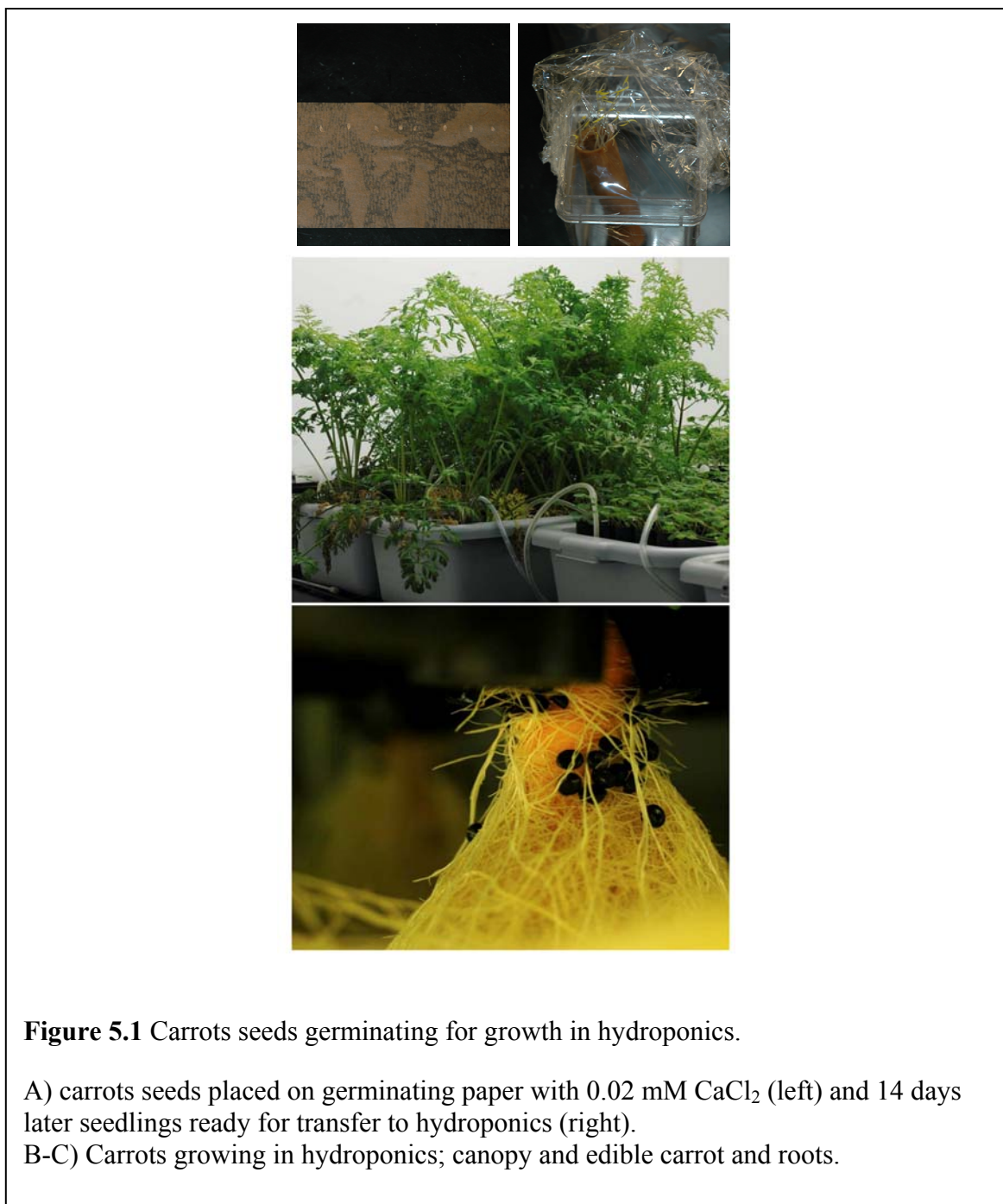
The creation of genetically modified plants with increased nutritional benefits is an expanding field (Freese and Schubert, 2004; DellaPenna, 2007). The term "nutritional genomics" has been used to describe various studies which implement some form of plant biochemistry, genomics or human nutrition. Transgenic plants are frequently analyzed for changes in plant metabolism and this is often where the experimentation ends. Ideally, these genetically modified plants need to be labeled and used in controlled animal and human feeding studies to assess nutritional impacts. To date, this type of analysis to assess the nutrient value of transgenic foods is minimal.

Here I analyze transgenic carrots which express increased levels of a plant calcium transporter for improved bioavailable calcium utilizing both mice and human feeding trials. The experimental design provides a rigorous platform to validate the nutritional impact of engineered foods. Furthermore, our findings offer a unique mechanism to enhance bioavailable calcium levels in numerous agriculturally important crops.

Materials and Methods

Mice feeding study

Animal protocols were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Mice were treated as previously described (Morris et al. 2007). C57-BL6 (Charles River Labs, Wilmington, MA) mice were housed in cages with ad libitum access to water and food (AIN93G diet) (Reeves et al., 1993). The mice ate approximately 3.5 to 4 grams of diet per day at six to seven weeks of age (n = 120). The mice were stratified and held without food for 24 hrs and separated into treatment groups using a randomized block design. After 24 hr, 3.5g of each respective diet was placed in a glass food dish and placed into the cage. The amount of diet fed to each mouse represented 14.0 kcal, which is similar to the 14.4 to 16 kcal/d that control mice of identical age consume. This amount of diet also insured that the mice ate the majority of the meal over the allotted 24 hrs. All mice were killed with CO_2 after 24 hrs of feeding and placed in a cooler at 4° C for 24 hrs. The remaining diet, if any, was placed in a bag and saved for analysis.

Figure 5.1 Carrots seeds germinating for growth in hydroponics.

A) carrots seeds placed on germinating paper with 0.02 mM $CaCl_2$ (left) and 14 days later seedlings ready for transfer to hydroponics (right).
B-C) Carrots growing in hydroponics; canopy and edible carrot and roots.

Growth of carrots and preparation of ^{45}Ca labeled plants

Carrots (*Daucus carota* L 'Danver') and *sCAX1* expressing carrots (sCAX1-1 and sCAX1-2; Park et al., 2004) seeds were germinated on paper and germinated plants transferred to hydroponic growth containers (Figure 5.1A; Grusak et a., 1996; Grusak, 1997). Carrot plants were grown in hydroponics for 60 days and then the solution was supplemented with 1μCi ^{45}Ca/L (Figure 5.1B). The carrots were then grown an additional 30 days.

The plant material was harvested and dried at 25° C. Once dried, the material was frozen in liquid nitrogen and ground to a powder with a mortar and pestle.

Preparation of ^{42}Ca labeled plants

Carrots were germinated and grown as described above. After 90 days the solution was supplemented with 5mg ^{42}Ca/L for the control carrots and 2.5mg ^{42}Ca/L for the *sCAX1* expressing carrots. This label was added to 21L of hydroponic solution and the carrots were grown until all the solution was absorbed by the plants. A second round of labeling was done with same concentrations of ^{42}Ca, but in a volume of 10L of hydroponic solution. The carrots were grown until all the label from the second solution was taken up by the plants. This two part labeling took 12-14 d. Fresh carrots were harvested, weighed, sliced and stored at -20°C.

One 0.25 to 0.50 in. latitudinal slice from, control and *sCAX1* expressing carrots, were taken for analysis. This slice was first dried at 70°C for 24 hrs and then ashed at 700°C for 15 hrs (Thermolyne Furnace, Barnsted International, Dubuque, IW). The carrots were then dissolved in 3N HCl and neutralized with 1N NaOH. The samples

were then dried for 24 hr at 70°C and re-suspended in 0.1N HCl. Total calcium was determined by ICP (Franson, 1989) and ^{42}Ca was determined by mass spectrometry.

Oxalic acid determination

Oxalic acid concentration of the carrot samples was determined by methods outlined in Nakata and McConn (2000).

Diet preparation

Initially, we determined the nutritional composition of carrots for the following constituents: fiber, total protein, calcium, potassium and magnesium (Sheldrick, 1986; Franson, 1989; Komarek, 1993). Two diet mixes were then obtained from Research Diets (New Brunswick, NJ) and used for mice feeding. These mixes allowed for the addition of 1.0g of control carrot and 0.5 g of *sCAX1* carrot to 2.5 g and 3.0g of prepared diet. This 3.5g diet was nutritionally equivalent (including 5.0mg/g calcium) to the standard AIN93G diet eaten by mice during a given 24 hrs period (Reeves et al., 1993). Prior to feeding, diets were mixed to homogeneity with 2ml of dH$_2$O. For the extrinsically labeled diet, the ^{45}Ca label was a component in the water.

Mice bioavailability analysis

The mice bones were analyzed as previously described (Morris et al. 2007). Briefly, the two hind limbs (femur, tibia, and fibula) were removed and the bones ashed in a muffle furnace (Thermolyne Furnace, Barnsted International, Dubuque, IW). The activity of ^{45}Ca incorporated in the bones was determined as previously described (Weaver et al., 1987).

Approximately 95% of the mice ate the entire labeled diet. If any diet was left uneaten, it was processed and analyzed as previously described. The ^{45}Ca activity in the uneaten diet was then subtracted from the total amount of ^{45}Ca label in the diets.

Percent dose of ^{45}Ca in hind limbs was calculated using Excel (Microsoft, Redmond, WA) and statistical differences calculated using ANOVA in SPSS (Chicago, IL).

Results

Labeling carrots with ^{45}Ca^{2+} and stable isotopes

Previously, we had demonstrated that *sCAX1*-expressing carrots contain two-fold more calcium than vector control lines (Park et al., 2004). As a first step toward determining bioavailability, we labeled the edible portions of theses carrots. The percent ^{45}Ca activity of the administered dose which accumulated in the edible carrot tissues was 14.86 % for control and 30.83% for *sCAX1* expressing carrots (Table 5.1). Using stable isotopes, we measured the concentration of ^{42}Ca enrichment in the edible portions of the control carrots at 2.5%% and the *sCAX1* expressing carrots at 2.4% (Table 5.1). The differences in percentages between the radio and stable label treatment could be related to altering the labeling procedure for the stable isotope. In a test label experiment with the stable isotope using the same labeling time table as the listed for the radio isotope label the carrots had an enrichment of 4% for the controls and 8% for *sCAX1* expressing carrots. To our knowledge this is first report of calcium labeling using radioisotope or stable isotope in carrots to study calcium bioavailability. This two fold increase in

radioisotope enrichment is consistent with the overall two fold increase in calcium in the

sCAX1 expressing carrots (Park et al., 2004).

Table 5.1. Labeling efficiency of radio and stable calcium isotopes in hydroponically grown carrots
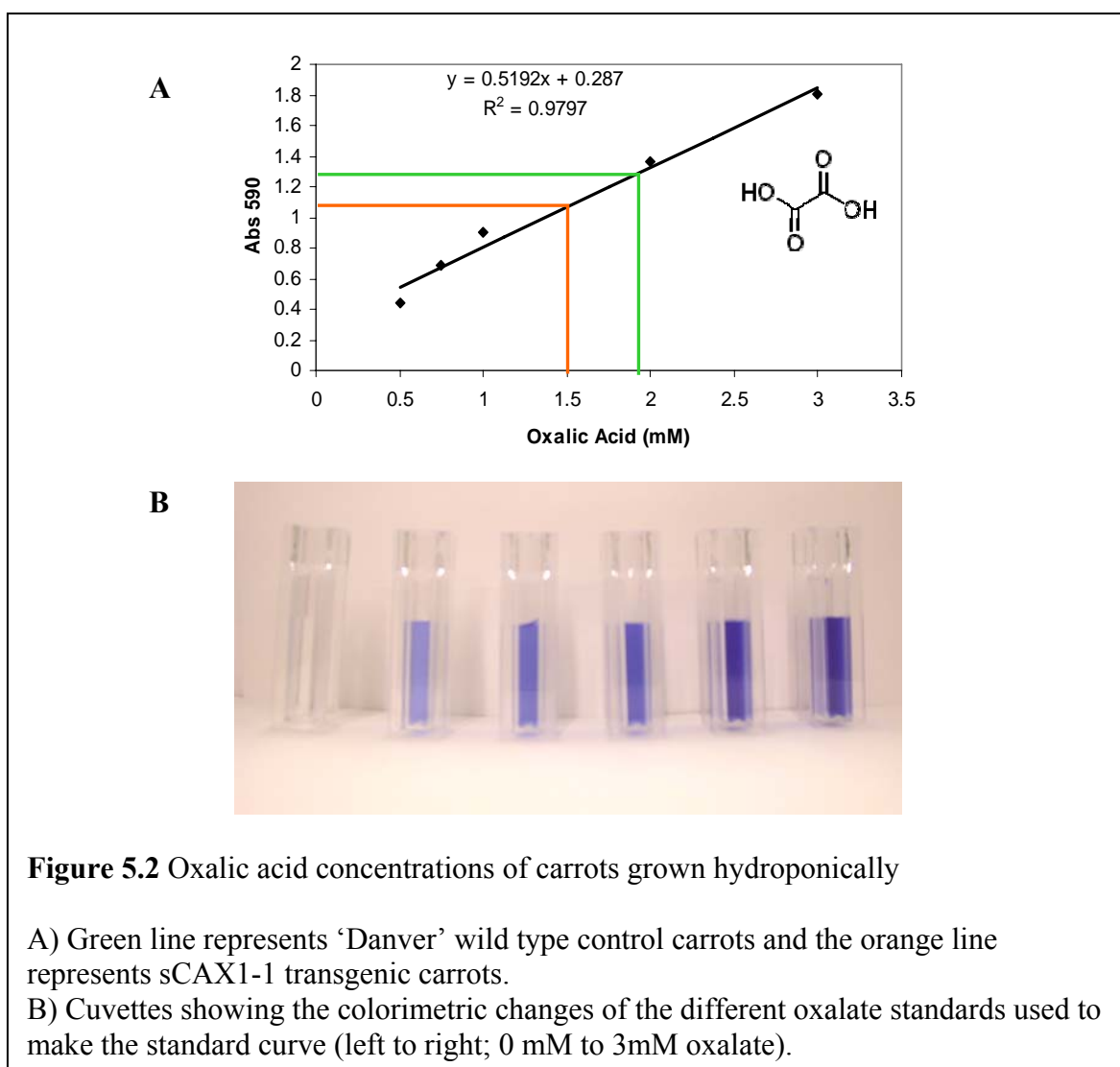
Carrot type	^{45}Ca Activity (%)[1]	^{42}Ca Enrichment (%)[2]
control	14.86	2.5
sCAX1 expressing carrot	30.83	2.4

[1] ^{45}Ca isotope activity remaining in edible carrot portion
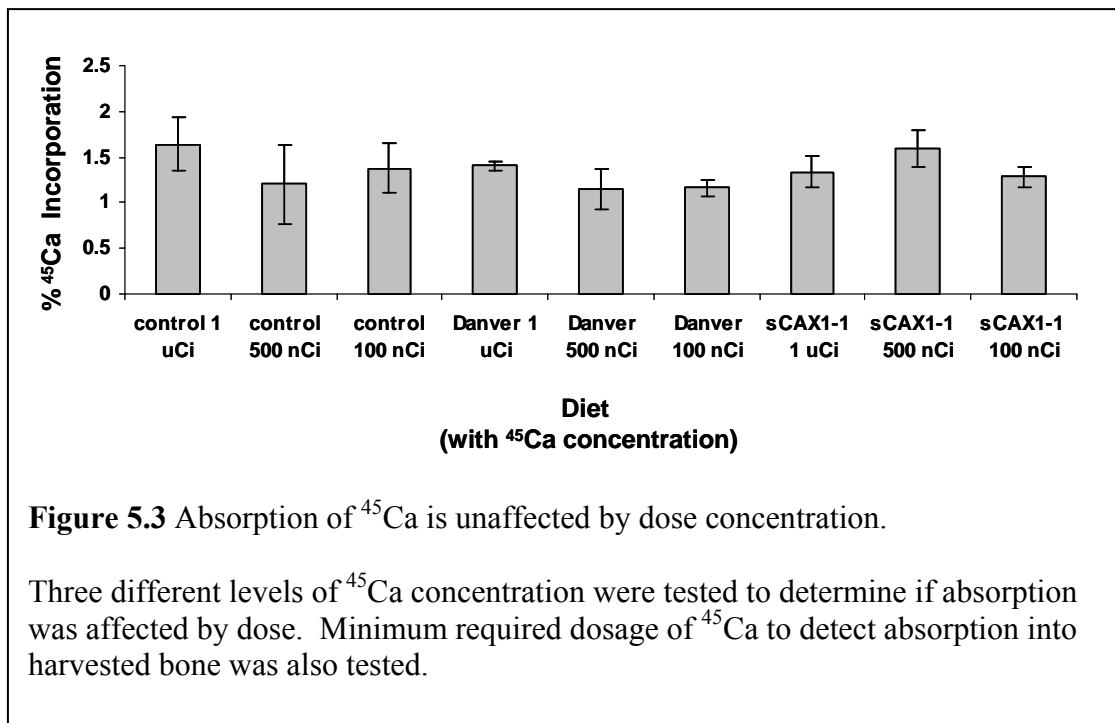[2] Total calcium in edible carrot portion supplied by the ^{42}Ca stable isotope

Mice fed sCAX1-expressing carrots

Previously we have used both extrinsic and intrinsic ^{45}Ca labeling of diets in

mouse feeding study models to measure tracer incorporation into hind limbs (Morris et

al., 2007). We have validated this mouse model using various diets and our results

compared favorably to previous feeding studies in rats (Weaver et al., 1987). Here, mice

were fed both extrinsic and intrinsically labeled diets containing control and *sCAX1*

expressing carrots. Potentially *sCAX1* expression may alter Ca partitioning within the

plant. Initially we determined if *sCAX1* expression altered oxalic acid concentration, a

known inhibitor of calcium absorption (Weaver et al., 1987). The oxalate concentration

was 1.88 +/- 0.08 mM for the control carrots compared to 1.46 +/- 0.10 mM for the

sCAX1 expressing carrots (Figure 5.2). This suggests *sCAX1* expression does not cause

Figure 5.2 Oxalic acid concentrations of carrots grown hydroponically

A) Green line represents 'Danver' wild type control carrots and the orange line represents sCAX1-1 transgenic carrots.
B) Cuvettes showing the colorimetric changes of the different oxalate standards used to make the standard curve (left to right; 0 mM to 3mM oxalate).
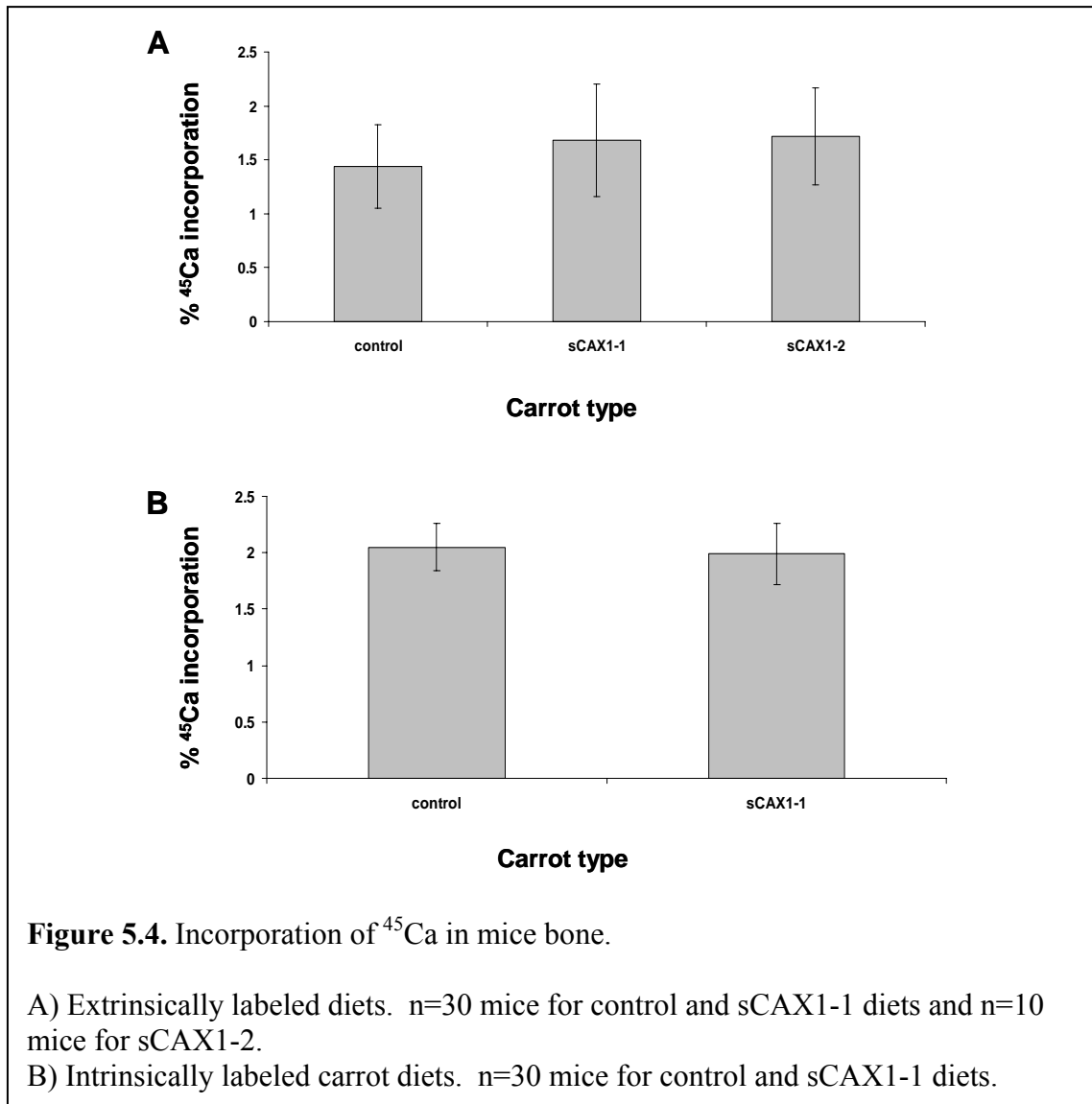
an increase in oxalate levels. However, these measurements do not directly address

calcium bioavailability issues.

To study the bioavailability we initially measured calcium incorporation by

extrinsically labeling the carrots. Extrinsic labeling can give meaningful bioavailability

data for foods like milk (Abrams, 2003; Avalos Mishaan et al, 2004); however, it does

Figure 5.3 Absorption of ^{45}Ca is unaffected by dose concentration.

Three different levels of ^{45}Ca concentration were tested to determine if absorption was affected by dose. Minimum required dosage of ^{45}Ca to detect absorption into harvested bone was also tested.

not work for spinach (Weaver et al., 1987). In general, extrinsic labeling should be expected to work for any food in which the various food calcium species are readily exchangeable. Since we are interested in addressing this exchangeable issue with the *sCAX1* expressing carrots, we made side-by-side tests of the intrinsically and extrinsically labeled carrots. First we made comparisons of the carrots to normal mice chow using extrinsically labeled diets. Using three different concentrations of label showed no difference in ^{45}Ca incorporation into mice bone (Figure 5.3). In the feeding studies, both control and *sCAX1* expressing extrinsically labeled carrots did not show any difference in ^{45}Ca absorption. The extrinsically labeled control carrots had

Figure 5.4. Incorporation of ^{45}Ca in mice bone.

A) Extrinsically labeled diets. n=30 mice for control and sCAX1-1 diets and n=10 mice for sCAX1-2.
B) Intrinsically labeled carrot diets. n=30 mice for control and sCAX1-1 diets.

incorporation of 1.45% of the ^{45}Ca activity compared to 1.68% for sCAX1-1 and 1.72% for sCAX1-2. (Figure 5.4A).

I then sought to compare the extrinsic labeling to the intrinsically labeled diets. Intrinsic labeling is more useful when studying the bioavailability of labeled calcium diets using vegetables. Many vegetable contain antinutritients which can interfere with calcium absorption in the gut (Heaney et al., 1998). These compounds can bind the calcium either freely and some plants also can bind calcium during the growth process (Heaney et al., 1998; Hanes et al., 1999a; Hanes et al., 1999b). In the case of spinach, a poor source of bioavailable calcium, the incorporation of a ^{45}Ca tracer in bone is reduced by 50% in the intrinsically labeled diets compared to the extrinsic diets when fed to rats or mice (Weaver et al., 1987 and Morris et al., 2007). Although my carrots contained very low concentrations of oxalic acid we still measured calcium bioavailability from the intrinsically carrots. In the intrinsically labeled diets, the incorporation from the control carrots was 2.05% compared to 1.99% for the *sCAX1-1* expressing carrots (Figure 5.4B). This data demonstrate that *sCAX1* expression does not negatively impact the absorption of calcium from the carrots.

Discussion

Most Americans do not get enough calcium in their diet (Mackenzie et al., 2001). To help compensate for this deficiency one strategy is to increase the calcium content of the foods they do eat. Recent work has shown that molecular breeding can increase the calcium content of popular vegetables. Tomatoes, carrots and potatoes have all been

modified to contain more calcium in their edible portions (Park et al., 2004; Park et al., 2005a; Park et al., 2005b). Previously, we have shown an approximate two fold increase in calcium from 30mg Ca/100g fwt in control carrots to 60mg Ca/100g fwt in the *sCAX1* expressing carrots. This increase in plant calcium is due to high level expression of a vacuolar localized calcium transporter (Hirschi, 1999). An additional benefit with these *sCAX1* expressing carrots is there was no increase in oxalic acid. This acid can be detrimental to calcium absorption from plants (Weaver et al., 1987; Weaver and Heaney, 1991; Morris et al., 2007). Although the calcium is increased in the carrots it would be difficult for most Americans to meet their requirements exclusively from these foods (Kirby et al., 1982). Here I have shown the ability to marginally improve the bioavailable calcium content of a staple food and when applied to a wide variety of fruits and vegetables could lead to more calcium consumption in the diet.

Testing the nutritional qualities of genetically modified foods is a rigorous process. Any initial mineral nutrition study requires labeling of the foods with either radioisotope for animal studies or stable isotopes for human trials. To date, there have been no reports of stable isotopes used to study the effects of nutrient availability from genetically modified foods. Most of the isotope research has been done with established plants or hybrid varieties (Patterson and Veillon, 2001; Weil, 2005). Here I have developed a labeling protocol for genetically modified carrots using both radioisotopes and stable isotopes to study nutrient bioavailability. Here I have shown that ^{45}Ca activity is two fold higher in the radioisotope labeled modified carrots and stable ^{42}Ca

enrichment is similar in both modified carrots compared to the control. To my knowledge these calcium labeling techniques are the first to be applied to carrots.

Aside from nutritional benefits, the use of genetic engineering to increase calcium levels could improve plant productivity and extend product shelf life. Calcium has long been used to combat many post harvest issues (Raz and Fluhr, 1992). Apples are immersed in a calcium solution to maintain firmness in shipping and prolong shelf life (Dris and Niskanen, 1999). Application of calcium solutions to plums (Alcaraz-Lopez et al., 2003) and pear fruits is used to increase firmness (Klein and Fergusson, 1987). Calcium is also added to soil to reduce the incidences of pathogen attack on potato tubers (Clough, 1994; Olsen et al., 1996) and to combat heat stress (Kleinhenz and Palta, 2002). All of these preventative measures require application of calcium containing solution to the soil or fruits involving time and manpower. Recently *sCAX1* expression has been used to increase calcium level in tomatoes which increased fruit firmness and prolong shelf. (Park et al., 2005a). This genetic engineering of increases in calcium over wide variety of fruits and vegetables could positively impact plant productivity, while simultaneously decreasing labor costs.

CHAPTER VI

CONCLUSIONS

Better understanding of plant cation transporters is useful to plant researchers by increasing the scope of known gene functions, as well as, expanding the ability of scientists to alter nutritionally important cations in the plants. The overall goal of this project had 2 parts. The first part was aimed at using molecular biology to understand and characterize the function of cation transporters from Arabidopsis. The second portion was to test if plants altered in calcium partitioning or calcium concentration had increased levels of bioavailability calcium.

The first section of research detailed a wide scale, systematic approach to infer function of plant cation transporters by using the GUS reporter system. Varying promoter fragments from 18 different genes, some fragments contained sequences 3' of the start codon, in Arabidopsis were analyzed at different ages and in response to different exogenous stresses. The results showed some interesting phenotypes as well as fragments of the coding region which might affect function of the transporter. Using this type of approach allowed us to surmise which cations certain gene products might transport or play a role in regulating within Arabidopsis. Those specific phenotypes will be useful starting points for further investigation.

In Chapter III, the goal of that research project was to take one specific cation transporter from the *CCX* family and characterize its function. The *CCX* family in Arabidopsis is a newly identified family of genes and little is known about the function

of the five genes within this family. By employing numerous molecular biology techniques along with several heterologous expression systems, such as yeast (*S. cereivseai)* and tobacco, I was able to determine various structural and functional properties of *At*CCX3 and *At*CCX4. *At*CCX3 appears to be a vacuolar localized transporter involved in K^+, Na^+ and Mn^{2+} homeostasis. The properties exhibited by *At*CCX3 were different from other previously characterized cation transporters in Arabidopsis and suggests that this gene and gene family have a novel and specific function within Arabidopsis. Further experiments and analysis of other family members will be needed to fully understand the role these genes play in Arabidopsis growth and development, along with how the genes within this family interplay with other cation transporters and regulators *in planta*.

The goal of the remaining research projects was to test the effectiveness, in a mammalian system, of molecular manipulations which are thought to improve the nutritional quality of the plant. The aim of the first project was to determine if a mutation abolishing oxalate crystal formation increased bioavailable calcium. Using a mouse model, I was able to show a 25% increase in calcium incorporation into bone. This corroborated that the plant biochemical change resulted in a gain in bioavailable calcium. Once this mutated gene is isolated, further studies in other nutritionally plants susceptible to oxalate crystal production can be manipulated to possible increase the levels of bioavailable calcium within the tissue. The second project showed that carrots modified to contain 2-fold higher calcium levels were not altered in calcium

bioavailability. The ability to assess molecular changes in a model mammalian system is crucial to improving the nutritional quality of food crops.

In summary, this research encompassed two unique aspects by utilizing molecular biology to better understand cation transport in plants and determining if modification intended to improve the calcium bioavailability actually accomplished that objective. There is increasing emphasis placed on increasing plant food consumption in the human diet, mainly fruits and vegetables. There is one caveat, in that fruits and vegetables are not great sources of calcium but molecular modification can help to lessen this effect. Reduction in calcium antinutrients and increasing calcium concentration appear to be vital areas of future research.

REFERENCES

Abrams, S.A. (2003). Using stable isotopes to assess the bioavailability of minerals in food-fortification programs. Forum Nutr. **56:** 312-313.

Aiello, D.P., Fu, L., Miseta, A., Sipos, K., and Bedwell, D.M. (2004). The Ca^{2+} homeostasis defects in a pgm2Δ strain of *Saccharomyces cerevisiae* are caused by excessive vacuolar Ca^{2+} uptake mediated by the Ca^{2+}-ATPase Pmc1p. J. Biol. Chem. **279**: 38495-38502.

Alcaraz-Lopez, C., Botia, M., Alcaraz, C.F., and Riquelme, F. (2003). Effects of foliar sprays containing calcium, magnesium and titanium on plum (*Prunus domestica* L.) fruit quality. J. Plant Physiol. **160:** 1441-1446.

Alexander, M.P. (1969). Differential staining of aborted and nonaborted pollen. Stain Technol. **44:** 117-22.

Ali, R., Zielinski, R.E., and Berkowitz, G.A. (2006). Expression of plant cyclic nucleotide-gated cation channels in yeast. J. of Exp. Bot. **57:** 125-138.

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, O., von Bodman, S., and Berkowitz, G.A. (2007). Death don't have no mercy and neither does calcium: Arabidopsis cyclic nucleotide gated channel2 and innate immunity. Plant Cell **19:** 1081-1095.

Allen, D.G., Blinks, J.R., and Prendergast, F.G. (1977). Aequorin luminescence: Relation of light emission to calcium concentration—a calcium-independent component. Science **195:** 996-998.

Allen, G.J., Kwak, J.M., Chu, S.P., Llopis, J., Tsein, R.Y., Harper, J.F., and Schroeder, J.I. (1999). Cameleon calcium indicator reports cytoplasmic calcium dynamics in Arabidopsis guard cells. Plant J. **19:** 735-747.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R. et al., (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653-657.

Apse, M.P., Aharon, G.S., Snedden, W.A., and Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. Science **285**: 1256-1258.

Apse, M.P., Sottosanto, J.B., and Blumwald, E. (2003). Vacuolar cation/H^+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of *At*NHX1, the Arabidopsis vacuolar Na^+/H^+ antiporter. Plant J. **36:** 229-239.

Arazi, T., Kaplan, B., Sunkar, R., and Fromm, H. (2000). Cyclic-nucleotide- and Ca^{2+}/calmodulin-regulated channels in plants: Targets for manipulating heavy metal tolerance, and possible physiological roles. Biochem. Doc. Trans. **28:** 471-475.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1998). Current Protocols in Molecular Biology. (New York: Greene Publishing Associates/Wiley Interscience).

Avalos Mishaan, A.M., Zavaleta, N., Griffin, I.J., Hilmers, D.C., Hawthorne, K.M., and Abrams S.A. (2004). Bioavailability of iron and zinc from a multiple micronutrient-fortified beverage. J Pediatr. **145:** 26-31.

Axelsen, K.B., and Palmgren, M.G. (2001). Inventory of the superfamily of P-type ion pumps in Arabidopsis. Plant Physiol. **126:** 696-706.

Bachrach, L.K. (2001). Acquisition of optimal bone mass in childhood and adolescence. TRENDS in Endocrinology & Metabolism. **12:** 22-28.

Baekgaard, L., Luoni, L., De Michelis, M.I., and Palmgren, M.G. (2006). The plant plasma membrane Ca^{2+} pump ACA8 contains overlapping as well as physically separated autoinhibitory and calmodulin-binding domains. J Biol. Chem. **281:** 1058-1065.

Balagué, C., Lin, B., Alcon, C., Flottes, G., Malmström, S., Köhler, C., Neuhaus, G., Pelletier, G., Gaymard, F., and Roby, D. (2003). HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell **15:** 365-379.

Barkla, B.J., and Pantoja, O. (1996). Physiology of ion transport across the tonoplast of higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47:** 159-184.

Baxter, I., Tchieu, J., Sussman, M.R., Boutry, M., Plamgren, M.G., Gribskov, M., Harper, J.F., and Axelsen, K.B. (2003). Genomic comparison of P-type ATPase ion pumps in Arabidopsis and rice. Plant Physiology **132:** 618-628.

Becker, D., Geiger, D., Dunkel, M., Roller, A., Bertl, A., Latz, A., Carpaneto, A., Dietrich, P., Roelfsema, M.R.G., Voelker, C., Schmidt, D., Mueller-Roeber, B., Czempinski, K., and Hedrich, R. (2004). *At*TPK4, an Arabidopsis tandem-pore K+ channel, poised to control the pollen membrane voltage in a pH- and Ca^{2+} -dependent manner. Proc. Natl. Acad. Sci. USA **101:** 15621-15626.

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signaling. Nat. Rev. Mol. Cell Biol. **1:** 11-21.

Blaustein, M.P., and Lederer, W.J. (1999). Sodium/calcium exchange: Its physiological implications. Physiol Rev. **79:** 763-854.

Blumwald, E., and Poole, R.J. (1986). Kinetics of Ca/H antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris* L. Plant Physiol. **80:** 727-731.

Bock, K.W., Honys, D., Ward, J.M., Padmanaban, S., Nawrocki, E.P., Hirschi, K.D., Twell, D., and Sze, H. (2006). Integrating membrane transport with male gametophyte development and function through transcriptomics. Plant Physiol. **140:** 1151-1168.

Bohn, T., Davidsson, L., Walczyk, T., and Hurrell, R.F. (2004). Fractional magnesium absorption is significantly lower in human subjects from a meal served with an oxalate-rich vegetable, spinach, as compared with a meal served with kale, a vegetable with low oxalate content. Bri J Nutr. **91:** 601-606.

Bonjour, J.P. (1996). Bone mineral acquisition in adolescence. In Osteoporosis, R. Marcus, ed (San Diego: Academic Press), pp. 465-476.

Bonza, M.C., Luoni, L., and DeMichelis, M.I. (2004). Functional expression in yeast of an N-deleted form of *At*-ACA8, a plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana*, and characterization of a hyperactive mutant. Planta **218:** 814-823.

Bothwell, J.H.F., and Ng, C.K.-Y. (2005). The evolution of Ca^{2+} signaling in photosynthetic eukaryotes. New Phytologist **16:** 21-38.

Braam, J. (2005). In touch: Plant responses to mechanical stimuli. New Phytologist **165:** 373-385.

Bradshaw, H.D. (2005). Mutations in CAX1 produce phenotypes characteristic of plants tolerant to serpentine soils. New Phytol. **167:** 81-88.

Bryant, R.J., Cadogan, J., and Weaver, C.M. (1999). The new dietary reference intakes for calcium: Implications for osteoporosis. J Am. Col. Nutr. **18:** 406S-412S.

Bugbee, B. (1999). Engineering plants for spaceflight environments. Gravit. Space Biol. Bull. **12:** 67-74.

Cai, X., and Lytton, J. (2004). The cation/Ca^{2+} exchanger superfamily: Phylogenetic analysis and structural implications. Mol. Biol. Evol. **21:** 1692-1703.

Carter, C., Songqin P., Zouhar J., Avila E.L., Girke, T., and Raikhel, N.V. (2004). The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. Plant Cell **16:** 3285-3303.

Cashman, K.D. (2002). Calcium intake, calcium bioavailability and bone health. Br J Nutr. **87:** S169-177.

Catalá, R., Santos, E., Alonso, J.M., Ecker, J.R., Martínez-Zapater, J.M., and Salinas, J. (2003). Mutations in the Ca^{2+}/H^+ transporter CAX1 increase CBF/DREB1 expression and the cold-acclimation response in Arabidopsis. Plant Cell **15:** 2940-2951.

Cervetto, L., Lagnado, L., Perry, R.J., Robinson, D.W., and McNaughton, P.A. (1989). Extrusion of calcium from rod outer segments is driven by both sodium and potassium gradients. Nature. **337:** 740-743.

Chan, C.W.M., Schorrak, L.M., Smith Jr., R.K., Bent, A.F., and Sussman, M.R. (2003). A cyclic nucleotide-gated ion channel, *CNGC2,* is crucial for plant development and adaptation to calcium stress. Plant Physiology **132:** 728-731.

Chen, X., Chang, M., Wang, W., and Wu, B. (1997). Cloning of a Ca^{2+}-ATPase gene and the role of cytosolic Ca^{2+} in the gibberellin-dependant signaling pathway in aleurone cells. Plant J. **11:** 363-371.

Cheng, N-H., Pittman, J.K., Shigaki, T., and Hirschi, K.D. (2002). Characterization of CAX4, an Arabidopsis H^+/cation antiporter. Plant Physiol. **128:** 1245-1254.

Cheng, N-H., and Hirschi, K.D. (2003). Cloning and characterization of CXIP1, a novel PICOT domain-containing Arabidopsis protein that associates with CAX1. J. Biol. Chem. **278:** 6503-6509.

Cheng, N-H, Pittman, J.K., Barkla, B., Shigaki, T., and Hirschi, K.D. (2003). The Arabidopsis cax1 mutant inhibits impaired ion homeostasis, development, and hormonal responses and reveals interplay among vacuolar transports. Plant Cell **15:** 347-364.

Cheng, N-H. Pittman, J.K., Zhu, J.K., and Hirschi, K.D. (2004a). The protein kinase SOS2 activates the Arabidopsis H^+/Ca^{2+} antiporter CAX1 to integrate calcium transport and salt tolerance. J. Biol. Chem. **279:** 2922-2926.

Cheng, N-H., Liu, J.Z., Nelson, R.S., and Hirschi, K.D. (2004b). Characterization of CXIP4, a novel Arabidopsis protein that activates the H^+/Ca^{2+} antiporter, CAX1. FEBS Lett. **559:** 99-106.

Cheng, N-H., Pittman, J.K., Shigaki, T., Lachmansingh, J., LeClere, S., Lahner, B., Salt, D.E., and Hirschi, K.D. (2005). Functional association of Arabidopsis CAX1 and CAX3 is required for normal growth and ion homeostasis. Plant Physiol. **138:** 2048-2060.

Cheng, N-H., Liu, J.Z., Brock, A., Nelson, R., and Hirschi, K.D. (2006). *At*GRXcp, an Arabidopsis chloroplastic glutaredoxin, is critical for protection against protein oxidative damage. J. Biol. Chem. **281:** 26280-26288.

Cheung, A.Y., Chen, C.Y., Tao, L.Z., Andreyeva, T., Twell, D., and Wu, H.M. (2003). Regulation of pollen tube growth by Rac-like GTPases. J Exp Bot. **54:** 73-81.

Chigri, F., Hormann, F., Stamp, A., Stammers, D.K., Bolter, B., Soll, J., and Vothknecht, U.C. (2006). Calcium regulation of chloroplast protein translocation is mediated by calmodulin binding to Tic32. Proc. Natl. Acad. Sci. USA **103:** 16051-16056.

Chiu, J.C., Brenner, E.D., DeSalle, R., Nitbach, M.N., Colmes, T.C., and Coruzzi, G.M. (2002). Phylogenetic and expresión análisis of the glutamate receptor-like gene family in *Arabidopsis thaliana*. Mol. Biol. Evol. **19:** 1066-1082.

Chung, W.S., Lee, S.H., Kim, J.C., Heo, W.D., Kim, M.C., Park, C.Y., Park, H.C., Lim, C.O., Kim, W.B., Harper, J.F., and Cho, M.J. (2000). Identification of calmodulin-regulated soybean Ca^{2+}-ATPase (SCA1) that is located on the plasma membrane. Plant Cell **12:** 1393-1407.

Clough, G.H. (1994). Potato tuber yield, mineral concentration, and quality after calcium fertilization. J. Am. Soc. Hortic. Sci. **119:** 175-179.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Cronin, S.R., Rao, R., and Hampton, R.Y. (2002). Cod1/Spf1p is a P-type ATPase involved in ER function and Ca2+ homeostasis. J. Cell Boil. **157:** 1017-1028.

Cunningham, K.W., and Fink, G.R. (1996). Calcineurin Inhibits *VCX1*-Dependent H^+/Ca^{2+} exchange and induces Ca^{2+}-ATPases in *Saccharomyces cerevisiae*. Mol. Cell Biol. **16:** 2226-2237.

Dabitz, N., Hu, N.J., Yusof, A.M., Tranter, N., Winter, A., Daley, M., Zschörnig, O., Brisson, A., and Hofmann, A. (2005). Structural determinants for plant annexin-membrane interactions. Biochem. **13:** 16292-16300.

Darley, C.P., van Wuytswinkel, O.C., van der Woode, K., Mager, W.H., and de Boer, A.H. (2000). *Arabidopsis thaliana* and *Saccharomyces cerevisiae* NHX1 genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers. Biochem J. **351:** 241-249.

Davenport, R. (2002). Glutamate receptors in plants. Annals of Botany **90:** 549-557.

Davletova, S., Rizhsky, L., Liang, H., Zhong, S., Oliver, D.J., Coutu, J., Shulaey, V., Schlauch, K., and Mittler, R. (2005). Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of Arabidopsis. Plant Cell **27:** 59-65.

DellaPenna, D. (2007). Biofortification of plant-based food: Enhancing folate levels by metabolic engineering. Proc. Natl. Acad. Sci. USA **104:** 3675-3676.

Demidchik, V., Shabala, S.N., Coutts, K.B., Tester, M.A.,and Davies, J.M. (2003). Free oxygen radicals regulate plasma membrane Ca^{2+}- and K+-permeable channels in plant root cells.   Journal of Cell Science **116:** 81-88.

Dode, L., Andersen, J.P., Raeymaekers, L., Missiaen, L., Vilsen, B., and Wuytack, F. (2005). Functional comparison between secretory pathway Ca^{2+}/Mn^{2+}-ATPase (SPCA) 1 and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 1 isoforms by steady-state and transient kinetic analyses. J. Biol. Chem. **280:** 39124-39134.

Dong, H., Light, P.E., French, R.J., and Lytton, J. (2001). Electrophysiological characterization and ionic stoichiometry of the rat brain K^+-dependent Na^+/Ca^{2+} exchanger, NCKX2. J Biol Chem. **276:** 25919-25928.

Dris, R., and Niskanen, R. (1999). Calcium chloride sprays decrease physiological disorders following long-term cold storage of apple. Plant Foods Hum Nutr. **54:** 159-171.

Eide, D.J., Clark, S., Nair, T.M., Gehk, M., Gribskov, M., Guerinot, M.L., and Harper, J.F. (2005). Characterization of the yeast ionome: A genome-wide analysis of nutrient mineral and trace element homeostasis in *Saccharomyces cerevisiae*. Genome Biol. **6:** R77.

Ellis, F.R., Holesh, S., and Ellis, J.W. (1972). Incidence of osteoporosis in vegetarians and omnivores. Am J Nutr. **25:** 555-558.

Ettinger, W.F., Clear, A.M., Fanning K.J., and Peck, M.L. (1999). Identification of a Ca^{2+}/H^+ antiport in the plant chloroplast thylakoid membrane. Plant Physiol **119:** 1379-1386.

Felsenstein, J. (1989). PHYLIP –Phylogeny inference package (version 3.2). Cladistics **5:** 164-166.

Fitzpatrick, L.M., and Keegstra, K. (2001). A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. Plant J. **27:** 59-65.

Fox, T.C., and Guerinot, M.L. (1998). Molecular biology of cation transport in plants. Annu Rev. Plant Physiol. Plant Mol. Biol. **49:** 669-696.

Foyer, C.H., DellaPenna, D., and Van Der Straeten, D. (2006). A new era in plant metabolism research reveals a bright future for bio-fortification and human nutrition. Physiologia Plantarum **126:** 289-290.

Franceschi, V.R., and Nakata, P.A. (2005). Calcium oxalate in plants: Formation and function. Ann Rv Plnt Biol. **56:** 41-71.

Franson, M.A.H. (1989). Metals by plasma emission spectroscopy. In Standard Methods for the Examination of Water and Wastewater. (Washington, D.C.: American Public Health Association), pp. 3-38.

Freese, W., and Schubert, D. (2004). Safety testing and regulation of genetically engineered foods. Biotechnol Genet Eng Rev. **21:** 299-324.

Fu, H-H., and Luan, S. (1998). AtKUP1: A dual-affinity K^+ transporter from Arabidopsis. Plant Cell **10:** 63-73.

Furuichi, T., Cunningham, K.W., and Muto, S. (2001). A putative two pore channel AtTPC1 mediates Ca2+ flux in Arabidopsis leaf cells. Plant Cell Physiol.**42:** 900-905.

Geisler, M., Frangne, N., Gomes, E., Martinoia, E., and Palmgren, M.G. (2000). The ACA4 gene of Arabidopsis encodes a vacuolar membrane calcium pump that improves salt tolerance in yeast. Plant Physiol. **124:** 1814-1827.

Gennari, C. (2001). Calcium and vitamin D nutrition and bone disease of the elderly. Public Health Nutr. **4:** 547-59.

Golovkin, M., and Reddy, A.S.N. (2003). A calmodulin-binding protein from Arabidopsis has an essential role in pollen germination. Proc. Natl. Acad. Sci. USA **100:** 10558-10563.

Gonzalez, A., Koren'kov, V., and Wagner, G.J. (1999). A comparison of Zn^{2+}, Mn^{2+}, Cd^{2+}, and Ca^{2+} transport mechanisms in oat root tonoplast vesicles. Physiol. Plant **106:** 203-209.

Gorecka, K.M., Konopka-Postupolska, D., Hennig, J., Buchet, R., and Pikula, S. (2005). Peroxidase activity of annexin 1 from *Arabidopsis thaliana*. Biochem.Biophys. Res. Commun. **336:** 868-75.

Grusak, M.A., Pezeshgi, S., O'Brien, K.O., and Abrams, S.A. (1996). Intrinsic ^{42}Ca-labelling of green bean pods for use in human bioavailability studies J. Sci. Food Agric. **70:** 11-15.

Grusak, M.A. (1997). Intrinsic stable isotope labeling of plants for nutritional investigations in humans. J Nutr Biochem. **8:** 164 –71.

Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiology **141:** 312-322

Halliwell, B., and Gutteridge, J.M.C. (2006). Free Radicals in Biology and Medicine. Ed 4 (Oxford: Clarendon Press), pp. 105-245.

Hanes, D.A., Weaver, C.M., Heaney, R.P., and Wastney, M.E. (1999a). Absorption of calcium oxalate does not require dissociation in rats. J Nutr. **129:** 170-173.

Hanes, D.A., Weaver, C.M., and Wastney, M.E. (1999b). Calcium and oxalic acid kinetics differ in rats. J Nutr. **129:** 165-169.

Harper, J.F., Hong, B., Hwang, I., Guo, H.Q., Stoddard, R., Huang, J.F., Palmgren, M.G., and Sze, H. (1998). A novel calmodulin-regulated Ca2+-ATPase (ACA2) from Arabidopsis with an N-terminal autoinhibitory domain. J. Biol. Chem. **273:** 1099-1106.

Haro, R., Banuelos, M.A., Senn, M.E., Barrero-Gil, J., and Rodriquez-Navarro, A. (2005). HKT1 mediates sodium uniport in roots. Pitfalls in the expresión of HKT1 in yeast. Plant Physiol. **139:** 1495-1506.

Harper, J.F., Breton, G., and Harmon, A. (2004). Decoding Ca^{2+} signals through plant protein kinases. Annu. Rev. Plant Biol. **55:** 263-288.

Heaney, R.P., Weaver, C.M., and Recker, R.R. (1988). Calcium absorbability from spinach. Am J Clin Nutr. **47:** 707-709.

Hepler, P.K. (2005). Calcium: A central regulator of plant growth and development. The Plant Cell **17:** 2142-2155.

Hetherington, A.M., and Brownlee, C. (2004) .The generation of Ca^{2+} signals in plants. *Annu. Rev. Plant Biol.* **55:** 401-427.

Hirschi, K.D., Zhen, R.G., Cunningham, K.W., Rea, P.A., and Fink, G.R. (1996). CAX1, an H^+/Ca^{2+} antiporter from Arabidopsis. Proc. Natl. Acad. Sci. USA **6:** 8782-8786.

Hirschi, K.D. (1999). Expression of Arabidopsis *CAX1* in Tobacco: Altered calcium homeostasis and increased stress sensitivity. Plant Cell **11:** 2113-2122.

Hirschi, K.D. (2001). Vacuolar H^+ /Ca^{2+} transport: Who's directing the traffic? Trends in Plant Sciences **6:** 100-104.

Hirschi, K.D. (2004). The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol. **136:** 2438-2442.

Holdaway-Clarke, T.L., Weddle, N.M., Kim, S., Robi, A., Parris, C., Kunkel, J.G., and Hepler, P.K. (2003). Effect of extracellular calcium, pH and borate on growth oscillations in Lilium formosanum pollen tubes. J. Exp. Bot. **54:** 65-72.

Hua, B.-G., Mercier, R.W., Leng, Q., and Berkowitz, G.A. (2003). Plants do it differently. A new basis for potassium/sodium selectivity in the pore of an ion channel. Plant Physiol. **132:** 1353-1361.

Huang, L., Berkelman, T., Franklin, A.E., and Hoffman, N.E. (1993). Characterization of a gene encoding a Ca^{2+}-ATPase-like protein in the plastid envelope. Proc. Natl. Acad. Sci. USA **90:** 10066-10070.

Hull A., Vij R., and Celenza, J.L. (2000). *Arabidopsis* cytochrome P450s that catalyze the first sep of tryptophan-dependent indol-3-acetic acid biosynthesis. Proc. Natl. Acad. Sci. USA **97:** 2379-2384.

Hwang, I., Sze, H., and Harper, J.F. (2000). A calcium-dependant protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of Arabidopsis. Proc. Natl. Acad. Sci. USA **23:** 6224-6229.

Institute of Medicine. (1997) Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. (Washington, DC: National Academy press).

Kabak, B., Dobson, A.D., and Var, I. (2006). Strategies to prevent mycotoxin contamination of food and animal feed: A review. Crit. Rev.Food Sci. Nutr. **46:** 593-619.

Kabala, K., and Klobus, G. (2005). Plant Ca^{2+} -ATPases. Acta Physiologiae Plantarum **27:** 559-574.

Kamiya, T., and Maeshima, M. (2004). Residues in internal repeats of the rice cation/H^+ exchanger are involved in the transport and selection of cations. J. Biol. Chem. **279:** 812-819.

Kamiya, T., Akahori, T., and Maeshima, M. (2005). Expression profile of the genes for rice cation/H$^+$ exchanger family and functional analysis in yeast. Plant and Cell Physiology **46:** 1735-1740.

Kang, K-J., Kinjo, T.G., Szerencsei, R.T., and Schnetkamp, P.P.M. (2005). Substitution of a single residue, Asp575, renders the NCKX2 K$^+$-dependent Na$^+$/Ca^{2+} exchanger independent of K$^+$. J. Biol. Chem. **280:** 6823-6833.

Kasai, M., and Muto, S. (1990). Ca^{2+} pump and Ca^{2+}/H$^+$ antiporter in plasma membrane vesicles isolated by aqueous two-phase partitioning from corn leaves. J. Membr. Biol. **114:** 133-142.

Kass, G.E., and Orrenius, S. (1999). Calcium signaling and cytotoxicity. Environ. Health Perspect. **107:** 25-35.

Kauffman, K.J., Pridgen, E.M., Doyle, F.J., Dhurjati, P.S., and Robinson, A.S. (2002). Decreased protein expression and intermittent recoveries in BiP levels results from cellular stress during heterologous protein expression in *Saccharomyces cerevisiae.* Biotechnol. Prog. **18:** 942-950.

Kaupp, U.B., and Seifert, R. (2002). Cyclic nucleotide-gated ion channels. Physiol. Rev. **82:** 769-824.

Kellermayer, R., Aiello, D.P., Miseta, A., and Bedwell, D.M. (2003). Extracellular Ca^{2+} sensing contributes to excess Ca^{2+} accumulation and vacuolar fragmentation in a pmr1Δ mutant of *S. cerevisiae.* Journal of Cell Science **116:** 1637-1646.

Kirby, E.A., and Pilbeam, D.J. (1982). Calcium as a plant nutrient. Plant Cell Environ. **7:** 397-405.

Klein, J.D., and Ferguson, I.B. (1987). Effect of high temperatures on calcium uptake by suspension cultured pear fruits cells. Plant Physiol. **84:** 153-156.

Kleinhenz, M.D., and Palta, J.P. (2002). Root zone calcium modulates the response of potato plants to heat stress. Physiol Plant. **115:** 111-118.

Komarek, A.R. (1993). An improved filtering technique for the analysis of neutral detergent and acid detergent fiber utilizing the filter bag technique. Publication #101. Ankom Company®, Fairport, NY 14450.

Koren'kov, V., Park, S., Cheng, N-H, Sreevidya, C., Lachmansingh, J., Morris, J., Hirschi, K., and Wagner, G.J. (2007). Enhanced Cd^{2+}-selective root-tonoplast-transport in tobaccos expressing Arabidopsis cation exchangers. Planta **225:** 403-411.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer E.L.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J. Mol. Biol. **305:** 567-580.

Krysan, P.J., Young, J.C., and Sussman, M.R. (1999). T-DNA as an insertional mutagen in Arabidopsis. Plant Cell **11:** 2283-2290.

Kurusu, T., Sakurai, Y., Miyao, A., Hirochika, H., and Kuchitsu, K. (2004). Identification of a putative voltage-gated Ca^{2+}-permeable channel (OsTPC1) involved in Ca^{2+} influx and regulation of growth and development in rice. Plant and Cell Physiology **45:** 693-702.

Kurusu, T., Yagala, T., Miyao, A., Hirochika, H., and Kuchitsu, K. (2005). Indentification of a putative voltage-gated Ca^{2+} channel as a key regulator of elicitor-induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. Plant J. **42:** 798-809.

Labayen, I., Diez, N., Parra, M.D., Gonzalez, A., and Martinez, J.A. (2004). Time-course changes in macronutrient metabolism induced by a nutritionally balanced low-calorie diet in obese women. Int J Food Sci Nutr. **55:** 27-35.

Lacombe, B., Becker, D., Hedrich, R., DeSalle, R., Hollman, M., Kwak, J.M., Schroeder, J.I., Le Novere, N., Nam, H.G., Spalding, E.P., Tester, M., Turano, F.J., Chiu, J., and Coruzzi, G. (2001). The identity of plant glutamate receptors. Science **25:** 1486-1487.

Lahner, B., Gong, J., Mahmoudian, M., Smith, E.L., Abid, K.B., Rogers, E.E., Guerinot, M.L., Harper, J.F., Ward, J.M., McIntyre, L., Schroeder, J.I., and Salt, D.E. (2003). Genomic scale profiling of nutrient and trace elements in Arabidopsis thaliana. Nat. Biotech. **21:** 1215-1221.

Lee, S.H., Kim, M.H., Park, K.H., Earm, Y.E., and Ho, W.K. (2002). K^+-dependent Na^+/Ca^{2+} exchange is a major Ca^{2+} clearance mechanism in axon terminals of rat neurohypophysis. J Neurosci. **22:** 6891-6899.

Lemtiri-Chlich, F., and Berkowitz, G.A. (2004). Cyclic adenosine monophosphate regulates calcium channels in the plasma membrane of *Arabidopsis* leaf guard and mesophyll cells. J. Biol. Chem. **279:** 35306-35312.

Leng, Q., Mercier, R.W., Hua, B.-G., Fromm, H., and Berkowitz, G.A. (2002). Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol **128:** 400-410.

Levchenko, V., Konrad, K.R., Dietrich, P., Roelfsema, M.R.G., and Hedrich, R. (2005). Cytosolic abscisic acid activates guard cell anion channels without preceding Ca^{2+} signals. Proc. Natl. Acad. Sci. USA **102:** 4203-4208.

Levine, R.L., Williams, J.A., Stadtman, E.R., and Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol. **233:** 346-357.

Li, J., Zhu. S., Song, X., Shen, Y., Chen, H., Yu, J., Yi, K., Liu, Y., Karplus, V.J., Wu, P., and Deng, X.W. (2006). A rice glutamate receptor-like gene is critical for the division and survival of individual cells in the root apical meristem. Plant Cell **18:** 340-349.

Liang, F., and Sze, H. (1998). A high-affinity Ca^{2+} pump, ECA1, from the endoplasmic reticulum is inhibited by cyclopiazonic acid but not by thapsigargin. Plant Physiol. **118:** 817-825.

Limtiri-Chlieh, F., MacRobbie, E.A.C., Webb, A.A.R., Manison, N.F., Brownlee, C., et al. (2003). Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. Proc. Natl. Acad. Sci. USA **100:** 10091-10095.

Liu, G.J., Martin, D.K., Gardner, R.C., and Ryan, P.R. (2002). Large Mg^{2+}-dependent currents are associated with the increased expression of ALR1 in Saccharomyces cerevisiae. FEMS Microbiol Lett. **213:** 231-237.

Luo, G.Z., Wang, H.W., Huang, J., Tian, A.G., Wang, Y.J., Zhang, J.S., and Chen, S.Y. (2005). A putative plasma membrane cation/proton antiporter from soybean confers salt tolerance in Arabidopsis. Plant Mol Biol. **59:** 809-20.
Luoni, L., Bonza, M.C., and De Michelis, M.I. (2000). H^+/Ca^{2+} exchange driven by the plasma membrane Ca^{2+}-ATPase of Arabidopsis thaliana reconstituted in proteoliposomes after calmodulin-affinity purification. FEBS Lett. **482:** 225-230.

MacDiarmid, C.W., and Gardner, R.C. (1998). Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. J. Biol. Chem. **273:** 1727-1732.

Maeshima, M. (2000). Vacuolar H(+)-pyrophosphatase. Biochem. Biophys. Acta. **1465:** 37-51.

Malmstrom, S., Askerlund, P., and Palmgren, M.G. (1997). A calmodulin-stimulated Ca^{2+}-ATPase from plant vacuolar membranes with putative regulatory domains at its N-terminus. FEBS Lett. **6:** 324-328.

Marschner, H. (1995). Mineral Nutrition of Higher Plants. (London: Academic Press).

Marty, F. (1999). Plant vacuoles. Plant Cell **11:** 587-599.

Maser, P., Thomine, S., Schroeder, J.I., Ward, J.M., Hirschi, K., Sze, H., Talke, I.N., Amtmann, A., Maathuis, F.J., Sanders, D., Harper, J.F., Tchieu, J., Gribskov, M., Persans, M.W., Salt, D.E., Kim, S.A., and Guerinot, M.L. (2001). Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol. **126:** 1646-1667.

Massey, L.K., Palmer, R.G., and Horner, R.T. (2001). Oxalate content of soybean seeds (Glycine max: Leguminosae), soyfoods, and other edible legumes. J Agric food Chem. **49:** 4262-4266.

Matcovic, V., and Lilich, J. (1993). Calcium requirement for growth. Are current recommendations adequate. Nutr. Rev. **51:** 171-180.

Medvedev, S.S. (2005). Calcium signaling system in plants. Russian Journal of Plant Physiology **52**: 249-270.

Mei, H., Zhao, J., Pittman, J.K., Lachmansingh, J., Park, S., and Hirschi, K.D. (2007). *In planta* regulation of the Arabidopsis Ca^{2+}/H^+ antiporter CAX1. J. Exp. Bot. (In press).

Meyerhoff, O., Muller, K., Roelfsema, M.R., Latz, A., Lacombe, B., Hedrich, R., Dietrich, P., and Becker, D. (2005). AtGLR3.4, a glutamate receptor channel-like gene is sensitive to touch and cold. Planta **222:** 418-427.

Miedema, H., de Boer, A.H., Pantoja, O. (2003). The gating kinetics of the slow vacuolar channel. A novel mechanism for SV channel functioning. J. of Mem. Biol. **194:** 11-20.

Miseta, A., Kellermayer, R., Aiello, D.P., Fu, L., and Bedwell, D.M. (1999). The vacuolarCa^{2+}/H^+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S.cerevisiae*. FEBS lett. **21:** 132-136.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. **7:** 405-410

Mori, I.C., and Schroeder, J.I. (2004). Reactive oxygen species activation of plant Ca^{2+} channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant Physiol. **135:** 702-708.

Morris, J., Nakata, P., McConn, M., Brock, A., and Hirschi, K.D. (2007). Increased calcium bioavailability in mice fed genetically engineered plants lacking calcium oxalate. Plant Mol. Biol. **64:** 613-618.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissues. Physiol. Plant. **15:** 473-497.

Nakamura, K., Niimi, M., Niimi, K., Colmes, A.R., Yates, J.E., Decottiginies, A., Monk, B.C., Goffeau, A., and Cannon, R.D. (2001). Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transport. Antimicrob. Agents Chemother. **45:** 3366-3374.

Nakata, P.A., and McConn, M.M. (2000). Isolation of *Medicago truncatula* mutants defective in calcium oxalate crystal formation. Plant Physiol. **124:** 1097-1104.

Nakata, P.A., and McConn, M.M. (2003). Calcium oxalate crystal formation is not essential for growth of *Medicago truncatula*. Plant Physiol Biochem. **41:** 325-329.

Nakata, P.A., and McConn, M.M. (2006). A genetic mutation that reduces calcium oxalate content increase availability in *Medicago truncatula*. Func. Plant Biol. **33:** 703-706.

Nass, R., Cunningham, K.W., and Rao, R. (1997). Intracellular sequestrations of sodium by a novel Na^+/H^+ exchanger in yeast is enhanced by mutations in the plasma membrane H^+-ATPase. Insights into mechanisms of sodium tolerance. J. Biol. Chem. **272:** 26145-26152.

Nass, R., and Rao, R. (1998). Novel localization of a Na^+/H^+ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis. J. Biol. Chem. **273:** 21054-21060.

Nathan, D.F., Vos, M.H., and Lindquist, S. (1999). Identification of *SSF1* and *HCH1* as multicopy suppressors of a *Saccharomyces cerevisiae* Hsp90 loss-of-function mutation. Proc. Natl. Acad. Sci. USA **96:** 1409-1414.

Navazio, L., Bewell, M.A., Siddiqua, A., Dickinson, G.D., Galione, A., and Sanders, D. (2000). Calcium release from the endoplasmic reticulum of higher elicited by the NAP metabolite nicotinic acied adenine dinucleotide phosphate. Proc. Natl. Acad. Sci. USA **97:** 8693-8698.

Nayyar, H. (2003). Calcium as environmental sensor in plants. Current Science **84:** 893.

New, S.A. (2001). Exercise, bone and nutrition. Proc Nutr Soc. **60:** 265-74.

Ng, C.K.-Y., McAinsh, M.R., Gray, J.E., Hunt, L., Leckie, C.P., Mills, L., and Hetherington, A.M. (2001). Calcium-based signaling systems in guard cells. New Phytologist **151:** 109.

Ng, C.K.-Y., and McAinsh, M.R. (2003). Encoding specificity in plant calcium signaling: Hot-spotting the ups and downs and waves. Annals of Botany **92:** 477-485.

Nieves, J.W. (2003). Calcium, vitamin D, and nutrition in elderly adults. Clin Geriatr Med. **19:** 321-35.

Oldroyd, G.E., and Downie, J.A. (2006). Nuclear calcium changes at the core of symbiosis signaling. Curr. Opin. Plant Biol. **9:** 351-357.

Olsen, N.L., Hiller, L.K., and Mikitzel, L.J. (1996). The dependence of internal brown spot development upon calcium fertility in potato tubers. Potato Res. **39:** 165-178.

Pardo, J.M., and Serrano, R. (1989). Structure of a plasma membrane H^+-ATPase gene from the plant *Arabidopsis thaliana*. J. Biol. Chem. **264:** 8557-8562.

Park, S. , Kim, C., Pike, L., Smith, R., and Hirschi, K. (2004). Increased calcium in carrots by expression of an Arabidopsis H^+/Ca^{2+} transporter. Molecular Breeding **14:** 275-282.

Park, S., Cheng, N-H., Pittman, J.K., Yoo, K.S., Park ,J., Smith, R.H., and Hirschi, K.D. (2005a). Increased calcium levels and prolonged shelf life in tomatoes expressing Arabidopsis H^+/Ca^{2+} transporters. Plant Phys. **139:** 1194-12061.

Park, S., Kang, T.S., Kim, C.K., Han, J.S., Kim, S., Smith, R.H., Pike, L.M., and Hirschi, K.D. (2005b). Genetic manipulation for enhancing calcium content in potato tuber. J. Agric. Food Chem. **53:** 5598-5603.

Patterson, K.Y., and Veillon, C. (2001) Stable isotopes of minerals as metabolic tracers in human nutrition research. Exp Biol Med **226:** 271-282.

Peiter, E., Maathuis, F.J.M., Mills, L.N., Knight, H., Pelloux, J., Hetherington, A.M., and Sanders, D. (2005). The vacuolar Ca^{2+} -activated channel TPC1 regulates germination and stomatal movement. Nature **434:** 404-408.

Philippar, K., Ivashikina, N., Ache, P., Christian, M., Luthen, H., Palme, K., and Hedrich, R. (2004). Auxin activates KAT1 and KAT2, two K^+-channel genes expressed in seedlings of *Arabidopsis thaliana*. Plant J. **37:** 815-8-27

Pittman, J.K. and Hirschi, K.D. (2001). Regulation of *CAX1*, an Arabidopsis Ca^{2+}/H^+ antiporter: Identification of an N-terminal autoinhibitory domain. Plant Physiol. **127:** 1020-1029.

Pittman, J.K., Shigaki, T., Cheng, N-H., and Hirschi, K.D. (2002). Mechanism of N-terminal autoinhibition in the Arabidopsis Ca^{2+}/H^+ antiporter CAX1. J. Biol. Chem. **277:** 26452-26459.

Pittman, J.K., and Hirschi, K.D. (2003). Don't shoot the second messenger: Endomembrane transporters and binding proteins modulate cytosolic Ca^{2+} levels. Curr. Opin. Plant Biol. **6:** 257-262.

Pittman, J.K., Shigaki, T., Marshall, J.L., Morris, J.M., Cheng, N-H., and Hirschi, K.D. (2004a). Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter. Plant Mol. Biol. **56:** 959-971.

Pittman, J.K., Cheng, N-H., Shigaki, T., Kunta, M., and Hirschi, K.D. (2004b). Functional dependence on calcineurin by variants of the *Saccharomyces cerevisiae* vacuolar Ca2+/H+ exchanger Vcx1p. Mol. Microbiol. **24:** 1104-16.

Plieth, C. (2005). Calcium: Just another regulator in the machinery of life? Ann. Bot. **96:** 1-8.

Prenen, J.A.C., Boer, P., and Dorhout Mees, E.J. (1984). Absorption kinetics of oxalate from oxalate-rich food in man. Am J Clin Nutr. **40:** 1007-1010.

Prokić, L., Jovanović, Z., Stikić, R., and Vućinić, Ž. (2005). The mutual effect of extracellular Ca^{2+}, abscisic acid, and pH on the rate of stomatal closure. Ann. N.Y. Acad. Sci. **1048:** 513-516.

Qu, H.Y., Shang, Z.L., Zhang, S.L., Liu L.M., and Qu, J.Y. (2007). Identification of hyperpolarization-activated calcium channels in apical pollen tubes of *Pyrus pyrifolia*. New Phytol. **174:** 524-536.

Ratajczak, R. (2000). Structure, function and regulation of the plant vacuolar H^+-translocating ATPase. Biochim Biophys Acta. **1465:** 17-36.

Raz, V., and Fluhr, R. (1992) Calcium requirement for ethylene-dependent responses. Plant Cell. **4:** 1123-1130.

Reddy, V.S., and Reddy, A.S.N. (2004). Proteomics of calcium-signaling components in plants. Phytochemistry **65:** 1745-1776.

Reeves, P.G., Nielsen, F.H., and Fahey Jr., G.C. (1993). AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. **123:** 1939-1951.

Rentel, M.C., and Knight, M.R. (2004). Oxidative stress-induced calcium signaling in Arabidopsis. Plant Physiology **135:** 1471-1479.

Salt, D.E., and Wagner, G.J. (1993). Cadmium transport across tonoplast vesicles from oat roots. J. Biol. Chem. **268:** 12297-12302.

Samaj, J., Muller, J., Beck, M., Bohm, N., and Menzel, D. (2006). Vesicular trafficking, cytoskeleton and signaling in root hairs and pollen tubes.   Trends Plant Sci. **11:** 594-600.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Press).

Sanders, D., Pelloux, J., Brownlee, C., and Harper, J.F. (2002). Calcium at the crossroads of signaling. Plant Cell **14:** S401-S417.

Schiøtt, M., Romanowsky, S.M., Bækgaard, L., Jakobsen, M.K., Palmgren, M.G., and Harper, J.F. (2004). A plant plasma membrane Ca^{2+} pump is required for normal pollen tube growth and fertilization. Proc. Natl. Acad. Sci. USA **101:** 9502-9507.

Schiøtt, M., and Palmgren, M.G. (2005). Two plant Ca^{2+} pumps expressed in stomatal guard cells show opposite expression patterns during cold stress. Physiologia Plantarum **124:** 278-281.

Schumaker, K.S., and Sze, H. (1985). Ca^{2+}/H^{+} antiport system driven by the proton electrochemical gradient of a tonoplast H^{+}-ATPase from oat root. Plant Physiol **79:** 1111-1117.

Scrase-Field, S.A.M.G., and Knight, M.R. (2003). Calcium: Just a chemical switch? Current Opinion in Plant Biology **6:** 500-506.

Sessions, A., Burker, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., et al. (2002). A high-through-put *Arabidopsis* reverse genetics system. The Plant Cell **14:** 2985-2994.

Shang, Z.-I., Ma, L., Zhang, H., He, R., Wang, X., Cui, S., and Sun, D. (2005). Ca^{2+} influx into lily pollen grains through a hyperpolarization-activated Ca^{2+} -permeable channel which can be regulated by extracellular CaM. Plant and Cell Physiol. **46:** 598-608.

Sheldrick, B.H. (1986). Test of the Leco CHN-600 Determinator for soil carbon and nitrogen analysis. Can J Soil Sci. **66:** 543-545.

Shigaki, T., and Hirschi, K.D. (2000). Cloning and Characterization of CAX-like Genes in Plants: Implications for functional diverstiy. Gene **257:** 291-298.

Shigaki, T., Cheng, N-H., Pittman, J.K., and Hirschi, K.D. (2001). Structural determinants of Ca^{2+} transports in the Arabidopsis H^+/Ca^{2+} antiporter CAX1. J Biol. Chem. **276:** 43152-43159.

Shigaki, T., Pittman, J.K., and Hirschi, K.D. (2003). Manganese specificity determinants in the Arabidopsis metal/H^+ antiporter CAX2. J. Biol. Chem. **278:** 6610-6617.

Shigaki, T., Kole, M., Ward, J.M., Sze, H., and Hirschi, K.D. (2005). Cre-*loxP* recombination vectors for the expression of Riken Arabidopsis full-length cDNAs in plants. Biotech. **39:** 301-303.

Shigaki, T., Rees, I., Nakhleh, L., and Hirschi, K.D. (2006). Identification of three distanct Phylogenetic groups of CAX cation/proton antiporters. J. Mol. Evol. **63:** 815-825.

Shigaki, T., and Hirschi, K.D. (2006). Diverse functions and molecular properties emerging for CAX cation/H^+ exchangers in plants. Plant Biol. **8:** 419-429.

Singh, P.P., Kothari, L.K., Sharma, D.C., and Saxena, S.N. (1972). Nutritional value of foods in relation to their oxalic acid content. Am J Clin Nutr. **25:** 1147-1152.

Sivitz, A.B., Reinders, A., Johnson, M.E., Krentz, A.D., Grof, C.P., Perroux, J.M., and Ward, J.M. (2007). Arabidopsis sucrose transporter AtSUC9. High affinity transport activity, intragenic control of expression, and early flowering mutant phenotype. Physiol. Plant. **143:** 188-98.

Sonnhammer, E.L.L., von Heijne, G., and Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. Proceedings of ISMB **6:** 175-182.

Sørensen, T.L-M., Møller, J.V., and Nissen, P. (2004). Phosphoryl transfer and calcium ion occlusion in the calcium pump. Science **304:** 1672-1675.

Subbaiah, C.C., and Sachs, M.M. (2000). Maize *cap1* encodes a novel serca-type calcium-ATPase with a calmodulin-binding domain. J. Biol.Chem. **28:** 21678-21687.

Sze, H., Li, X., and Palmgren, M.G. (1998). Energization of plant cell membranes by H$^+$-pumping ATPases. Regulation and biosynthesis. Plant Cell **11:** 677-690.

Sze, H., Liang, F., Hwang, I., Curran, A.C., and Harper, J.F. (2000). Diversity and regulation of plant Ca^{2+} pumps; insights from expression in yeast. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 433-462.

Sze, H., Padmanaban, S., Cellier, F., Honys, D., Cheng, N-H., Bock, K.W., Conéjéro, G., Li, X., Twell, D., Ward, J.M., and Hirschi, K.D. (2004). Expression patterns of a novel *AtCHX* gene family highlight potential roles in osmotic adjustment and K$^+$ homeostasis in pollen development. Plant Physiol. **136:** 2532-2547.

Tanouye, M.A., Ferrus, A., and Fujita, S.C. (1981). Abnormal action potentials associated with the *Shaker* complex locus of *Drosophilia*. Proc. Natl. Acad. Sci. USA **78:** 6548-6552.

Tarczynski, M.C., Jensen, R.G., and Bohnert, H.J. (1992). Expression of a bacterial mtlD gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Natl. Acad. Sci. USA **89:** 2600-2604.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4680.

Thuleau, P., Leclerc, C., Xiiong, T.C., Mazars, C., Leclerc, C., and Moreau, M. (2003). Luminous plant and animals or the expression of aequorin and "chameleon" probes: A new light in calcium signaling. J. Soc. Biol.**197:** 291-300.

Ton, V.-K., and Rao, R. (2004). Functional expression of heterologous proteins in yeast: Insights into Ca^{2+} signaling and Ca^{2+} -transporting ATPases. Am. J. Physiol. Cell Physiol. **287:** C580-C589.

Vanoevelen, J., Dode, L., Van Baelen, K., Fairclough, R.J., Missiaen, L., Raeymaekers, and L., Wuytack. (2005). The secretory pathway Na$^+$/ Ca^{2+} -ATPase 2 is a golgi-localized pump with high affinity for Ca^{2+} ions. J.Biol. Chem. **280:** 22800-22808.

Wallis, J.W., Cherbet, G., Brodsky, G., Rolfe, M., and Rothstein, R. (1989). A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. Cell **58:** 409-419.

Wang, Y.-F., Fan, L.-M., Zhang, W.-Z., Zhang, W., and Wu, W.-H. (2004). Ca^{2+} - permeable channels in the plasma membrane of Arabidopsis pollen are regulated by actin microfilaments. Plant Physiol. **136:** 3892-3904.

Wang, Y.-J., Yu, J.-N., Chen, T., Zhang, Z.-G., Hao, Y.-J., Zhang, J.-S., and Chen, S.-Y. (2005). Functional analysis of a putative Ca^{2+} channel gene *TaTPC1* from wheat. J. Exp. Biol. **56:** 3051-3060.

Weaver, C.M., Martin, B.R., Ebner, J.S., and Krueger, C.A. (1987). Oxalic acid decreases calcium absorption in rats. J Nutr. **117:** 1903-1906.

Weaver, C.M., Proulx, W.R., and Heaney, R. (1990). Choices for achieving adequate dietary calcium with a vegetarian diet. Am. J. Clin. Nutr. **70:** 543s-548s.

Weaver, C.M., and Heaney, R.P. (1991) Isotopic exchange of ingested calcium between labeled sources. Evidence that ingested calcium does not form a common absorptive pool. Calcif Tissue Int. **49:** 244-7.

Weaver, C.M., and Plawecki, K.L. (1994). Dietary calcium: Adequacy of a vegetarian diet. Am. J. Clin. Nutr. **59:** 1238S-1241S.

Webb, M.A. (1999). Cell-Mediated crystallization of calcium oxalate in plants. Plant Cell. 751-761.

Weil, J.H. (2005) Are genetically modified plants useful and safe? IUBMB Life. **57:** 311-314.

White, P.J., Pineros, M., Testr, M., and Ridout, M.S. (2000). Cation permeability and selectivity of a root plasma membrane calcium channel. J. Membr. Biol. **174:** 71-83.

White, P.J., and Broadley, M.R. (2003). Calcium in plants. Annals of Botany **92:** 487-511.

Wimmers, L.E., Ewing, N.N., and Bennett, A.B. (1992). Higher plant Ca^{2+}-ATPase: primary structure and regulation of mRNA abundance by salt. Proc. Natl. Acad. Sci. USA **89:** 9205-9209.

Winkfein, R.J., Szerencsei, R.T., Kinjo, T.G., Kang, K., Perizzolo, M., Eisner, L., and Schnetkamp, P.P.M. (2003). Scanning mutagenesis of the alpha repeats and of the transmembrane acidic residues of the human retinal cone Na/Ca-K exchanger. Biochemistry **42:** 543-552.

Wu, Z., Liang, F., Hong, B., Young, JC., Sussman, M.R., Harper, J.F., and Sze, H. (2002). An endoplasmic reticulum-bound Ca^{2+}/Mn^{2+} pump, ECA1, supports plant growth and confers tolerance to Mn^{2+} stress. Plant Physiol.**130:** 128-137.

Xiong, L., Schumaker, K.S., and Zhu, J.K. (2002). Cell signaling during cold, drought, and salt stress. Plant Cell **14:** S165-S183.

Xiong, T.C., Borque, S., Lecourieux, D., Amelot, N., Grat, S., Briere, C., Mazars, C., Puqin, A., and Ranjeva, R. (2006). Calcium signaling in plant cell organelles delimited by a double membrane. Biochem. Biophys. Acta. **1763:** 1209-1215.

Xu, C., Rice W.J., He, W., and Stokes, D.L. (2002). A structural model for the catalytic cycle of Ca^{2+}-ATPase. J Mol. Biol. **8:** 201-211.

Yang, M., Jensen, L.T., Gardner, A.J., and Culotta, V.C. (2005). Manganese toxicity and *Saccharomyces cerevisiae* Mam3p, a member of the ACDP (ancient conserved domain protein) family. Biochem J. **386:** 479-487.

Yang, T., and Poovaiah, B.W. (2003). Calcium/calmodulin-mediated signal network in plants. Trends in Plant Science **8:** 505-512.

Yokoi, S., Quintero, F.J., Cubero, B., Ruiz, M.T., Bressan, R.A., Hasegawa, P.M., and Pardo, J.M. (2002). Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J. **30:** 529-539.

Yoshioka, K., Moeder, W., Kang, H.G., Kachroo, P., Masmoudi, K., Berkowitz, G., and Klessig, D.F. (2006). The chimeric Arabidopsis cyclic nucleotide-gated ion channel 11/12 activates multiple pathogen resistance responses. Plant Cell **18:** 747-763.

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Morris, J.L., Hawthorne, K., Hotze, T., Abrams, S., and Hirschi, K.D. Increased calcium bioavailability in mice and humans fed carrots modified in calcium transport activity (in preparation).

Morris, J., and Hirschi, K.D. (2007). Chapter II: Calcium transporters: From fields to the table. In: Plant Membrane and Vacuolar Transporters. (1st edition): (Oxfordshire, UK: CAB International).

Morris J.L., Nakata, P., McConn, M., Brock, A., and Hirschi, K.D. (2007). Increased calcium bioavailability in mice fed genetically engineered plants lacking calcium oxalate (Cover) Plant Mol. Biology **64:** 613-618.

V. Koren'kov, V., Park, S.H., Cheng, N.H., Sreevidya, C., Lachmansingh, J. Morris, J.L., Hirschi, K.D., and Wagner, G.J. (2006). Enhanced Cd^{2+-}selective root-tonoplast-transport in tobaccos expressing Arabidopsis cation exchangers. Planta **225:** 403-411

Park, S.H., Li, J., Pittman, J.K., Berkowitz, G., Undurraga, S., Yang, H., Unduragga, S., Morris, J.L., Hirschi, K.D., and Gaxiola, R.A. (2005). Up-regulation of a vacuolar H^+-PPase as a strategy to engineer drought resistant crop plants. Proc. Natl. Acad. Sci. USA **102:** 18830-18835.

Pittman, J.K., Shigaki, T., Marshall, J.L., Morris, J.L., Cheng, N.H., and Hirschi, K.D. (2004). Functional and regulatory analysis of the *Arabidopsis thaliana* CAX2 cation transporter. Plant Mol. Biol. **56:** 959-971.

Park, S.H., Morris, J.L., Park, J.E.,. Hirschi, K.H., and Smith, R.H. (2003). An efficient and genotype independent method to transform tomato using Agrobacterium. J Plant Physiol. **160:** 1253-1257.