PHENOTYPE ANALYSIS OF TOBACCO LINES EXPRESSING A DEREGULATED ARABIDOPSIS CA-ATPASE (ACA2)

A Thesis

by

SEAN MICHAEL THOMPSON

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

MASTER OF SCIENCE

December 2010

Major Subject: Molecular and Environmental Plant Sciences

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Approved by:

Co-Chairs of Committee,	Kendal D. Hirschi
	Jean H. Gould
Committee Member,	Bhimanagouda S. Patil
Chair of Interdisciplinary Faculty,	Dirk B. Hays

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ABSTRACT

Phenotype Analysis of Tobacco Lines Expressing a Deregulated Arabidopsis Ca-ATPase (ACA2). (December 2010)

Sean Michael Thompson, B.S., Texas A&M University - Corpus Christi

Co-Chairs of Advisory Committee: Dr. Kendal D. Hirschi
Dr. Jean H. Gould

Functional foods go beyond simply supplying nutrients and are increasingly becoming a focus in the prevention and treatment of disease; however, the benefits of biofortified crops to human nutrition have not been well demonstrated. Modern breeding, molecular genetics, and biotechnology are currently focusing on how to improve the nutritional content in foods. Potatoes, carrots, and lettuce are popular vegetables eaten today and are targets in developing nutrient dense crops (biofortification). Biofortification of vegetables to increase calcium (Ca) in the diet has had promising results. Here we describe the current standing of nutrient biofortification of crops.

Ca distribution within the plant cell moderates critical functions from signaling to growth and development and can affect overall plant vigor. The endoplasmic reticulum (ER) located Ca-ATPase ACA2 (*Arabidopsis* Ca-ATPase, isoform 2) is thought to play a role in intracellular calcium homeostasis. In yeast studies; a truncated pump (Δ80-ACA2) lacking the N-terminal region is about 10-fold more active than the full-length ACA2 pump. Single point mutations have been shown to increase activity of ACA2 in

yeast as well. Previously in our lab, human feeding studies demonstrated that increased Ca accumulation and bioavailability in transgenic plants accompanied increased activity of the deregulated vacuolar Ca / H^+ antiporter CAX1 (Cation Exchanger 1) termed sCAX1.

In this study, transgenic tobacco plants expressing deregulated Ca transporters are compared. The phenotypes of deregulated vacuolar localized CAX and the ER localized ACA2 are compared. These results suggest deregulation of ACA2 may provide an additional tool to utilize in altering the calcium accumulation in agriculturally important crops.

DEDICATION

This thesis is dedicated to my childhood and lifelong hero, Kathi L. Thompson.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Kendal D. Hirschi, Jean H. Gould, and Bhimanagouda S. Patil for their guidance and support throughout the course of this research. I thank my mentor Dr. Toshiro Shigaki for his steady direction and understanding. Additional thanks go to the faculty and staff of the Vegetable and Fruit Improvement Center at Texas A&M University for daily support.

I would like to thank Dr. Hirschi for the opportunity to contribute figures and figure legends to the publication entitled, Nutrient Biofortification of Crops.

Acknowledgement is given to Dr. Toshiro Shigaki for developing the DNA constructs for plant transformation. Ms. Nallely Sanchez conducted the screening of transgenic tobacco lines for ion sensitivity and assisted in gene expression analysis. A special thanks to my friend and senior lab colleague, Mr. Murli Manohar, for his daily feedback and support. I would also like to express my appreciation to Jonathan Aguilar for his assistance both in the lab and greenhouse.

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CHAPTER I

INTRODUCTION: NUTRIENT BIOFORTIFICATION OF CROPS*

Introduction

More than half of the world's population is deficient in calcium (Ca), iron (Fe), iodine (I), magnesium (Mg), selenium (Se) or zinc (Zn) (Graham et al., 2007; White and Broadley, 2009). The consumption of plants, directly or via livestock, containing inadequate concentrations of particular minerals causes these deficiencies. Agronomic and genetic strategies can increase the delivery of bioavailable minerals (Figure 1.1) (White and Broadley, 2009; Morris *et al.*, 2008). Although the focus is predominately on Ca, the framework discussed here should be generally applicable to boosting the levels of other elements in agriculturally important crops.

A recent panel of the world's foremost economists deemed plant biofortification, the process of increasing the bioavailable concentration of an element in foods, as one of the preeminent global challenges (www.copenhagenconsensus.com). Furthermore, the economists predicted tremendous benefits compared to costs associated with developing this technology. Indeed, genome projects are providing novel approaches for identifying plant genes of nutritional importance.

This thesis follows the style of Journal of Experimental Botany.

The term "nutritional genomics" has been coined to describe this work at the interface of plant genomics and human nutrition (DellaPenna, 1999; Mayer et al., 2008; Zhu et al., 2007; Zhu and Shimamoto, 2007). However, work done to measure the nutrient value of these engineered plant foods to date is minimal (Adams et al., 2002; Hambidge et al., 2005; Hirschi, 2008; Mendoza et al., 1998).

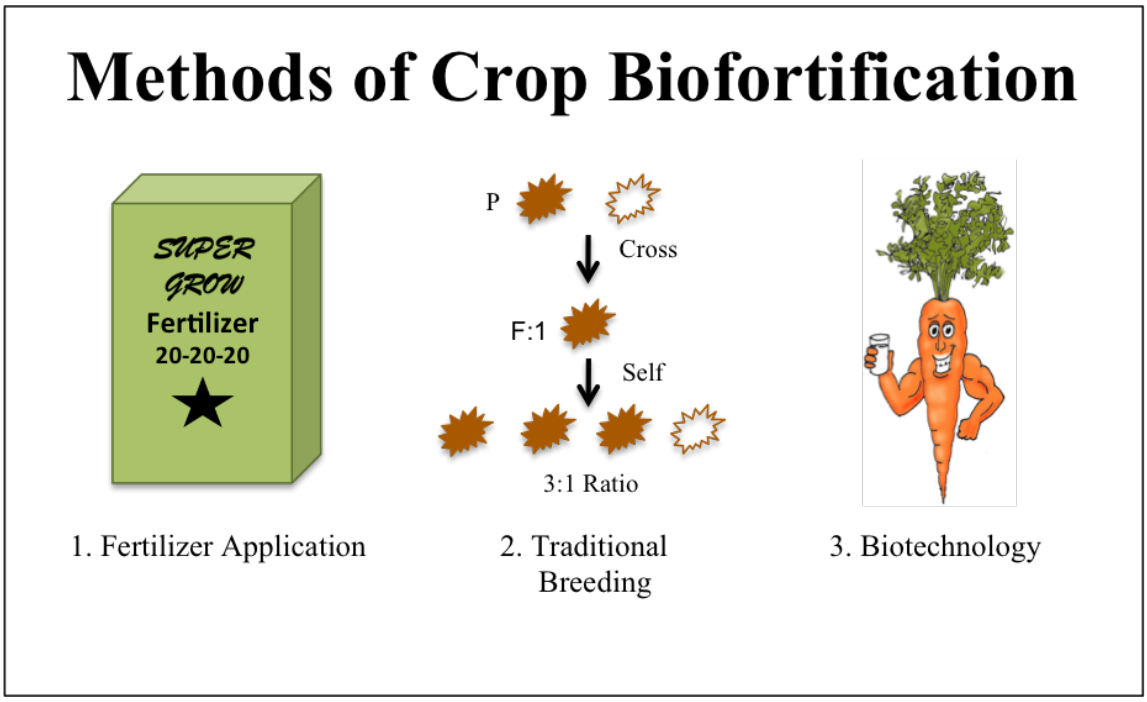

Figure 1.1 Methods of developing nutrient dense crops. (1) Application of fertilizers during plant growth can be used to increase specific nutrients into crops. (Woods et al.) Traditional breeding is an effective and proven approach to increasing specific nutrients in closely related crops. (3) Biotechnology can be used to increase the nutrient content of crops by expressing genes from unrelated organisms.

Genetically Modified Foods: Farmers and Consumers

The debate over genetically modified foods is being decided on the ground. Global demands for both food and fuel have sold farmers on transgenic technologies. In America, despite some consumer concerns, our nation eats more genetically modified foods than any other country. The possibilities associated with transgenic approaches keep plant biologists and production agriculture optimistic despite the current political and economic landscape that is not completely receptive to this technology (Freese and Schubert, 2004; ISLI, 2008; Johnson et al., 2007; Powell, 2007; Weil, 2005). Even with these current limitations, the potential for genetic modifications to alleviate hunger and nutrient deficiencies warrants advocacy of this technology among both scientists and citizens. Furthermore, despite the hurdles, if you have been to the grocery store lately, odds are you have eaten genetically modified foods.

The integration of genetically modified foods into modern agriculture and our current food supply can be exemplified by the work of the St. Louis based biotechnology company Monsanto (Hindo, 2007). Although humans do not directly consume the majority of Monsanto crops, there are plenty of ways for people to ingest them indirectly. For example, the majority of Monsanto's genetically modified corn is used to make animal feed and ethanol but a portion goes into the food supply as corn syrup and cornstarch, as well as helping to make some corn tortilla chips. Also, the bulk of the biotech cotton feeds the textile industry. However, cotton-by products do end up in the food chain as cottonseed oil, which can be used to make mayonnaise and margarine. Another illuminating example is papaya. An estimated 80% of the papayas from Hawaii

are genetically engineered. This technology is publically licensed and sold to farmers through a not-for-profit group. Although these are just a few examples, in the U.S. about 70% of all "formulated foods"(processed food with more than one ingredient) contain GMO's (Figure 1.2).

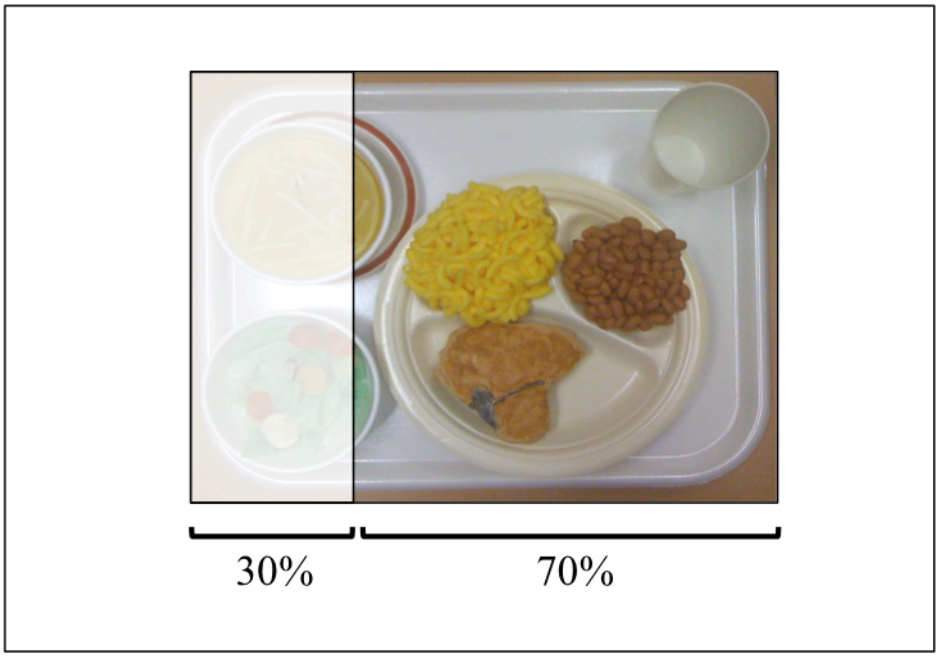

Figure 1.2 A biotechnologist's view of a school lunch. Genetically modified ingredients have been introduced into nearly 70 percent of processed foods available in the United States; including snacks, cereals, and hot dogs (Bren, 2003).

The work outlined here may promote positive viewpoints among consumers regarding genetically modified foods. Our studies detailed below addressing the bioavailability of Ca were well received by the media, including news articles by the BBC and NPR (press articles can be found at

http://www.bcm.edu/cnrc/faculty/kendalh.htm). An article in a London Telegraph (http://www.telegraph.co.uk/scienceandtechnology/science/sciencenews/3321694/GM-carrot-may-help-treat-osteoporosis.html) talking about our work contained the subheading: "Europe 'will be forced to re-think on GM crops'".

Biofortification and Nutritional Studies

Biofortified foods offer a potentially powerful intervention tool that targets the most vulnerable people (resource-poor women, infants and children). However, few studies have measured the most important parameter to determine the eventual successes of conventionally bred foods or genetically modified lines; namely, are these foods actually functional foods (Powell, 2007).

The most notable example of this gap between the technology of the transgenic plants and measuring nutritional efficacy is the case of "Golden Rice", engineered to produce beta-carotene in the edible portion of the grain (Adams et al., 2008). Seven plus years after the introduction of "Golden Rice", scientists are still pondering its nutritional benefits; however, some recent preliminary findings are encouraging (Krawinkel, 2007; Tang et al., 2009). While the first human bioavailability studies have just been completed, we are left to consider the timing of these events (Figure 1.3). From one vantage point, it was prudent to wait on the bioavailability studies, since the initially engineered versions of "Golden Rice" did, in fact, have very little beta-carotene (Nestle, 2001). On the other hand, how can so much time and effort be invested in this technology without performing fundamental studies regarding nutritional benefits?

Unfortunately, the lack of nutritional assessment in biofortified foods is the norm rather than the exception. Different carotenoid-enriched foods (DellaPenna, 2007) as well as crops enriched with other micronutrients such as vitamin E (Ajjawi and Shintani, 2004) and folate (Bekaert et al., 2008) are further examples where the genetically modified foods have not been adequately assessed at the nutritional level.

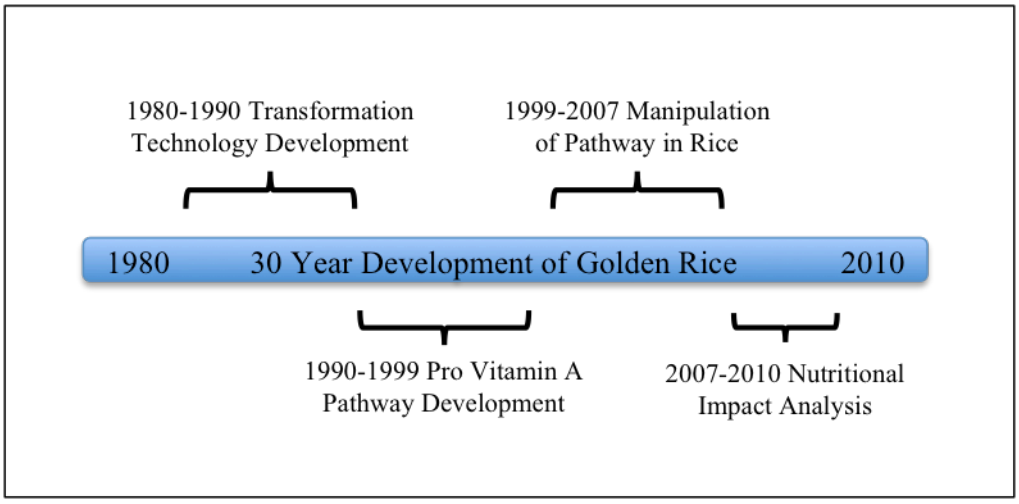

Figure 1.3 Vitamin A fortification of "Golden Rice" timeline. Technological developments in the 1980s allowed for different genes to be expressed in rice- a process termed transformation. The carotenoid pathway was characterized and the genes identified during the 1990s. The engineering of different genes responsible for carotenoid biosynthesis in rice was optimized by 2007. In the last several years' nutritional studies have been initiated to assay the effectiveness of the food (Tang et al., 2009). The ecological impact of "Golden Rice" is currently under study.

A notable exception to the lack of nutritional studies is the work manipulating phytic acid content in crops (Adams et al., 2002; Hambidge et al., 2005; Raboy, 2001). As detailed below, phytic acid is the storage form of phosphorus in seeds. Human feeding studies were done that compared the absorption of Ca from tortilla meals prepared from low-phytate maize with that from meals prepared from maize with typical phytate content. Mean fractional absorption of Ca from tortillas prepared from the low-phytate maize was significantly greater than that from tortillas prepared from the control maize. This type of approach serves as a paradigm for the integration of plant biology and human nutritional studies.

Targets for Ca Biofortification

Popular vegetables among American consumers, namely potatoes, carrots and lettuce, are excellent targets for enhancing Ca content (Table 1.1, Figure 1.4). On average, Americans consume only 3.3 servings of vegetables a day (www.fruitandveggiesmatter.gov/; http:apps.nccd.cdc.gov/5ADaySurveillance/). Currently, less than 25% of the US population reaches the adequate daily consumption of five daily portions of fruits and vegetables. These statistics argue for the development of strategies to increase the levels of health promoting compounds, such as Ca in the vegetables that people consume in substantial amounts.

The dark green vegetables (broccoli, bok choy, collard greens, kale, etc.), which may be high in dietary Ca, represent only 0.2 of these daily servings. The number one vegetable eaten by Americans is the potato followed by iceberg lettuce. In terms of Ca

content, these vegetables are not as desirable as some of the dark leafy greens (Table 1.1). However, rather then trying to alter consumer preferences, our work here will enhance the Ca content of vegetables that are already popular among consumers.

Figure 1.4 Targets for Ca biofortification. (A) Potatoes, (B) carrots and (C) lettuce are targets for Ca biofortification because they are currently low in Ca content and are popular among American consumers.

Potatoes

Traditionally potatoes have been grown and consumed in Europe and North America. Recently, there has been a dramatic increase in potato production and demand in Asia, Africa and Latin America. In fact, Asia now consumes almost half of the world's potato supply, but its huge population means that consumption per person is approximately 25kg. The heartiest potato eaters remain Europeans (85kg) and North Americans (60 kg). Per capita consumption is lowest, but increasing, in Africa and Latin America.

Potatoes are eaten all over the world in an assortment of different dishes. Potatoes are high in carbohydrate content (19%), mostly in the form of starch, that

makes it such a good source of energy, but the potato also provides a fair amount of protein (2%). In addition, a medium-sized potato can provide about a third of the recommended daily allowance (RDA) of vitamin C, around a fifth of the RDA of vitamin B6 and about 30 percent of the RDA of Fe, as well as small amounts of thiamin, riboflavin, folate, niacin, Mg, phosphorus, and Zn. An average sized baked potato (including skin) contains approximately 26 mg of Ca and is not a good source of Ca (Table 1.1).

Table 1.1 Comparison of absorbable dietary Ca in traditional and fortified foods. Ca content and bioavailability varies among foods. Fortified vegetables have the potential to positively impact consumers whose current diet is deficient in Ca (Titchenal and Dobbs, 2007). * Potential change in Ca absorption if Ca oxalate were removed from spinach.

Food	Serving Volume	Serving Weight (g)	Ca Content (mg) / Serving	Ca Fractional Absorption (%)	Ca Absorbed (mg) / Serving
Milk	1 cup	245	285	32	91
Kale	1 cup	118.5	47	49	23
Spinach	1 cup	118.5	244	5	12
Spinach (-Ca OX)	1 cup	118.5	244	59*	144*
Carrots	1 cup	128	42	53	22.26
Ca Fortified Carrots	1 cup	128	420	26	109.2
Lettuce	1 cup	72	13	49	6.37
Ca Fortified Lettuce	1 cup	72	130	25	32.5
Potato	1 med	173	26	22	5.72
Ca Fortified Potato	1 med	173	260	11	28.6

Carrots

Carrots are one of the most popular vegetables in the United States (Simon and Goldman, 2007). Although consumption varies by ethnicity, age, and income, the average American eats 4.98 kg of carrots per year. Carrots can be eaten in a variety of ways and raw carrots have become a popular ready-to-eat snack food. Since the 1980s, baby carrots (carrots that have been peeled and cut into uniform cylinders) have transformed the way people eat carrots. Carrots are an excellent source of beta-carotene or pro vitamin A, which can be converted by the human body into an active form. In addition, they are a very good source of vitamin C, vitamin K, dietary fiber and potassium. Ca content in carrots is approximately 42 mg/cup and is currently a fair source of Ca (Table 1.1).

Lettuce

Lettuce is an attractive dietary option for enhancing consumption of dietary Ca. Like carrots, lettuce can be eaten raw (http://www.foodreference.com/html/artlettuce.html). Per capita consumption of all lettuce varieties has been increasing since 1960. In 2004 total lettuce consumption reached a record high of 15.65 kg per capita. Some lettuces (especially iceberg) have been specifically bred to remove the bitterness from their leaves. These lettuces have a high water content with very little nutrient value. However, lettuce is rich in vitamin K (as much as 167 µg/head but currently low in Ca (13 mg/1 cup shredded) and is not a good source of Ca (Table 1.1).

In our studies modified potatoes, carrots and lettuce are being used to translate knowledge from model systems to crops that can benefit consumers. Alternative vegetables that we have considered modifying (after discussions with dieticians) include tomatoes and corn; however, these plants have proven recalcitrant to the approaches used in our studies (Park et al., 2005). Preliminary studies suggest Ca biofortification efforts using potatoes; carrots and lettuce should concentrate on fertilizer applications and genetic engineering approaches. There is limited genetic variation among potatoes, carrot and lettuce varieties in terms of Ca content but these plants can be easily transformed to facilitate genetic engineering approaches. Additionally, we have working relationships in place with carrot and potato breeders at Texas A&M University (Park et al., 2004a). These advantages have already allowed design and implementation of an experimental pipeline to genetically alter these crops and measure bioavailability (discussed later); (Morris et al., 2008). The inability to transform many crops, such as spinach, makes these plants recalcitrant to our experimental approaches.

Antinutrients

Ca present in plant foods typically exists as a complex with oxalate, phytate, fiber, lactate, fatty acid, protein, and other anions (Mendoza *et al.*, 1998; Linder, 1991; Wilson and Clifford, 1990). Many plants are particularly high in total Ca, but it is often sequestered by an antinutrient. Antinutrients, although not toxic, are plant compounds that decrease the nutritional value of a food, usually by making an essential nutrient unavailable or indigestible.

Phytic Acid

Grains and legumes are foods often rich in phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate), the storage form of phosphorus in seeds. This compound binds to mineral nutrients such as I and Zn, forming salts that are excreted. This can contribute to mineral depletion and deficiency. To solve this problem, the U. S. Department of Agriculture and others have isolated cereal and legume low-phytic acid mutations and have used these to breed low-phytate hybrids, cultivars and lines of maize (*Zea mays*), barley (*Hordeum vulgare*), rice (*Oryza sativa*) and soybean (*Glycine max*). Seed phytic acid is reduced in these crops by 50–95%.

A maize mutant in the synthesis of phytic acid during seed maturation has been used to study the consequences of the lack of this important reserve substance on seed survival. Data on germination, free Fe levels, free radical relative abundance, protein carbonylation level, damage to DNA, and other parameters were recorded on seeds of maize and of an isogenic low phytic acid mutant (lpa1-241), either unaged or incubated for 7 days in accelerated ageing conditions (46 °C and 100% relative humidity). The lpa1-241 mutant, compared to the control showed a lower germination capacity, which decreased further after accelerated aging. Whole lpa1-241 mutant kernels contained about 50% more free or weakly bound Fe than control ones and showed a higher content of free radicals, mainly concentrated in embryos. These findings suggest antioxidant activity for phytic acid through Fe complexation. Therefore, a role in plant seed development can be assigned to phytic acid, that is, protection against oxidative stress

during maturation. These studies suggest that removal of phytic acid may have negative consequences for plant development.

Oxalate

Oxalate is also an 'antinutrient'; it sequesters Ca in a state that may render it unavailable for nutritional absorption (Figure 1.5); (Weaver et al., 1987). In support of this, Ca absorption appears to be inversely proportional to the oxalic acid content in food (Table 1.1); (Weaver et al., 1987). Whilst spinach contains between 23.8 and 26.7 mg/g Ca, oxalate content is high and drastically reduces Ca bioavailability; however, kale which contains between 26.3 and 27.6 mg/g Ca has low oxalate levels and much higher bioavailable concentrations of Ca. (Weaver et al., 1987); a notable exception to this correlation is soybeans, where oxalate levels are high (35mg/g) (Massey et al., 2001); but bioavailable Ca is also high (total Ca 27.7 mg/g) (Massey et al., 2001).

Previous studies have strongly suggested that Ca when sequestered in the form of Ca oxalate is unavailable for nutritional absorption (Franceschi and Nakata, 2005; Heaney et al., 1988; Weaver, 1990). These studies lacked precision because the genetic mechanisms underlying the inherent differences in the plants were too numerous to accurately determine the antinutrient(s).

In order to clarify these nutritional studies, comparisons were made in the Ca absorption from isogenic lines of a plant that differed in a single gene, which mediates oxalate crystal content (Morris *et al.*, 2007a; Nakata and McConn, 2000a). Using genetic analysis and mice fed labeled diets; this experimental platform shows that plants lacking Ca oxalate crystals are better sources of bioavailable Ca (Figure 1.5). However, oxalate crystals are also an important factor in deterring insects from eating the plants (Korth et al., 2006). Recent work has shown that the size and shape of oxalate crystals are important factors in determining effects on insect growth (Korth et al., 2006). If manipulation of Ca oxalate is to be used in developing improved nutritional qualities and insect resistance in plants, then controlling not only the overall amount, but also the size and shape of crystals, could be valuable traits.

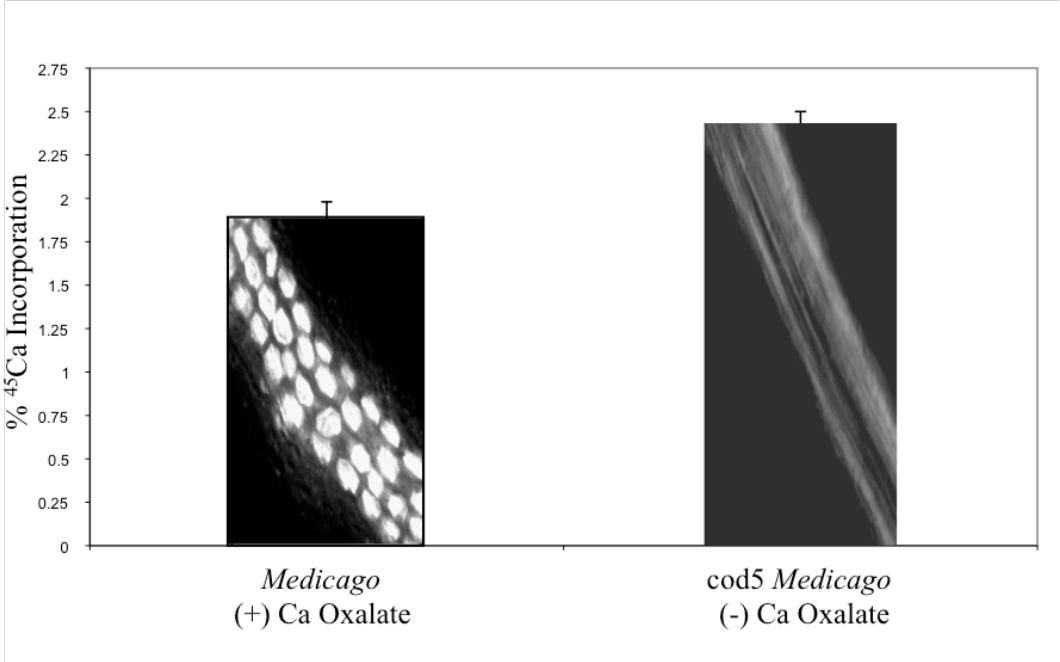

Figure 1.5 Ca oxalate crystals impair Ca absorption in mice feeding studies. A representative portion of a leaf is shown using partially polarized light. Spinach cleared of chlorophyll displays Ca oxalate crystals (bright crystal structures) that are not present in the kale. Mice given diets containing the cod5 mutant lacking Ca oxalate crystals (Nakata and McConn, 2000b) have a higher percent of Ca incorporation into bones (Morris *et al.*, 2007b).

Got Veggies? Enhancing Ca Content in Popular Vegetables

We will concentrate on the tools that we have developed and outline the approaches we used to combine these technologies to produce potatoes, carrots and lettuce that have high levels of dietary Ca.

Biofortification: Increasing Ca Transport Activity

Heightened activity of a plant Ca transporter doubles Ca content in potatoes, carrots and lettuce. In its simplest form, this strategy can be compared to nutrient mining; Ca is transported from the soil into the edible portions of plants. Specifically, one approach is to manipulate plant endomembrane transporters to increase Ca transport. In animal cells and in yeast, capactitative Ca entry (CCE) mechanisms are activated when vacuolar Ca transporters are highly expressed (Pittman and Hirschi, 2001). In plants, there is evidence to suggest that expression of a gene from an unrelated plant species may be able to bypass some of the endogenous regulatory elements in the recipient plant. This may produce higher activity of a given gene product (Diener and Hirschi, 2000). With this in mind, we have expressed an Arabidopsis Ca transporter (CAX) in lettuce, carrots and potatoes to increase Ca content (Figure 1.6). In each case, these modifications did not appear to alter the levels of antinutrients, perturb growth, development or fertility (Park et al., 2004a).

In the potatoes, carrots and lettuce, we have increased Ca content and verified this was caused by expression of a single copy of the CAX cassette (Morris et al., 2008). In carrots, genetic crosses verified this is a heritable trait. Using both biochemical

analysis and microscopic studies, we noted no alteration in oxalate levels, a potential antinutrient.

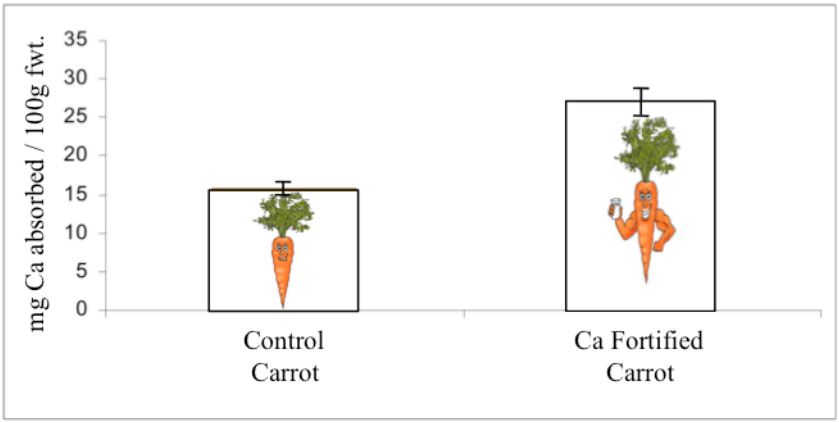

Figure 1.6 Ca absorption in human subjects fed control and Ca fortified carrots. Increase in total Ca absorbed by human subjects from control carrot lines and those fortified using biotechnology. Increased expression of a Ca transporter from Arabidopsis increases total bioavailable Ca. Increased Ca in these fortified carrots demonstrates the potential of this technology to address Ca deficiencies in today's diets.

Ca Absorption Measurements Using Mouse Feeding Studies

The use of radioisotopes in mice feeding studies is a rapid and economically feasible method of getting preliminary results regarding a net gain in total Ca absorbed from biofortified foods (Figure 1.7). The mouse has become the pre-eminent mammalian model animal because of the underlying biological similarity to humans, the

emerging genomic sequence data and the ability to manipulate the mouse genome in a targeted or random fashion (Nguyen and Xu, 2008). Some of the most elegant mice genetics has been directed at the study of bone development (Provot et al., 2008; Wagner and Karsenty, 2001). For example, studies using mice mutants elucidated the role of leptin as a potent inhibitor of bone formation. Long-term nutrition studies that are directed at understanding gene for gene interactions between the food and the 'consumer' must utilize malleable genetic systems on both ends of the equation. For example, if a plant is expressing high levels of a Ca transporter, in the near future it will be interesting to observe how mice defective in Ca bone deposition, respond to diets containing genetically altered food.

We have modified protocols previously used in rat feeding studies for analyzing Ca bioavailability in mice (Morris *et al.*, 2007b). Our data on Ca absorption with $CaCl_2$, CaC_2O_4 and spinach (both extrinsic and intrinsically labeled) compare favorably to similar studies in rats and thus establish the equivalence between the two in vivo models (Weaver et al., 1987). For example, in the published rat studies, absorption values around 1.8% for both $CaCl_2$ and $CaCO_3$ diets and only 0.2% from CaC_2O_4. In our work, we showed absorption of 45Ca from $CaCl_2$ and $CaCO_3$ diets were 1.21% and 1.17%, compared to only 0.07% from CaC_2O_4. While the precise absorption values are not identical to those in rats (Weaver et al., 1987), the findings from both studies suggest that oxalic acid binds Ca, rendering it unavailable for absorption.

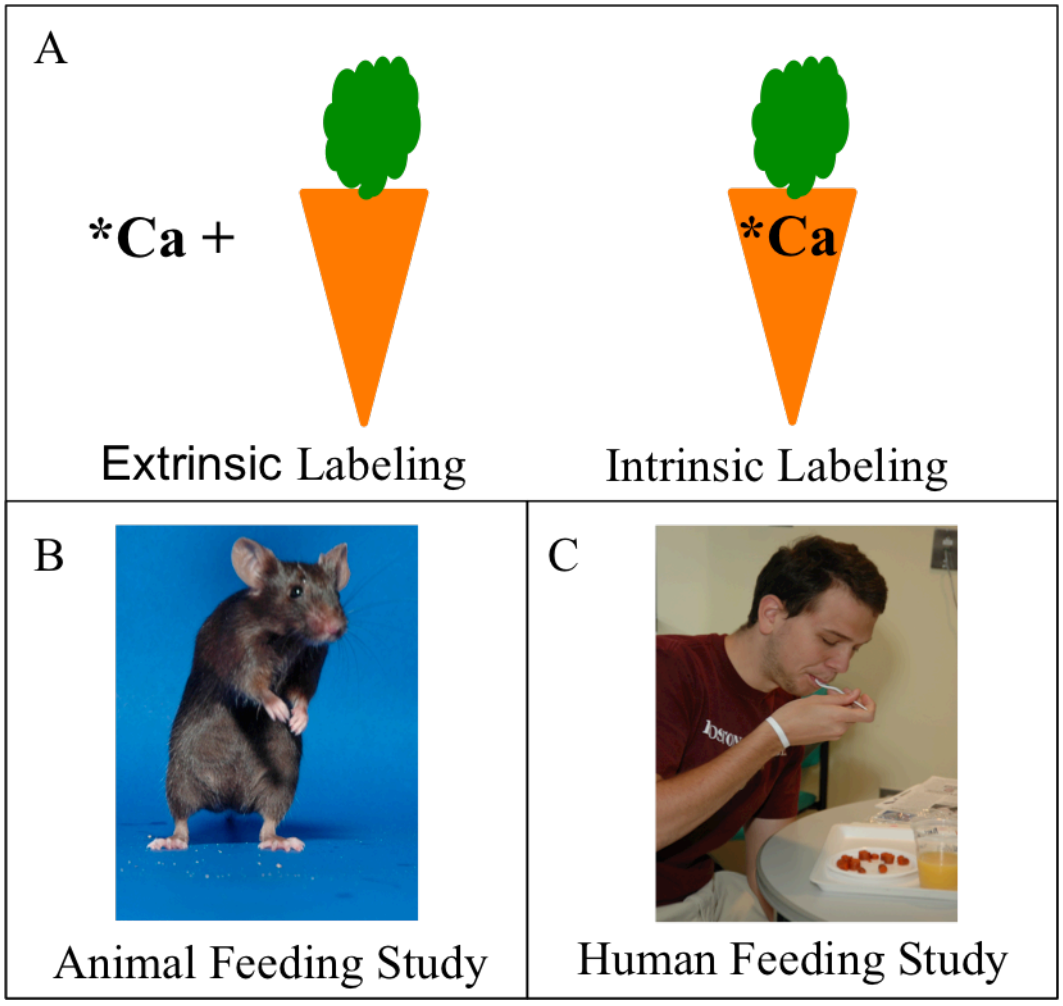

Figure 1.7 Design for testing bioavailability in biofortified foods. (A) Extrinsic and Intrinsic labeling of food. Extrinsic labeling adds the tracer (*) to the food whereas intrinsic labeling incorporates the label into the plant matrix. (B) Initially, modified plants are labeled with inexpensive radioactive tracers and fed to animals. (C) Human feeding studies using safe, but expensive, stable isotopes are used if the animal work shows promise (Hirschi, 2008).

As mentioned in the introduction, modifying plants to reduce the concentration of antinutrients have been shown to increase bioavailable mineral content (Adams *et al.*, 2002; Hambidge *et al.*, 2005; Mendoza *et al.*, 1998; Morris *et al.*, 2007a). This lab has also conducted studies concerning the role of Ca oxalate crystals in *Medicago truncatula* variants fed to mice (Morris *et al.*, 2007b). *M. truncatula* is the model plant species representing the legumes , due to its small diploid genome, self-fertility, rapid generation time, prolific seed production, and is it is amenable to genetic transformation. The Ca oxalate deficient (cod) mutant, cod5, of *M. truncatula* contains identical Ca concentrations to wild-type, but scanning electron microscopy showed that the mutants contained no oxalate crystals (Morris *et al.*, 2007b). For our feeding studies, diets using wild type *M. truncatula* (oxalate crystals) were compared to the cod5 oxalate crystal mutant based diets. We fed equal numbers of male and female mice four Ca-labeled diets: *M. truncatula* extrinsically or intrinsically labeled, and cod5 extrinsically or intrinsically labeled by radioisotope 45Ca (*Ca). Extrinsic labeling adds the tracer to the food before ingestion, whereas for intrinsic labeling plants are grown in the presence of the label to incorporate the tracer into the plant matrix (Figure 1.7). Using both diets, absorption of the tracer was determined one day after consumption. In the intrinsically labeled diets, Ca absorption was 22.87% ($p < 0.001$) higher in mice fed cod5 (Morris *et al.*, 2007b). We postulate from our findings that during the formation of crystals in Medicago, much like oxalate crystals in spinach, Ca is bound in a non-bioavailable form. To our knowledge this study presents the first genetic evidence to demonstrate the

nutritional impact of removing oxalate crystals from food. This work also demonstrates the usefulness of the mouse model for Ca absorption studies.

Ca Absorption in Humans

Ultimate demonstration of our hypothesis regarding nutritional benefits to alterations in Ca content requires human studies. The use of stable isotopes makes this feasible and safe as a method for determining a net gain in total Ca absorbed.

Although animal models provide evidence related to bioavailability, there are fundamental differences in the mechanism of Ca absorption between humans and small animals. In particular, humans utilize a greater proportion of Ca absorption in the upper small intestine than small animals (Abrams, 2003). Recently, we have analyzed the bioavailability of the Ca from modified carrots in a human study (Morris et al., 2008). We chose young adults who were healthy and represent a typical population that might utilize vegetable sources to obtain a substantial amount of their Ca intake. In addition, unlike studies done with children, we could more easily obtain their informed consent.

Through both mouse and human feeding studies we have demonstrated that sCAX1-expressing carrots have increased Ca bioavailability (Figure 1.8). Although there is a 10% reduction in absorbed Ca from the sCAX1-expressing carrots; the total concentration of Ca absorbed from the sCAX1-expressing carrots is 42 ± 2% higher compared to an equal quantity of control carrots. In the human feeding trials, our working hypothesis to explain the differences between controls and sCAX1 lines, is that not all the Ca sequestered in the vacuole by ectopic expression of sCAX1 is bioavailable,

it may be conjugated to phytates, phosphates or other antinutrients within the edible carrot.

To the best of our knowledge, our findings represent the first report to directly evaluate the nutritional consequence of transgenic foods in both animal and human feeding studies. We establish unequivocally that modifying a single plant Ca transporter improves plant Ca bioavailability. Additionally, we can now use this approach to further boost Ca levels in a variety of different foods.

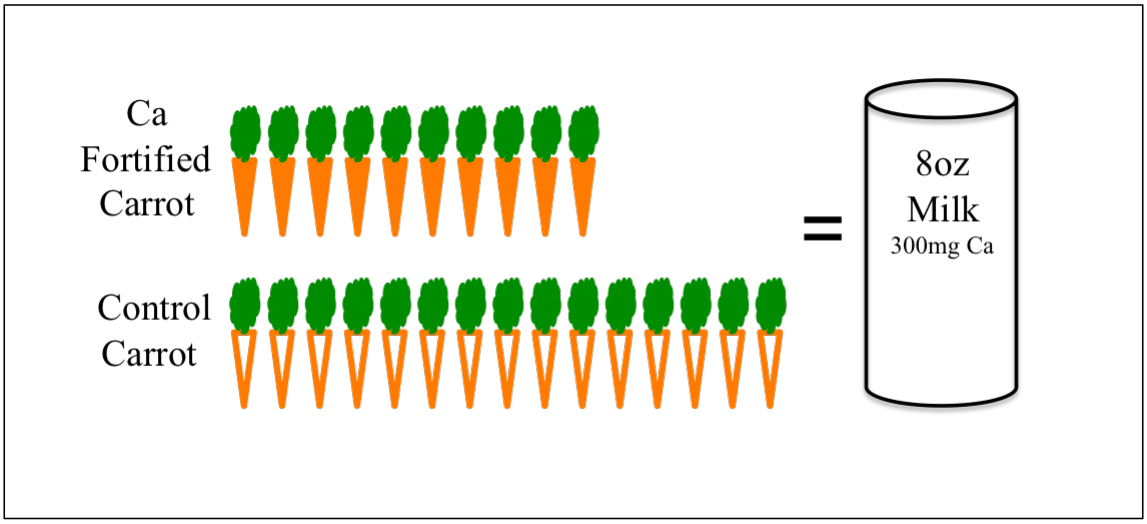

Figure 1.8 Serving sizes of carrots, Ca fortified carrots, and milk required to obtain 300 mg of Ca. One 8oz serving (240mL) of milk is comparable to 10 (650g) Ca fortified carrots or 15 (1000g) control carrots (Hawthorne et al., 2009).

Impact of This Research

Single servings of our biofortified vegetables could significant contribute to dietary Ca intake. These improved vegetables could be widely consumed, making them a substantial component of the Ca intake of Americans. Historically nutritional problems have centered on the inadequate intake of certain vitamins and minerals resulting in nutritional deficiencies (Frazao, 1999). Currently, nutritional problems in America are driven by the discovery of strong links between nutrition and chronic diseases.

A majority of Americans are interested in improving their diets. Evidence suggests that many are changing their diets and attempting to move closer to established dietary recommendations (Hornick et al., 2008). However, the direction and magnitude of these changes vary considerably, both among individuals and among food groups. For example, survey data suggest a trend toward lower fat diets. However, the same data show that individuals are not increasing their consumption of fruits and vegetables as recommended, and that the prevalence of obesity is rising (CDC, 2008; Wareham et al., 2005).

Government assistance can also have some influence on consumer dietary choices (Ralston et al., 2008). Food assistance programs can affect the amount and the types of foods consumed by low-income populations. However, the long-term changes on the consumer diet, especially after leaving the program, have so far been uncertain. Certainly, greater food expenditure does not necessarily imply a more healthful diet. For the more narrowly targeted programs, such as the School Lunch Program, nutrient intake is typically increased (at least while the recipient remains in the program). For example,

children in schools with restricted snack availability had significantly higher frequency of fruit and vegetable consumption than children in schools without restricted snack availability. These recent findings suggest that a restrictive snack policy should be part of a multi-faceted approach to improve children's diet quality (Gonzalez et al., 2009).

Here we are seeking to provide substantial benefit to the public at no inconvenience and minimal cost to the consumer. This passive method does not require consumer knowledge, understanding, or commitment to change food consumption behavior.

Increasing the dietary Ca levels five-fold in potatoes; lettuce and carrots could have a significant affect on total Ca consumption in the United States without requiring altering dietary habits. The U.S. Dietary Reference Intake (DRI) for Ca is linked to a person's age and stage of life, so among adolescents these vegetables contribute even less to their daily Ca requirements. The DRI for Ca for 19 to 30 year old adults is an adequate intake (AI) level of 1000mg/day. If Americans continue to eat 60 kg of potatoes a year (approximately 150 medium size potatoes) they would receive a net benefit of approximately 3,432 mg of absorbed Ca from these alterations (4290 mg in the biofortified potatoes vs. 858 mg in the varieties found today (Table 1.1). For lettuce, if Americans continue to eat 14.4 kg of lettuce per year (approximately 200 servings of 1 shredded cup) the net gain in Ca would be 5,226 mg from this food (6,500 mg in the biofortified vs. 1,274 in the standard variety (Table 1.1). For carrots, if Americans consume 4.989 kg of carrots per year (39 servings of chopped carrots) that would increase Ca consumption by 3390 mg (4258 mg from the biofortified carrots compared

with the 868 mg from standard carrots (Table 1.1). These back-of-the-envelope calculations and predictions assume optimal preparation of the vegetables to ensure Ca bioavailability. While bearing in mind these caveats, these Ca biofortified vegetables could contribute approximately 3-5% of the DRI of Ca for a wide array of Americans.

Future Studies

The ability to substantially increase bioavailability of Ca in vegetables through a combination of biofortification efforts will be pursued. Vegetables, namely potatoes, lettuce and carrots, containing single gene alterations in Ca transport activity will be grown in hydroponic conditions to accumulate significantly higher levels (>10X) of Ca. The benefit associated with increasing nutrient bioavailability in foods already regularly consumed is in the potential to impact those most in need. Increasing Ca content in vegetables provides potential increased calcium in the diet without changing the diet.

We anticipate that in pilot mice feeding studies, the biofortified vegetables will have at least five-fold more bioavailable Ca than the respective control vegetables. In humans, we also assume that total Ca absorption from a serving of biofortified vegetables will be increased at least five-fold compared to controls. In sensory analysis tests, the biofortified vegetables should be equivalent in taste and texture to the non-biofortified vegetables. We have developed nutritional readouts that can be used to quantify differences among plant-based diets that differ in the location of Ca within the plant matrices. This positions us to rapidly iterate between nutrient partitioning within

the plant matrices and bioavailability to devise strategies to further improve the nutritional status of a multitude of agriculturally important crops.

Closing Remarks

Changing metabolic functions may impact plant growth and productivity. For example, changes in metal content could alter various enzyme and protein functions. It is important to establish if a alteration in plant metabolism is cost effective. A useful plant improvement should increase nutrient content while keeping cultivation and production costs affordable.

The tests used to analyze genetically modified foods should mimic clinical trials with a novel pharmacological agent. With the novel foods, after nutritional efficacy of the food is proven comes the difficult task of determining the collateral effects. Interactions with nutrients in the plant matrices, allergic responses to the consumer and altering plant-stress responses are some of the measurements that need to be performed. As mentioned earlier, removal of the antinutrient Ca oxalate crystals causes these modified plants to be more nutritious but reduces the plant's defense to insect chewing (Korth et al., 2006). For consumer confidence, the most important thing the scientific community must do is be careful in our analysis of these foods before they become available to consumers.

Demand for any biofortified food must drive the product through developmental stages and to offset associated cost increases (Freese and Schubert, 2004; ISLI, 2008; Johnson et al., 2007; Powell, 2007; Weil, 2005). For this to occur, the health benefits

must be apparent to the consumer. The first steps in this process include nutritional studies in both animal and human feeding trials.

Breeding approaches can be used to enhance the nutritional qualities of foods (Bouis, 2000; Grusak and Cakmak, 2005). However, breeding alone will not be an adequate approach because of the limitations of particular plant species (Jeong and Guerinot, 2008). Breeding and molecular genetics were elegantly combined to characterize an important wheat gene associated with grain protein, Zn and Fe content (Uauy et al., 2006). Wild durum (pasta) wheat has shorter grain maturation periods and higher protein, zinc and iron contents than domesticated wheats. The version of the gene found in a wild ancestor of durum wheat was isolated, sequenced and compared to the version in modern domesticated wheats. The domesticated wheats have an inactive form of the gene thus explaining their lower nutrient content relative to the wild durum wheat. The active version can now be incorporated by breeding or genetic engineering to increase the protein, zinc and iron contents of domesticated wheats. This is likely an example of the optimal experimental approach in biofortification- where research is directed primarily at breeding and genetic modifications will occur only when necessary.

CHAPTER II

PHENOTYPE ANALYSIS OF TOBACCO LINES EXPRESSING A DEREGULATED ARABIDOPSIS CA-ATPASE (ACA2)

Introduction

Plants, like all organisms, rely on calcium (Ca) as a regulator of growth, development, and cell function (Dayod *et al.*, 2010). Additionally, Ca contributes as a scaffold to tissue structure (De Roeck et al., 2010). For humans consuming primarily plant-based diets, inadequate Ca content in the foods can negatively impact growth and development (Zhu et al., 2008). Improving Ca content in foods could ameliorate dietary Ca deficiencies in at risk populations (Hirschi, 2009).

Altering transport of specific nutrients into the edible portions of plants is one strategy to increase their nutritional value and improve shelf life (Conn and Gilliham, 2010). Ca transport proteins located within different compartments of the plant cell have been identified (Hirschi *et al.*, 1996; Harper *et al.*, 1998). Some preliminary evidence suggests manipulation of these proteins can alter Ca levels in plants (Hirschi, 1999; Park et al., 2004b). However, there are limited examples of phenotypic studies analyzing how different transporters alter plant growth, development, stress tolerance, and Ca content.

The *Arabidopsis thaliana* Cation Exchanger 1 (CAX1) was the first vacuolar Ca / H+ antiporter to be identified in plants (Figure 2.1) (Hirschi et al., 1996). A *Saccharomyces cerevisiae* yeast strain lacking the high affinity Ca-ATPase and low affinity Ca/ H+ antiporter is sensitive to high Ca in the growth medium. CAX1 was isolated from *A. thaliana* by suppression of this yeast mutant (Hirschi et al., 1996). CAX1 is regulated via an N-terminal autoinhibitory domain; removal of this region is termed sCAX1 and is constitutively active in both yeast and plants (Pittman and Hirschi, 2001; Mei *et al.*, 2007). In tobacco expressing sCAX1, overall Ca content is increased (Hirschi, 1999). However, Ca deficiency symptoms such as tip burning become more prevalent as do stress sensitivities (Hirschi, 1999). We speculate the Ca deficiency symptoms are the result of Ca being sequestered within the vacuole, making it inaccessible for cellular function. Although deregulated CAX works to increase total Ca, the collateral damage caused by sCAX1 expression in some plants requires that other tools be developed to alter plant Ca content.

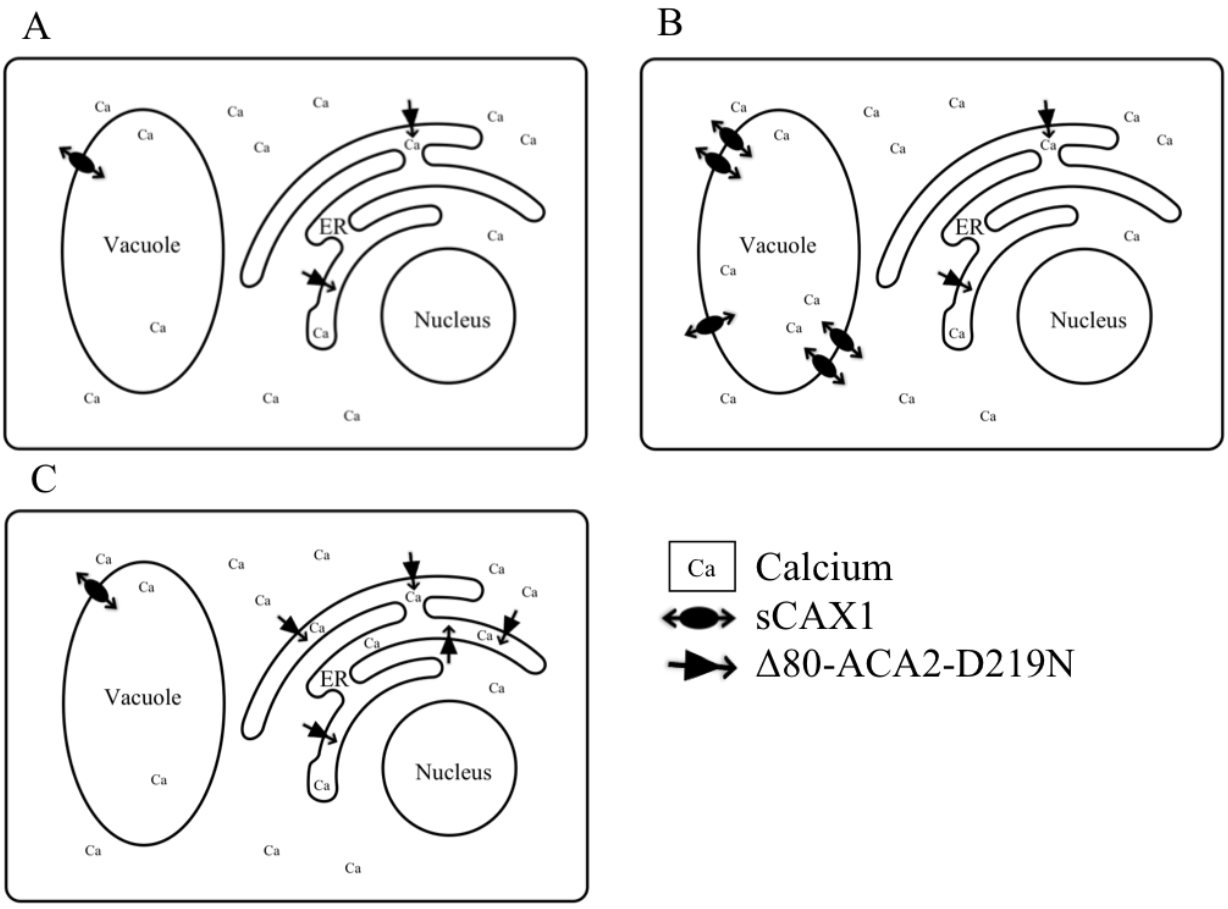

Figure 2.1 Potential ways of manipulating calcium content in a normal cell. (A) The vacuole, endoplasmic reticulum (ER), nucleus and calcium are depicted. Although many transporters and binding proteins are present in the cell only those engineered in this study are shown. (B) The vacuole is shown with increased expression of the calcium antiporter sCAX1. (C) The ER is depicted with increased expression of the Ca-ATPase Δ80-ACA2-D219N.

The endoplasmic reticulum (ER) localized *Arabidopsis thaliana* Ca-ATPase ACA2, also regulates Ca in plants (Figure 2.1) (Harper et al., 1998). Removal of the N-terminus, which contains a calmodulin (CaM) binding domain, termed (Δ80-ACA2), constitutively activates the transporter in yeast making it 10-fold more active than ACA2 (Figure 2.2) (Hwang et al., 2000). A point mutation of ACA2 also affects the stalk connecting the ATPase catalytic and transmembrane domain (Figure 2.2) (Curran et al., 2000). The mutation also produces a CaM independent hyperactive pump ACA2-D219N (Curran et al., 2000) but this mutation does not appear to be as active in yeast (Curran et al., 2000). Coupling the point mutation in the ACA2 gene with removal of 80 amino acids from the N-terminal region (Δ80-ACA2-D219N) yields ACA2 calmodulin independent and is most active in yeast (Figure 2.2) (Hwang et al., 2000). However, expression of these ACA2 variants in plants and the impact of these deregulated transporter on plant growth, stress sensitivities and Ca content has not been analyzed.

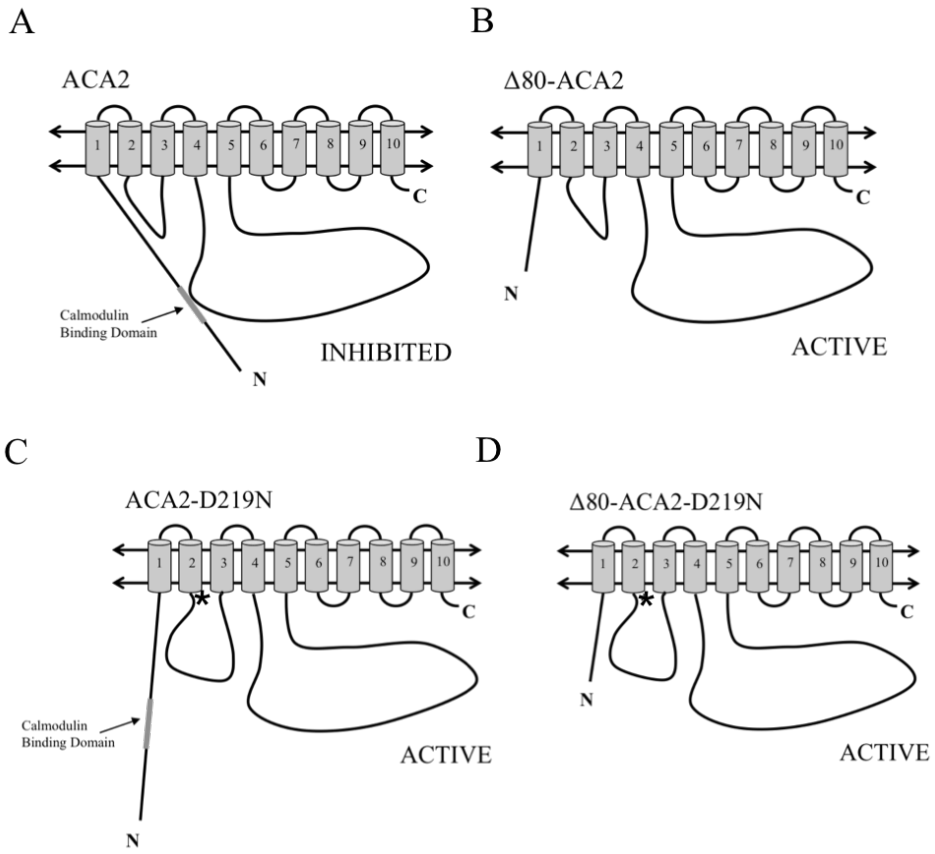

Figure 2.2 ACA2 model of regulation by N-terminus and point mutation. (A) ACA2 is inhibited by an interaction of an N-terminal region with the hydrophilic loop. The binding of calmodulin results in a conformational change blocking the autoinhibitory domain from its interaction with the hydrophilic loop, thus activating the pump. (B) An N-terminal truncated pump (Δ80-ACA2) is therefore constitutively active and unresponsive to calmodulin. (C) Single point mutation (*) (D219N) produces ACA2-D219N a change in the hydrophilic loop activating the pump. (D) Deregulation by both truncation and point mutation yields Δ80-ACA2-D219N. N and C refer to the N-terminal amino and carboxyl termini of the transporter.

In this study transgenic tobacco plants expressing deregulated Ca transporters are compared. The phenotypes of deregulated vacuolar localized CAX and the ER localized ACA2 are compared. These results suggest deregulation of ACA2 may provide an additional tool to utilize in altering the calcium accumulation in agriculturally important crops.

Materials and Methods

Plasmids, DNA Constructs and Plant Transformation

The coding regions of ACA2-D219N and Δ80-ACA2-D219N were amplified by PCR to add *Sfi*I restriction sites (underlined) at both ends, using the primers ACA2-F (5' -GAATTCGGCCAAATCGGCCATGGAGAGTTACCTAAACGAG- 3'), Δ80-ACA2-F (5' -GAATTCGGCCAAATCGGCC**ATG**AGTGACTACACTGTCCCTGAAG- 3'), and ACA2-R (5' -GAATTCGGCCCTTATGGCCTCAAACGGGAATCGTCTTCAG- 3'). To make the Δ80-ACA2-D219N construct, a start codon (bold) was incorporated on the Δ80-ACA2-F primer before the 81st codon of ACA2. The template for PCR was a plasmid containing an ACA2-D219N insert (obtained from Jeffrey Harper, University of Nevada). The amplified fragment was first cloned into a pCRII-TOPO PCR cloning vector (Invitrogen) and the sequence was verified for the absence of errors. Subsequently, the insert was sub-cloned into a modified pBI121 (Colnetech Laboratories, USA) binary vector containing *Sfi*I cloning sites, expression was driven by the 35S calflower mosaic virus (CaMV 35S) promoter. The recombinant plasmids and

vector control were transformed into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen, CA, USA).

Tobacco (*Nicotiana tabacum*, Cv. KY14) plants were transformed following published procedures (Tarczynski et al., 1992), along with the empty vector as a control. In brief, KY14 tobacco seeds were surface sterilized and germinated on ½ strength Murashige and Skoog media (MS). Leaf disk (5cm) were cut and precluded for 24 hours on ½ MS (Murashige and Skoog, 1962). Leaf disk were inoculated by dipping them in *Agrobacterium* culture containing the desired construct and blotted on Whatman filter paper. Following 24-hour, co cultivation, leaf disk were then transferred to MS media containing appropriate selection agents and supplemented with hormones to induce callus growth. Proliferating callus was transferred to MS media containing appropriate selection agents and shoot induction. Regenerated shoots were recovered and placed on rooting media containing appropriate selection agents. Following the formation of roots, the putative transgenic plants were transferred to soil and grown to seed in greenhouse conditions.

DNA and RNA Isolation: Southern and Northern Blot Analysis

For Southern analysis, tobacco genomic DNA was extracted from leaf tissue as previously described (Kang and Yang, 2004). DNA (5-10 µg) was digested with HindIII or EcoR1, fragments separated by electrophoresis and blotted onto Hybond N+ membrane (Amersham Biosciences, NJ, USA). For Northern analysis, total RNA was extracted from tobacco leaves as previously described (Hirschi, 1999). Total RNA

(10μg) was separated on a 1.5% agarose gel containing formaldehyde, as previously described (Mei et al., 2007). PCR reactions were performed to make probes for both Southern and Northern blot analysis, using ACA2-specific primer set. The sequences of the ACA2 primers were 5′- CGTGATTTGGATAAAGAGAAG -3′ and 5′- GCTTCATTAGCAAACTCGTTG -3′. Membranes were pre-hybridized at 65ºC in 7% (w/v) SDS and 0.25 M Na2HPO4, and then hybridized overnight at 65ºC in the same solution containing the probe labeled with 32P-dCTP using a random primed labeling kit (GE Healthcare, USA). Membranes were washed twice for 30 min each with 2X SSC and 1% SDS at 65ºC and then washed again for 30 min each with 0.1×SSC and 0.5% (w/v) SDS at 65ºC. Membranes were exposed to a phospho screen over night and imaged using a GE Storm Phospho Imager.

Plant Materials and Growth Conditions

Tobacco cultivar KY14 seeds were surface-sterilized, germinated, and grown on half-strength MS medium (Murashige and Skoog, 1962) containing 2% sucrose solidified with 0.8% agar. Petri dishes were sealed with 3M surgical tape and incubated at 25°C under cool-fluorescent illumination. For the ion sensitivity assays, 5-day-old vector control and transgenic seedlings grown under normal conditions were transferred onto half-strength MS medium and the identical medium supplemented with various metal ions as previously described (Hirschi, 1999). Greenhouse plants were grown in 3L pots with Metro Mix- 700 soil. Once a week the plants were watered with Miracle-Grow (Scotts Miracle-Gro Products) and once a month they were watered to saturation with

20mM calcium chloride. Greenhouse temperatures were maintained between 25°C and 40°C. Second generation plants were grown in the greenhouse under similar conditions.

Ion Sensitivity Analysis

Surface-sterilized tobacco seeds were plated on standard ½ MS media and germinated in a temperature-controlled incubator at 25°C with 16h illumination. First generation plants were screened on ½ MS media containing 100mg/L Kanamycin. Plants surviving selection were transferred to standard ½ MS media and ½ MS media supplemented with the appropriate ion after 14 days. Following 10 days of treatment, plants were compared and images were taken using a Nikon Cool Pix digital camera.

Results

CAX1 and ACA2 Variants Expressed in Transgenic Tobacco

We are interested in understanding the phenotype of tobacco lines expressing deregulated ER Ca-ATPases ACA2 and to compare and contrast these lines to previously characterized lines expressing the deregulated vacuolar Ca/H+ transporter sCAX1. Removal of the N-terminal 80 amino acids constitutively activates ACA2. Furthermore, mutation of cytosolic exposed residue D219 result in a de-regulated pump (Curran et al.). In this study, sCAX1, ACA2-D219N and Δ80-ACA2-D219N were expressed in tobacco cultivar KY14. Previously it has been shown that expression of sCAX1 in tobacco produces tip burning, cold sensitivity and altered stress responses

(Hirschi, 1999). Here, sCAX1, ACA2-D219N and Δ80-ACA2-D219N were expressed in tobacco cultivar KY14. Coding regions of ACA2-D219N and Δ80-ACA2-D219N were inserted into the PBI121 cloning vector under and transformed in to *agrobacterium* LBA4404. ACA2-D219N, Δ80-ACA2-D219N, and vector only constructs were incorporated in to KY14 by an *agrobacterium*-mediated transformation of leaf disk. Plants positive for selection were evaluated in this study. The transgenic sCAX1 tobacco line was obtained from previous studies (Hirschi, 1999).

Southern blot analysis was preformed to confirm transgenic tobacco lines did not contain multiple insertions Northern blot analysis was conducted on Independent transgenic tobacco lines harboring ACA2-D219N and 80-ACA2-D219N (Figure 2.3). These analyses suggested the expression of ACA2-D219N and Δ80-ACA2-D219N were comparable and that meaningful comparisons could be made between the ACA2 lines.

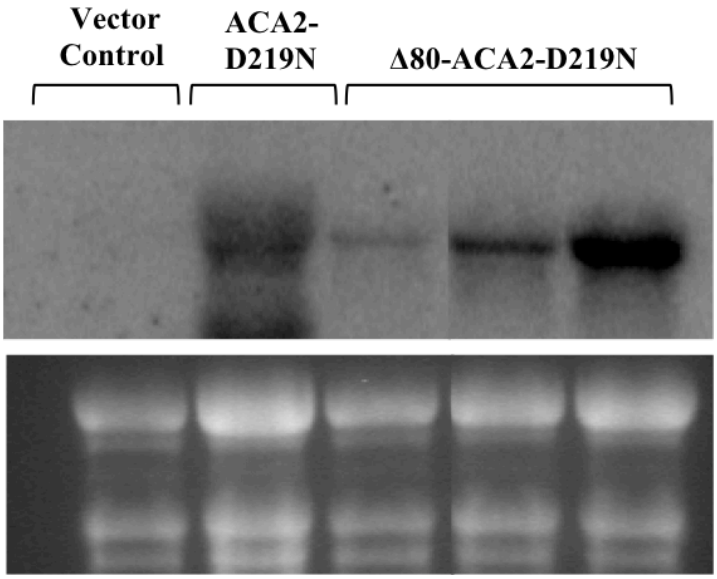

Figure 2.3 Northern blot analysis of ACA2-D219N and Δ80-ACA2-D219N in tobacco. ACA2-D219N, and Δ80-ACA2-D219N transcripts were detected by Northern blot analysis. Ten micrograms of total RNA from tobacco leaves were hybridized with the ACA2 cDNA probe. Ethidium bromide-stained rRNA bottom panel) is shown as a loading control.

Phenotypes of Tobacco Lines Expressing ACA2 Variants

Phenotypes were compared among tobacco lines expressing sCAX1, ACA2-D219N, and Δ80-ACA2-D219N. ACA2-D219N transgenic tobacco lines were indistinguishable from the vector control. However, the Δ80-ACA2-D219N transgenic lines displayed necrotic spots at different time intervals during growth and deployment of the plants. The necrotic lesions were most prevalent in young rapidly dividing

tissues. Over time the phenotype diminished in older leaves that were once similarly affected (Figure 2.4). Following transfer from culture to soil, all the sCAX1 tobacco plants expressed calcium deficiency phenotypes (tip burning and curling of the leafs) as described previously (Hirschi, 1999). Approximately half the putative transgenic Δ80-ACA2-D219N (12 of 22 independent lines) plants displayed the necrotic spots in their young leaves. The putative transgenic ACA2-D219N (10 total independent lines) tobacco plants were indistinguishable from vector controls. All tobacco lines were fertile and produced the T1 generation of seed. The phenotype of the T1 generation expressing ACA2-D219N was similar to the vector expressing controls. Surprisingly, the T1 generation of the Δ80-ACA2-D219N tobacco lines did not exhibit the necrotic phenotype, seen earlier in the T0 generation. Northern blot analysis suggested expression of the Δ80-ACA2-D219N, gene in the T1 and T0 generations were similar.

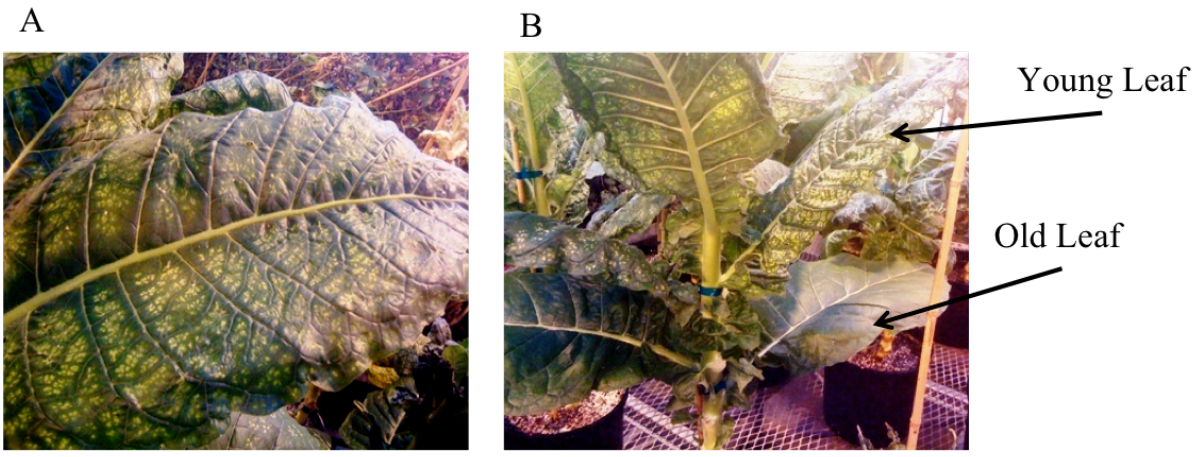

Figure 2.4 Phenotype of tobacco (T0) expressing Δ80-ACA2-D219N. (A) Transgenic T0 plants harboring Δ80-ACA2-D219N displayed necrotic spots in young leaves. (B) The necrotic phenotype seen in young leaves was not seen in the older leaves.

Ion Sensitivity of Transgenic Tobacco

Transgenic T1 tobacco seedlings expressing Δ80-ACA2-D219N, displayed an increased sensitivity to increased magnesium (Mg) on ion-supplemented media. However, the T1 ACA2-D219N seedlings did not display altered sensitivity, and were similar to the vector control plants. Ion sensitive phenotypes were described for sCAX1 in transgenic tobacco seedlings (Hirschi, 1999). Previously, sCAX1 transgenic tobacco seedlings display increased sensitivity to magnesium (Mg) and sodium (Na) as well as low Ca (Hirschi, 1999). T1 Δ80-ACA2-D219N plants showed ion sensitivity by decreased growth. Sensitivity of sCAX1 lines was seen in decreased growth and tip burning of the leaves (Fig. 2.5).

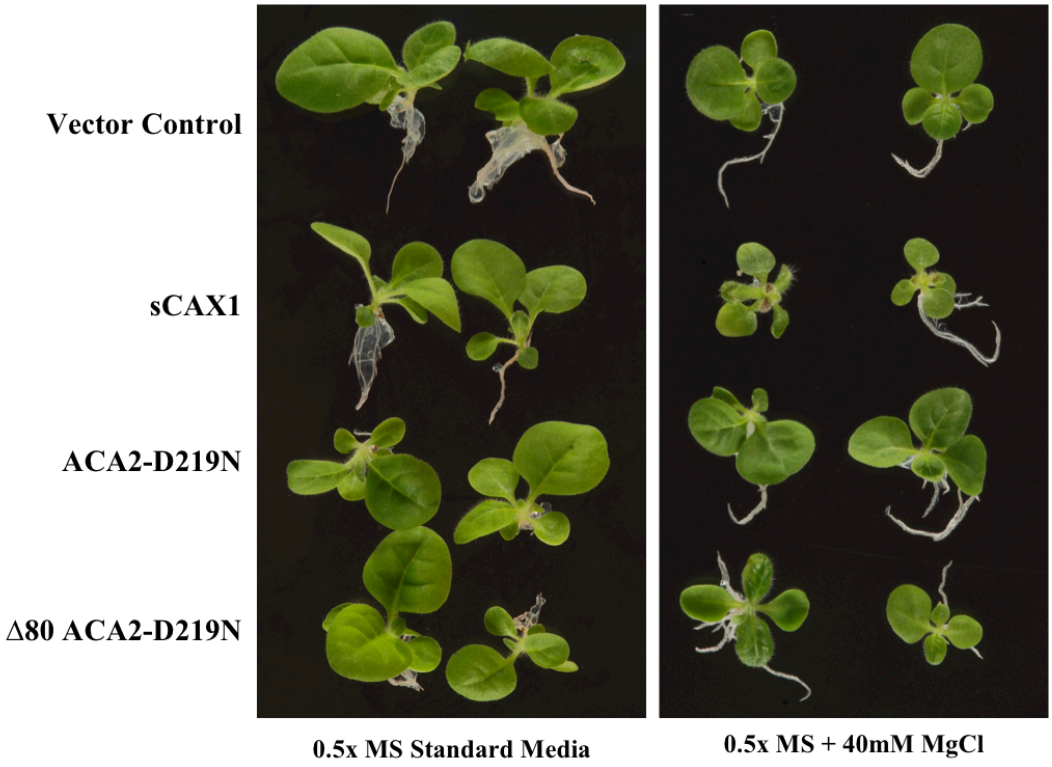

Figure 2.5 Ion sensitivity phenotypes of vector-, sCAX1-, ACA2-D219N-, and Δ80 ACA2-D219N- in T1 generation tobacco seedlings. Plants were transferred 21 d after germination to standard media (0.5x MS), and standard media (0.5x MS) with 40mM MgCl. All plants were then grown for 14 d.

Discussion

High-level activity of plant transporters in yeast often requires the removal of N-terminal autoinhibitory domains (Pittman and Hirschi, 2001; Harper *et al.*, 1998). Here we suggest that these same N-terminal regulatory elements alter activity of ACA2 in tobacco. Interestingly, the deregulation of ACA2 by point mutation (Fig. 2.2) did not produce a distinct phenotype like the truncated ACA2 and CAX transporters when expressed in tobacco (Pittman and Hirschi, 2001; Hirschi, 1999; Hwang *et al.*, 2000). These results validate yeast expression assays as a tool to dissect plant transport function. Furthermore, our studies infer that ectopic expression of both ACA2 and CAX1 activators may be alternate means of boosting transporter function in *planta.*

Steep gradients of Ca exist across the plant tonoplast and ER (Hepler, 2005). ACA2 and CAX1 have distinct roles in Ca transport across these membranes. The ER localized ACA2 is a Ca-ATPase with a high affinity, low capacity (K_M = 0.67 μm) that may fine-tune Ca concentrations around the ER (Hwang et al., 2000; Sanders et al., 1999). CAX1 is a vacuolar localized, low affinity (K_M = 10-15 μm) and high capacity Ca/H+ antiporter that appears to function predominantly when cytosolic Ca concentrations are high (Hirschi et al., 1996). The regulation of Ca content into these organelles has proven to be important for plant growth and development. Arabidopsis knockout mutants defective in vacuolar Ca-ATPase function, display apoptotic-like lesions in leaf tissues (Boursiac *et al.*, 2010). Furthermore, disruption of vacuolar Ca/H+ transporters appears to alter plant growth and stress sensitivities but displays phenotypes that are distinct from lesions in Ca-ATPase activity (Cheng et al., 2005). These genetic

studies underline the concept that despite similar locations transporters have distinct biological functions.

Tobacco plants expressing Δ80-ACA2-D219N exhibit necrotic lesions in the young leaf tissues (Fig. 2.5). Over time the lesions become less prevalent and are repaired completely. The deregulated CAX1 calcium deficiency phenotype can be conditionally suppressed under high Ca concentrations. The differences in phenotypes are distinct further suggesting the transporters have different roles in plant adaptations. The loss of necrotic spots over time in T0 transformants and the absence of this phenotype in the T1 generation raises questions regarding the ability of tobacco to buffer the expression of this deregulated Ca transporter. Gene expression was confirmed by northern analysis in the T1 generation. We are puzzled by the fact the T1 generation tobacco does not show a phenotype, one potential explanation of this variation could be environmental conditions being different from growth in the first to second generation. Experimenting with different soils, altering the pH and ultimately altering the plant's ability to acquire specific nutrients are future directions in understanding this possible conditional phenotype. Necrotic spots observed in our study have close similarity to potassium deficiencies seen in the leaves of plants. Although the phenotypes are similar they differ in the plants ability to recover the damaged tissue over time. This suggest there is a mineral deficiency being created by the over expression of Δ80-ACA2-D219N that is compensated for by the plant. Increased expression of genes, altering plant ion homeostasis would affect the phenotype. Increasing or decreasing the plant of specific

nutrients we could identify possible mechanisms the plant is employing to regulate the damage being caused by what we think is an excess of Ca in the ER.

Modulating ER Ca-ATPase transport may be a means of altering Ca content in crops; however, this study suggests care must be taken in modulating expression. In this study, ACA2 when deregulated and expressed in tobacco appears to be detrimental to the plant while expression of the transporter in less active forms does not appear to alter plant growth and development (Fig 2.5). These findings emphasize a need to further investigate Ca regulation within the plant cell. Ectopic expression of transporters to alter Ca partitioning within the plant could play a role in future biofortification efforts. Emerging technologies such as X-ray Micro Analysis (XMRA) could help to define these changes by allowing visualization of Ca within the plant matrices (Punshon et al., 2009). Understanding the distribution of Ca within the cell could also aid in conceptualizing how Ca transporters alter storage of nutrients and ultimately provide insights into improving biofortification strategies.

Conclusion

The phenotype comparison of sCAX1, ACA2-D219N, and Δ80 ACA2-D219N-expressing tobacco plants is incomplete. However, this study lays a foundation for understanding calcium distribution and partitioning and its effect on plant physiology.

CHAPTER III

SUMMARY

Using biotechnological approaches, I have attempted to characterize the physiological impact of expression of the deregulated Ca-ATPase ACA2 in comparison with that of sCAX1 a vacuolar Ca / H+ antiporter in transgenic tobacco. Tobacco was used to express ACA2 variants in *planta*. The results showed the truncated ACA2 mutants yielded a distinct phenotype in comparison to others lacking an N-terminal truncation. Further experiments and analysis will be needed to fully understand the role this gene plays in calcium sequestration and physiological development.

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