

# Expression of Arabidopsis *CAX1* in Tobacco: Altered Calcium Homeostasis and Increased Stress Sensitivity

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Calcium ( $\text{Ca}^{2+}$ ) efflux from the cytosol modulates  $\text{Ca}^{2+}$  concentrations in the cytosol, loads  $\text{Ca}^{2+}$  into intracellular compartments, and supplies  $\text{Ca}^{2+}$  to organelles to support biochemical functions. The  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter *CAX1* (for *CALCIUM EXCHANGER 1*) of Arabidopsis is thought to be a key mediator of these processes. To clarify the regulation of *CAX1*, we examined *CAX1* RNA expression in response to various stimuli. *CAX1* was highly expressed in response to exogenous  $\text{Ca}^{2+}$ . Transgenic tobacco plants expressing *CAX1* displayed symptoms of  $\text{Ca}^{2+}$  deficiencies, including hypersensitivity to ion imbalances, such as increased magnesium and potassium concentrations, and to cold shock, but increasing the  $\text{Ca}^{2+}$  in the media abrogated these sensitivities. Tobacco plants expressing *CAX1* also demonstrated increased  $\text{Ca}^{2+}$  accumulation and altered activity of the tonoplast-enriched  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter. These results emphasize that regulated expression of  $\text{Ca}^{2+}/\text{H}^{+}$  antiport activity is critical for normal growth and adaptation to certain stresses.

## INTRODUCTION

Transient increases in cytosolic free calcium ( $\text{Ca}^{2+}$ ) concentrations are essential for the conversion of signals, such as red light, touch, cold shock, and pathogen infection, into adapted biological responses (Knight et al., 1991, 1996; Poovaiah and Reddy, 1993; Bowler et al., 1994; Sanders et al., 1999). Stress conditions, such as salinity, also induce cytosolic  $\text{Ca}^{2+}$  accumulation. During these biological responses,  $\text{Ca}^{2+}$  may be mobilized from the plant vacuole, an important storage compartment for  $\text{Ca}^{2+}$ , to act as an intracellular signaling ion (Alexandre et al., 1990; Allen and Sanders, 1995; Barkla and Pantoja, 1996; Marty, 1999). However, little is known about the contribution of individual  $\text{Ca}^{2+}$  transporters in the tonoplast (vacuolar membrane) in establishing  $\text{Ca}^{2+}$  homeostasis during and after signal transduction events and stress responses.

Physiological data suggest an elaborate interplay between concentrations of  $\text{Ca}^{2+}$  and those of numerous ions within the plant.  $\text{Ca}^{2+}$  deficiency in plants may cause death and blackening of the growing points, but this is rarely the result of an absolute lack of soil  $\text{Ca}^{2+}$  (Marschner, 1995). More often, the cause is low  $\text{Ca}^{2+}$  concentrations in relation to other ions present in the soil. Supplemental  $\text{Ca}^{2+}$  is known to mitigate the adverse effects of salinity on plant growth (LaHaye and Epstein, 1969, 1971; Epstein, 1998; Liu and Zhu, 1998). In addition,  $\text{Ca}^{2+}$  may be necessary for

maintaining adequate  $\text{K}^{+}$  transport (Sussman et al., 1994). Thus, precise modulation of cytosolic to vacuolar  $\text{Ca}^{2+}$  ratios may be important for plants to adapt to numerous ion imbalances.

A concentration gradient of  $\text{Ca}^{2+}$  is established across the tonoplast by high-capacity  $\text{Ca}^{2+}/\text{H}^{+}$  exchange activity (Schumaker and Sze, 1985; Blumwald and Poole, 1986). Previously, an Arabidopsis gene, *CAX1* (for *CALCIUM EXCHANGER 1*), was identified by its ability to suppress mutants of yeast defective in vacuolar  $\text{Ca}^{2+}$  transport (Hirschi et al., 1996). In addition, *CAX1* biochemical activities in yeast vacuoles correlate well with those described for the vacuolar  $\text{Ca}^{2+}/\text{H}^{+}$  antiport activities from plant sources (Hirschi et al., 1996). A homolog of *CAX1* from mung bean specifically localizes to the plant vacuole (Ueoka-Nakanishi et al., 1999). When cytosolic  $\text{Ca}^{2+}$  concentrations are low, the yeast vacuolar  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter appears to be responsible for maintaining  $\text{Ca}^{2+}$  homeostasis and may attenuate the propagation of  $\text{Ca}^{2+}$  signals (Cunningham and Fink, 1996; Miseta et al., 1999). However, the role of *CAX1* in plant growth, ion homeostasis, and signal transduction is unknown.

In yeast, expression of either *CAX1*, the lower affinity  $\text{Ca}^{2+}/\text{H}^{+}$  transporter, or *CAX2* can compensate for the absence of the endogenous vacuolar  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter (Hirschi et al., 1996). This functional redundancy of *CAX1* and *CAX2* suggests that loss-of-function mutations of *CAX1* in plants may not reveal a perceived phenotype because of the presence of other genes compensating for its absence. In classic genetics, the alternative to loss-of-function mutations is the creation of dominant gain-of-function mutations. Evidence

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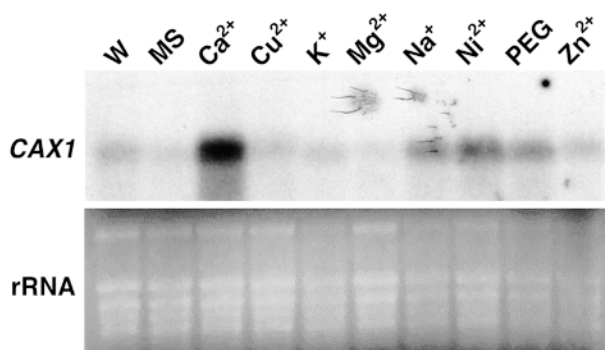
suggests, however, that heterologous expression of genes from related organisms might be a more efficient and fruitful approach to the production of dominant-like gene activities. In fungi, heterologous expression of signaling molecules led to stimulus-independent, constitutively active phenotypes (Milne and Weaver, 1993; Singh et al., 1994). Similar dominant-like interactions have been documented for highly related plant orthologous myc transcription factors when overexpressed in tobacco (Payne et al., 1999), which suggests that plant-plant heterologous expression of *CAX1* might be a useful and efficient way to generate constitutively active phenotypes.

Here, *CAX1* gene expression in response to various stimuli is characterized. In the presence of excess  $\text{Ca}^{2+}$ , Arabidopsis *CAX1* RNA is highly expressed. Details of the growth characteristics, ion sensitivities, and chilling response of transgenic tobacco plants expressing *CAX1* are also reported. The phenotypes obtained are reversible if  $\text{Ca}^{2+}$  is added to the plant growth media, thereby demonstrating involvement of *CAX1* in  $\text{Ca}^{2+}$  homeostasis.

## RESULTS

### *CAX1* Expression in Arabidopsis

Examination of the environmental stimuli that induce *CAX1* expression should increase our understanding of the role this transporter plays in general ion homeostasis and stress



**Figure 1.** *CAX1* RNA Expression in Response to Ion Imbalances.

RNA was isolated from whole Arabidopsis plants 16 hr after watering with various solutions (W, water; MS, nutrient media;  $\text{Ca}^{2+}$ , 80 mM  $\text{CaCl}_2$ ;  $\text{Cu}^{2+}$ , 0.1 mM  $\text{CuCl}_2$ ;  $\text{K}^+$ , 80 mM  $\text{KCl}$ ;  $\text{Mg}^{2+}$ , 50 mM  $\text{MgCl}_2$ ;  $\text{Na}^+$ , 80 mM  $\text{NaCl}$ ;  $\text{Ni}^{2+}$ , 0.1 mM  $\text{NiCl}_2$ ; PEG, 10% polyethylene glycol 3350 solution (118 mOsm/kg);  $\text{Zn}^{2+}$ , 5 mM  $\text{ZnCl}_2$ ). The blot was hybridized with the *CAX1* cDNA. Ethidium bromide-stained rRNA before transfer is shown at bottom.

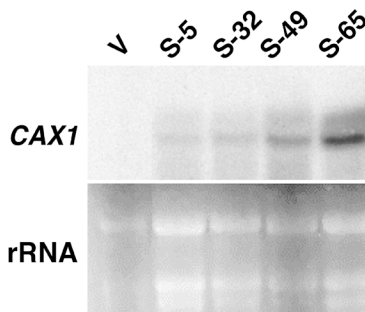
responses. Previous reports establishing that several putative P-type  $\text{Ca}^{2+}$ -ATPases from plants are induced by sodium ( $\text{Na}^+$ ) stress (Perez-Prat et al., 1992; Wimmers et al., 1992) have provided further insight into the role of  $\text{Ca}^{2+}$  in salt adaptation. Thus, RNA gel blot analyses were performed to determine how ion imbalances and various stresses affect *CAX1* RNA accumulation. As shown in Figure 1, *CAX1* RNA was moderately induced by  $\text{Na}^+$ , nickel ( $\text{Ni}^+$ ), and osmotic stress but appeared to be highly induced (>12-fold) by  $\text{Ca}^{2+}$  in the media. *CAX1* RNA accumulated in response to  $\text{Ca}^{2+}$  in a dose-dependent manner, with maximal response at the highest concentration tested (150 mM; data not shown). Given that none of the initial ion imbalances presented the same osmotic effect as 80 mM  $\text{Ca}^{2+}$ , plants also were treated with a range of  $\text{Mg}^{2+}$  concentrations (10 to 150 mM) to mimic the osmotic effect of  $\text{Ca}^{2+}$ . In these experiments, no dose-dependent induction of *CAX1* RNA was observed (data not shown). The plant hormones abscisic acid, auxin, and gibberellin, at concentrations of 0.1  $\mu\text{M}$ , also did not induce *CAX1* expression after a 16-hr incubation (data not shown).

### Expression of *CAX1* in Transgenic Plants

Attempts to exacerbate normal calcium homeostasis in plants were made by constitutive expression of *CAX1* in Arabidopsis. Transgenic plants expressing *CAX1* were created by using a conventional cauliflower mosaic virus 35S promoter vector construct. *CAX1* overexpression in Arabidopsis plants was expected either to attenuate endogenous *CAX1* transcripts by a gene-silencing phenomenon or to exaggerate *CAX1* expression. In fact, *CAX1* overexpression in Arabidopsis was found by RNA gel blot analysis to augment the basal amounts of *CAX1* expression. Despite the increased amounts of *CAX1* RNA in these transgenic lines, no change in plant growth or development was detected (K.D. Hirschi, unpublished data). Lack of a *CAX1*-specific antibody, however, prevented actual quantification of alterations of *CAX1* protein amounts.

Alternatively, the Arabidopsis *CAX1* gene was heterologously expressed in tobacco (cultivar KY160). Transgenic lines of tobacco were generated with a *CAX1* open reading frame (ORF) expressed in either the sense or antisense orientation in front of the 35S promoter. As a control, a transgenic line harboring only the expression vector (designated V) was used. For sense expression, four independent transgenic lines (S-65, S-49, S-32, and S-5) were used.

Expression of *CAX1* RNA was measured in  $T_2$  transgenic lines by RNA gel blot analysis. As shown in Figure 2, *CAX1* RNA accumulated in all 35S::*CAX1* transgenic lines. *CAX1*-specific RNA was also detected in all antisense lines tested (data not shown). The inability to detect an endogenous transcript of the tobacco *CAX1* homolog in the vector transgenic lines attests to the high-stringency hybridization conditions used.



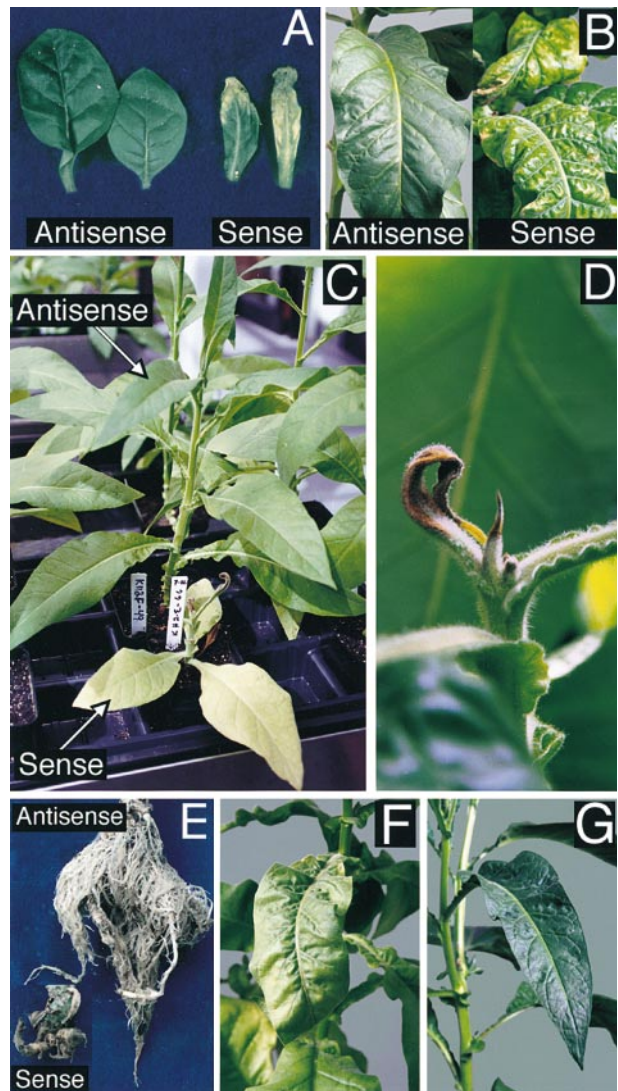
**Figure 2.** Expression of *CAX1* in Transgenic Tobacco Plants.

Ten micrograms of total RNA extracted from fully expanded leaves of 6-week-old  $T_2$  plants was analyzed by RNA gel blotting. The blot was hybridized with the *CAX1* cDNA probe. Transgenic lines expressing the vector (V) alone do not express *CAX1* RNA. Sense (S-5, S-32, S-49, and S-65) lines denote 5'–3' expression of the *CAX1* ORF by using the 35S promoter (35S::*CAX1*). Ethidium bromide-stained rRNA before transfer is shown at bottom.

### *CAX1* Expression in Tobacco Disturbs Normal Vigor

Preliminary examination suggested that heterologous *CAX1* expression in tobacco had disrupted normal  $Ca^{2+}$  metabolism. Figure 3 demonstrates that 48 of the 60 primary transformants expressing the sense-oriented *CAX1* formed necrotic lesions after 3 weeks under sterile conditions (Figure 3A). After transfer to soil, the same *CAX1*-transformed plants appeared to recover. Several weeks later, however, the leaves of all of the 48 primary transformants that initially displayed necrotic lesions began to redevelop altered leaf morphology. After several more weeks, the leaves were chlorotic and twisted (Figure 3B), the severity of these symptoms varying between the lines. Twenty-five of the 48 lines that displayed leaf necrosis also developed terminal buds that turned black and died (Figures 3C and 3D). Further examination of the root area among these stunted plants revealed not only reduced root mass but also, in ~50% of the stunted plants (i.e., 12 of the original 60 primary transformants), almost no root formation (Figure 3E). In contrast, the 50 transgenic plants expressing antisense-oriented *CAX1* displayed growth phenotypes indistinguishable from the 10 vector-control transgenic plants.

The 35S::*CAX1* lines were selected for further study on the basis of their  $T_1$  phenotype and their ability to make seeds. Only seven of the original 48 transgenic lines that displayed altered morphology also maintained adequate fertility. In contrast, >90% of the antisense-oriented *CAX1* and vector-containing transgenic lines maintained complete fertility. The same necrosis of the original transformants revisited the  $T_2$  generation of *CAX1* transformants. When grown from seed in tissue culture, the  $T_2$  plants appeared normal and unperturbed for the first 5 weeks, after which their leaves began to display visible lesions.  $T_2$  *CAX1*-transformed plants



**Figure 3.** Phenotypes of Tobacco Plants Expressing *CAX1* Genes.

Sense lines denote expression of the *CAX1* ORF. Antisense lines contain the ORF in the opposite orientation.

(A) Leaf phenotype of *CAX1*-expressing lines obtained by using a 35S promoter. Leaves are from plants grown in tissue culture.

(B) Leaf phenotype of *CAX1*-expressing lines after several weeks in the greenhouse.

(C) Size differences in *CAX1*-expressing plants. The 35S::*CAX1* sense plant (in front) is stunted. The plant in the background is expressing *CAX1* in the antisense confirmation. This plant is the same size as vector control plants (not shown).

(D) Magnified view of a 35S::*CAX1* plant similar to (C).

(E) Root phenotype of *CAX1*-expressing plants. The sense roots are significantly stunted.

(F) Leaf of 10-week-old vector control plant grown for 2 weeks without  $Ca^{2+}$ .

(G) Leaf of 10-week-old *CAX1*-expressing plant given  $Ca^{2+}$  supplementation.

that were sown and grown in the greenhouse also displayed necrotic lesions on their leaves as well as their apical meristems after 6 weeks of growth. Once again, the T<sub>2</sub> *CAX1*-expressing plants displayed a visible reduction in root mass as well as reduced fertility.

### Ca<sup>2+</sup> Suppresses *CAX1*-Induced Symptoms

The symptoms of the *CAX1*-expressing plants, particularly the altered leaf morphology, could be phenocopied in both the vector control and antisense-oriented *CAX1*-expressing lines by growth in the absence of Ca<sup>2+</sup> (Figure 3F). Therefore, we tested whether daily watering of plants with 2 mM CaCl<sub>2</sub> (a sevenfold greater daily concentration of Ca<sup>2+</sup> than in the normal nutrient solution) could ameliorate an apparent depletion of Ca<sup>2+</sup> in tobacco attributable to heterologous expression of Arabidopsis *CAX1*. Primary transformants and T<sub>2</sub> plants displayed altered leaf morphology and necrotic lesions on the leaves and apical meristems, regardless of the media used; however, adding Ca<sup>2+</sup> to the medium delayed the onset and severity of these symptoms, the extent of the suppression varying among the individual lines. In some cases, this supplementation allowed *CAX1*-transformed plants to develop almost wild-type leaf morphology (Figure 3G).

### *CAX1* Expression Induces Ion Sensitivity

Ca<sup>2+</sup> homeostasis is an important component of stress responses (Bressan et al., 1998; Sanders et al., 1999). As observed here, *CAX1* expression was induced when Arabidopsis plants were watered with high concentrations of Ca<sup>2+</sup>; perturbations of other ions, however, caused no substantial alterations in *CAX1* expression (Figure 1). This suggests that plants regulate *CAX1* expression in response to various stimuli. Conceivably, constitutive *CAX1* expression may alter the ion sensitivity of transgenic plants. Transgenic seeds germinated on standard media were transferred to various media when the seedlings were similar in size and vigor to the control plants (Figure 4A). If allowed to grow in standard media, the *CAX1*-transformed plants were only slightly smaller than vector controls (Figure 4B). More than 200 T<sub>2</sub> seeds were analyzed from multiple 35S::*CAX1* lines; >85% of the plants were hypersensitive to Mg<sup>2+</sup> and K<sup>+</sup> salts at concentrations that failed to disturb the growth of the control plants (Figures 4C and 4D). *CAX1*-expressing plants were also sensitive to excess Na<sup>+</sup> in the media; however, the effects on phenotype were not as dramatic as those seen with Mg<sup>2+</sup> (Figure 4E) and were comparable with those seen with K<sup>+</sup> (data not shown). The growth of *CAX1*-expressing plants was not enhanced by above-normal concentrations of Ca<sup>2+</sup>, even though the plants were hypersensitive to Ca<sup>2+</sup>-depleted media (Figures 4F and 4G). In none of the tests for ion sensitivity did the 100 transgenic plants expressing antisense-oriented *CAX1* display a condition similar to the *CAX1*



**Figure 4.** Ion Sensitivity of *CAX1*-Expressing Plants.

Two vector control plants are shown at left and two *CAX1*-expressing plants (35S::*CAX1*) at right.

(A) Plants grown in standard media immediately after transfer to various media (pretreatment).

(B) Plants transferred to standard media and grown for 10 days.

(C) Plants transferred to standard media supplemented with 50 mM MgCl<sub>2</sub> and grown for 10 days.

(D) Plants transferred to standard media supplemented with 100 mM KCl and grown for 10 days.

(E) Plants transferred to standard media supplemented with 50 mM NaCl and grown for 10 days.

(F) Plants transferred to standard media supplemented with 100 mM CaCl<sub>2</sub> and grown for 10 days.

(G) Plants transferred to standard media without Ca<sup>2+</sup> and grown for 10 days.

(H) Plants transferred to standard media supplemented with 50 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> and grown for 10 days.

(I) Plants transferred to standard media supplemented with 100 mM KCl and 2 mM CaCl<sub>2</sub> and grown for 10 days.

sense-oriented transgenic plants or different from the vector control transgenic plants (data not shown).

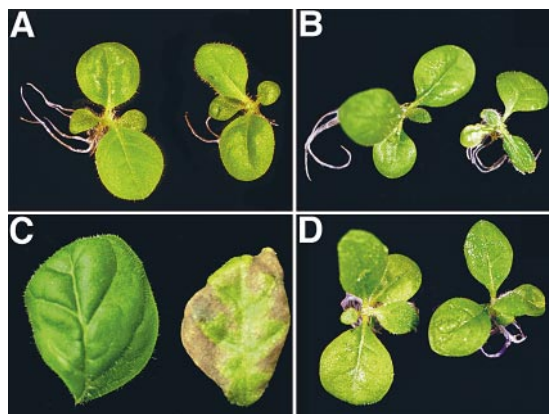
### Ca<sup>2+</sup> Restores Ion Homeostasis

In light of the apparent Ca<sup>2+</sup> deficiency accompanying heterologous expression of *CAX1* in tobacco, we tested

whether or not the ion sensitivities imposed by *CAX1* expression could be attributed to such a deficiency. The transgenic plants expressing *CAX1* and the vector controls were germinated on standard media and then transferred to standard media plus 100 mM KCl or 50 mM MgCl<sub>2</sub>, as described above, but were also supplemented with 2 mM CaCl<sub>2</sub>. As shown in Figures 4H and 4I, adding Ca<sup>2+</sup> greatly reduced the ion sensitivities of the *CAX1*-expressing plants. Analyzing >200 T<sub>2</sub> seeds from multiple 35S::*CAX1* lines showed that under these growth conditions <20% of the plants showed any perturbations in growth.

### *CAX1* Expression Induces Increased Cold-Shock Sensitivity

A brief increase in cytosolic Ca<sup>2+</sup> is one of several transient responses that occur when plants are chilled (Minorsky, 1989). Acclimation to subsequent freezing is partly the result of a modified calcium response to this stress condition (Knight et al., 1996). Cold damage in plants has been speculated to result from a lack of Ca<sup>2+</sup> homeostasis (Minorsky, 1985). To determine whether *CAX1* expression causes phenotypic alterations in plant responses, we examined the sensitivity of *CAX1*-expressing lines to chilling. As shown in Figure 5A, 3-week-old *CAX1*-expressing plants, although slightly smaller than the controls, did not display any visible growth defect. Both 3-week-old vector control and *CAX1*-



**Figure 5.** Cold-Shock Sensitivity of *CAX1*-Expressing Plants.

Vector control plants are shown at left and *CAX1*-expressing plants (35S::*CAX1*) at right.

(A) Plants grown in standard media for 21 days (before cold treatment).

(B) Twenty-seven-day-old plants 6 days after cold treatment.

(C) Leaves of plants at 21 days after cold treatment.

(D) Twenty-seven-day-old plants grown in standard media supplemented with 2 mM CaCl<sub>2</sub>, seen 6 days after cold treatment.

expressing plants were subjected to a mild cold treatment. Six days after the cold shock, ~50% of 200 *CAX1*-expressing plants (50 plants from each of the T<sub>2</sub> plants analyzed in Figure 2) that were chilled developed necrotic lesions that were not found on any of the 200 vector or antisense transgenic plants analyzed (Figures 5B and 5C). *CAX1*-expressing plants also appeared to grow slightly more slowly after the cold treatment (data not shown). Addition of exogenous Ca<sup>2+</sup> reduced the chilling sensitivity of these plants. When 2 mM CaCl<sub>2</sub> was added to the standard media, only ~15% of the 200 *CAX1*-expressing plants developed necrotic lesions when cold-shocked (Figure 5D).

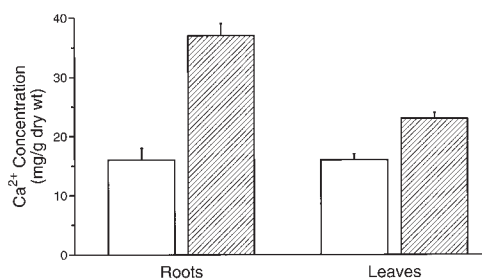
### Ca<sup>2+</sup> Accumulation in *CAX1*-Expressing Plants

To ascertain whether *CAX1* expression altered total Ca<sup>2+</sup>, presumably through sequestration into the vacuole, we measured the total accumulation of Ca<sup>2+</sup> in the roots and leaves of transgenic plants. As shown in Figure 6, *CAX1*-expressing plants contained almost twice as much total Ca<sup>2+</sup> in root tissue as did the vector control plants. Leaves of *CAX1*-expressing plants contained 30% more total Ca<sup>2+</sup> than did plants expressing the vector alone.

### Ca<sup>2+</sup>/H<sup>+</sup> Antiport in *CAX1*-Expressing Plants

Because expression of *CAX1* in yeast restored vacuolar Ca<sup>2+</sup>/H<sup>+</sup> antiport activity to yeast strains deficient in this transporter (Hirschi et al., 1996), Ca<sup>2+</sup>/H<sup>+</sup> antiport activity was measured in tonoplast-enriched vesicles from transgenic tobacco roots to determine whether *CAX1* expression caused altered Ca<sup>2+</sup>/H<sup>+</sup> transport. Proton gradients (acidic inside the vesicles) were formed artificially by adding nigericin to K<sup>+</sup>-loaded vesicles (Salt and Wagner, 1993). As shown in Figure 7A, treatment with nigericin caused a rate of proton accumulation (monitored with <sup>14</sup>C-methylamine) similar to those reported in oat and observed in tobacco (Schumaker and Sze, 1985; V. Korenkov and G.J. Wagner, unpublished results). Addition of CaCl<sub>2</sub> (10 to 100 μM) caused efflux of methylamine, an indication that Ca<sup>2+</sup> was exchanged (antiported) with H<sup>+</sup> (Schumaker and Sze, 1985).

Tonoplast-enriched vesicles were prepared from plants watered with either nutrient solution or Ca<sup>2+</sup>. In the absence of Ca<sup>2+</sup>, the endogenous Ca<sup>2+</sup>/H<sup>+</sup> antiporter may be weakly expressed (Figure 1); regardless of the environmental conditions, however, the 35S::*CAX1* transgene should produce constitutive amounts of *CAX1*. As shown in Figure 7B, *CAX1*-expressing transgenic plants watered with nutrient solution were capable of accumulating Ca<sup>2+</sup> to steady state concentrations of ~14 nmol/mg protein, compared with ~7 nmol/mg protein for the vector controls. When tonoplast-enriched vesicles were prepared from transgenic plants watered with Ca<sup>2+</sup>, the *CAX1*-expressing plants were capable of accumulating steady state concentrations of Ca<sup>2+</sup> to ~16



**Figure 6.** Calcium Concentrations in Roots and Leaves of Transgenic Plants.

Ca<sup>2+</sup> content of vector controls (open bars) and *CAX1*-expressing plants (hatched bars) grown in standard media was determined by atomic absorption spectrophotometry. Data represent the means ( $\pm$ SD) of three independent assays. wt, weight.

nmol/mg protein; the comparable value for the vector controls was  $\sim$ 14 nmol/mg protein (Figure 7C). Under both growth conditions, the amounts of Ca<sup>2+</sup>/H<sup>+</sup> antiport activity varied in the *CAX1*-expressing plants, being greater in S-65 plants than in S-49 or S-32 plants (data not shown); this coincided with the greater abundance of *CAX1* transcripts in S-65 plants (Figure 2). Despite some variation among lines, the *CAX1*-expressing plants always expressed at least 30% more Ca<sup>2+</sup>/H<sup>+</sup> antiport activity than did control plants watered with nutrient solution.

In our attempts to measure the kinetic properties of Ca<sup>2+</sup>/H<sup>+</sup> transport activity as a function of Ca<sup>2+</sup> concentration, variations between experiments made it difficult to obtain precise measurements of apparent  $K_m$  and  $V_{max}$ . However, a dependence on Ca<sup>2+</sup> concentration and a saturation in Ca<sup>2+</sup> uptake were observed. Preliminary experiments indicated an apparent  $K_m$  for Ca<sup>2+</sup> of  $\sim$ 15  $\pm$  5  $\mu$ M and a  $V_{max}$  of 13  $\pm$  5 nmol of Ca<sup>2+</sup> mg<sup>-1</sup> protein min<sup>-1</sup>, values in general agreement with those reported for Ca<sup>2+</sup>/H<sup>+</sup> in plants (Schumaker and Sze, 1985). In addition, the *CAX1* sense- and antisense-expressing lines did not appear to differ from the vector controls in these kinetic measurements (data not shown).

## DISCUSSION

### Exogenous Ca<sup>2+</sup> Induces *CAX1* RNA

The induction of *CAX1* expression in plants with exogenous Ca<sup>2+</sup> is consistent with the *CAX1* protein playing a role in loading the vacuole with Ca<sup>2+</sup> (Figure 1). The plant vacuole contains millimolar concentrations of Ca<sup>2+</sup>, whereas Ca<sup>2+</sup> in the cytosol is submicromolar under resting conditions. In plants and fungi, this asymmetric distribution is achieved by P-type Ca<sup>2+</sup>-ATPases and Ca<sup>2+</sup>/H<sup>+</sup> antiport systems (Cunningham and Fink, 1996; Ferrol and Bennett,

1996; Malmström et al., 1997; Ueoka-Nakanishi et al., 1999). The vacuolar Ca<sup>2+</sup>-ATPase has a high affinity for Ca<sup>2+</sup> compared with the antiporter (Ueoka-Nakanishi et al., 1999). In response to a Ca<sup>2+</sup> load, the vacuolar antiporter may lower cytosolic Ca<sup>2+</sup> content to a few micromolar, and the Ca<sup>2+</sup>-ATPase then may act to further lower the cytosolic Ca<sup>2+</sup> pools. Although the Ca<sup>2+</sup>/H<sup>+</sup> antiporter may have some role in controlling the cytosolic Ca<sup>2+</sup> concentrations during signal transduction events (see below), the Ca<sup>2+</sup>-specific induction of *CAX1* RNA is too slow to account for any portion of this rapid redistribution of Ca<sup>2+</sup>.

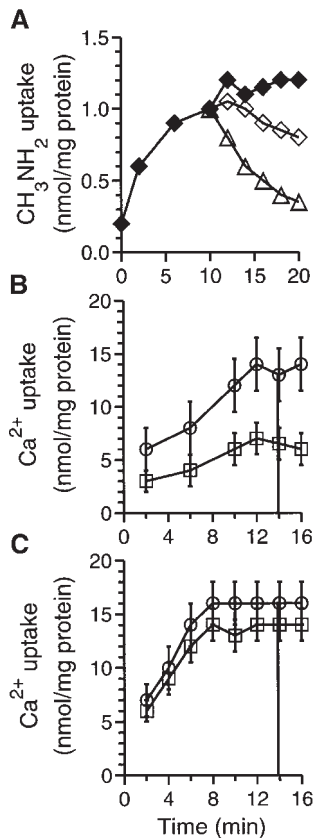
The data presented here demonstrate that *CAX1* also could be weakly induced by Na<sup>+</sup>, osmotic shock, and Ni<sup>+</sup>, indicating a need for future studies to address the specificity of *CAX1* transport and mechanisms of *CAX1* induction. Potentially, *CAX1* may have a role in Ni<sup>+</sup> transport; however, oats do not appear to possess a tonoplast high-affinity Ni<sup>+</sup>/H<sup>+</sup> transporter (Gries and Wagner, 1998).

### *CAX1* Phenotypes: Importance of Regulated Gene Expression

The results presented here show that constitutive expression of *CAX1*, a putative vacuolar Ca<sup>2+</sup>/H<sup>+</sup> antiport, can alter growth, increase the sensitivity of the plant to various ions, and alter the plant response to chilling. Expression of *CAX2*, an antiporter with different ion affinities, did not produce these phenotypes (K.D. Hirschi, unpublished data). Furthermore, the antisense *CAX1* constructs produced no visible or biochemical differences from the vector controls in any of our studies. The lack of sequence similarity between the Arabidopsis *CAX1* gene and the tobacco homolog (Figure 2) may explain these results.

The phenotypes described here may reflect the inability to modulate Ca<sup>2+</sup>/H<sup>+</sup> antiport activity in certain cells and during certain events. In fact, given the expression pattern of *CAX1* in Arabidopsis (Figure 1), the *CAX1*-expressing tobacco plants were expressing amounts of Ca<sup>2+</sup>/H<sup>+</sup> antiport activity that may be appropriate only in the presence of high concentrations of Ca<sup>2+</sup> in the soil. In *CAX1*-expressing plants, the tonoplast-enriched Ca<sup>2+</sup>/H<sup>+</sup> activity was independent of the watering conditions (Figures 7B and 7C). In contrast, the vector control plants, when watered with Ca<sup>2+</sup>, demonstrated tonoplast-enriched Ca<sup>2+</sup>/H<sup>+</sup> antiport activity nearly equal to that of the *CAX1*-expressing plants. Thus, the *CAX1*-expressing plants appeared to possess constitutively high amounts of Ca<sup>2+</sup>/H<sup>+</sup> antiport activity.

Constitutive Ca<sup>2+</sup>/H<sup>+</sup> antiport activity in transgenic tobacco plants appears to cause stress sensitivities. Organisms usually are rendered sensitive to various ions through defects in transporters and regulatory proteins. In yeast, a defect in a transport protein causes increased metal sensitivity (Li and Kaplan, 1998). In plants, a defect in a putative regulatory Ca<sup>2+</sup> sensor causes hypersensitivity to salt (Liu and Zhu, 1998).



**Figure 7.** K<sup>+</sup>/Nigericin-Dependent Methylamine and Ca<sup>2+</sup> Uptake Activity in Tonoplast-Enriched Vesicles from Tobacco Roots.

(A) K<sup>+</sup>/nigericin was used to generate a ΔpH that was monitored as the accumulation of <sup>14</sup>C-methylamine (filled diamonds). After ~10 min, Ca<sup>2+</sup> was added to a final concentration of 10 μM (open diamonds) or 100 μM (open triangles). Methylamine equilibration was monitored by direct filtration (Schumaker and Sze, 1985). The data shown are representative of the >10 experiments that were performed with tonoplast-enriched vesicles from various transgenic plants. Data are presented as nanomoles of CH<sub>3</sub>NH<sub>2</sub> mg<sup>-1</sup> of protein, after subtraction of the control values (obtained in the absence of nigericin).

(B) and (C) Ca<sup>2+</sup> uptake activity in tonoplast-enriched vesicles from vector control tobacco roots (open squares) and CAX1-expressing roots (open circles) watered with nutrient solution (B) or CaSO<sub>4</sub> (C). Ca<sup>2+</sup> uptake was monitored by using the direct filtration method (Schumaker and Sze, 1985). After 14 min, Triton X-100 was added to a final concentration of 0.03% to dissipate the accumulation of Ca<sup>2+</sup>. Results are from assays of two tonoplast preparations with the vector control and three tonoplast preparations from transgenic line S-65 (35S::CAX1) with both the nutrient water solution and CaCl<sub>2</sub> watering treatments. Data represent the means (±SD) of these assays. Although the Ca<sup>2+</sup> uptake varied with each line tested, similar results were obtained with S-49 and S-32 lines assayed in the same manner (data not shown). These results are presented as nanomoles of Ca<sup>2+</sup> mg<sup>-1</sup> of protein, after subtraction of the control values (absence of nigericin).

Ca<sup>2+</sup> partitioning may be altered by expression of CAX1 in the transgenic plants. This model is supported by the constitutive amounts of tonoplast-enriched Ca<sup>2+</sup>/H<sup>+</sup> activity and the increased total Ca<sup>2+</sup> accumulation (Figures 6 and 7). Future experiments will have to specifically address localized fluctuations in Ca<sup>2+</sup> and the possibility that CAX1 functions at other membranes in the plant cell to cause these changes in Ca<sup>2+</sup> homeostasis.

The phenotypes of CAX1-expressing plants are consistent with Ca<sup>2+</sup> deficiencies. In crop plants, Ca<sup>2+</sup>-deficient symptoms include necrosis of young leaves, often followed by necrosis of the apical meristem (Scaife and Turner, 1984); roots may appear brownish and short. These phenotypes are very similar to those seen in the CAX1-expressing plants (Figure 3). Furthermore, Ca<sup>2+</sup>-deficient crop plants often are diagnosed by their increased sensitivity to other cations (Scaife and Turner, 1984). The CAX1-expressing transgenic tobacco plants studied here were hypersensitive to K<sup>+</sup> and Mg<sup>2+</sup> (Figures 4C and 4D). The high concentrations of these ions may compete with Ca<sup>2+</sup> for uptake and may induce Ca<sup>2+</sup> deficiency-like symptoms (Figure 3G). The ability to suppress the growth defects and ion sensitivities of CAX1-expressing plants with exogenous Ca<sup>2+</sup> strongly suggests that these plants are deficient in Ca<sup>2+</sup> (Figures 3G, 4H, 4I, and 5D).

#### Chilling Sensitivity: Ca<sup>2+</sup> Deficiency or Altered Ca<sup>2+</sup> Signaling?

The cold-shock sensitivity data presented here suggest that even in optimal growth media, CAX1-expressing plants have altered Ca<sup>2+</sup> homeostasis (Figure 5). These growth phenotypes may be caused by alterations in the availability of Ca<sup>2+</sup> at the plasma membrane. Displacement of membrane-associated Ca<sup>2+</sup> can impair membrane stability (Läuchli, 1990). In fact, Ca<sup>2+</sup> may protect membranes from the effects of high salt and cold shock (LaHaye and Epstein, 1969; Lynch and Steponkus, 1987).

Alternatively, the chilling sensitivity may be attributable to a dampening in the cytosolic burst after this environmental stimulus. In plants, Ca<sup>2+</sup> is mobilized from the vacuole (Alexandre et al., 1990; Gilroy et al., 1993; Allen and Sanders, 1995; Trewavas and Malhó, 1997). In fact, cold-induced vacuolar release of calcium has been demonstrated in tobacco and Arabidopsis (Knight et al., 1996). Thermodynamic considerations argue that the vacuolar Ca<sup>2+</sup>-ATPase is necessary to account for the restoration of resting concentrations of cytosolic Ca<sup>2+</sup> (Malmström et al., 1997); however, localized increases in cytosolic Ca<sup>2+</sup> can reach micromolar concentrations (McAinsh and Hetherington, 1998). Thus, a major function of the vacuolar Ca<sup>2+</sup>/H<sup>+</sup> antiporter may be to reduce cytosolic Ca<sup>2+</sup> concentrations after they have been increased as a result of external stimuli (Ueoka-Nakanishi et al., 1999). Conceivably, constitutive expression of CAX1 in transgenic plants

may perturb these transient concentrations of cytosolic  $\text{Ca}^{2+}$  during adapted biological responses.

In summary, a critical finding in this study is the ability to significantly perturb the growth parameters of a plant through the constitutive expression of a single transport protein. The *CAX1*-expressing plants displayed altered phenotypes and increased stress sensitivities, emphasizing that modulated expression of a  $\text{Ca}^{2+}/\text{H}^{+}$  antiporter is important for normal growth and several biological responses.

## METHODS

### Plant Materials

*Arabidopsis thaliana* ecotype Columbia was used in this study. For stress treatment, surface-sterilized seeds were grown on one-half strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2% (w/v) sucrose and 1% (w/v) agar, pH 5.7 (standard media), for 3 weeks and then transferred to a water bath containing the appropriate stressor. Osmolality of the 10% polyethylene glycol 3350 (PEG) solution was measured with an osmometer (Wescor, Logan, UT). Tobacco cultivar KY160 was also used in this study. Tobacco plants were grown in a greenhouse as previously described (Grusak and Pomper, 1999). For optimal growth, plants in the greenhouse were watered three times a day, with soil-saturating volumes of a nutrient solution containing 3 mM  $\text{KNO}_3$ , 0.275 mM  $\text{CaCl}_2$ , 0.25 mM  $\text{MgSO}_4$ , and 0.25 mM  $\text{KH}_2\text{PO}_4$ . For studies of calcium deficiency, the  $\text{CaCl}_2$  was omitted from this mixture. For  $\text{Ca}^{2+}$  supplementation and measurement of total  $\text{Ca}^{2+}$  content, a soil-saturating volume of 2 mM  $\text{CaCl}_2$  was added daily.

Surface-sterilized tobacco seeds were plated on standard media and maintained in a temperature-controlled room at 25°C with continuous cool-fluorescent illumination. To produce a cold shock, we placed the plants germinated on standard media at 4°C for 24 hr at 21 days after plating, then returned the plants to 25°C for 6 or 21 days. For ion sensitivity analysis, surface-sterilized seeds were germinated in standard media; 14 days after plating, they were transferred to standard media supplemented with the appropriate ion. To make media deficient in  $\text{Ca}^{2+}$ , we deleted the  $\text{CaCl}_2$  from the nutrient solution and added sucrose and agar, as given for the standard media. Standard media plus 2 mM  $\text{CaCl}_2$  was used to suppress the *CAX1*-conferred phenotypes.

Most experiments were performed with segregating  $T_2$  populations of tobacco lines S-65 and S-49. Phenotypes did not drastically differ among the cauliflower mosaic virus 35S::*CAX1*-expressing plants. Antisense line AS-46 was used in most experiments; however, the phenotypes displayed by antisense and vector control lines were indistinguishable in all experiments performed.

### Plant Transformations

Standard techniques of DNA cloning were performed as described by Ausubel et al. (1998). The coding region of *CAX1* was cloned into pBin19 (Clontech, Palo Alto, CA), which contained the cauliflower mosaic virus 35S promoter fragment. The recombinant plasmids, or vector controls, were introduced into *Agrobacterium tumefaciens* LB4404 (Life Technologies, Grand Island, NY). Tobacco leaf disc

transformation was conducted according to published procedures by using tobacco strain KY160 (Tarczynski et al., 1992). Transformants were selected on standard media containing 100  $\mu\text{g}/\text{mL}$  kanamycin. Approximately 60 primary transformants harboring the 35S::*CAX1* construct were placed in soil.

### RNA Extraction and RNA Gel Blot Analysis

RNA was isolated from *Arabidopsis* plants (leaves, stems, and roots) and tobacco leaves, according to previously published procedures (Niyogi and Fink, 1992). After electrophoresis on a 1% agarose gel in formaldehyde, total RNA was blotted onto Hybond N+ membrane (Amersham), according to the manufacturer's recommendations. The full-length *CAX1* cDNA was radiolabeled with  $^{32}\text{P}$ -dCTP by using a random-primed labeling kit (Amersham). Blots were hybridized at 65°C according to the method of Church and Gilbert (1984). Blots were washed three times (15 min each time) in  $0.1 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS at 65°C, and hybridization was visualized by autoradiography. Results were quantified with a PhosphorImager SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### Calcium Analysis

Fifty-day-old tobacco plants were grown in the greenhouse as described in Plant Materials. The young leaves were harvested, and the roots were rinsed in water and harvested. The leaves and roots were dried at 70°C for 3 days. At least 0.3 g (dry weight) of roots was analyzed individually; roots weighing less were pooled to yield the required weight. The tissues then were wet digested, and the digestate was resuspended as described in Grusak (1994). Total  $\text{Ca}^{2+}$  content per gram of dry mass was determined by atomic absorption spectrophotometry (model 2100; Perkin-Elmer, Norwalk, CT).

### Isolation of Sealed Tonoplast-Enriched Vesicles

To determine tonoplast transport properties of individual transgenic plant types in studies such as this one, we also determined transport activity in vesicles prepared from soil-grown tobacco plants. Tobacco roots (without the tap root) teased from soil by gentle washing with water yielded vesicles with good transport activity (V. Korenkov, K.D. Hirschi, and G.J. Wagner, unpublished results). After root isolation, the procedure of Salt and Wagner (1993) was used with some modifications (Gonzales et al., 1999). All steps were conducted at 4°C, and when used, DTT and phenylmethylsulfonyl fluoride (PMSF) were added just before the buffer was used. Fifty-day-old tobacco plants were watered with  $0.5 \times \text{MS}$  medium or 10 mM  $\text{CaSO}_4$  for the last 7 days of growth. Roots were harvested (40 g) and soaked for 10 min in homogenization buffer (3 mL/mg roots) containing 250 mM mannitol, 20 mM Hepes adjusted with Bis-Tris propane (BTP) to pH 7.4, 6 mM EGTA, 0.5% polyvinylpyrrolidone-40, 0.1% BSA, 0.1 mM PMSF, and 1 mM DTT. Half of the root samples (i.e., ~20 g) were transferred to a cold mortar holding 40 mL of homogenization buffer, cut into 2-cm pieces, and homogenized with a mortar and pestle. The remaining root samples were divided and homogenized the same way, and the root suspension from all portions was filtered through four layers of cheesecloth. The supernatants were decanted and centrifuged for 5 min at 1000g, followed immediately by 10 min at 12,000g. The homogenates were decanted and centrifuged for 30



min at 60,000g. The supernatants were discarded, and the pellet was resuspended in 250 mM mannitol, 2.5 mM Hepes-BTP, pH 7.2, 0.1% BSA, 0.1 mM PMSF, and 0.1 mM DTT. The resuspended pellet then was layered onto a 10-mL solution containing 6% (w/v) dextran, 250 mM mannitol, and 2.5 mM Hepes-BTP, pH 7.2. The resulting gradient was centrifuged for 2 hr at 70,000g. The visible band at the mannitol-dextran interface (vesicles) was collected.

### Loading of Vesicles with Potassium

Vesicles from the dextran solution were recovered and diluted to 36 mL with K<sup>+</sup>-loading buffer containing 50 mM mannitol, 2.5 mM Hepes-BTP, pH 7.2, 150 mM KCl, 0.1% BSA, 0.1 mM PMSF, and 0.1 mM DTT. After incubation at 10°C for 1 hr, vesicles were sedimented at 90,000g for 30 min. The pellet was resuspended in 100 to 200  $\mu$ L K<sup>+</sup>-loading buffer to a final concentration of 1 to 2 mg protein/mL. Protein analysis was performed as previously described (Salt and Wagner, 1993).

### Ca<sup>2+</sup> Transport Assay

<sup>45</sup>Ca<sup>2+</sup> uptake was measured by a filtration assay as previously described (Schumaker and Sze, 1985). The loaded vesicle preparations were pelleted and resuspended in buffer without KCl just before use, and an aliquot was diluted into a reaction mixture containing 250 mM mannitol, 25 mM Hepes-BTP, pH 7.0, with or without 5  $\mu$ M nigericin. After incubation with 10  $\mu$ M <sup>45</sup>Ca<sup>2+</sup> (American Radiolabeled Chemicals, St. Louis, MO) at 4°C for the indicated period of time, the samples were filtered through prewetted HATF Millipore filters (0.45  $\mu$ m pore size; Millipore Corp., Bedford, MA) and then washed in resuspension buffer containing 0.1 mM CaCl<sub>2</sub>. The filters were dried, and the radioactivity was determined by liquid scintillation counting. Results are presented as nanomoles of Ca<sup>2+</sup> taken up per milligram of protein. Each transgenic line was tested for Ca<sup>2+</sup> transport at least twice. Absolute rates of Ca<sup>2+</sup> transport varied with each preparation; however, the difference between CAX1-expressing lines and vector controls was always >30% when plants were watered with nutrient solution.

### Methylamine Accumulation

$\Delta$ pH generation in vesicles (acid inside) was determined by <sup>14</sup>C-methylamine (American Radiolabeled Chemicals) accumulation by using the direct filtration method previously described (Churchill and Sze, 1983). All vesicles appeared to be well sealed, as evidenced by maintenance of the steady state gradient in the control. The rate and magnitude of proton dissipation in response to Ca<sup>2+</sup> were concentration dependent. Absolute rates of methylamine accumulation varied with each preparation.

### ACKNOWLEDGMENTS

I thank Maricar Miranda, Nathaniel Wilganowski, and Jean Sunega for technical support, and George Wagner and Victor Korenkov for assistance establishing the Ca<sup>2+</sup> transport assays. I am also grateful to Bonnie Bartel, Andrew Diener, Toshiro Shigaki, and George

Wagner for critical reading of the manuscript. This work was funded in part by the National Institutes of Health (Grant Nos. CHRC 5 P30 and 1R01 GM 57427). This work is a publication of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS), and the Children's Nutrition Research Center (Department of Pediatrics, Baylor College of Medicine, Houston, TX); it has been partially funded by the USDA/ARS under Cooperative Agreement No. 58-6250-6001. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Received June 29, 1999; accepted August 31, 1999.

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**Expression of Arabidopsis *CAX1* in Tobacco: Altered Calcium Homeostasis and Increased Stress Sensitivity**

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*Plant Cell* 1999;11;2113-2122

DOI 10.1105/tpc.11.11.2113

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