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Mutations in the Ca^{2+}/H^{+} Transporter CAX1 Increase *CBF/DREB1* Expression and the Cold-Acclimation Response in Arabidopsis

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Transient increases in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) are essential for plant responses to a variety of environmental stimuli, including low temperature. Subsequent reestablishment of $[Ca^{2+}]_{cyt}$ to resting levels by Ca^{2+} pumps and C-REPEAT BINDING FACTOR/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR 1 (Ca^{2+}/H^{+}) antiporters is required for the correct transduction of the signal. We have isolated a cDNA from Arabidopsis that corresponds to a new cold-inducible gene, *RARE COLD INDUCIBLE4* (*RCI4*), which was identical to *CALCIUM EXCHANGER 1* (*CAX1*), a gene that encodes a vacuolar Ca^{2+}/H^{+} antiporter involved in the regulation of intracellular Ca^{2+} levels. The expression of *CAX1* was induced in response to low temperature through an abscisic acid–independent pathway. To determine the function of CAX1 in Arabidopsis stress tolerance, we identified two T-DNA insertion mutants, *cax1-3* and *cax1-4*, that display reduced tonoplast Ca^{2+}/H^{+} antiport activity. The mutants showed no significant differences with respect to the wild type when analyzed for dehydration, high-salt, chilling, or constitutive freezing tolerance. However, they exhibited increased freezing tolerance after cold acclimation, demonstrating that CAX1 plays an important role in this adaptive response. This phenotype correlates with the enhanced expression of *CBF/DREB1* genes and their corresponding targets in response to low temperature. Our results indicate that CAX1 ensures the accurate development of the cold-acclimation response in Arabidopsis by controlling the induction of *CBF/DREB1* and downstream genes.

INTRODUCTION

Ca^{2+} is used by most cells to convert external signals into cytosolic information, which can drive processes that are required for full responses to a particular stimulus. Change in the cytosolic concentration of free Ca^{2+} ($[Ca^{2+}]_{cyt}$) is the basis for Ca^{2+} serving as a second messenger (Sze et al., 2000). Transient increases in $[Ca^{2+}]_{cyt}$ are assumed to mediate a wide variety of biotic and abiotic signals. Biotic stimuli include the hormones abscisic acid (ABA) and gibberellins and fungal elicitors (Bush and Jones, 1988; Knight et al., 1991; McAinsh and Hetherington, 1998). Abiotic signals include red, blue, and UV/B light, each acting via different transduction pathways (Shacklock et al., 1992; Frohnmeyer et al., 1998; Baum et al., 1999), touching, hyperosmotic stress, high salt, and high and low temperatures (Knight et al., 1991, 1997; Gong et al., 1998). Different messages can be encoded by changing the magnitude, duration, localization, or frequency of the $[Ca^{2+}]_{cyt}$ spike (Ghosh and Greenberg, 1995; Sanders et al., 1999). Precise regulation of $[Ca^{2+}]_{cyt}$ is essential to develop adequate responses to specific stimuli (Sze et al., 2000). Increases in $[Ca^{2+}]_{cyt}$ result mainly from Ca^{2+} influx through permeable channels in the plasma membrane and/or Ca^{2+} discharge from internal stores (Piñeros and Tester, 1997; White, 1998). After Ca^{2+} influx, efflux systems to internal stores and out of the cell restore $[Ca^{2+}]_{cyt}$ to unstimulated levels via Ca^{2+} pumps and C-REPEAT BINDING FACTOR/DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR 1 (Ca^{2+}/H^{+}) exchangers (Knight, 2000).

Influx mechanisms have received considerable attention because their regulation is of primary importance to initiate a Ca^{2+} signal (Sanders et al., 2002). By contrast, less consideration has been given to the role of efflux systems in Ca^{2+} signaling. An important question is whether efflux systems help to shape the dynamic form of a Ca^{2+} spike and, thereby, help to define the information encoded in the signal. The diversity of Ca^{2+} pumps and H^{+}-coupled Ca^{2+} cotransporters suggests that these transporters could participate in determining the overall amplitude, duration, and frequency of Ca^{2+} signals. In the case of Ca^{2+} pumps, some studies have provided indications of their role in Ca^{2+} signaling. For instance, the frequency of repetitive Ca^{2+} waves induced by inositol triphosphate increased when the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) pump was overexpressed in *Xenopus* oocytes (Camacho and Lechleiter, 1993). Furthermore, the overexpression of SERCA1, -2a, and -2b isoforms in oocytes provoked the dispersion of Ca^{2+} waves, limiting their propagation in the cytoplasm (Lechleiter et al., 1998). In addition, a deregulated form of ACA4, a vacuolar Ca^{2+}-ATPase from Arabidopsis, has been reported to confer increased tolerance to salt stress when expressed in a yeast mutant with nonfunctioning endogenous

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Ca^{2+} pumps and grown on Ca^{2+}-depleted medium (Geisler et al., 2000). Regarding Ca^{2+}/H^+ antiporters, their role in Ca^{2+} signaling (decoding the appropriate response) has not been the subject of many studies and requires further attention.

CALCIUM EXCHANGER 1 (*CAX1*) was the first plant gene encoding a Ca^{2+}/H^+ antiporter to be cloned. It was identified by screening a cDNA library from Arabidopsis for clones able to complement a yeast mutant defective in vacuolar Ca^{2+} transport (Hirschi et al., 1996). A cDNA from mung bean (*VCAX1*) that showed high sequence identity with *CAX1* was cloned subsequently (Ueoka-Nakanishi et al., 1999). Arabidopsis appears to have up to 10 genes that encode cation/H^+ antiporters closely related to CAX1 (Maser et al., 2001), although the functions of most of them remain unknown. CAX1 seems to be localized in the vacuolar membrane (Cheng et al., 2003) and has high Ca^{2+} transport capacity and low Ca^{2+} affinity (Shigaki et al., 2001). It has been proposed that CAX1 may play a role in reducing cytosolic Ca^{2+} concentration to resting levels after a $[Ca^{2+}]_{cyt}$ increase in response to external stimuli (Hirschi, 1999). Transgenic tobacco plants overexpressing *CAX1* show increased sensitivity to chilling temperatures (Hirschi, 1999), suggesting that it can play a role in plant adaptation to this environmental condition. Mutations in *CAX1* reduce tonoplast Ca^{2+}/H^+ antiport activity and vacuolar-type H^+-translocating ATPase activity while increasing tonoplast Ca^{2+}-ATPase activity (Cheng et al., 2003). *cax1* mutants (*cax1-1* and *cax1-2*) are not affected in their sensitivity to chilling temperatures, but they exhibit altered plant development and perturbed hormone sensitivities (Cheng et al., 2003). The expression of *CAX1* is highly induced in response to exogenous Ca^{2+} and nitrate (Hirschi, 1999; Wang et al., 2000).

By screening a cDNA library from cold-acclimated etiolated seedlings of Arabidopsis with a subtracted probe enriched in cold-induced transcripts (Jarillo et al., 1994), we identified different *RARE COLD INDUCIBLE* (*RCI*) genes (Jarillo et al., 1994; Capel et al., 1997; Llorente et al., 2002). One of these genes, *RCI4*, was identical to *CAX1*. Here, we report that *CAX1* is induced in response to low temperature and that this regulation is mediated through an ABA-independent pathway. The characterization of two T-DNA insertion mutants, *cax1-3* and *cax1-4*, demonstrated that they are not affected in their constitutive capacity to tolerate freezing temperature, dehydration, chilling, or high salt. Interestingly, however, they exhibit an increased ability to cold acclimate, which correlates with an enhanced expression of *CBF*/*DREB1* genes and downstream targets in response to low temperature. These results indicate that CAX1 plays an essential role in the cold-acclimation response by controlling *CBF*/*DREB1* expression, likely by ensuring the proper control of Ca^{2+} homeostasis under low-temperature conditions.

RESULTS

The Expression of *CAX1* Is Induced Transiently in Leaves in Response to Low Temperature

RCI4 was isolated by screening a cDNA library prepared from cold-acclimated (4°C, 7 days) etiolated seedlings of Arabidopsis with a subtracted cDNA probe enriched in cold-induced transcripts. Comparison of the nucleotide sequence of *RCI4* with sequences available in the databases revealed 100% identity with *CAX1*, a gene that encodes a vacuolar Ca^{2+}/H^+ antiporter (Hirschi et al., 1996; Cheng et al., 2003).

The expression of *CAX1* in response to low temperature was first characterized in etiolated seedlings of Arabidopsis. Because *CAX1* is a component of a gene family (Maser et al., 2001), a specific *CAX1* probe was obtained to avoid cross-hybridizations. The specificity of this probe was determined by DNA gel blot analysis (data not shown). RNA gel blot hybridizations indicated that *CAX1* expression was induced transiently in etiolated seedlings, reaching a maximum level after 12 to 24 h of cold treatment (Figure 1A). Expression analysis performed in Arabidopsis plants showed that *CAX1* transcripts also were induced transiently in leaves when exposed to low temperature (Figure 1B). To determine the expression pattern of *CAX1* in different organs of Arabidopsis, total RNA from roots, leaves, stems, flowers, and siliques from unstressed or stressed (4°C, 24 h) plants were subjected to RNA gel blot hybridizations with the specific *CAX1* probe. Results revealed that under unstressed conditions, *CAX1* transcripts were present at low levels in all organs analyzed. In response to low temperature, *CAX1* transcripts did not experience apparent changes in stems and flowers, whereas they increased in leaves and decreased in roots and siliques (Figure 1C). A probe that recognizes the *KIN1* gene from Arabidopsis, the expression of which is induced by cold, dehydration, high-salt, and ABA treatments (Kurkela and Borg-Franck, 1992), was used as a positive control for low-temperature treatments. These data indicate that the expression of *CAX1* is induced specifically in leaves in response to low temperature and that this induction is regulated transiently.

CAX1 Expression Is Regulated Negatively by Dehydration and Not Affected by High Salt or ABA

Because many cold-inducible genes also are responsive to exogenous ABA and osmotic stresses (Thomashow, 1999), the effect of dehydration, high salt, and ABA on *CAX1* mRNA accumulation was analyzed. Given the expression of *CAX1* in response to low temperature (see above), this study was performed in leaves. Although *CAX1* transcript accumulation was unaffected by NaCl and ABA treatments, it was reduced notably below control levels by dehydration (Figure 2A). *KIN1* was used as a positive control for these treatments.

To determine whether the regulation of *CAX1* expression by cold and dehydration is mediated by ABA, the accumulation of corresponding mRNAs in response to both stresses was analyzed in ABA-deficient (*aba1*) and ABA-insensitive (*abi1*) mutants. Figure 2B shows that the expression of *CAX1* in response to low temperature and dehydration was identical in the mutants and in the wild type. Thus, the regulation of *CAX1* expression by low temperature and dehydration was not mediated by ABA. *KIN1* was used as a positive control for these experiments.

Identification of T-DNA Insertion Mutants in *CAX1*

To define the function of CAX1, a reverse-genetics approach was used. Two different transgenic lines containing T-DNA in-

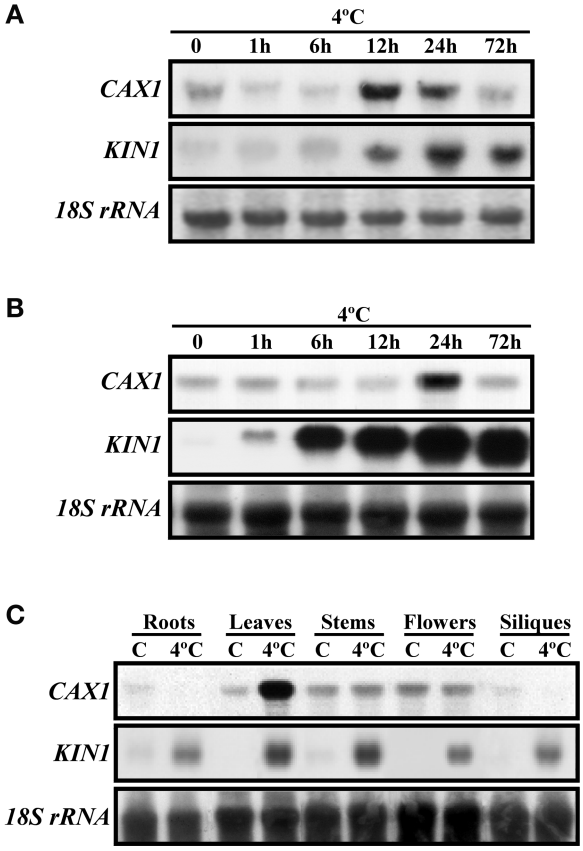


Figure 1. Accumulation of *CAX1* Transcripts in Response to Low Temperature.

(A) RNA gel blot hybridization with total RNA (10 μg) from 4-day-old etiolated seedlings exposed to 4°C for the indicated times.
(B) RNA gel blot hybridization with total RNA (10 μg) from leaves of 3-week-old plants exposed to 4°C for the indicated times.
(C) RNA gel blot hybridization with total RNA (10 μg) from roots, rosette leaves, stems, flowers, and siliques of 8-week-old plants exposed to 4°C for 1 day.
Cold treatment efficacy and equal RNA loading were controlled with probes for *KIN1* and *18S rRNA*, respectively.

sertions in the third intron of *CAX1* were identified (Figure 3A). In both cases, they had single T-DNA insertions (data not shown) that were predicted to disrupt *CAX1* expression. This finding was confirmed by RNA gel blot hybridization (Figure 3B), indicating that these new *CAX1* alleles (*cax1-3* and *cax1-4*) are null or severely hypomorphic. No obvious morphological differences were found between wild-type and mutant plants.

To verify that *cax1-3* and *cax1-4* mutants had reduced CAX1 activity, three different sets of experiments were performed. First, we measured the tolerance of the mutants to Ca^{2+}. It has been reported that yeast mutants defective in VCX1, an antiporter homologous with CAX1 (Hirschi et al., 1996), are more sensitive to Ca^{2+} in the growing medium than is the wild type (Cunningham and Fink, 1996). We reasoned that the absence of CAX1 also might enhance the Ca^{2+} sensitivity of our mutants. Wild-type and *cax1* plants grown on standard medium were transferred to medium supplemented with increasing concentrations of $CaCl_2$ (20 to 100 mM) for 7 days. Ca^{2+} sensitivity was estimated as the percentage of initial fresh weight remaining after treatment. Wild-type and *cax1* plants showed no significant differences in their initial fresh weight values (data not shown). After 7 days, the levels of added $CaCl_2$ that caused a fresh weight loss of 50% in the *cax1* mutants was 80 mM. At this [Ca^{2+}], wild-type plants experienced no loss of fresh weight (Figure 4A), demonstrating that the mutants have enhanced sensitivity to Ca^{2+}. These significant differences were very apparent at the morphological level, with *cax1* mutants displaying a highly stressed phenotype compared with the wild type (Figure 4B).

We also determined the accumulation of total Ca^{2+} in *cax1* mutants. Because the CAX1 antiporter is involved in Ca^{2+} homeostasis (Hirschi, 1999), alterations in its activity are expected to change Ca^{2+} partitioning. As shown in Figure 5A, *cax1-3* and *cax1-4* accumulate 46 and 35% less Ca^{2+} in their leaves, respectively, than does the wild type. Given the high [Ca^{2+}] in the central vacuole and the fact that this occupies 80 to 90% of the whole plant cell (Marty, 1999; Ueoka-Nakanishi et al., 1999), total Ca^{2+} accumulation can be considered an indirect measure of the vacuolar Ca^{2+} content. Therefore, it can be assumed that

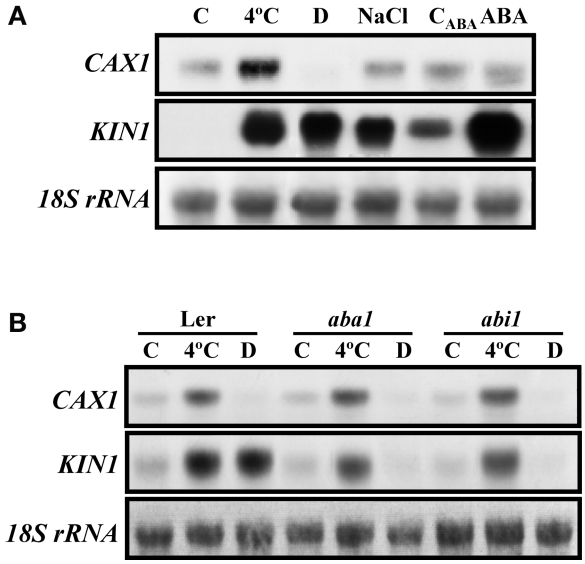


Figure 2. Accumulation of *CAX1* Transcripts in Response to Different Treatments and in ABA-Deficient and ABA-Insensitive Mutants.

(A) RNA gel blot hybridization with total RNA (10 μg) obtained from 3-week-old leaves grown at control temperature (C), exposed for 1 day at 4°C (4°C), dehydrated until they lost 50% of their fresh weight (D), or treated with 250 mM NaCl (NaCl), with 100 μM ABA (ABA), or with the ABA solvent (C_{ABA}).
(B) RNA gel blot hybridization with total RNA (10 μg) obtained from 3-week-old leaves of Landsberg *erecta* (*Ler*), *aba1*, and *abi1* plants grown at control temperature (C), exposed for 1 day at 4°C (4°C), or dehydrated until they lost 50% of their fresh weight (D).
Cold treatment efficacy and equal RNA loading were controlled with probes for *KIN1* and *18S rRNA*, respectively.

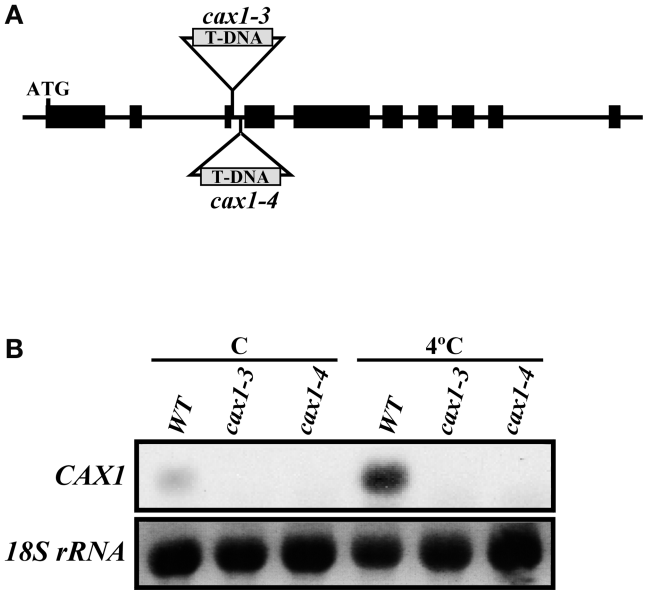


Figure 3. Localization of T-DNAs and Accumulation of *CAX1* Transcripts in *cax1-3* and *cax1-4*.

(A) Scheme of the *CAX1* gene. Large and small boxes represent exons and introns, respectively. ATG indicates the start codon. T-DNA insertions corresponding to *cax1-3* and *cax1-4* are shown. The scheme is not drawn to scale.

(B) RNA gel blot hybridization with total RNA (10 μg) obtained from 3-week-old leaves of Col wild-type (WT), *cax1-3*, and *cax1-4* plants grown at 20°C (C) or exposed for 1 day at 4°C (4°C). Equal RNA loading was controlled with a probe for *18S rRNA*.

cax1 mutants have a lower level of vacuolar Ca^{2+} than do wild-type plants, which suggests that Ca^{2+} partitioning is affected by *cax1* mutations.

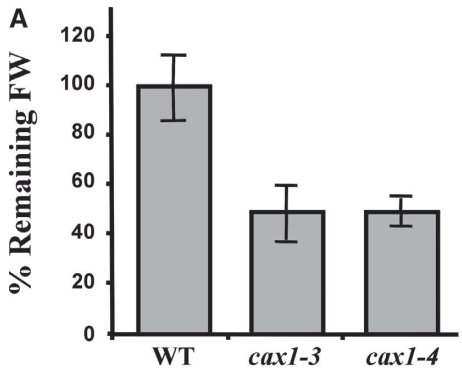
Finally, we measured Ca^{2+}/H^+ antiport activity in vacuole-enriched membrane vesicles isolated from roots of wild-type and *cax1* plants treated previously with 100 mM $CaCl_2$. Figure 5B shows that the activity detected in vesicles prepared from *cax1-3* and *cax1-4* plants is ~50% lower than the activity found in wild-type vesicles. Similar results were obtained with vacuole-enriched membrane vesicles isolated from leaves of plants pretreated with 100 mM $CaCl_2$ (Figure 5B). Together, these results demonstrate that the loss of the *CAX1* transcripts in *cax1-3* and *cax1-4* causes a reduction in Ca^{2+}/H^+ antiport activity.

cax1 Mutants Show an Enhanced Cold-Acclimation Response

Ca^{2+} is a second messenger in plant responses to different abiotic stresses, including high salt, dehydration, and low temperatures (Knight et al., 1991, 1997), and changes in $[Ca^{2+}]_{cyt}$ constitute the basis for encoding such responses (Knight, 2000). To explore a possible role of Ca^{2+}/H^+ antiporters in Ca^{2+}-mediated stress responses, *cax1-3* and *cax1-4* were analyzed for salt, dehydration, chilling, and freezing tolerance.

Salt tolerance was estimated by determining the fresh weight of 3-week-old wild-type and *cax1* plants after growing for 2 weeks in a medium containing 100 mM NaCl. Dehydration was induced by maintaining 3-week-old plants on dry filter paper for 1 day without watering. The rate of dehydration was established as the percentage of initial fresh weight that remained after treatment. Chilling injury was assessed by placing 4-day-old wild-type and mutant seedlings at 4°C for 5 weeks. No significant differences between *cax1* and wild-type plants were found in any case (data not shown).

Tolerance to freezing was examined in 3-week-old wild-type and *cax1* plants before and after cold acclimation (4°C, 7 days). In all cases, freezing tolerance was determined as the percent-



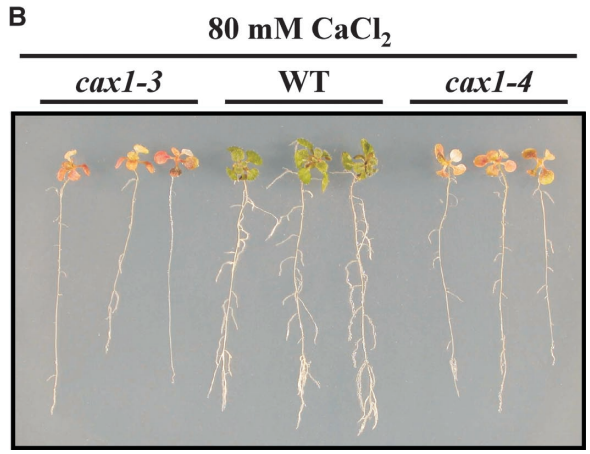


Figure 4. Ca^{2+} Tolerance in *cax1-3* and *cax1-4*.

(A) Tolerance to Ca^{2+} was estimated as the percentage of initial fresh weight (FW) that remained after transferring 3-week-old Col wild-type (WT), *cax1-3*, and *cax1-4* plants to a medium containing 80 mM $CaCl_2$ for 7 days. Data are expressed as means of three independent experiments with 20 plants each. Bars indicate standard errors. In all cases, values obtained from wild-type and mutant plants were significantly different ($P < 0.05$) as determined by Student's *t* test.

(B) Representative wild-type and mutant plants after $CaCl_2$ treatment.

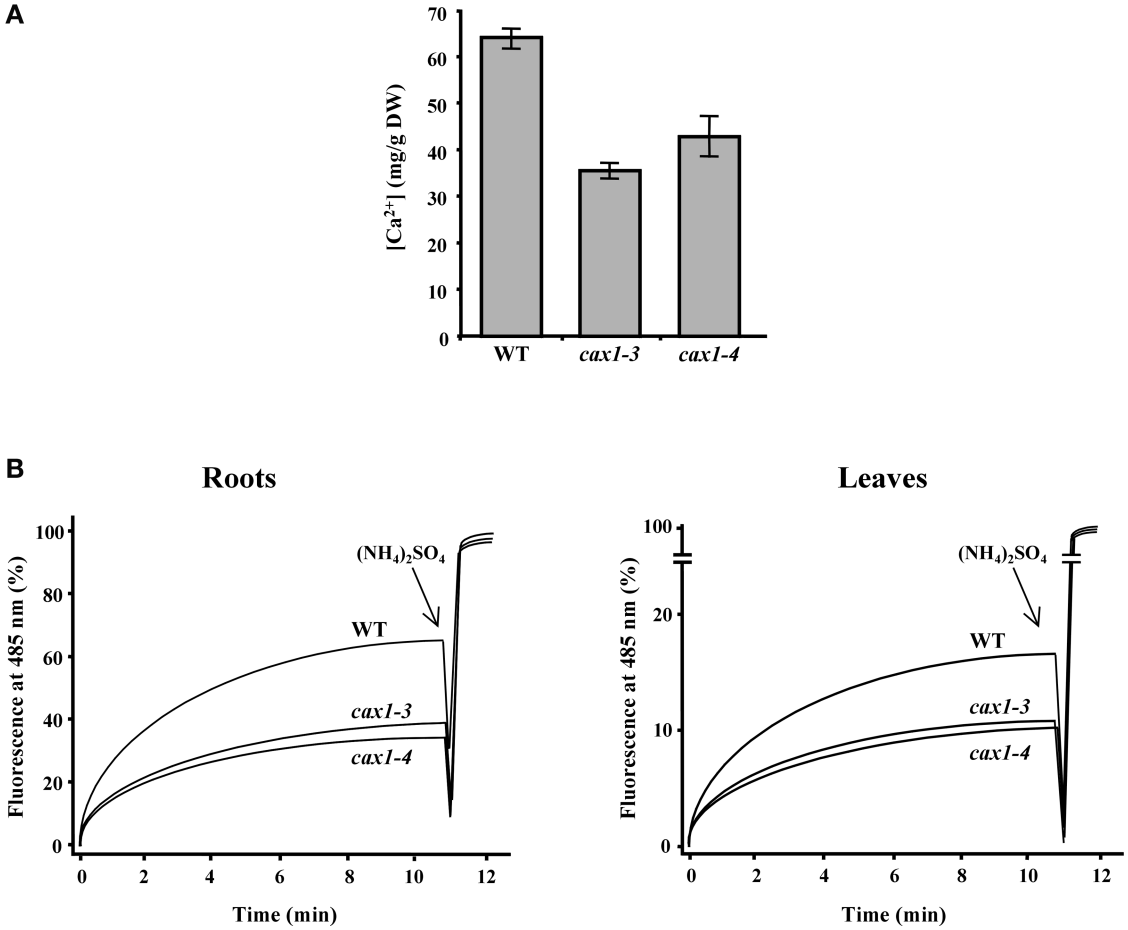


Figure 5. Ca^{2+} Concentration and Ca^{2+}/H^{+} Antiport Activity in *cax1-3* and *cax1-4*.

(A) Ca^{2+} content of leaves from 3-week-old Col wild-type (WT), *cax1-3*, and *cax1-4* plants was determined by atomic absorption spectrophotometry. Data are expressed as means of three independent experiments. Bars indicate standard errors. In all cases, values obtained from wild-type and mutant plants were significantly different ($P < 0.05$) as determined by Student's *t* test. DW, dry weight.

(B) Ca^{2+}/H^{+} antiport activity was measured in vacuole-enriched membrane vesicles from roots and leaves of Col wild-type, *cax1-3*, and *cax1-4* plants pretreated with 100 mM $CaCl_2$ by monitoring the quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence. The percentage of fluorescence recovered during a 12-min time course is represented. At 11 min, $(NH_4)_2SO_4$ (25 mM) was added for full recovery of fluorescence. Representative results from five replicate experiments are shown. Each replicate experiment was performed using independent membrane vesicle preparations.

age of surviving plants after exposure to different freezing temperatures for 1 h. Figure 6A shows that nonacclimated *cax1* and wild-type plants had similar capacities to tolerate freezing. The LT_{50} (temperature that causes 50% lethality) value was −4.6°C in both cases. By contrast, *cax1-3* and *cax1-4* plants displayed significantly greater freezing tolerance than did wild-type plants after cold acclimation (Figure 6B). The LT_{50} values of acclimated wild-type and *cax1* plants were estimated to be −8.0 and −8.9°C, respectively. The increased freezing tolerance manifested by the mutants with respect to the wild type after cold acclimation was very apparent (Figure 6C). Therefore, *cax1* mutations enhanced the freezing tolerance of Arabidopsis after cold acclimation. These data indicate that CAX1 negatively controls the cold-acclimation response.

cax1 Mutants Show Enhanced Induction of *CBF/DREB1* Genes in Response to Low Temperatures

Because *cax1* mutations affected the ability of Arabidopsis to cold acclimate, we investigated whether they also could influence cold-induced gene expression. The accumulation of transcripts corresponding to several genes whose expression is regulated by low temperatures through different pathways (*KIN1* [Kurkela and Franck, 1990], *COR47* [Gilmour et al., 1992], *RAB18* [Lang and Palva, 1992], *LTI78* [Nordin et al., 1993], and *AtP5CS2* [Strizhov et al., 1997]) was examined by RNA gel blot analysis. Under control conditions, wild-type and mutant plants always showed very similar expression patterns (Figure 7A). In response to cold, the levels of all messengers increased in both

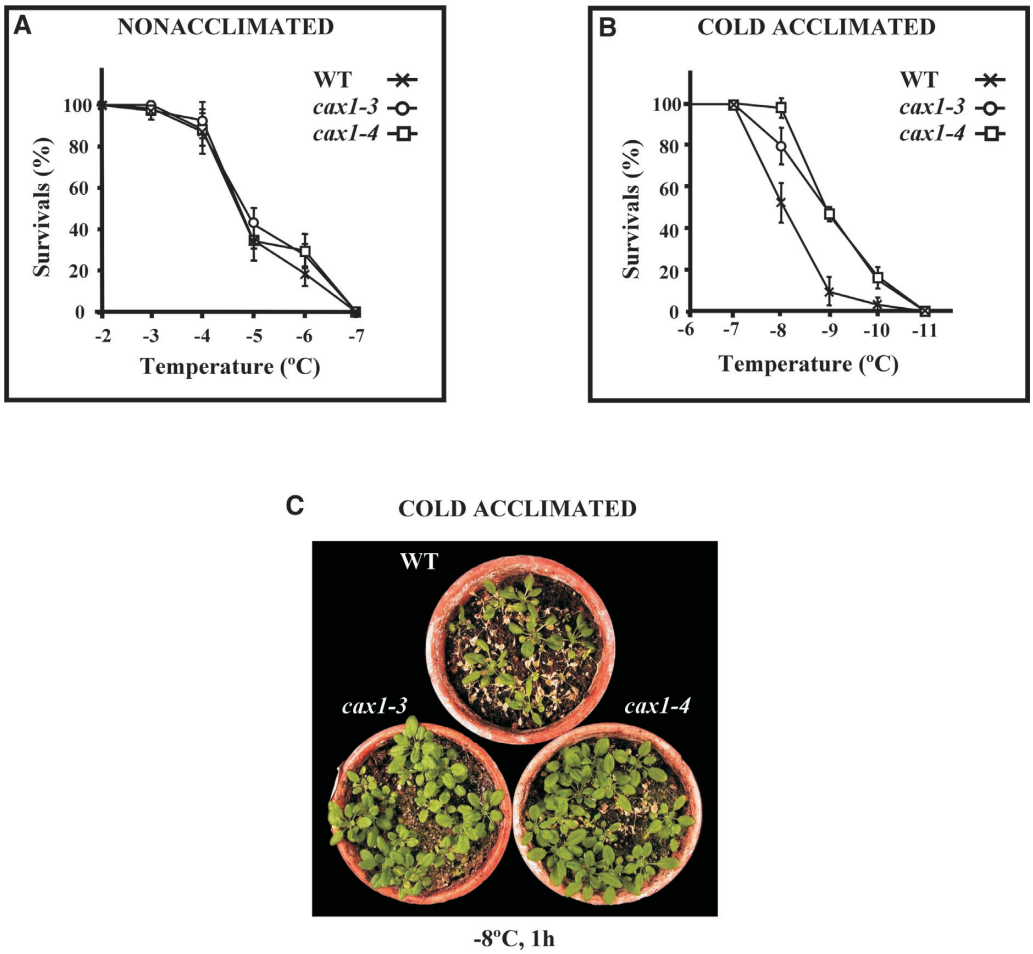


Figure 6. Freezing Tolerance of *cax1-3* and *cax1-4*.

Three-week-old Col wild-type (WT), *cax1-3*, and *cax1-4* plants were exposed to different freezing temperatures for 1 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 2 weeks of recovery under unstressed conditions. In **(A)** and **(B)**, data are expressed as means of three independent experiments with 50 plants each. Bars indicate standard errors.

(A) Freezing tolerance of nonacclimated wild-type and *cax1* plants.

(B) Freezing tolerance of cold-acclimated (4°C, 7 days) wild-type and *cax1* plants.

(C) Representative cold-acclimated wild-type and *cax1* plants after being exposed at −8°C for 1 h and recovering for 2 weeks at 20°C.

wild-type and mutant plants (Figure 7A). However, in *KIN1*, *LTI78*, *COR47*, and *AtP5CS2*, these levels were considerably higher in *cax1* mutants than in the wild type (Figure 7A). Interestingly, all of these genes are considered to be targets of the CBF/DREB1 transcriptional activators (Gilmour et al., 2000). The cold induction of *RAB18*, which has not been described as a CBF/DREB1 target, was similar in mutants and the wild type (Figure 7A).

The overexpression of *CBF/DREB1* genes in transgenic Arabidopsis activates the expression of downstream *CBF/DREB1* targets, which in turn promotes freezing tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). Thus, one hypothesis is that the increased induction of *CBF/DREB1* target genes displayed by *cax1* mutants in response to low temperature may be caused by an increased induction of the *CBF/DREB1* genes themselves. RNA gel blot experiments using *CBF1/DREB1B*-, *CBF2/DREB1C*-, and *CBF3/DREB1A*-specific probes showed that the cold induction of the three genes was enhanced by the *cax1* mutations (Figure 7B). The cold induction of *AtERF4* and *AtERF5*, two genes that encode transcription factors that are regulated by low temperature and ethylene (Fujimoto et al., 2000), was not affected in *cax1* plants (Figure 7B). Together, these results indicate that CAX1 acts to negatively control the expression of *CBF/DREB1* genes and their corresponding downstream targets.

DISCUSSION

This work describes the functional characterization of CAX1, a vacuolar Ca^{2+}/H^{+} antiporter, in response to abiotic stresses.

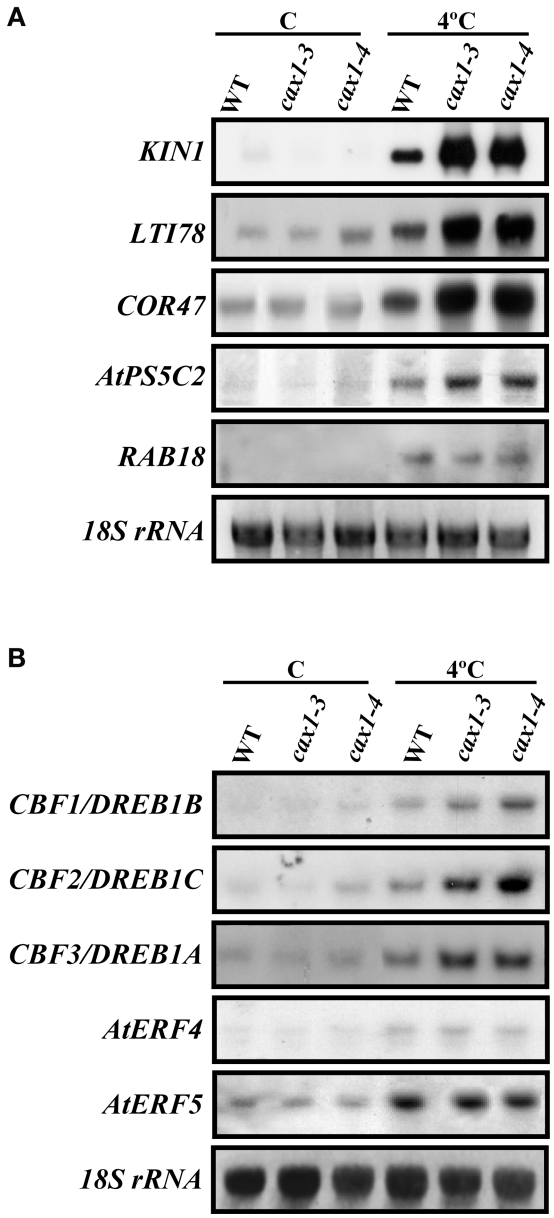


Figure 7. Transcript Levels of Cold-Inducible Genes in *cax1-3* and *cax1-4*.

(A) RNA gel blot hybridization with total RNA (10 μg) obtained from 3-week-old Col wild-type (WT), *cax1-3*, and *cax1-4* plants grown under control conditions (C) or exposed for 1 day at 4°C (4°C).

(B) RNA gel blot hybridization with total RNA (20 μg) obtained from 3-week-old Col wild-type, *cax1-3*, and *cax1-4* plants grown under control conditions (C) or exposed for 1 h at 4°C (4°C).

Equal RNA loading was controlled with a probe for *18S rRNA*.

Expression analysis revealed that *CAX1* was subjected to an intricate regulation. Under control conditions, *CAX1* transcripts accumulated at basal levels in all organs analyzed. In response to low temperature, these levels increased transiently in etiolated seedlings and leaves of adult plants and decreased in roots and siliques. Furthermore, *CAX1* expression was inhibited by dehydration but was not affected by ABA and high-salt treatments. Although the significance of this expression pattern is not clear at present, it indicates that during Arabidopsis development and in response to different adverse environmental situations, the regulation of *CAX1* is accomplished through several ABA-independent signal transduction pathways. In addition, *CAX1* has been reported to be induced in response to exogenous Ca^{2+} (Hirschi, 1999) and nitrate (Wang et al., 2000). Recent studies have shown that *CAX1* also is regulated at the post-transcriptional level by an autoinhibitory N-terminal region (Pittman et al., 2002). The implication of this regulation in CAX1 function remains to be determined.

We have identified two T-DNA insertion mutants, *cax1-3* and *cax1-4*, that are null or highly hypomorphic for *CAX1* expression as judged by mRNA levels. Both mutants accumulated lower levels of total Ca^{2+} than did wild-type plants and were hypersensitive to Ca^{2+} in the growth medium, and their Ca^{2+}/H^+ antiport activity was reduced by 50%. *cax1-3* and *cax1-4* plants responded differentially to salt, dehydration, chilling, and freezing treatments. In fact, after being exposed to high salt, dehydration, or chilling temperatures, they showed no significant differences with respect to the wild type. These results are identical to those reported by Cheng et al. (2003) after characterizing the *cax1-1* and *cax1-2* alleles. Compared with the wild type, *cax1-3* and *cax1-4* displayed no differences in their constitutive freezing tolerance. However, their freezing tolerance after cold acclimation was significantly greater than that of cold-acclimated wild-type plants, indicating that CAX1 negatively controls the cold-acclimation response. Interestingly, it was reported that transgenic tobacco plants overexpressing *CAX1* are hypersensitive to chilling stress, suggesting a role for this Ca^{2+}/H^+ antiporter in the plant response to low temperatures (Hirschi, 1999). Our results demonstrate that CAX1 does not play a general role in the development of Arabidopsis tolerance to abiotic stresses but has a specific function in cold acclimation, which is consistent with the fact that *CAX1* expression is induced specifically in response to low temperature. Although the origin of this specificity remains unknown, it is tempting to speculate that it is related to the cold-induced Ca^{2+} signature.

The question arises of why *cax1* mutants have an increased ability to cold acclimate. Expression analysis revealed that this capability is correlated with an enhanced expression of the *CBF/DREB1* genes and their corresponding downstream targets in response to low temperature. The expression of other cold-inducible genes that have not been reported to be regulated by CBF/DREB1 transcriptional activators was not altered in cold-treated *cax1* plants. The *CBF/DREB1* genes encode a small family of transcriptional activators that have been reported to play important roles in freezing tolerance and cold acclimation in Arabidopsis (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). They are induced specifically and transiently by low temperature, and this induction precedes that of downstream cold-inducible genes, which results in an increase in freezing tolerance (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). The data presented here indicate that CAX1 negatively controls the cold induction

of the CBF/DREB1 regulon and, thereby, the cold-acclimation response.

A working model for CAX1 function in low-temperature signaling during cold acclimation is presented in Figure 8. When plants are exposed to low temperature, a transient increase in $[Ca^{2+}]_{cyt}$ is induced, generating a Ca^{2+} signal (Knight et al., 1991). This signal, conveniently decoded and transduced by downstream effectors such as AtCBL1 (Kudla et al., 1999), CDPKs (Martin and Busconi, 2001), or CIPK3 (Kim et al., 2003), induces the expression of the *CBF/DREB1* genes as well as that of *CAX1*. Subsequently, CBF/DREB1 transcription factors activate the expression of downstream target genes, which in turn, together with other cold-inducible genes regulated through additional signaling pathways, promote freezing tolerance. Consistent with this part of the model, the expression of some *CBF/DREB1* target genes under low-temperature conditions has been reported to be mediated by the cold-induced increase in $[Ca^{2+}]_{cyt}$ (Knight et al., 1996; Tähtiharju et al., 1997; Townley and Knight, 2002). Moreover, the induction of *CBF/DREB1* genes in response to low temperature is inhibited in the presence of the Ca^{2+} chelator EGTA, suggesting that their expression also is dependent on cold-induced $[Ca^{2+}]_{cyt}$ increases (R. Catalá and J. Salinas, unpublished results). The expression of *CAX1*, which has been reported to be induced by Ca^{2+} (Hirschi, 1999) in cooperation with other Ca^{2+} transporters (i.e., Ca^{2+}/H^+ exchangers and Ca^{2+}-ATPases), would contribute to the reestablishment of resting levels of cytosolic Ca^{2+} and *CBF/DREB1* expression. In agreement with this notion, Townley and Knight (2002) reported that the overexpression of *CaM3*, an Arabidopsis gene that encodes the calmodulin CaM3, inhibits the cold induction of *LTI78* and *KIN1*, and they suggested that this inhibition is caused by the activation of a Ca^{2+}-ATPase by CaM3. Thus, we propose that CAX1 plays a role in controlling the correct restoration of $[Ca^{2+}]_{cyt}$ levels after the transient increase induced by the cold signal, which is essential for an adequate response, including the accurate expression of *CBF/DREB1*. It is conceivable that mutations that affect CAX1 activity may perturb that restoration, leading to an increase in $[Ca^{2+}]_{cyt}$ that under cold conditions would result in the overaccumulation of *CBF/DREB1* transcripts and the subsequent increase in the induction of target genes and freezing tolerance.

The model presented suggests that Ca^{2+}/H^+ antiport activity may increase in Arabidopsis in response to low temperature. If so, this increase would be reduced in *cax1* mutants. The possibility exists, however, that *CAX1* is induced in response to low temperature just to maintain steady state levels of CAX1 activity when Arabidopsis copes with this environmental constraint. Under our experimental conditions, no significant differences in Ca^{2+}/H^+ antiport activity were detected among vacuole-enriched membrane vesicles from wild-type plants grown under control conditions and vesicles isolated from wild-type plants exposed to 4°C (Figure 9A). Similarly, we detected no differences between vesicles isolated from the *cax1* mutants before and after low-temperature treatment (Figure 9A). Nevertheless, these results cannot exclude the possibility that Ca^{2+}/H^+ antiport activity could be induced during cold acclimation. In fact, Ca^{2+}/H^+ antiport activity can be measured directly in vacuole-enriched membrane vesicles from Arabidopsis only after pretreatment of the plants with 100 mM $CaCl_2$ (Pittman et al., 2002), which could mask the cold induction. When wild-type or mutant plants were pretreated with 50 mM $CaCl_2$, the Ca^{2+}/H^+ antiport activity measured was lower in all cases, but no cold induction was detected (Figure 9B).

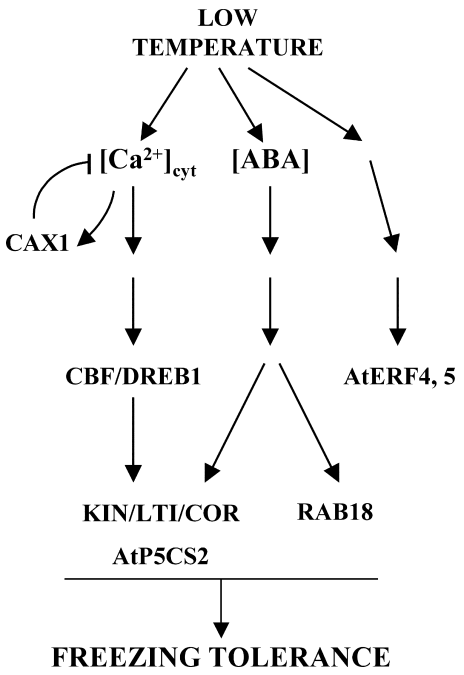


Figure 8. Hypothetical Model for CAX1 Function in Low-Temperature Signaling.

In summary, we demonstrate that CAX1 participates in the development of the cold-acclimation response in Arabidopsis by negatively controlling the induction of *CBF/DREB1* and downstream genes, probably by ensuring the proper control of Ca^{2+} homeostasis in response to low temperature. Two other negative effectors of the cold-acclimation response, PP2C and HOS1, were identified recently (Lee et al., 2001; Tähtiharju and Palva, 2001), indicating that this adaptive process is subject to a precise control that includes active negative regulation. Although further studies are needed to address the participation of other Ca^{2+} transporters in low-temperature signaling and the mechanisms by which $[Ca^{2+}]_{cyt}$ regulates *CBF/DREB1* expression, our findings provide new insights to advance our understanding of the molecular basis of the cold-acclimation response.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana ecotypes Columbia (Col) and Landsberg *erecta* and abscisic acid (ABA)–deficient (*aba1-1*) and ABA-insensitive (*abi1-1*) mutants were used in this study. Etiolated seedlings were obtained from seeds sown under sterile conditions in Petri dishes containing mineral nutrient solution (Haughn and Somerville, 1986) solidified with 0.8% (w/v) agar. Etiolation

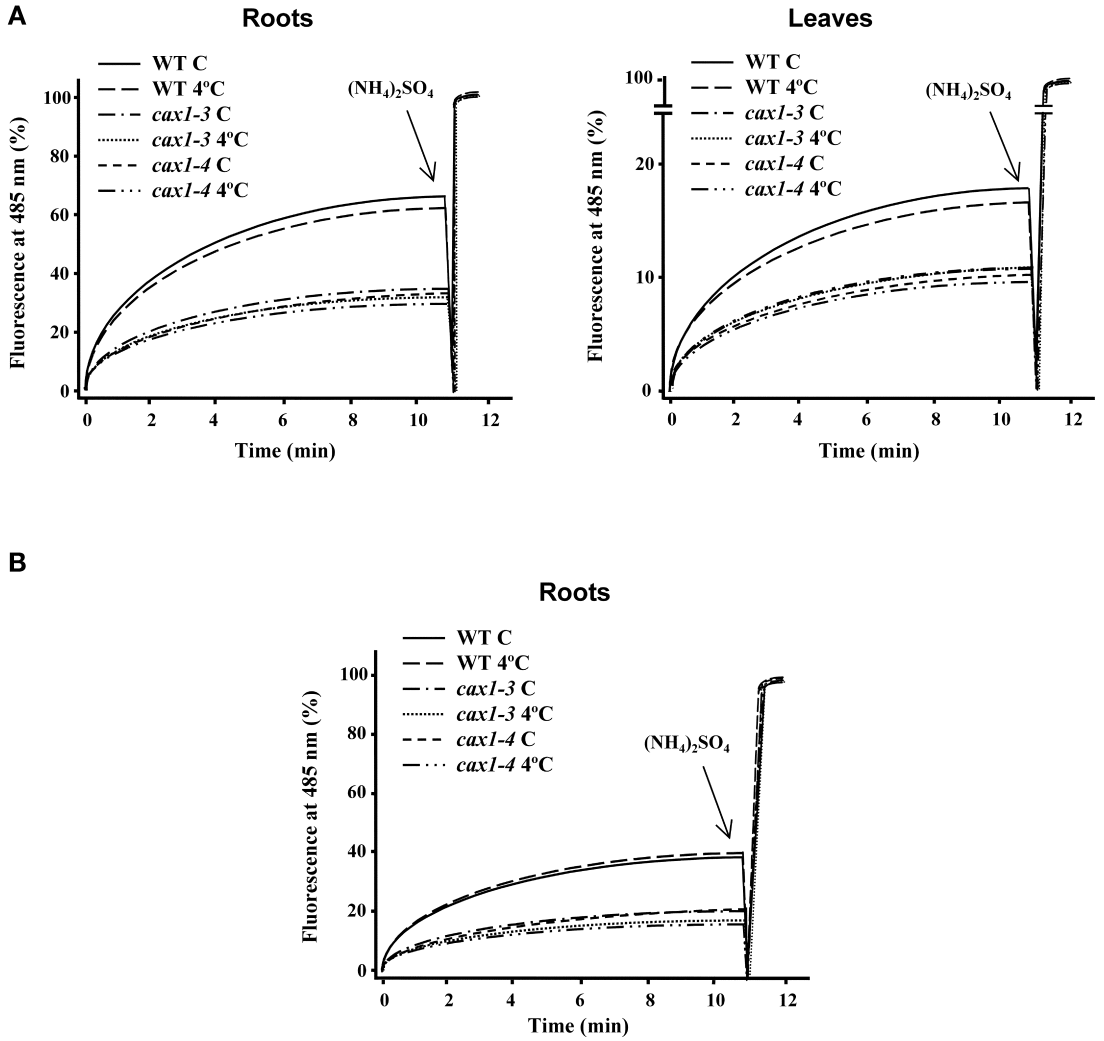


Figure 9. Ca^{2+}/H^{+} Antiporter Activity in Wild-Type, *cax1-3*, and *cax1-4* Plants in Response to Low Temperature.

Ca^{2+}/H^{+} antiport activity was measured in vacuole-enriched membrane vesicles from roots and leaves of Ca^{2+}-treated Col wild-type (WT), *cax1-3*, and *cax1-4* plants grown at 20°C (C) or exposed for 1 day at 4°C (4°C) by monitoring the quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence. The percentage of fluorescence recovered during a 12-min time course is represented. At 11 min, $(NH_4)_2SO_4$ (25 mM) was added for full recovery of fluorescence. Representative results from three replicate experiments are shown. Each replicate experiment was performed using independent membrane vesicle preparations.

(A) Antiport activity in vesicles from roots and leaves of plants pretreated with 100 mM $CaCl_2$.

(B) Antiport activity in vesicles from roots of plants pretreated with 50 mM $CaCl_2$.

was achieved by covering plates with aluminum foil. Plants for Ca^{2+}-, dehydration-, chilling-, and salt-tolerance assays, as well as for the preparation of vacuole-enriched membrane vesicles, were obtained from seeds sown under sterile conditions in Petri dishes containing GM medium (MS medium [Murashige and Skoog, 1962] supplemented with 1% sucrose) solidified with 0.8% (w/v) agar. Soil-grown plants were obtained by sowing seeds in pots containing a mixture of organic substrate and vermiculite (3:1, v/v) and irrigating the pot with water and mineral nutrient solution medium once per week. Unless specified otherwise, in all cases, 3-week-old plants grown at 20°C under long-day photoperiods (16 h of cool-white fluorescent light; PPFD of 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) were used for experiments.

Low-temperature treatments were performed by transferring 4-day-old etiolated seedlings and plants to a growth chamber set to 4°C for different periods in the dark or under the light and photoperiodic conditions described above. Low-temperature treatments in different organs were performed in 8-week-old plants. Dehydration was induced by removing plants from soil and allowing them to lose 50% of their initial fresh weight. Salt stress was accomplished by watering plants with 250 mM NaCl for 24 h. For ABA treatment, plants were sprayed with 100 μM ABA. The ABA stock solution (100 mM) was prepared in DMSO, and control treatments were given with water containing the same final concentration as the ABA solvent. After the treatments, seedlings and plants used for RNA gel blot hybridizations were frozen immediately in liquid N_2 and stored at −80°C until their use.

Tolerance to Ca^{2+} and NaCl was checked by transferring plants to new Petri dishes containing agar medium supplemented with different

concentrations (20, 40, 60, 80, and 100 mM) of $CaCl_2$ or 100 mM NaCl. Ca^{2+} and NaCl tolerance were estimated based on the percentage of initial fresh weight remaining after 7 and 14 days of treatment, respectively. Dehydration tolerance was investigated by removing plants from the medium, placing them on dry filter paper, and allowing them to develop for 1 day without watering. The rate of dehydration was estimated as the percentage of initial fresh weight remaining after treatment. Chilling was imposed by placing Petri dishes containing 4-day-old seedlings at 4°C under long-day photoperiods (35 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 5 weeks. Tolerance to chilling was determined as the percentage of plants surviving after the treatment. Freezing tolerance was analyzed by exposing nonacclimated and cold-acclimated (4°C, 7 days) plants to 4°C for 30 min in darkness and subsequently allowing the temperature to decrease at a rate of 2°C/h. The final desired freezing temperature was maintained for 1 h, and then the temperature was increased again to 4°C at the same rate. After thawing at 4°C for 12 h in the dark, plants were returned to the original growth conditions. Tolerance to freezing was determined as the percentage of plants surviving after 2 weeks of recovery in control conditions.

Preparation of Vacuole-Enriched Membrane Vesicles and Ca^{2+}/H^+ Antiport Activity Assays

Vacuole-enriched membrane vesicles were prepared from root or leaf tissues obtained from plants pretreated with 50 or 100 mM $CaCl_2$ for 18 h before harvest, according to Cheng et al. (2003). Ca^{2+}/H^+ antiport activity was measured by monitoring the formation and dissipation of pH gradients across membrane vesicles, essentially as described by Schumaker and Sze (1986). Vesicles (50 μg) were incubated in a buffer containing 0.3 M sorbitol, 5 mM 1,3-bis(Tris[hydroxymethyl]methylamino) propane–Mes, pH 7.6, 1 mM Mg^{2+}-ATP, 100 mM KCl, 3 mM $MgSO_4$, and 100 nM validomycin. Ca^{2+}-dependent pH gradient dissipation was initiated by the addition of 10 μM $CaCl_2$ and measured by monitoring the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (Molecular Probes, Eugene, OR) for 12 min. Fluorescence quenching was monitored in a thermostatted cell at 25°C using a fluorescence spectrometer (model RF-540; Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 415 and 485 nm, respectively. After 11 min, $(NH_4)_2SO_4$ was added to a final concentration of 25 mM to dissipate the pH gradient and determine total fluorescence recovery.

Molecular Biology Methods

CAX1 was isolated by screening a cDNA library prepared from cold-acclimated etiolated seedlings of Arabidopsis with a subtracted cDNA probe enriched in cold-induced transcripts as described by Jarillo et al. (1994). Databases were searched for sequence similarities using the Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (Altschul et al., 1997).

Total RNA was isolated as described by Logemann et al. (1987). Restriction digestion, cloning, and DNA and RNA gel blot hybridization were performed according to standard procedures (Sambrook et al., 1989). The *CAX1*-specific probe was obtained by PCR from genomic DNA of Arabidopsis ecotype Col using the primers 5′-TGTCGTCACTGCAACAGGAGGA-3′ and 5′-GACATTCATAGATAGTTCATTGC-3′. The specific probes for *KIN1* (Kurkela and Borg-Franck, 1992), *LTI78* (Nordin et al., 1993), *COR47* (Gilmour et al., 1992), *AtP5CS2* (Strizhov et al., 1997), *RAB18* (Lang and Palva, 1992), *CBF1/DREB1B*, *CBF2/DREB1C*, and *CBF3/DREB1A* (Medina et al., 1999), and *AtERF4* and *AtERF5* (Fujimoto et al., 2000) were obtained by PCR from genomic DNA of ecotype Col with the following primers: *KIN1* (5′-GGCACCACACTCCCTTTAG-3′ and 5′-GAATATAAGTTTGGCTCGTC-3′); *LTI78* (5′-ACCATAATACATCAAAGAC-3′ and 5′-CGGGATTTGACGGAGAACC-3′); *COR47* (5′-TGGCTGAGGAGTACAAGAACAACG-3′ and 5′-CTTCACCGATCCAACAGCTCTTCT-3′); *AtP5CS2* (5′-CGCGGATCCCTCGTTCTCTCGTGTTTTCG-3′ and 5′-GCGGATCCGACATCAGCAGAGAAAGAGAG-3′); *RAB18* (5′-CCCCTGCAGTCCATATCCGAAACCGGA-3′ and 5′-GGGGAATTCACGTACCGAGCTAGAGCTGG-3′); *CBF1/DREB1B* (5′-GTGACGTGTCGCTTTGGAGTTAC-3′ and 5′-GTGAAGCAAAGAAGTAGAAAACG-3′); *CBF2/DREB1C* (5′-TCGAGGGAGATGATGACGTGTCCT-3′ and 5′-TATTTTGATTTGTTGCTTATGG-3′), *CBF3/DREB1A* (5′-CGACGGCGATGATGACGACGTA-3′ and 5′-GCATTTAAGAATAGCCCACACT-3′); *AtERF4* (5′-TATCCGAGAATGGCCAAG-3′ and 5′-GACTGAGAGAGAGAGAGG-3′); and *AtERF5* (5′-ATGGCGACTCCTAACGAAG-3′ and 5′-CGTCAGCATACACATCGTTC-3′). Equal RNA loading was monitored using an EcoRI fragment from the *18S rRNA* as a probe (Tremousaygue et al., 1992). RNA samples from each experiment were analyzed on at least two independent blots, and each experiment was repeated at least twice.

Isolation of T-DNA Insertion Mutants in *CAX1*

A total of 60,000 Arabidopsis T-DNA insertion lines (J.M. Alonso and J.R. Ecker, unpublished data) were screened for *cax1* mutants by PCR. Specific primers for the left and right borders of the T-DNA and for *CAX1* (5′-CTTTGGGGATTCGGCAATGCATGAT-3′ and 5′-CGGATATTAGCGATTCCTCCACAGAA-3′) were used to identify mutant lines.

Total Ca^{2+} Content Analysis

Leaves from 3-week-old Col, *cax1-3*, and *cax1-4* plants watered with 2 mM $CaCl_2$ for 2 days were frozen in liquid N_2 and lyophilized. At least 0.25 mg of these materials was treated with 0.5 mL of 1 M HCl for 1 day. Supernatants were collected after centrifugation at 13,000*g* for 10 min, and total Ca^{2+} content was determined by atomic absorption spectrophotometry (model 2380; Perkin-Elmer, Norwalk, CT).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact J. Salinas, salinas@inia.es.

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