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Low-Temperature Signal Transduction: Induction of Cold Acclimation-Specific Genes of Alfalfa by Calcium at 25°C

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To study the role of calcium in cold acclimation, we examined the relationship between calcium influx and accumulation of transcripts of two *cas* (cold acclimation–specific) genes of alfalfa, *cas15* and *cas18*. Whereas a decline in temperature from 25 to 15°C had little effect on the influx of extracellular $^{45}Ca^{2+}$, an increasing influx was observed when the temperature was lowered further. The influx of $^{45}Ca^{2+}$ at 4°C was nearly 15 times greater than at 25°C. The addition of calcium chelators or of calcium channel blockers, which have been shown to prevent cold acclimation, inhibited the influx of extracellular $^{45}Ca^{2+}$ as well as the expression of *cas* genes at 4°C. The addition of a calcium ionophore or a calcium channel agonist to nonacclimated cells caused the influx of extracellular $^{45}Ca^{2+}$ and induced the expression of *cas* genes at 25°C. These results suggest that a cold-induced calcium influx plays an essential role in cold acclimation. To further study the role of calcium, we isolated two sequences corresponding to calcium-dependent protein kinases. The transcript level of one of them was markedly upregulated at 4°C. We propose a sequence of signaling events that is likely to occur early during cold acclimation and leads to the expression of *cas* genes and the development of freezing tolerance.

INTRODUCTION

As winter approaches, many plants acquire freezing tolerance during exposure to low, nonfreezing temperatures (cold acclimation) (Levitt, 1980; Guy, 1990). In the laboratory, freezing tolerance can be induced by cold acclimation at 2 to 5°C over a period of days or weeks. During cold acclimation, various metabolic changes occur and specific genes are expressed (Guy, 1990). Many cold-induced genes have been isolated and their expression characterized in several plant species (see Guy, 1990). Despite this progress, the mechanisms by which low-temperature signals are perceived and transduced into biochemical responses are still not known. A variety of experimental approaches has shown that signaling for the induction of some of the genes depends on low temperature but not on abscisic acid (ABA) (Mohapatra et al., 1989; Nordin et al., 1993; Dallaire et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). From their analysis of *cis* elements of stress-induced genes of Arabidopsis, Yamaguchi-Shinozaki and Shinozaki (1994) have proposed the existence of a second messenger for ABA-independent stress signaling. The identity of this proposed second messenger, however, is not yet known.

The cold-induced genes of alfalfa that we have reported (Monroy et al., 1993a; Wolfraim and Dhindsa, 1993; Wolfraim et al., 1993) are not induced by ABA or other stresses (Mohapatra et al., 1989) and are thus cold acclimation–specific (*cas*) genes. The level of expression of *cas* genes is strongly and positively correlated ($r \geq 0.965$) with the degree of cold-induced freezing tolerance in several cultivars of alfalfa (Mohapatra et al., 1989), making them reliable reporters for studies on low-temperature signaling. Moreover, our recent studies have shown a correlation between calcium availability, *cas* gene expression, and the development of freezing tolerance (Monroy et al., 1993b), implicating calcium as an important second messenger in low-temperature signal transduction.

Calcium is known to function as a second messenger in a variety of organisms, including plants. Cytosolic calcium has been shown to be regulated in response to a number of stimuli (Trewavas and Gilroy, 1991; Gilroy et al., 1993), and basic components of a calcium signaling system, such as calcium transporters, calmodulin (CaM), CaM-dependent enzymes, and calcium-dependent protein kinases (CDPKs), have been found in plants (Trewavas and Gilroy, 1991; Roberts and Harmon, 1992; Bush, 1993). The involvement of calcium in low-temperature signaling during cold acclimation can be inferred indirectly from the observed transient changes of cytosolic calcium in response to cold shock (Knight et al., 1991) and from electrophysiological studies of the modulation of calcium channel activity by low temperature (Ding and Pickard, 1993). However, more significant evidence for the involvement of calcium as a second messenger comes from the observation that calcium chelators, calcium channel blockers, and inhibitors of CDPKs prevent cold acclimation (Monroy et al., 1993b). Thus, it has been proposed that calcium functions as a second messenger in response to chilling (Minorsky, 1989; Knight et al., 1991) and cold acclimation (Dhindsa et al., 1993; Ding and Pickard, 1993; Monroy et al., 1993b).

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To understand the role of a calcium signaling system during cold acclimation of alfalfa, we have focused our attention on the early stages of cold acclimation. Specifically, we have monitored the influx of extracellular $^{45}Ca^{2+}$ into protoplasts as a function of temperature and the effects of chemicals that modify this calcium influx on *cas* gene expression. We used the transcript levels of two *cas* genes, *cas15* and *cas18*, as reporters of the relative role of calcium in low-temperature signal transduction. *cas15* encodes a putative nuclear protein, and its induction by cold is rapid and independent of continued protein synthesis (Monroy et al., 1993a). The other gene, *cas18*, encodes a protein related to the late embryogenesis abundant/ABA-responsive/dehydrin family of proteins, and its cold induction is relatively slower (Wolfraim et al., 1993). We show that (1) temperatures generally used to induce cold acclimation caused a greater than 15-fold increase in the influx of extracellular $^{45}Ca^{2+}$ into the protoplast; (2) inhibitors of this cold-induced calcium influx also inhibited *cas* gene expression at 4°C; (3) the need for the influx of extracellular calcium in the induction of *cas* genes during cold acclimation is transitory; and (4) chemicals inducing the influx of extracellular $^{45}Ca^{2+}$ at 25°C also induced *cas* genes at 25°C but that the *cas* transcripts became unstable after a few hours. We conclude that the influx of extracellular calcium alone is sufficient to induce *cas* genes but that low temperature is required to promote the stability of *cas* transcripts. In addition, to further extend our understanding of the signaling role of calcium, we monitored the expression of genes encoding calcium binding proteins and found two CDPK genes whose expression is regulated by low temperature.

RESULTS

Low Temperature–Induced Calcium Influx

In plants, the major calcium storage sites are the cell wall and the vacuole (Trewavas and Gilroy, 1991; Bush, 1993). We have previously shown that exogenously added calcium chelators inhibit cold acclimation (Monroy et al., 1993b). Because the chelators are not expected to enter the cell, we considered the possibility that cell wall calcium may be involved. We first examined the influx of extracellular $^{45}Ca^{2+}$ into alfalfa protoplasts at different temperatures. Alfalfa protoplasts are suitable for such experiments because they are devoid of calcium-rich cell walls.

Figure 1A shows that as the temperature declined below 15°C, the influx of extracellular $^{45}Ca^{2+}$ into protoplasts increased, reaching a maximum at 4 to 7°C. This maximum was 15 to 20 times greater than at 25°C. The efflux of preloaded $^{45}Ca^{2+}$ was also examined. Preloading was carried out at 25°C for 20 min, at which time the influx was 200 pmol cm^{-2}. The efflux was then allowed at either 4 or 25°C for 20 min. The efflux was 12 pmol cm^{-2} at 25°C and 9 pmol cm^{-2} at 4°C. In relation to the influx during preloading, the efflux was 6% at 25°C and 4.5% at 4°C. We concluded that the observed cold-induced increase in influx was not due to inhibition of the efflux through calcium pumps.

We then examined the time course of the influx of extracellular $^{45}Ca^{2+}$ into protoplasts that were maintained at 4 or 25°C. The results are shown in Figure 1B. The kinetics of the $^{45}Ca^{2+}$ influx were similar to those reported by others for carrot protoplasts (Rincon and Boss, 1987; Graziana et al., 1988). The influx was rapid during the first 15 to 20 min and slowed considerably to a lower steady state rate thereafter. The influx was nearly 20-fold greater at 4 than at 25°C during the first 15 to 20 min.

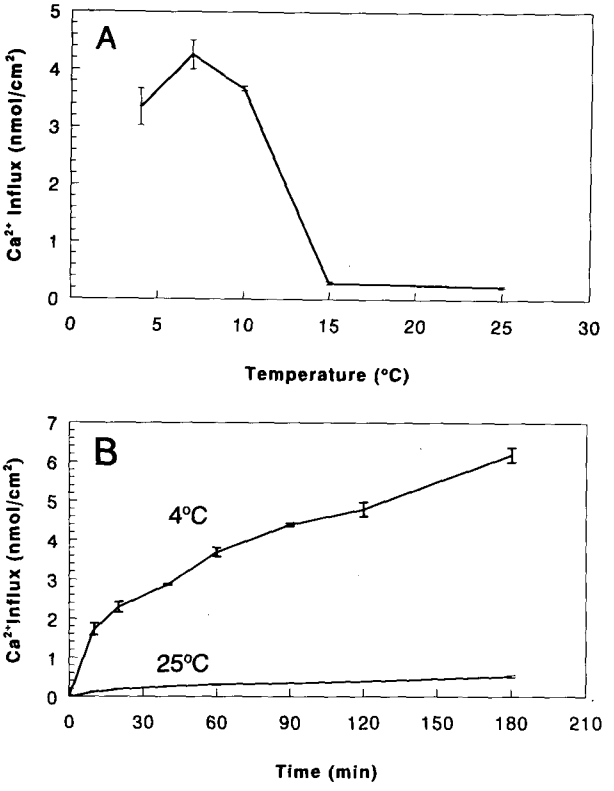

Figure 1. The Effect of Temperature on the Influx of Extracellular $^{45}Ca^{2+}$ into Alfalfa Protoplasts.

(A) Protoplasts prepared from cell suspension cultures incubated with 0.1 mM $^{45}CaCl_2$ at the temperatures indicated. The influx time was 20 min.

(B) Time course of $^{45}Ca^{2+}$ influx into alfalfa protoplasts at 4 and 25°C. At the termination of uptake, unincorporated $^{45}CaCl_2$ was removed by cation exchange chromatography. Radioactivity was then determined by liquid scintillation spectrometry. Each datum point is the mean of three replicates. The standard error of the mean is shown where it exceeds the size of the symbol.

Calcium Channels and Cold-Induced Gene Expression

In plants, more than half of total tissue calcium is located in the apoplast, and a large fraction of this apoplastic calcium is bound to the cell wall (Cleland et al., 1990), leaving only a small fraction of the apoplastic calcium free and available for influx into the protoplast. Because low temperature dramatically increases the influx of extracellular calcium into the protoplast (Figure 1), we considered it worthwhile to examine the involvement of free apoplastic calcium in *cas* gene expression. We reasoned that if influx of this calcium through the plasma membrane plays a primary role in *cas* gene expression, then its prevention by chelation or by blockage of calcium channels should inhibit the *cas* transcript accumulation. First, we assessed the effects of calcium channel blockers La^{3+}, nitrendipine, and verapamil and of the calcium chelator 1,2-*bis*(*o*-aminophenoxy)ethane *N,N,N′,N′*-tetraacetic acid (BAPTA) on $^{45}Ca^{2+}$ influx into protoplasts at 4°C, the temperature at which cold acclimation is generally conducted. As shown in Figure 2A, all but nitrendipine strongly inhibited cold-induced $^{45}Ca^{2+}$ influx. The inhibition was the greatest with BAPTA and the lowest with nitrendipine. We then examined the effects of these inhibitors of calcium influx on *cas* gene expression in cell suspension cultures. The effects of the calcium chelator BAPTA and of the three calcium channel blockers, La^{3+}, nitrendipine, and verapamil, on transcript levels of *cas15* and *cas18* are shown in Figure 2B. BAPTA almost completely inhibited the cold induction of *cas* genes. The inhibition due to different calcium channel blockers was variable, being the highest with verapamil and the lowest with nitrendipine. Densitometric measurement of the hybridization spots on the autoradiogram showed that the inhibition due to BAPTA was more than 90%, whereas that due to calcium channel blockers varied from 20% for nitrendipine to more than 65% for verapamil. In contrast, the level of ubiquitin mRNA, used as a control, was not affected by any of the treatments. Thus the data in Figure 2 show that the treatments that inhibited the influx of extracellular $^{45}Ca^{2+}$ into the cytosol also caused a corresponding inhibition of *cas* gene expression.

To study further the significance of the relationship between calcium influx and *cas* gene expression, we examined the temporal nature of the requirement for calcium influx in *cas* gene expression to determine whether this requirement is transient or permanent during cold acclimation. We studied the effects of the time of addition of BAPTA or ruthenium red on the accumulation of *cas* transcripts during cold acclimation. BAPTA is expected to chelate and thus render the cell wall calcium unavailable for influx. Ruthenium red is known to inhibit calcium release from intracellular stores (Knight et al., 1992). Transcripts of both *cas15* and *cas18* were similarly affected. Therefore, only the results for *cas15* transcript levels are shown in Figure 3. Effects of BAPTA varied with the time of its addition. The accumulation of *cas15* transcripts was strongly inhibited when BAPTA was applied 2 hr before or at the initiation of cold acclimation but was decreasingly effective when applied later. Thus, when BAPTA was applied 4 hr after the start of cold acclimation, *cas* gene expression was not affected. In contrast, a similar treatment with ruthenium red at any time before or during cold acclimation had little effect on *cas* transcript accumulation. Therefore, these experiments indicate that the need for elevated intracellular calcium during cold acclimation is transient and confirm the conclusion from other experiments (Figures 1 and 2) that calcium influx occurs primarily from the apoplast.

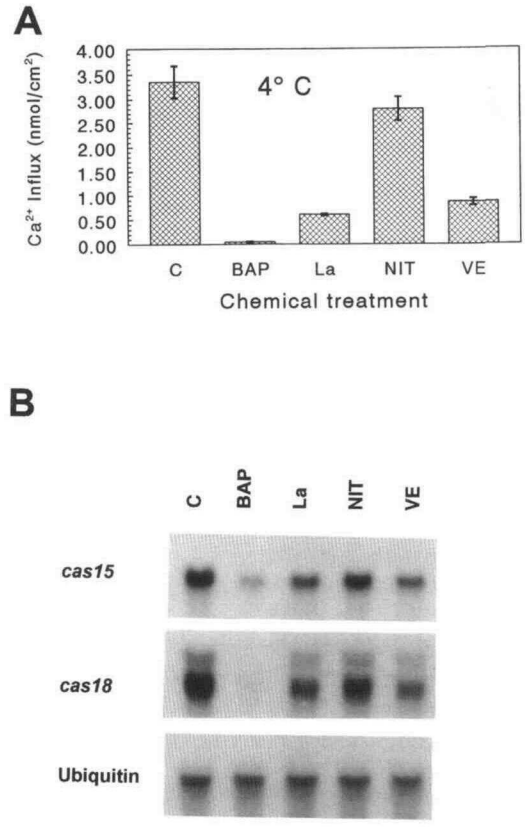

Figure 2. Effects of a Calcium Chelator and Calcium Channel Blockers on Cold-Induced Calcium Influx and on Accumulation of Cold Acclimation–Specific mRNAs.

(A) Protoplasts prepared from cell suspension cultures were first incubated for 30 min at 25°C either without (C) or with 2 mM BAPTA (BAP), 1 mM La^{3+} (La), 0.1 mM nitrendipine (NIT), or 0.1 mM verapamil (VE), and then for 20 min at 4°C with $^{45}CaCl_2$. Unincorporated $^{45}CaCl_2$ was removed by cation exchange chromatography, and radioactivity was determined by scintillation spectrometry. Each datum point is the mean of three replicates ± SE.

(B) Aliquots (20 mL) of cell suspension cultures were acclimated to 4°C for 24 hr either in the absence (C) or in the presence of 2 mM BAPTA, 1 mM La^{3+}, 0.1 mM nitrendipine, or 0.1 mM verapamil. Total RNA was extracted and analyzed by RNA gel blot hybridization with cDNA probes of the genes indicated to the left. *cas15* and *cas18* transcripts accumulated specifically in response to cold acclimation, whereas ubiquitin transcripts accumulated constitutively and served as the control. This experiment was repeated three times, and the results were similar each time.

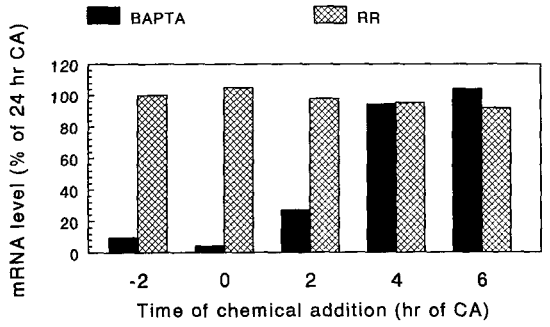

Figure 3. Time-Dependent Effects of BAPTA and Ruthenium Red on the Cold-Induced Accumulation of *cas15* mRNA.

Aliquots (20 mL) of cell suspension cultures were treated with 2 mM BAPTA (solid bars) or 50 μM ruthenium red (crosshatched bars) at the times shown on the x-axis. Chemically treated cells were acclimated at 4°C for a total of 24 hr. Total RNA was extracted and analyzed by RNA gel blot hybridization; the level of *cas15* mRNA was determined by densitometry. RNA levels are expressed as a percentage of control levels, untreated cells cold acclimated at 4°C for 24 hr (CA). This experiment was repeated at least two times with similar results.

Calcium Induces *cas* Genes at 25°C

We attempted to separate the relative roles of calcium and low temperature by experimentally elevating the level of intracellular calcium at 25°C by treating cells with the calcium ionophore A23187 or with the calcium channel agonist Bay K8644. First, the effects of these chemicals on $^{45}Ca^{2+}$ influx were analyzed in protoplasts maintained at 25°C. Data in Figure 4A show that although $^{45}Ca^{2+}$ influx at 25°C was small, it could be significantly increased by treating the cells with the calcium channel agonist Bay K8644 or the ionophore A23187. We then examined the effects of these chemicals on *cas* gene expression in cell suspension cultures. Results presented in Figure 4B show that either of these chemicals induced *cas* gene expression in cells at 25°C. However, this induction did not take place when cells were pretreated with BAPTA. The level of ubiquitin mRNA was not affected by any of the treatments. Thus, regulation of *cas* transcript levels might be specifically affected by the elevated calcium influx induced by A23187 or Bay K8644. The level of induction by the calcium channel agonist or the ionophore at 25°C during the first 6 hr was greater than that by low temperature during the same time period. After 6 hr, however, the transcript levels in cells treated with the ionophore or the channel agonist declined, and by 24 hr they were much lower than in the cold-acclimated cells. Therefore, it appears that the influx of extracellular calcium alone is sufficient to induce but not to sustain the *cas* gene expression.

Low Temperature and Expression of Genes Encoding Calcium Binding Proteins

To understand the role of elevated cytosolic calcium, we considered it worthwhile to examine the levels of transcripts for CaM and CDPKs during early stages of cold acclimation. Moreover, CaM transcripts have been shown to change in response to some stresses (Braam and Davis, 1990). Initial attempts to detect transcripts of CaM and CDPKs by RNA gel blot analysis using heterologous probes yielded ambiguous results (data not shown). Therefore, alfalfa CaM and a pool of protein kinases were amplified from RNA by polymerase chain reaction (PCR). As shown in Figure 5A, CaM transcripts were readily detectable in nonacclimated cells, and their levels did not change during 24 hr of cold acclimation. To monitor CDPK transcript levels, pools of protein kinase sequences containing the conserved amino acid motif RDLKPEN were first amplified by PCR. These pools were then probed with a soybean CDPK cDNA insert. Figure 5B shows that at least three distinct CDPK sequences were readily detectable in nonacclimated cells. However, unlike CaM, the relative levels of the amplified CDPK sequences were differentially regulated by low temperature. This can be better appreciated by the quantitation of the hybridization signal intensities from these sequences shown in Table 1. Levels of the largest putative CDPK sequence, 1.3 kb in length, increased by more than eightfold within 3 hr of cold acclimation. Its levels then declined, and at 24 hr of cold acclimation, it was only ~2.5-fold compared to the level in the nonacclimated tissue (zero cold acclimation). Levels of the 0.93-kb PCR product slowly but consistently declined during cold acclimation, decreasing to less than 25% of the original level after 24 hr of cold acclimation. Levels of the 0.67-kb PCR product showed a small but progressive increase during 24 hr of cold acclimation, registering levels more than twofold higher compared to zero time. Thus, transcripts for calcium signaling proteins are present in nonacclimated cells, but the relative levels of specific CDPKs change during 24 hr of cold acclimation.

To confirm that the signals detected had originated in authentic CDPKs, the corresponding DNAs were purified from agarose gels and amplified by PCR using primers designed to amplify sequences between subdomains VIb and IX of the catalytic domain of plant CDPKs (Harper et al., 1991, 1993; Suen and Choi, 1991; Kawasaki et al., 1993). Products of the predicted lengths (205 to 210 bp) were obtained from the 1.3- and 0.93-kb transcripts but not from the 0.67-kb putative CDPK. Sequence analysis revealed that all of the PCR products from the 1.3-kb sequence were nearly identical. A search of data bases by using BLAST-P software (Altschul et al., 1990) showed that this product, designated MSCK1 (*Medicago sativa* calcium kinase1), was highly similar to plant CDPKs. On the other hand, the PCR products obtained from the 0.93-kb DNA were more heterogeneous, comprising 50% sequences unrelated to CDPKs. The other half corresponded to the product designated MSCK2, which is distinct from MSCK1 but highly similar to plant CDPKs.

The alignment of deduced amino acid sequences of the two MSCK clones and the corresponding region of other plant CDPKs are shown in Figure 6. Scores from BLAST-P searches indicated that the probe, soybean CDPK, was 67 and 62% identical to MSCK2 and MSCK1, respectively. Interestingly, these scores revealed that MSCK1 is more related to the rice CDPK

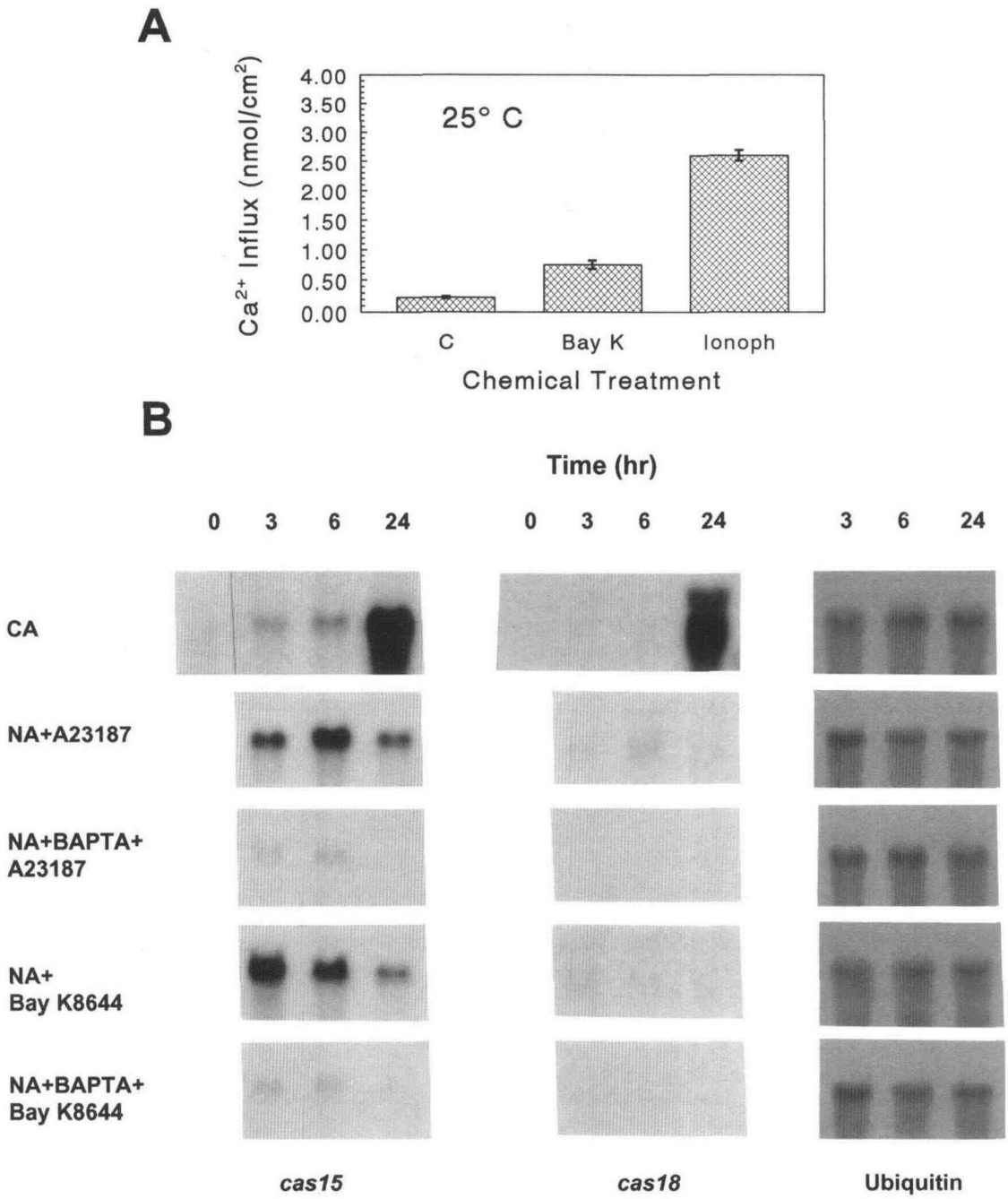

Figure 4. Effects of A23187 and Bay K8644 at 25°C on the Influx of Extracellular Calcium and on *cas* Gene Expression.

(A) Effects of A23187 and Bay K8644 on influx of extracellular calcium. Protoplasts from nonacclimated cell suspension cultures were incubated for 30 min at 25°C without (C) or with 100 μM Bay K8644 (Bay K) or 50 μM A23187 (Ionoph) and then for 20 min at 25°C with $^{45}CaCl_2$. Unincorporated $^{45}CaCl_2$ was removed by cation exchange chromatography, and radioactivity was determined by scintillation spectrometry. Each datum point is the mean of three replicates ± SE.

(B) Effects of A23187 and Bay K8644 on the expression of *cas15* and *cas18* in nonacclimated cells (NA) at 25°C. For comparison, cold acclimation–induced expression of these genes is also shown (CA). Aliquots (20 mL) of cell suspension cultures were incubated at 25°C with 50 μM A23187 or 100 μM Bay K8644 for the times indicated. To test the specificity of these chemicals, 2 mM BAPTA was added to the cells 1 hr before the addition of A23187 or Bay K8644. Total RNA was extracted and analyzed by RNA gel blot hybridization with cDNA probes of the genes indicated at the bottom. The experiment was repeated three times, and the results were similar each time.

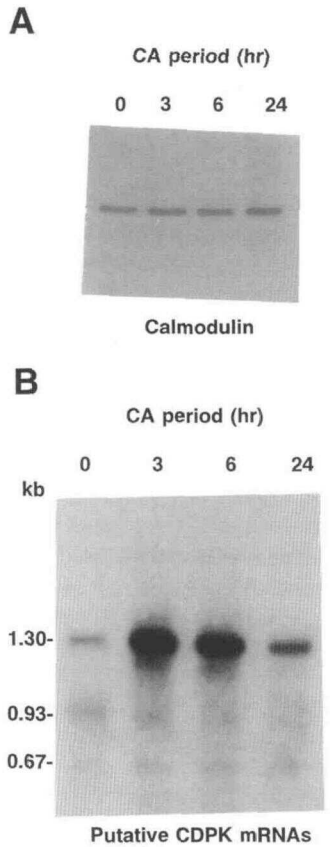

Figure 5. Analysis of the Cold-Induced Levels of mRNAs for CaM and CDPKs by PCR Amplification of cDNAs.

Total RNA isolated from cells cold acclimated for the durations indicated was subjected to reverse transcription to produce cDNAs that were then amplified by PCR.

(A) CaM sequences were amplified from total RNA using specific primers corresponding to the reported sequence of alfalfa CaM cDNA (Barnett and Long, 1990). Depicted is a negative of the PCR products separated on agarose gels and visualized by ethidium bromide staining.

(B) Protein kinase sequences were amplified using a degenerate primer corresponding to subdomain VI of plant protein kinases (Hanks and Quinn, 1991) and an oligo(dT) primer. The PCR products were separated on agarose gels, blotted, and hybridized to a radiolabeled PCR product comprising subdomains VI to IX of soybean CDPK (Harper *et al.*, 1991).

CA, cold acclimation.

(67%) than to MSCK2 (63%), whereas MSCK2 is by far more related to the carrot CDPK (89%).

DISCUSSION

Calcium has been implicated as a second messenger of plant responses to a variety of external stimuli (Trewavas and Gilroy, 1991; Bush, 1993; Gilroy et al., 1993). Thus, levels of cytosolic calcium increase in response to wind and cold shock (Knight et al., 1991, 1992). Our study definitively shows that a cold-induced calcium influx may play an essential role in low-temperature signal transduction leading to *cas* gene expression and cold acclimation.

In plants, more than half of the tissue calcium is located in the apoplast, where a large proportion of it is bound by the cell wall (Cleland et al., 1990). Determinations of apoplastic calcium as a proportion of total calcium have been successful in plants with large cells, such as *Chara*, but those in plants with normal cell sizes are problematic (Reid and Smith, 1992a). For example, the segment-loading method successfully used with *Chara* (Reid and Smith, 1992a), in which the individual cells can measure several centimeters long, is difficult to use for alfalfa cells that are only ~40 μm in diameter. Similarly, concentration of the free apoplastic calcium is not known. The estimates vary from 10 μM to 10 mM (Macklon, 1975; Raven, 1985), which is an unacceptable range of variation. Thus, it is not surprising that researchers have resorted to using protoplasts to study calcium influx in higher plant cells. The kinetics of $^{45}Ca^{2+}$ influx observed in our study are similar to those reported for protoplasts by others (Rincon and Boss, 1987; Graziana et al., 1988). In nature, the total amount of tissue calcium is unlikely to change with short-term exposure to cold. However, cold-induced redistribution of preexisting calcium has been demonstrated in transgenic tobacco expressing the calcium-reporting protein aequorin (Gilroy et al., 1993). In our study, the effects of calcium channel blockers and the results shown in Figure 1 clearly indicate that cold affects calcium transport. Because we detected no cold-induced efflux of preloaded $^{45}Ca^{2+}$, the cold-induced influx is probably unidirectional, as has been observed in *Chara* (Reid and Smith, 1992b). Our study demonstrates the need for calcium influx in the induction of *cas* genes. A limitation of our study is that it tells little about calcium concentration in various cellular compartments. Such determinations must await the transformation of alfalfa cells with the aequorin protein directed to different cellular compartments.

The source of calcium influx has been shown to vary with the type of environmental stimulus. Thus, calcium is released from the cell wall in the case of cold shock but from the

Table 1. Results of the Densitometric Quantitation of the Hybridization Signals of Figure 5 To Show the Relative mRNA Levels of Putative CDPKs during Cold Acclimation

	Cold Acclimation Period (Hours)			
PCR Product (Length)	0	3	6	24
1 (1.30 kb)	100	845	735	263
2 (0.93 kb)	100	67	41	23
3 (0.67 kb)	100	138	191	224

mRNA levels at 3, 6, and 24 hr represent a percentage of those at zero time.

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                          VII              VIII              IX
                           ↓                ↓                ↓
MSCK1  RDLKPENFFFANKKETA-LKAIDFGLSVFFKPGERFNEIVGSPLYMAPEVLKRNYGPEVDIWSAGVIV
MSCK2  ********LLSS*DDG*A***T*******IEE*KVYRDM***AY*V*****H****K*I********L
  RIC  ********LLLDADDEFSV**************QV*R******Y*I*****EKR****A***T****L 263
  CAR  ********LLSS*DKD*M***T*******IEE*KVYRN****AY*V*****R*S**K*I********L 164
  ARA  ********L*VS*H*DSL**T******M****DDV*TDV****Y*V*****R*R****A*V******* 340
  SOY  ********L*DTID*D*K***T*******Y****S*CDV****Y*V*****RKL****S********L 224
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Figure 6. Alignment of Deduced Amino Acid Sequences of Alfalfa PCR Products MSCK1 and MSCK2 with CDPK Sequences from Rice, Carrot, Arabidopsis, and Soybean.

The reported CDPK sequences used in comparison are from rice (Kawasaki et al., 1993), carrot (Suen and Choi, 1991), Arabidopsis (Harper et al., 1993), and soybean (Harper et al., 1991). Asterisks denote identical amino acids, and the hyphen denotes a gap in the sequence. Roman numerals at the top indicate subdomains of the catalytic domain. The target amino acids of PCR are underlined. Numbers on the right indicate the amino acid position in each known sequence. RIC, rice; CAR, carrot; ARA, Arabidopsis; SOY, soybean.

intracellular stores (e.g., vacuole) in the case of touch and wind (Knight et al., 1992). Because the $^{45}Ca^{2+}$ influx observed in alfalfa protoplasts is sensitive to calcium chelators and chemicals known to block voltage-gated calcium channels (Narahashi and Herman, 1992) and because these chemicals are unlikely to enter the cell, cold-induced influx of $^{45}Ca^{2+}$ probably occurs from the cell wall through calcium channels on the plasma membrane. Nitrendipine, which causes only a small inhibition of cold-induced $^{45}Ca^{2+}$ influx, is known to be a much less effective calcium channel blocker in other systems as well (Graziana et al., 1988). Because the cold-induced $^{45}Ca^{2+}$ influx is not completely prevented by verapamil or La^{3+}, the possibility cannot be ruled out that some influx occurs through some other type of calcium channels or even through nonselective channels (Bush, 1993). The effects of temperature on $^{45}Ca^{2+}$ influx observed in our study are in agreement with those obtained in electrophysiological studies of onion skin cells by Ding and Pickard (1993). Calcium channel activity was found to increase with decreasing temperature, with maximum activity at ~6°C.

The steady state concentration of cytosolic calcium is tightly regulated and maintained at a low level. Thus, increases in cytosolic calcium in response to hormonal and physical stimuli are transient (Trewavas and Gilroy, 1991; Bush, 1993; Gilroy et al., 1993). For example, in the cotyledons of transgenic tobacco constitutively expressing the calcium-reporting protein aequorin, calcium increases in the cytosol within seconds in response to cold shock but is taken out of the cytosol within 3 min (Knight et al., 1991; Gilroy et al., 1993). Similar calcium transients are sufficient to activate calcium-dependent multifunctional protein kinases, such as protein kinase C (Nishizuka, 1992) and CaM kinase (Schulman et al., 1992), which amplify and extend the calcium signal. In agreement with other studies of calcium as a second messenger, our study shows that the need for calcium influx during initiation of cold acclimation (measured as *cas* transcript accumulation) is also transient.

How is the increased concentration of cytosolic calcium restored to the resting level after the signal cascade has been initiated during cold acclimation? We did not detect any cold-induced efflux of preloaded $^{45}Ca^{2+}$ from protoplasts. This suggests that some intracellular sequestering mechanism(s) may be operating. Levels of CaM transcripts increase in response to touch and wind (Braam and Davis, 1990), both of which release calcium from intracellular stores (Knight et al., 1992). In our study, cold did not increase CaM transcripts, and the calcium influx appears to have occurred from the cell wall. Therefore, CaM is unlikely to play a major role in sequestering the increased cytosolic calcium when cold acclimation is triggered. It is tempting to suggest that the calcium-sequestering role of calmodulin may be specifically associated with calcium release from intracellular stores. There is evidence that the vacuole plays an important buffering role in maintaining homeostasis of cytosolic calcium in fungal hyphae (Miller et al., 1990). It is likely that a similar mechanism for a short-term homeostasis of cytosolic calcium operates in higher plants. Ultimately, however, the cell must exclude the excess intracellular calcium through such normal means as calcium–ATPase or H^+/Ca^{2+} antiport (Miller et al., 1990).

Our study suggests a positive correlation between cold-induced calcium influx and cold-induced accumulation of *cas* transcripts. Both are inhibited strongly by BAPTA, verapamil, and La^{3+} and only slightly by nitrendipine. We have previously reported that La^{3+} and verapamil cause complete inhibition of cold acclimation (Monroy et al., 1993b). However, their inhibition of *cas* gene expression, which was examined in this study, is not complete. It is possible that the level of *cas* transcripts accumulated in the presence of these chemicals is insufficient to induce a measurable degree of freezing tolerance. Experiments to study the signal transduction events need to be conducted during initial stages of cold acclimation. However, freezing tolerance takes much longer to develop to an unambiguously measurable degree (Mohapatra et al., 1989; Wolfraim et al., 1993).

We showed that experimentally induced calcium influx caused an accumulation of *cas* transcripts at 25°C. This induction is likely to be mediated specifically by calcium because it is prevented by a pretreatment with BAPTA. Because BAPTA did not affect transcript accumulation when added 4 hr after the start of cold acclimation (Figure 3), it is unlikely to affect the level of preexisting mRNAs. Therefore, the effects of pretreatment with BAPTA as shown in Figure 4B are probably

exerted through an inhibition of *cas* transcript accumulation due simply to chelation of the free apoplastic calcium and making it unavailable for influx. That induction of *cas* genes by A23187 and Bay K8644 at 25°C declines with time suggests a role for as yet undetermined low temperature–promoted processes in sustaining the calcium-induced *cas* gene expression. Such processes may include low-temperature signaling pathways other than that of calcium, leading to either the inhibition of RNases or the expression of mRNA-stabilizing proteins.

The induction of *cas15* by A23187 or by Bay K8644 at 25°C was much higher than that of *cas18* (Figure 4). There are at least two possible explanations for this difference. First, *cas18* transcripts are much less stable at 25°C than are *cas15* transcripts. We have shown that *cas18* transcripts decline with a half-life of 20 min and become undetectable within 5 hr of deacclimation (Wolfraim et al., 1993). However, *cas15* transcripts decline slowly and remain detectable even after 24 hr (Monroy et al., 1993a). Second, *cas15* encodes a putative nuclear protein, and its induction does not require continued protein synthesis (Monroy et al., 1993a). It is possible that *cas15* plays a regulatory role so that *cas18* is expressed only when *cas15* expression reaches a threshold level.

To elicit responses, calcium transients must be efficiently linked either directly via CDPKs (Roberts and Harmon, 1992) or indirectly via CaM (Schulman et al., 1992) to a protein phosphorylation signaling cascade. Our previous studies have shown that the induction of *cas15* (Monroy et al., 1993a) and cold-dependent changes in protein phosphorylation rely solely on preexisting proteins (Monroy et al., 1993b). Based on these observations, we have suggested the involvement of cold-regulated protein kinases in cold acclimation (Monroy et al., 1993b; Dhindsa and Monroy, 1994). In this study, we isolated sequences of two CDPKs whose transcripts and presumably translation products exist in nonacclimated cells. Thus, both CDPKs can potentially participate in calcium signaling during cold acclimation. However, we found that with the onset of cold acclimation, their transcript levels were differentially regulated by low temperature. Whereas the transcripts of one of them, MSCK1, increased by more than eightfold within 3 hr at 4°C, those of the other, MSCK2, decreased in response to low temperature. The regulation of protein kinases by other stresses such as drought has been reported (Anderberg and Walker-Simmons, 1992). Thus, it appears likely that protein kinases are involved in the regulation of plant responses to environmental stress signals. Inheritance of such kinases could determine the ability of plants to develop stress resistance or adaptation.

How does low temperature trigger the activity of calcium channels? Although direct experimental evidence is lacking, several observations suggest that a low temperature–induced change in membrane fluidity may provide the trigger. (1) In this study, the most rapid increase in calcium influx was at temperatures below 15°C. Below this temperature, most plants also show decreased membrane fluidity (Levitt, 1980). (2) Chemicals such as sterols, known to alter membrane fluidity, also alter ligand binding to calcium channels (Mason et al., 1992). (3) Catalytic hydrogenation of membrane lipids, expected to decrease membrane fluidity, induces the cold-inducible gene *desaturase A* in the cyanobacterium *Synechocystis* (Vigh et al., 1993). The model presented in Figure 7, which is a modification of an earlier model (Dhindsa et al., 1993), is an attempt to integrate the aforementioned findings. We propose that the signaling pathway of low temperature–induced development of freezing tolerance comprises two major classes of events. First, there are events, such as a temperature-induced shift in membrane fluidity and cold-induced calcium influx, that occur in all plants, regardless of their capacity to develop freezing tolerance. For example, the cold-induced increase in cytosolic calcium has also been shown in tobacco (Knight et al., 1991, 1992), a freezing-sensitive plant. Second, there are events that specifically occur in cold acclimation–competent genotypes. In this second set of events, we envision the existence of as yet uncharacterized protein kinases/phosphatases and/or their substrates that are regulated by calcium and/or cold and lead to *cas* gene expression. Although our studies have suggested a direct effect of calcium, low temperature affects the conformation of a diversity of macromolecules (Frenkel, 1991) and promotes the stability of others such as *cas18* transcripts (Wolfraim et al., 1993). Thus, regulation of protein phosphorylation by calcium might be contingent on conformational

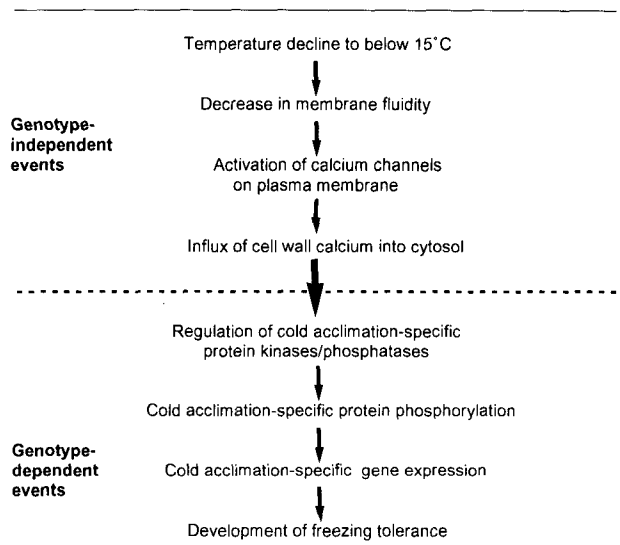

Figure 7. Proposed Sequence of Events Occurring during Low-Temperature Signal Transduction Leading to *cas* Gene Expression and Development of Freezing Tolerance.

According to the model proposed here, the cold-induced changes in membrane fluidity, activation of calcium channels, and entry of calcium into the cytosol are independent of the genotypic capacity for cold acclimation. However, the amplification of the calcium signal along the pathway to cold acclimation is genotype dependent and involves the action of specific protein kinases/phosphatases and their substrates, leading to *cas* gene expression and development of freezing tolerance.

responses or changes in the stability of proteins at low temperature. Finally, further downstream along the pathway, there are protein kinases/phosphatases, perhaps such as MSCK1, specifically involved in the development of freezing tolerance. Therefore, because of its potential implications for the bioengineering of plant frost tolerance, genotype-dependent signaling pathways constitute a logical target for future research.

METHODS

Plant Material

Conditions for growth and cold acclimation of alfalfa (*Medicago sativa* ssp *falcata* cv Anik) cell suspension cultures were as previously described (Monroy et al., 1993b; Wolfraim et al., 1993). For protoplast isolation, 20 mL of 6-day-old cells (2 to 3 g of fresh weight) was washed by filtration in buffer A (10 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 5.6, 0.4 M mannitol) and resuspended in 20 mL of the same buffer but containing 18.7 mg/mL cellulysin, 18.7 mg/mL cellulase, 19 mg/mL pectolyase Y23, and 19 mg/mL BSA. Cell wall digestion was allowed to proceed for 2.5 hr at 25°C with shaking (150 rpm). The suspension was filtered through Miracloth (Calbiochem, San Diego, CA), and the protoplasts were recovered from the eluate by centrifugation (200*g*). Crude protoplasts were resuspended in 5 mL of 20% Ficoll (Pharmacia, Sweden) in buffer A and placed in a centrifuge tube. On top of this layer, 5 mL of buffer A containing 10% Ficoll was layered followed by 5 mL of buffer A. This discontinuous gradient was subjected to centrifugation at 950*g* for 15 min, and protoplasts were recovered from the 0 to 10% Ficoll interface (Graziana et al., 1988). Protoplasts were washed twice with 10 mL of buffer A, and the protoplast pellet was recovered by centrifugation at 200*g*. Protoplasts were then resuspended in 1 to 2 mL of buffer A. All steps were carried out at 25°C. Purified protoplasts were stored at 25°C for 90 min before use.

Analysis of Calcium Influx

To determine calcium influx, protoplasts (150 to 300 protoplasts/μL) were incubated in buffer A containing 0.1 mM $^{45}CaCl_2$ (7.47 mCi/mg calcium) for different times or at different temperatures. At the termination of influx, 60-μL aliquots were withdrawn and applied to a minicolumn packed with 250 μL of macroporus AG MP-50 cation-exchange resin (Bio-Rad) to remove unincorporated $^{45}CaCl_2$. After 30 sec, the radioactive protoplasts were recovered by centrifugation (12,000*g* for 30 sec). Radioactivity was then determined by liquid scintillation spectrometry. Using this technique, more than 99% of unincorporated $^{45}CaCl_2$ was removed. The results of influx experiments are expressed in nanomoles of calcium per square centimeter. The surface area of the protoplasts was calculated using an average diameter of 40 μm. The data presented are means of three replicates. To determine calcium efflux, protoplasts were labeled as for influx at 25°C. After a 20 min labeling, radioactive protoplasts were recovered by centrifugation (200*g*) and washed twice in buffer A. Aliquots of the radioactive protoplasts were incubated for 0 or 20 min at various temperatures, then passed through the AG MP-50 minicolumn and quantified as described earlier. Efflux was the difference in protoplast radioactivity between the 0- and 20-min incubation periods.

Administration of Chemical Treatments

Cell suspension cultures were treated with calcium channel blockers as previously described (Monroy et al., 1993b). The calcium chelator 1,2-*bis*-(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA) (Tsien, 1980) was used at 2 mM in place of EGTA to overcome the weak calcium buffering of the latter at the pH of the culture medium, 5.6. Ruthenium red at 50 μM was used as an intracellular calcium channel blocker (Knight et al., 1992). The calcium ionophore A23187 (Klausner et al., 1979) and the calcium channel agonist Bay K8644 (Nowycky et al., 1985) were used at 50 and 100 μM, respectively. In experiments to test the specificity of the effects of the latter chemicals, calcium was rendered unavailable for influx by adding 2 mM BAPTA to the cells 1 hr before the addition of A23187 and Bay K8644.

Analysis of Relative Transcript Levels

The isolation, fractionation, hybridization, and densitometric quantitation of RNA were as previously described (Monroy et al., 1993b). The full-length sequences and expression patterns of the two cold acclimation–specific (*cas*) cDNAs used in these experiments have been published previously (Monroy et al., 1993b; Wolfraim et al., 1993). Levels of transcripts for calmodulin (CaM) and calcium-dependent protein kinases (CDPKs), which were judged at low abundance in alfalfa cells, were monitored by the enzymatic amplification of RNA by a modification of the two-step polymerase chain reaction (PCR) protocol (Barnard et al., 1994). Total RNA was isolated as previously described (Monroy et al., 1993b), but an additional precipitation using LiCl was performed to eliminate residual DNA. To amplify RNA, cDNA was synthesized by using total RNA, primer KSXT [5′-CGAGGTCGACGGTATCTCGAG-$(T)_{17}$-3′], and SuperScript H^- reverse transcriptase (Gibco BRL) following the protocol prescribed by the manufacturer. The cDNAs were then amplified by Taq DNA polymerase. The conditions for PCR were 94°C for 1 min, 50°C for 2 min, and 72°C for 1 min for three cycles, and 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min for 17 cycles. Under these conditions, the relative levels of amplified products were roughly the same in the range of 20 to 2000 ng of RNA input.

CaM sequences were amplified using the sense oligonucleotide 5′-GATAAGGATGGCGATGGTTGT-3′ and the complementary oligonucleotide 5′-GAGCTCAGCTGCAGAGATGAA-3′, corresponding to nucleotides 107 to 128 and 354 to 365, respectively, of the alfalfa CaM cDNA sequence (Barnett and Long, 1990). Partial sequence analysis of this product indicated that it had more than 95% sequence identity with the published sequence. Pools of protein kinase sequences were amplified using the sense oligonucleotide 5′-(A/C)GIGATTT(G/C)AAG-CCIGAGAAC-3′, corresponding to the conserved amino acid motif RDLKPEN (Hanks and Quinn, 1991), and primer KSXT (see aforementioned cDNA synthesis) as a complementary oligonucleotide. CDPK sequences were amplified using the sense primer that was used above for protein kinases and the complementary oligonucleotide 5′-TACA-(G/C)AATAACTCCAGC(A/G)C(T/A)GC(G/A)CTCC-3′, which corresponds to the amino acid motif (V/L)WSAGVILY of CDPKs (Harper et al., 1991, 1993; Suen and Choi, 1991; Kawasaki et al., 1993). Use of the latter combination of primers resulted in the amplification of a single band of ~210 bp from either of the cloned soybean (Harper et al., 1991) or carrot (Suen and Choi, 1991) CDPK. For analysis of putative CDPK transcripts, PCR products were resolved on 1.6% agarose gels, transferred to nylon membranes, and probed with a ^{32}P-labeled PCR gene fragment composed of amino acids 169 to 238 of soybean CDPK (Harper et al., 1991). The radioactivity of specific CDPK hybrids on

the blots was determined by using a PhosphorImager coupled with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Isolation and Sequencing of Alfalfa CDPKs

DNA fragments corresponding in size to those of the signals generated by hybridizing soybean CDPK to alfalfa protein kinase pools (see previous section) were purified by using the QIAEX gel extraction kit (Qiagen, Chatsworth, CA). CDPK sequences were then amplified from each of these fragments as described; the PCR fragments were extracted from agarose gels and cloned by the T-addition method (Finney, 1993) on the EcoRV site of pBluescript II KS– (Stratagene). Plasmid DNA from selected clones was purified by the QIA prep-spin kit (Qiagen), and both sense and complementary strands were sequenced by using the CircumVent thermal cycle sequencing kit of New England BioLabs (Beverly, MA).

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