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Signaling Events Leading to Crassulacean Acid Metabolism Induction in the Common Ice Plant[1]

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A rapid, semiquantitative reverse transcriptase-polymerase chain reaction assay was developed to investigate signal transduction events involved in the induction of Crassulacean acid metabolism (CAM) in detached common ice plant (*Mesembryanthemum crystallinum*) leaves. Transcript abundance of *Ppc1*, a gene encoding the CAM-specific isoform of phospho*enol*pyruvate carboxylase, increased rapidly in response to osmotic stress (dehydration and mannitol), ionic stress (NaCl), and exogenous abscisic acid treatment, but failed to accumulate in response to exogenous cytokinin or methyl jasmonate. Stress-induced accumulation of *Ppc1, GapC1, and Mdh1* transcripts was inhibited by pretreating leaves with the calcium chelator ethyleneglycol-bis(aminoethyl ether)-*N,N*′-tetraacetic acid, suggesting that extracellular calcium participates in signaling events leading to CAM induction. Treatment of unstressed detached leaves with ionomycin, a Ca^{2+} ionophore, and thapsigargin, a Ca^{2+}-ATPase inhibitor, enhanced *Ppc1* transcript accumulation, indicating that elevations in cytosolic [Ca^{2+}] are likely to participate in signaling CAM induction. Inhibitors of Ca^{2+}- or calmodulin-dependent protein kinases (N-[6-aminohexyl]-5-chloro-1-napthalenesulfonamide, Lavendustin C) and protein phosphatase 1 and 2A (okadaic acid) activity suppressed *Ppc1* transcript accumulation in response to ionic and osmotic stresses, as well as abscisic acid treatment. These results suggest that both protein phosphorylation and dephosphorylation events participate in signaling during CAM induction. In contrast, pretreatment with cyclosporin A or ascomycin, inhibitors of protein phosphatase 2B activity, stimulated *Ppc1* gene expression either directly or indirectly through promoting water loss.

CAM, an alternative CO_2 assimilating and concentrating mechanism present in more than 6% of vascular plant species, is found primarily in plants adapted to water-limited (deserts or epiphytic) habitats where daytime stomatal closure to reduce water loss can limit atmospheric CO_2 uptake (Winter and Smith, 1996). However, a number of aquatic vascular plant species, which occupy CO_2-limited environments arising from daytime competition from C3 species, also display CAM (Keeley, 1998). CAM plants employ PEP carboxylase (PEPC) as a "CO_2 pump" to elevate intracellular CO_2 concentrations in the vicinity of Rubisco, to suppress photorespiration and thereby improve their competitiveness under conditions of high light intensity, high temperatures, or low water and CO_2 availability. CAM plants display a great plasticity in the extent to which the pathway is expressed, largely under the control of environmental or developmental influences (Edwards et al., 1996; Cushman and Bohnert, 1999). In facultative CAM plants, such as the common ice plant (*Mesembryanthemum crystallinum*), high salinity, osmotic, or dehydration stress and the exogenous application of ABA cause the activity and gene expression of many enzymes involved in CAM (e.g. glycolysis, gluconeogenesis, and malate metabolism) to increase (Cushman et al., 1998; Cushman and Bohnert, 1999). Transcriptional activation is the primary mechanism controlling mRNA accumulation of CAM-associated genes (Cushman et al., 1989). In common ice plant the expression of a CAM-specific PEPC isoform can also be enhanced by high light intensity, light quality, and long photoperiods (McElwain et al., 1992; Cockburn et al., 1996), suggesting that phytochrome modulates the action of stress or plant growth regulators.

Various plant growth regulators have been implicated as signaling molecules that modulate CAM induction. Salinity and drought stress treatments cause marked increases of endogenous ABA amounts (Thomas et al., 1992; Taybi et al., 1995). Endogenous increases or exogenous application of ABA result in CAM induction (Dai et al., 1994; Taybi et al., 1995) by stimulating increased expression of key CAM enzymes such as PEPC (Chu et al., 1990; Dai et al., 1994; Taybi et al., 1995), enolase (Forsthoefel et al., 1995a), phosphoglyceromutase (Forsthoefel et al., 1995b), and vacuolar ATPase subunit c (Tsiantis et al., 1996). Other plant growth regulators such as cytokinins have been shown to either suppress or enhance PEPC expression depending on the mode of application (Schmitt and Piepenbrock, 1992; Thomas et al., 1992; Thomas and Bohnert, 1993; Dai et al., 1994; Peters et al., 1997). Cytokinin applied to roots causes an enhancement in PEPC expression, whereas foliar application of intact plants or feeding to detached leaves suppresses PEPC expression and prevents PEPC induction by drought or salinity stress (Schmitt and Piepenbrock, 1992; Dai et al., 1994; Peters et al., 1997). Endogenous cytokinin levels are negatively correlated with *Ppc1* transcripts during dehydration stress, suggesting that cytokinins act as negative effectors in the expression of CAM (Peters et al., 1997). Methyl jasmonate has also been shown to limit PEPC expression (Dai et al., 1994; Schmitt et al., 1996).

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Whereas CAM provides a useful paradigm to study the coordinate expression of stress-responsive genes, the perception and transduction of environmental stress or hormonal signals that trigger CAM induction remain poorly understood. Reductions in leaf water content and mesophyll cell turgor have been suggested to trigger expression of the CAM pathway (Winter and Gademann, 1991). Salinity stress also leads to reduced turgor pressures in root endodermal and cortical cells (Rygol and Zimmermann, 1990), which can initiate CAM induction (Winter and Gademann, 1991). Split root experiments suggest that roots perceive water stress and convey this information to leaves triggering a switch from C3 to CAM photosynthesis without detectable reductions in leaf turgor (Eastmond and Ross, 1997). However, water deficit in detached leaves can initiate CAM gene expression (Schmitt, 1990; Dai et al., 1994; Peters et al., 1997), suggesting that root-derived signals are not essential. Regardless of their source, the signals that trigger CAM induction are not fully understood. We have developed a sensitive reverse transcriptase-PCR (RT-PCR) assay to monitor *Ppc1* transcript abundance and rapidly identify possible second messengers and signaling components involved in the initial stages of CAM induction. We demonstrate that Ca^{2+}, Ca^{2+}-, or calmodulin (CaM)-dependent protein kinases, and protein phosphatase (PPs) are likely to participate in environmental stress- and ABA-mediated induction of CAM in common ice plant.

MATERIALS AND METHODS

Plant Material

Common ice plant (*Mesembryanthemum crystallinum*) seeds were germinated in Metromix 200 (Scotts Sierra Horticulture Products, Marysville, OH) in a growth chamber on a 12-h light (26°C)/12-h dark (18°C) cycle. Fluorescent and incandescent lighting provided a photon flux density of 450 to 500 μE M^{-2} s^{-1}. Ten-day-old seedlings were transplanted individually in 1-L Styrofoam pots containing Metromix 200 and irrigated once daily with 0.5× Hoagland solution 2.

Inhibitor Treatments

Leaves were removed from 5-week-old plants with a razor blade, and petioles were recut under water with a razor blade prior to submerging petioles in inhibitor solutions. Inhibitors were dissolved in DMSO and diluted in water to the appropriate final concentrations, as indicated in figure legends. Inhibitor treatments were conducted prior to exposing leaves to stress or plant growth regulator treatments, as indicated in figure legends. Following treatments, leaves were collected, immediately frozen in liquid N_2, ground to a fine powder, and stored at −80°C until use. Fresh weight changes of individual detached leaves were monitored during inhibitor treatments and the data presented are the means of three or more replicate experiments.

RNA Isolation

Total RNA was isolated by mixing 150 mg of ground, frozen leaf material in 1 mL of TRIzol Reagent (Life Technologies, Grand Island, NY). Chloroform (250 μL) was added and the extract was vortexed for 30 s and left at RT for 15 min. The RNA was then precipitated in the presence of 200 mM NaCl, 133 mM Na-Citrate, and 17% (v/v) isopropanol and pelleted by centrifugation at 14,000*g* for 30 min at 4°C. RNA pellets were washed with 1 mL of ice-cold 70% (v/v) ethanol, air-dried for 15 to 30 min, and resuspended in 50 μL of diethyl pyrocarbonate-treated water. RNA quality was assessed by formaldehyde-denaturing agarose gel electrophoresis (Sambrook et al., 1989).

Semiquantitative RT-PCR

After RNA isolation and quality determination, a 3-μg aliquot was treated with amplification grade DNase I (Life Technologies) according to manufacturer's instructions to eliminate DNA contamination. RNA were diluted in diethyl pyrocarbonate-water to 50 ng μL^{-1} and used for RT-PCR amplification. Single tube RT and PCR reactions were conducted in 25-μL reactions containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 μM $MgCl_2$, 10 mM DTT, 100 μM each dNTP, 400 nM forward primer, 400 nM reverse primer, 20 units of RNase Out (Life Technologies), 40 units of Superscript II RT (Life Technologies), 0.5 unit of *Taq* DNA polymerase (Fisher Scientific, Pittsburgh) synthetic Δ RNA mimic (3 pg of Δ *Ppc1* and 5 ng of Δ *Fnr1*), and 50 ng of DNase I-treated RNA. Reactions were conducted using a PTC-100 thermal cycler (MJ Research, Watertown, MA) at 50°C for 30 min, at 94°C for 2 min to denature the RT, followed by 21 PCR cycles: 95°C for 1 min, 94°C for 15 s, 50°C for 30 s, 72°C for 45 s, and a 5-min final extension step at 72°C. Under these conditions the linear phase of amplification occurred between 15 and 25 cycles; 21 cycles were used for all experiments.

Ppc1/Δ *Ppc1* primers (forward primer, 5′-CACTTAAACATGTCCTTGAG-3′ [Tm = 56°C], reverse primer: 5′-GAGCACACAGCAACAAAGA-3′ [Tm = 56°C]) produced a 556-bp amplicon from native mRNA and a 393-bp amplicon from a synthetic RNA homologous mimic. *Fnr1*/Δ *Fnr1* primers (forward primer, 5′-ATTGCCAGCAGGCCCTTG-3′ [Tm = 54°C], reverse primer, 5′-GAACCAGTCAATACCATCT-3′ [Tm = 54°C]) produced a 600-bp amplicon from native mRNA and a 406-bp amplicon from a synthetic Δ RNA homologous mimic.

Primers used to generate the 511-bp *GapC1* amplicon were: forward primer, 5′-GTCCATCAATGAAGGACT-3′ (Tm = 52°C) and reverse primer, 5′-GATAGCCATCTCATGATAG-3′ (Tm = 54°C). Primers used to generate the 495-bp *Mdh1* amplicon were: forward primer, 5′-CTCCTCAACTCAATACCC-3′ (Tm = 54°C) and reverse primer, 5′-GCCACTTCTAATGACACG-3′ (Tm = 54°C). PCR primers were designed to span an intron to distinguish PCR products generated from cDNA versus genomic DNA. However, since all RNA samples were treated with DNAase I prior to amplification, no PCR products derived from genomic DNA were ever observed. After amplifica-

tion, the reaction was resolved by electrophoresis on a 1.2% (w/v) agarose gel stained with ethidium bromide. Gel images were captured using a Gel-Doc 1000 DNA Gel Analysis and Documentation System (Bio-Rad Laboratories, Hercules, CA). DNA amplification products were quantitated using Molecular Analyst software (Bio-Rad Laboratories). RT-PCR results presented are for a representative leaf from three to four replicate experiments. RT-PCR assay results were validated by slot-blot hybridization experiments using EGTA, okadaic acid (OKA), 1-norokadaone (Nor), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), and cyclosporin A (CsA) treatments.

Semiquantitative RT-PCR reactions were conducted in the presence of a synthetic, competitor RNA (mimics) to control for reaction-to-reaction variations in RT and PCR conditions against which native transcript abundance can be normalized. Homologous, synthetic RNA mimics containing deletions of 163 bp (*Ppc1*) or 194 bp (*Fnr1*) generated by inverse PCR were synthesized using a RiboMax (Promega, Madison, WI) in vitro transcription system according to the manufacturer's instructions.

RESULTS

A semiquantitative RT-PCR assay was developed to rapidly monitor changes in transcript abundance of *Ppc1*, which encodes a CAM-specific isoform of PEPC, an early indicator of CAM induction in the common ice plant (Cushman et al., 1989). We first established experimental conditions to monitor induction of steady-state *Ppc1* transcripts under various stress treatments in detached common ice plant leaves (Schmitt, 1990; Dai et al., 1994; Peters et al., 1997). Exposure of detached leaves to dehydration, 0.4 M NaCl, and 0.8 M mannitol caused a rapid (<3 h) increase in *Ppc1* transcript abundance (Fig. 1A). Incubation of detached leaves in water failed to initiate *Ppc1* transcript accumulation until 12 h after detachment. Steady-state *Ppc1* transcripts were most effectively induced by dehydration stress reaching maximum accumulation within 3 to 6 h. NaCl (0.4 M) was also effective, however, maximal induction occurred more slowly than in dehydration-stressed leaves.

Mannitol treatment resulted in a lower amount of transcript accumulation, although the rapidity of induction was comparable to that observed for dehydration. ABA treatment resulted in less transcript accumulation than dehydration or NaCl induction, with maximal induction occurring after 9 h. Other plant growth regulators, including 6-BAP and methyl jasmonate, failed to cause an increase in *Ppc1* transcript abundance (data not shown) consistent with previous observations (Dai et al., 1994; Schmitt et al., 1996; Peters et al., 1997). As a control, the transcript abundance of *Fnr1*, a gene encoding Fd NADP+ reductase, was assessed using the same RNA samples and remained unchanged following stress or ABA treatments (Fig. 1B) consistent with earlier observations that *Fnr1* expression was

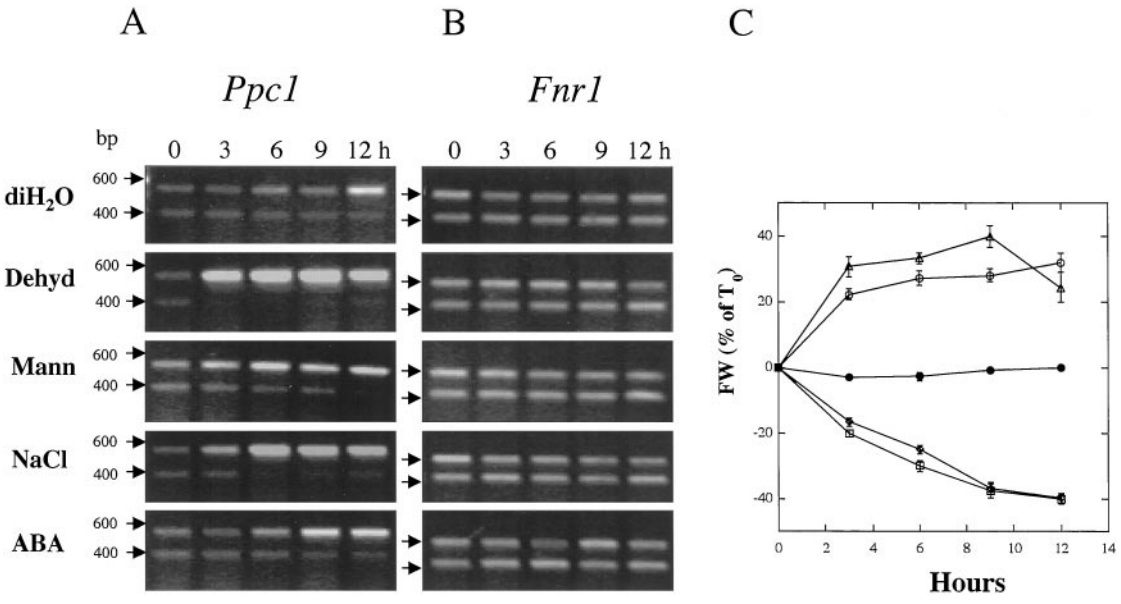

Figure 1. Stress-induced changes in *Ppc1* and *Fnr1* transcript accumulation in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were incubated in diH_2O, without water (Dehyd), incubated in 0.8 M mannitol (Mann), 0.4 M NaCl (NaCl), or 10 μM ABA for 0, 3, 6, 9, or 12 h. Endogenous *Ppc1* transcripts are represented by a 556-bp band, whereas, RT-PCR products arising from the synthetic Δ *Ppc1* RNA homologous mimic transcripts produced a 393-bp product. B, Ethidium bromide-stained gel of RT-PCR products of *Fnr1* and a homologous, synthetic Δ *Fnr1* RNA mimic. Control RT-PCR assays for native *Fnr1* transcripts resulted in a 600-bp product, whereas the synthetic Δ *Fnr1* RNA homologous mimic transcripts produced a 406-bp product. C, Fresh weight (FW) changes in detached leaves following different treatments: diH_2O, ○; without water, □; 0.8 M mannitol, ◇; 0.4 M NaCl, ●; 10 μM ABA, △. Data represent the mean values obtained from three or four independent experiments. Error bars indicate the SE. T_0, Time zero.

unaffected by salinity stress (Michalowski et al., 1989). *Fnr1* expression also serves as a convenient internal control to confirm equivalent amounts of input RNA analogous to ethidium bromide staining of RNA gels used for northern-blot analysis.

Under dehydration stress, detached leaves incubated under controlled conditions lost approximately 20% of their fresh weight within 3 h (Fig. 1D), as opposed to 2 h observed under greenhouse conditions (Schmitt, 1990). After 12 h of exposure to dehydration or osmotic stress (0.8 M mannitol) leaves lost 40% of their original fresh weight (Fig. 1C). In contrast, ionic stress caused a slight decline in fresh weight (3%) with leaves recovering most of their fresh weight after 9 to 12 h. Leaves held in water or treated with 10 μM ABA gained an average of 27% and 32% fresh weight, respectively.

The movement of Ca^{2+} down a concentration gradient through Ca^{2+}-permeable channels from extra- or intracellular stores represents one important mode of signal transduction of external stress signals (Bush, 1995; Sanders et al., 1999). Therefore, we investigated the role of calcium on the stress and ABA induction of *Ppc1* transcripts by testing various calcium chelators, ionophores, and inhibitors of calcium ATPase activity. A 3-h pretreatment of detached leaves with 5 mM EGTA, a chelator of extracellular Ca^{2+}, blocked *Ppc1* steady-state transcript accumulation under dehydration, NaCl, and mannitol stress, as well as ABA-treated and water control leaves (Fig. 2A). To confirm that the effects of EGTA treatment were not limited to *Ppc1* expression, the expression of two other genes that have been shown to undergo increased expression during CAM induction were examined. The expression of both *GapC1*, which encodes cytosolic NAD-glyceraldehyde-3-phosphate dehydrogenase (Ostrem et al., 1990), and *Mdh1*, which encodes cytosolic NAD-malate dehydrogenase (Ocheretina and Scheibe, 1997), were blocked by 5 mM EGTA (Fig. 2, C and D). This concentration of EGTA had no effect on *Fnr1* expression (Fig. 2B), although higher concentrations (10–20 mM) inhibited expression of all genes nonspecifically (data not shown). Specific inhibition of *Ppc1, GapC1, and Mdh1* induction by EGTA implicated the involvement of extracellular calcium in signaling increased CAM-specific gene expression. EGTA pretreatment enhanced water loss under dehydration, mannitol, and NaCl treatment and prevented leaf fresh weight gains in deionized water (diH_2O)- and ABA-treated leaves (Fig. 2E) consistent with the role of extracellular Ca^{2+} in mediating stomatal guard cell closure (Webb and Hetherington, 1997).

To provide further evidence that elevations of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{cyt}$) are involved in signaling CAM induction, detached leaves were treated with ionomycin, a calcium ionophore (Liu and Hermann, 1978), for 1, 3, 6, and 9 h in the absence of stress or ABA treatments. Although ionomycin treatment was observed to trigger increases in *Ppc1* transcript at earlier time points, results varied from different experimental replicates. Consistent *Ppc1* transcript accumulation was observed, however, after 9 h of treatment with 0.1 and 1 μM ionomycin (Fig. 3A). *Fnr1* expression remained unchanged by ionomycin treatment at all concentrations tested. These results suggest that an

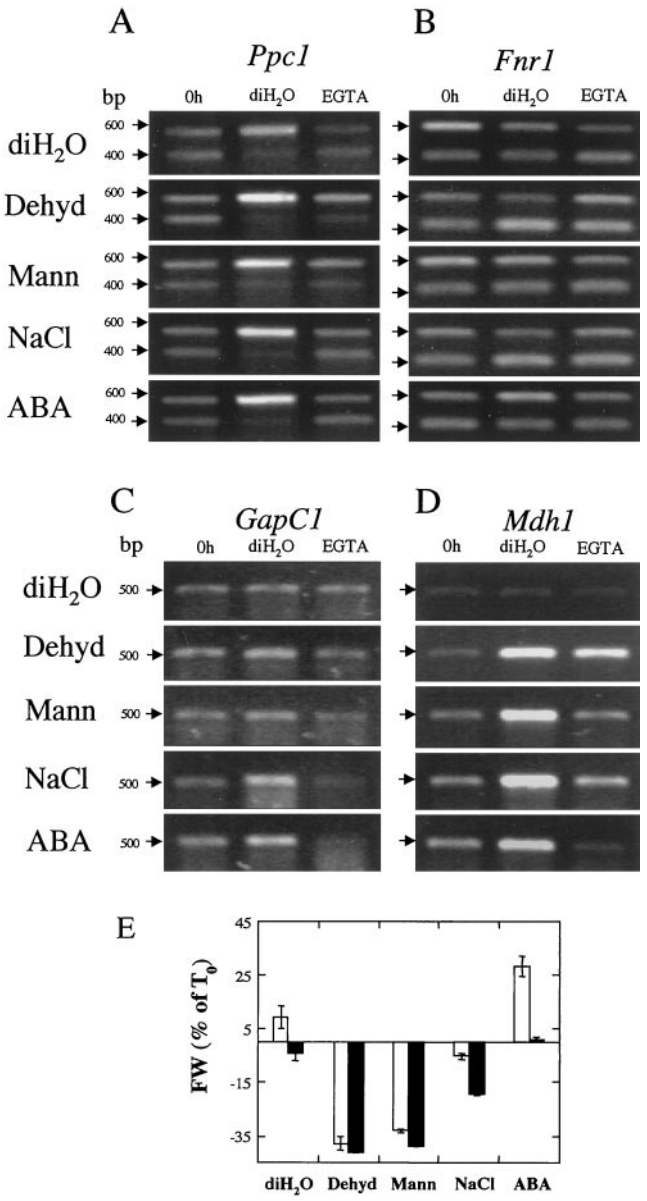

Figure 2. Effect of EGTA pretreatment on *Ppc1*, *GapC1*, *Mdh 1*, and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products from native PEPC transcripts (*Ppc1*) and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were subjected to diH_2O, dehydration (Dehyd), 0.8 M mannitol (Mann), and 0.4 M NaCl (NaCl) for 6 h or 10 μM ABA for 9 h as described in the Figure 1 legend, except that leaves were incubated in 5 mM EGTA for 3 h prior to these treatments. B, Control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts. Data shown are representative of three independent experiments. C, RT-PCR products from the cytosolic NAD-glyceraldehyde-3-phosphate dehydrogenase gene (*GapC1*). D, RT-PCR products from the cytosolic NAD-malate dehydrogenase gene (*Mdh1*). E, Fresh weight (FW) changes in detached leaves following different stress treatments in the absence (diH_2O, white bars) or presence (black bars) of 5 mM EGTA. Data represent the mean values obtained from three independent experiments. Error bars indicate the SE. T_0, Time zero.

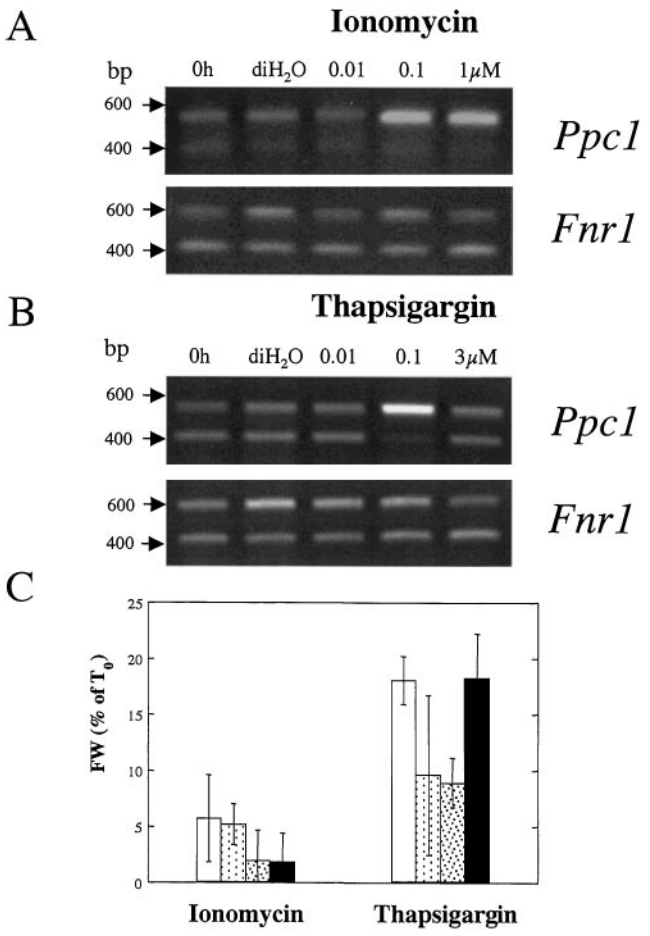

Figure 3. Effect of ionomycin and thapsigargin on *Ppc1* and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic (top) or control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts (bottom). Detached leaves were subjected to diH_2O for 0 h, 9 h (diH_2O), or to 0.01, 0.1, and 1 μM ionomycin for 9 h. Data shown are representative of three independent experiments. B, Identical to A, except that leaves were subjected to 0.01, 0.1, and 3 μM thapsigargin for 9 h. Data shown are representative of three independent experiments. C, Fresh weight (FW) changes in detached leaves following treatments in the absence (diH_2O, white bars) or presence of ionomycin and thapsigargin (0.01 μM, white bars with loose dot pattern; 0.1 μM, white bars with tight dot pattern; and 1 μM/3 μM, black bars). Data represent the mean values obtained from three independent experiments. Error bars indicate the SE. T_0, Time zero.

influx of extracellular Ca^{2+} may play a role in this signaling pathway. Ionomycin treatment had no significant effect on leaf fresh weight changes (Fig. 3C).

To confirm that Ca^{2+} participates in CAM induction signaling, leaves detached from well-watered plants were treated for 9 h with thapsigargin, an inhibitor of endomembrane Ca^{2+}-ATPases and a releaser of intracellular Ca^{2+} (Takemura et al., 1989; Thastrup et al., 1989). In water control leaves, a low concentration of thapsigargin (0.1 μM) was sufficient to induce *Ppc1* transcript accumulation in the absence of stress, whereas lower (0.01 μM) and higher concentrations (3 μM) were ineffective (Fig. 3B). In contrast, thapsigargin failed to induce *Fnr1* transcript accumulation within the concentration range tested (Fig. 3B). Dose dependency of the effects of thapsigargin on cytosolic free Ca^{2+} concentrations has been observed in mammalian cells. At nanomolar concentrations, thapsigargin induced sustained Ca^{2+} release, whereas at micromolar concentrations it decreased intracellular Ca^{2+} concentrations by blocking voltage-activated channels (Rossier et al., 1993). Although thapsigargin treatments cannot differentiate between intra- and extracellular Ca^{2+} sources, these results indicate that changes in intracellular Ca^{2+} are likely to contribute to *Ppc1* induction. Low concentrations of thapsigargin (0.01 and 0.1 μM) repressed water uptake, however, *Ppc1* transcripts accumulated only at the 0.1 μM concentration (Fig. 3C).

As a first step toward identifying potential downstream effectors of Ca^{2+} in the common ice plant, we tested specific inhibitors of Ca^{2+}-dependent/CaM-like domain protein kinases (CPKs) and CaM-dependent protein kinases (CaMKs) for their effect on steady-state *Ppc1* transcript accumulation. W7, a CaM antagonist and inhibitor of CPK (Harmon et al., 1987), inhibited *Ppc1* expression in response to dehydration, mannitol, salinity, and ABA treatments (Fig. 4A). W5 (*N*-(6-aminohexyl)-1-naphthalenesulfonamide), a structural analog of W7 having approximately 10-fold less activity (Hidaka et al., 1981), did not significantly inhibit *Ppc1* transcript accumulation. To obtain additional evidence that CPKs or CaMKs may participate in regulating *Ppc1* expression, we tested the effects of lavendustin C (C5, compound 5), a CaM kinase II inhibitor (O'Dell et al., 1991). *Ppc1* expression was specifically inhibited at 0.5 μM C5, although the effect was less dramatic than W7 (Fig. 4A). In all cases, *Fnr1* expression was not inhibited by W7/W5/C5 treatments ruling out general toxic effects on transcriptional processes (Fig. 4B). The observation that a less potent structural analog of W7 failed to interfere with *Ppc1* induction suggests that nonspecific toxic effects on signaling are also unlikely. Whereas results obtained with sulfonamide inhibitor studies must always be interpreted with caution due to possible inhibition of other processes, such as mitochondrial respiration (Miernyk et el., 1987), our results suggest that CaM and/or Ca^{2+}- or CaM-dependent protein kinase may participate in transducing Ca^{2+} signals during CAM induction. Although W7/W5/C5 treatments did significantly alter leaf fresh weight changes under certain treatment conditions, these changes were generally small and were negatively correlated with *Ppc1* transcript abundance (Fig. 4C).

To identify possible PP activities that might participate in CAM induction, OKA, an inhibitor of PP2A (and PP1) activities (Smith and Walker, 1996) was tested from 2.5 to 250 nM to establish a dose-response curve. Intermediate concentrations of OKA (20 nM) specifically inhibited *Ppc1* induction under all conditions tested (Fig. 5A), whereas *Fnr1* expression remained unaffected (Fig. 5B). A lower concentration (2.5 nM) had only a slight inhibitory effect, whereas a higher concentration (250 nM) resulted in the nonspecific inhibition of both *Ppc1* and *Fnr1* expression (data not shown). Furthermore, treatment with 20 nM Nor, a structural analog of OKA, had little effect on *Ppc1* tran-

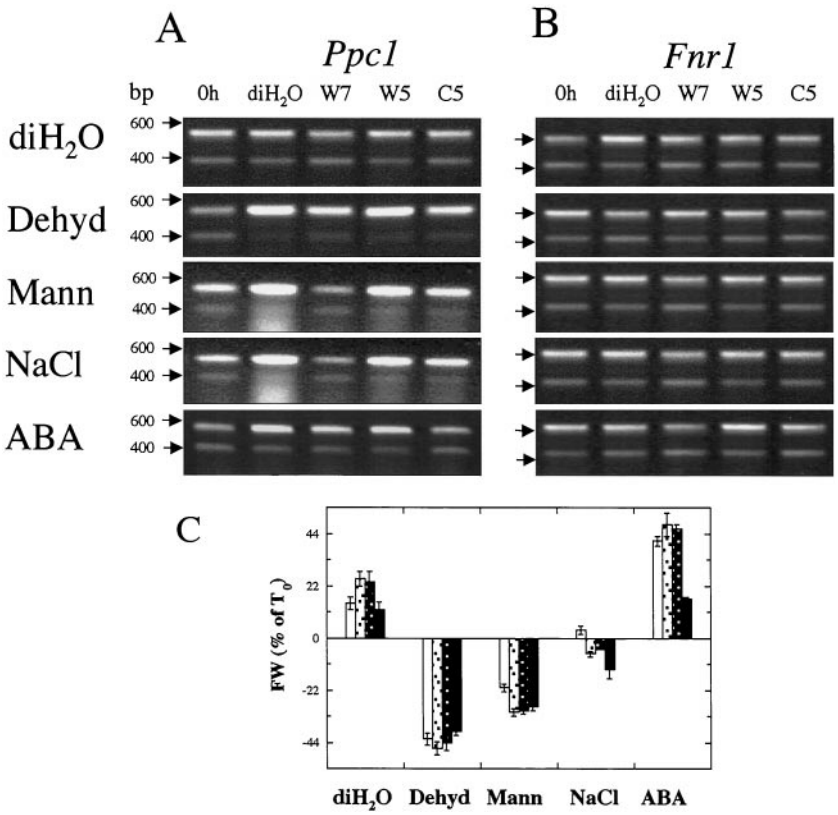

Figure 4. Effect of W7, W5, and C5 on *Ppc1* and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were subjected to diH_2O, dehydration (Dehyd), 0.8 M mannitol (Mann), and 0.4 M NaCl for 6 h or 10 μM ABA for 9 h as described in the Figure 1 legend, except that leaves were subjected to 50 μM W7 or W5, and 0.5 μM compound 5 (C5) for 3 h prior to these treatments. B, Control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts. Data shown are representative of three independent experiments. C, Fresh weight (FW) changes in detached leaves following different stress treatments in the absence (control, white bars (diH_2O) or presence of 50 μM W7 (white bars with black dots) or W5 (black bars with white dots), and 0.5 μM C5 (black bars). Data represent the mean values obtained from three independent experiments. Error bars indicate the SE. T_0, Time zero.

script expression (Fig. 5A), even at the highest concentration (250 nM) tested (data not shown). The sensitivity of *Ppc1* induction to OKA suggests that PP2A and/or PP1 may be involved in the CAM induction process, however, the concentrations used make it difficult to distinguish between these two classes of phosphatases. The concentration of OKA used in these experiments (20 nM) is unlikely to inhibit other classes of PPs, such as PP2B (calcineurin). OKA and Nor treatments had no significant effect on *Fnr1* accumulation (Fig. 5B). Although both OKA and Nor treatments did result in modest alterations in leaf fresh weight in certain instances, the changes observed were not positively correlated with changes in *Ppc1* or *Fnr1* mRNA accumulation (Fig. 5C).

Recent studies have implicated Ca^{2+}/CaM-dependent PP2B (calcineurin) as playing a pivotal role in signal cascades involving guard cell behavior (Luan et al., 1993; Allen and Sanders, 1995) and salt stress adaptation (Pardo et al., 1998). Disruption of the Arabidopsis SOS3 gene, which encodes a protein resembling yeast calcineurin subunit B and animal calcium sensor proteins that stimulate PPs or inhibit protein kinases, results in hypersensitivity to Na^+ and Li^+, suggesting that a signaling pathway involving calcineurin or related phosphatase activities regulates K^+ and Na^+ transport processes (Liu and Zhu, 1998). The requirement of Ca^{2+} for CAM signal transduction presents the possibility that Ca^{2+}-dependent PP activities participate in signaling events leading to CAM induction. To test the possible involvement of calcineurin in signaling CAM induction, detached leaves were treated with cyclosporin A (CsA), which forms cyclophilin-CsA complexes that inhibit Ca^{2+}/CaM-activated PP2B (Liu et al., 1992), prior to stress or ABA treatments. CsA stimulated *Ppc1* transcript accumulation in unstressed (control) leaves and caused a slight super-accumulation of transcripts in leaves exposed to dehydration stress (Fig. 6A). In contrast, *Fnr1* expression was largely unaffected by this treatment (Fig. 6B). Cyclosporin A treatment caused a large decrease (20%) in water content in diH_2O control leaves (Fig. 6C). Unlike most of the other inhibitors tested, CsA caused an average decrease of 6.8% in leaf fresh weights for stress and ABA treatments (Fig. 6C). CsA and FK506 block Ca^{2+}-induced inactivation of guard cell K^+ channels, which in turn prevents K^+ efflux and stomatal closure (Luan et al., 1993). Thus, enhanced *Ppc1* transcript accumulation in response to CsA may merely be an indirect effect of increased water loss due to a disruption in stomatal closure.

To determine if the stimulatory effects of CsA on *Ppc1* transcript accumulation were indeed indirect effects of changes in leaf water status, ascomycin, an analog of the immunosuppressant FK-506, which forms immunophilin (FK-506 binding protein)-FK-506 complexes that inhibit PP2B [Kawai et al., 1993], was tested. Ascomycin treatment stimulated *Ppc1* transcript accumulation in dehydration, mannitol and ABA treated leaves, but not in NaCl treated leaves (Fig. 7A). *Fnr1* transcripts remained largely unchanged by ascomycin treatments (Fig. 7B). Ascomycin treatment resulted in an average decrease of 5.6% in leaf fresh weights for mannitol and NaCl stress treatments, as well as diH_2O control leaves (Fig. 7C). Under conditions

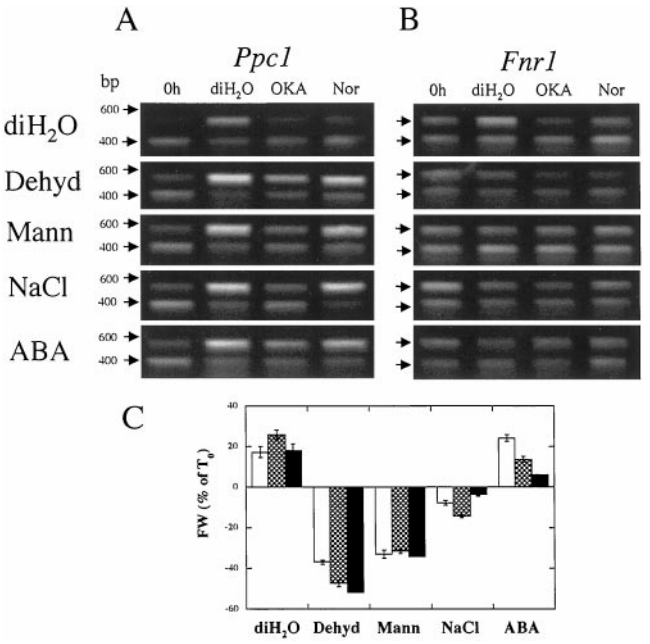

Figure 5. Effect of OKA and Nor on *Ppc1* and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were subjected to diH_2O, dehydration (Dehyd), 0.8 M mannitol (Mann), and 0.4 M NaCl for 6 h or 10 μM ABA for 9 h as described in the Figure 1 legend, except that leaves were treated with 20 nM OKA or Nor for 3 h prior to these treatments. B, Control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts. Data shown are representative of three independent experiments. C, Fresh weight (FW) changes in detached leaves following different stress treatments in the absence (control [diH_2O] white bars) or presence of 20 nM OKA (checkered bars or Nor (black bars). Data represent the mean values obtained from three independent experiments. Error bars indicate the SE. T_0, Time zero.

where ABA is produced in abundance (e.g. dehydration stress) or supplied exogenously, ABA-induced stomatal closure may limit the water loss promoting effects of Ascomycin resulting in little or no increase in fresh weight losses (Fig. 7C). In these instances, it is possible that ascomycin may exert a direct, stimulatory effect on *Ppc1* transcript accumulation.

DISCUSSION

Osmotic stress (dehydration and mannitol), ionic stress (NaCl), as well as ABA cause an increase in *Ppc1* transcript accumulation in detached leaves of the common ice plant. Previous studies have successfully used detached leaves to monitor gene expression or enzyme activity changes associated with CAM induction (Schmitt, 1990; Dai et al., 1994; Peters et al., 1997). Control leaves held in water alone showed a 27% increase in relative fresh weight and showed lower or unchanged amounts of *Ppc1* transcript accumulation up to 9 h post-detachment similar to an earlier report (Schmitt, 1990). *Ppc1* transcript accumulation was shown previously to arise primarily from increased transcription rates (Cushman et al., 1989). The increase in *Ppc1* transcripts after 9 h (Fig. 2, top left) or 12 h (Fig. 1, top left) due to slight decreases in fresh weight suggests that *Ppc1* induction is triggered by relative changes in the water status of the leaf, rather than the absolute water content. As expected, dehydration stress caused very rapid and strong induction of *Ppc1* transcripts consistent with previous observations (Schmitt, 1990; Taybi et al., 1995). Although mannitol treatment resulted in fresh weight losses similar to dehydration stress, the magnitude of *Ppc1* transcript accumulation was about one-tenth that observed in dehydrated leaves. This observation suggests that the extent of water loss alone does not strictly correlate with the magnitude of induction. This notion is reinforced by the observation that iso-osmolar NaCl treatment caused a strong increase in *Ppc1* transcript abundance with only a small decrease in fresh weight loss that is readjusted within a few hours (Fig. 1C). These observations, along with the distinct magnitude of response elicited by the different treatments, suggest that the signal transduction mechanisms of perceiving and/or responding to ionic stress differ from those of osmotic or dehydration stress. Furthermore, detached

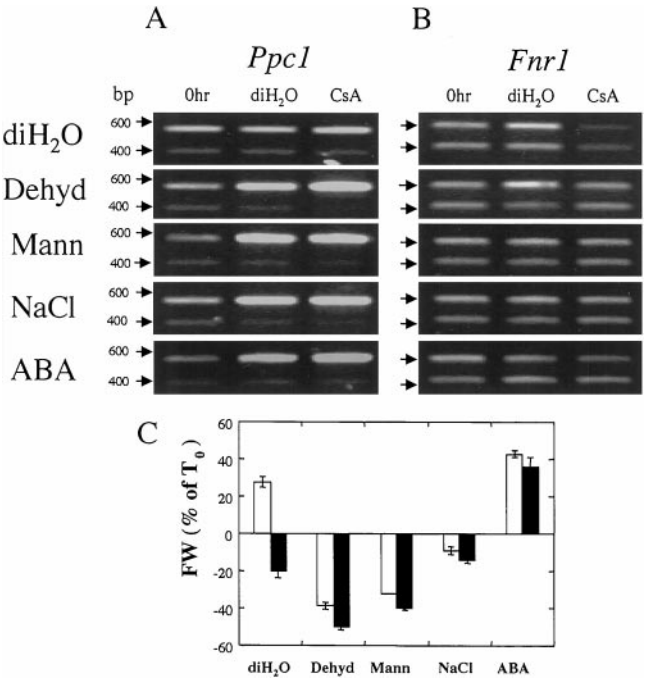

Figure 6. Effect of CsA on *Ppc1* and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were subjected to diH_2O, dehydration (Dehyd), 0.8 M mannitol (Mann), or 0.4 M NaCl for 6 h or 10 μM ABA for 9 h as described in the Figure 1 legend, except that leaves were treated with 50 μM (CsA) for 3 h prior to these treatments. B, Control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts. Data shown are representative of three independent experiments. C, Fresh weight (FW) changes in detached leaves following different stress treatments in the absence (diH_2O, white bars) or presence of 50 μM CsA (black bars). Data represent the mean values obtained from four independent experiments. Error bars indicate the SE. T_0, Time zero.

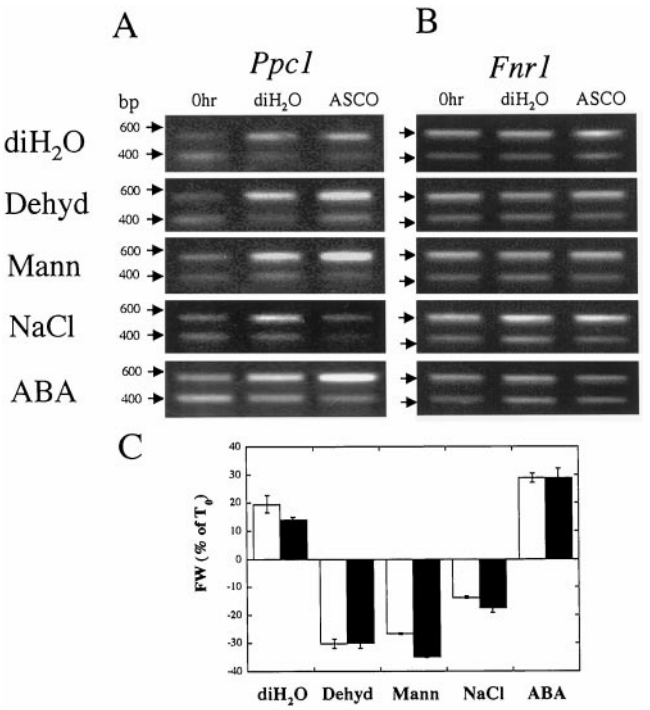

Figure 7. Effect of ascomycin on *Ppc1* and *Fnr1* transcript abundance in detached common ice plant leaves. A, Ethidium bromide-stained gel of RT-PCR products of *Ppc1* and a homologous, synthetic Δ *Ppc1* RNA mimic. Detached leaves were subjected to deionized water, dehydration (Dehyd), 0.8 M mannitol (Mann), or 0.4 M NaCl for 6 h or 10 μM ABA for 9 h as described in the Figure 1 legend, except that leaves were treated with 25 μM ascomycin (ASCO) for 3 h prior to these treatments. B, Control RT-PCR assays using primer sets for native *Fnr1* and synthetic Δ *Fnr1* transcripts. Data shown are representative of three independent experiments. C, Fresh weight (FW) changes in detached leaves following different stress treatments in the absence (diH_2O, white bars) or presence of 25 μM ascomycin (black bars). Data represent the mean values obtained from three independent experiments. Error bars indicate the SE. T_0, Time zero.

leaves can sense and respond to osmotic or ionic stress in the absence of root signals. Although root signaling can trigger CAM induction (Eastmond and Ross, 1997), changes in turgor pressure in leaves must also participate in signaling CAM induction (Winter and Gademann, 1991).

ABA induction of *Ppc1* transcripts was slower than other stresses and proceeded despite large increases (27%–33%) in relative fresh weight. This observation agrees with previous work showing that ABA-induced increases in the expression of CAM or key CAM enzymes does not require reductions in leaf fresh weight (Chu et al., 1990). These results also confirm previous reports that exogenous application of ABA to plants or detached leaves results in increased accumulation of PEPC transcripts (Thomas et al., 1992; Taybi et al., 1995), protein, and activity of CAM-specific isoforms of PEPC (Dai et al., 1994). Although ABA amounts were not determined in these experiments, it is well established that salinity and drought stress cause rapid increases in ABA amounts and that these increases precede the accumulation of *Ppc* transcripts (Thomas et al., 1992; Taybi et al., 1995). The delay in *Ppc1* transcript induction by exogenous ABA treatment suggests that ABA signaling operates through the same pathway as salinity, osmotic, or dehydration stress, but that flux through the pathway is simply slower or that ABA operates through a secondary, independent, or parallel signaling pathway.

Alterations in cytosolic $[Ca^{2+}]$ play an important role in transducing salinity or drought stress signals into cellular responses (Shinozaki and Yamaguchi-Shinozaki, 1997). Changes in $[Ca^{2+}]_{cyt}$ following osmotic shock of protoplasts or cell suspensions (Lynch et al., 1989; Takahashi et al., 1997) or intact plants (Knight et al., 1997) provide strong evidence for the involvement of Ca^{2+} in signaling stress responses. To our knowledge, results from this study provide the first evidence that changes in $[Ca^{2+}]_{cyt}$ participate in the induction of multiple CAM-specific genes and that such changes are also likely to initiate the induction of CAM in general. Preincubation of detached leaves with 5 mM EGTA to chelate extracellular Ca^{2+} abolished elevated *Ppc1*, *GapC1*, or *Mdh1* transcript accumulation following stress or ABA treatments. This result indicated that an influx of extracellular Ca^{2+} is likely to contribute, in part, to signaling events leading to CAM induction (Fig. 2). Ionomycin treatment of detached leaves from well-watered plants lends further support to the role of extracellular Ca^{2+} in CAM signaling (Fig. 3A).

Previous reports in Arabidopsis, using the calcium channel blockers lanthanum chloride, verapamil, and gadolinium, suggest that entry of extracellular Ca^{2+} into the cell under stress conditions may occur mainly through L-type Ca^{2+} channels and to a lesser extent through stretch-activated Ca^{2+} channels (Knight et al., 1997). Work is in progress to verify the existence and relative contribution of different classes of Ca^{2+} channels to CAM induction in the common ice plant. Preliminary results, however, showed inhibition of *Ppc1* expression with the calcium channel blockers lanthanum chloride and verapamil and suggested that L-type Ca^{2+} channels in common ice plant might also mediate Ca^{2+} entry into the cell during stress conditions. In addition to extracellular Ca^{2+} stores, intracellular sources of Ca^{2+} mobilized from the vacuole or ER may also contribute to altered $[Ca^{2+}]_{cyt}$ following osmotic stress.

The ability of thapsigargin to enhance *Ppc1* expression in the absence of stress or ABA treatments (Fig. 3B) suggests that intracellular and/or extracellular Ca^{2+} efflux can trigger the signaling events leading to CAM induction. By inhibiting Ca^{2+}-ATPase activity at the tonoplast, ER, or plasma membrane, thapsigargin is likely to disrupt normal Ca^{2+} sequestration resulting in *Ppc1* induction in the absence of stress derived signaling events. These results are similar to experiments performed using Arabidopsis, in which the incomplete inhibition of drought- and salt-induced increases in $[Ca^{2+}]_{cyt}$ by the calcium channel blocker lanthanum or EGTA suggested that internal and external Ca^{2+} stores contribute to $[Ca^{2+}]_{cyt}$ elevations following stress (Knight et al., 1997).

Ca^{2+} signals are sensed and converted into changes in gene expression through the activation of a collection of Ca^{2+} sensors, including CPKs, CaMKs, Ca^{2+}-dependent phosphatases (calcineurin), Ca^{2+}/CaM-regulated ion transporters, and other calcium-binding proteins (Sanders

et al., 1999). The signaling machinery responsible for decoding various Ca^{2+} signatures is also thought to play a role in controlling the specificity of responses (Lee et al., 1995, 1998; Szymanski et al., 1996). Partial inhibition of *Ppc1* transcript accumulation under stresses and ABA by the CaM/CPK antagonist W7 and the CaM kinase II inhibitor C5, but not by the less active analog W5, suggests that CaM and/or CPKs and CaMKs are likely to act as positive regulators in this response. Large multigene families of CPKs have been described in plants which exhibit diverse expression patterns implying equally diverse functions (Hong et al., 1996; Hrabak et al., 1996). Previous reports of salinity, dehydration, or osmotic stress or ABA-induced expression or activity of Ca^{2+}-binding proteins (Frandsen et al., 1996; Jang et al., 1998) or CPKs (Urao et al., 1994; Pestenácz and Erdei, 1996) suggest that these proteins may play important roles in Ca^{2+}-mediated signaling pathways. Treatment of isolated maize protoplasts with Ca^{2+} and Ca^{2+} ionophores can activate an ABA- and stress-responsive gene expression, presumably through the action of CPKs (Sheen, 1996). Selected CPKs, modified to contain a constitutively active catalytic domain, can act as positive regulators of ABA-mediated stress signal transduction when overexpressed in maize protoplasts (Sheen, 1996).

In the halotolerant, unicellular green alga *Dunaliella salina*, CaM antagonists trifluoperazine and W-7 reduce osmotic adaptation to hyperosmotic stress by inhibiting glycerol biosynthesis, suggesting that CaM or CPK participates in the signal transduction pathway activated during salinity stress adaptation (Ko and Lee, 1995). More recently, a guard cell CPK has been characterized that can phosphorylate the KAT1 potassium channel and thus is likely to participate in Ca^{2+}-regulated modulation of stomatal responses to environmental stimuli (Li et al., 1998). To further investigate the roles of CPKs involved in signaling gene expression changes leading to the induction of CAM or other stress adaptive processes, we have recently characterized a salinity- and drought-inducible CPK from common ice plant (Taybi and Cushman, 1998). However, more information, such as the physiological substrate(s) phosphorylated by this CPK, is needed before a functional role can be assigned.

The phosphorylation status of downstream signaling components is controlled not only by protein kinases, but also by the opposing action of PPs. In addition to protein kinase inhibitors, the use of specific PP inhibitors, such as OKA, can provide a powerful way to initially assess the involvement of phosphorylation/dephosphorylation events that may participate in CAM induction. PP2A and PP1 activities have been implicated in a wide range of plant metabolic processes. In CAM plants, protein synthesis-dependent dephosphorylation of PEPC by a PP2A down-regulates PEPC activity (Carter et al., 1990). Although results from inhibitor studies must be viewed with caution, our results show that OKA causes a specific, dose-dependent inhibition of *Ppc1* expression, while having no effect on *Fnr1* expression at the same concentration (Fig. 5). These results implicate the participation of PP2A/PP1 activities in CAM induction, possibly in a positive role.

Pharmacological and biochemical studies support the apparent involvement of Ca^{2+}-dependent calcineurin-like PP activity in regulating guard cell K^+ channel activity (Luan et al., 1993; Allen and Sanders, 1995). Enhanced salt tolerance in transgenic tobacco plants expressing a reconstituted, constitutively active yeast calcineurin indicates that Ca^{2+}/CaM-dependent calcineurin, as in yeast, are involved in osmotic stress adaptation in plants (Pardo et al., 1998). Calcineurin-like calcium sensor proteins that modulate the activities of protein kinases and phosphatases are also involved in salt adaptation (Liu and Zhu, 1998). Thus, we sought evidence for the involvement of calcium-dependent (Ser/Thr) PP2B (calcineurin) activity in CAM signaling transduction (Figs. 6 and 7). It is interesting that *Ppc1* expression was found to be enhanced by treating detached leaves with CsA or ascomycin, although this was probably an indirect effect due to excessive fresh weight loss from the leaves, since CsA is known to antagonize ABA-inhibited stomatal opening and ABA-induced stomatal closure (Hey et al., 1997). However, ascomycin treatment did not lead to an increase in fresh weight loss in dehydration and ABA-treated leaves, suggesting that *Ppc1* transcript accumulation may be a direct effect of this PP2B inhibitor. These results do not discount the possibility that calcineurin activity might negatively regulate stress signaling during CAM induction.

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