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Convergent Induction of Osmotic Stress-Responses[1]

Abscisic Acid, Cytokinin, and the Effects of NaCl

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ABSTRACT

In *Mesembryanthemum crystallinum*, salt stress induces the accumulation of proline and a specific isoform of the enzyme phosphoe*nol*pyruvate carboxylase (PEPCase) prior to the switch from C_3 to Crassulacean acid metabolism (CAM). To determine whether plant growth regulators initiate or imitate these responses, we have compared the effects elicited by NaCl, abscisic acid (ABA), and cytokinins using PEPCase and proline levels as diagnostic tools. Exogenously applied ABA is a poor substitute for NaCl in inducing proline and CAM-specific PEPCase accumulation. Even though ABA levels increase 8- to 10-fold in leaves during salt stress, inhibition of ABA accumulation does not affect these salt-induced responses. In contrast, the addition of cytokinins (6-benzylaminopurine, zeatin, 2-isopentyladenine) mimic salt by greatly increasing proline and PEPCase amounts. Endogenous zeatin levels remain unchanged during salt stress. We conclude: (a) The salt-induced accumulation of proline and PEPCase is coincident with, but is not attributable to, the rise in ABA or zeatin concentration. (b) For the first time, cytokinins and NaCl are implicated as independent initiators of a sensing pathway that signals leaves to alter PEPCase gene expression. (c) During stress, the sensing of osmotic imbalances leading to ABA, proline, and CAM-specific PEPCase accumulation may be mediated directly by NaCl.

NaCl effects on plant physiological responses, metabolism, and gene expression have been studied, yet little is known about how salt stress alters metabolism in higher plants. NaCl may act directly via osmotic or ionic sensory mechanisms, or it may act indirectly through mediators such as PGRs[2] to affect existing metabolic pathways, gene expression, and result in a coordinated response to osmotic stress. Experimental evidence suggests that the PGR ABA may initiate global physiological reactions in plants in response to environmental stress. ABA has been shown to participate in a number of plant processes, including the stimulation of stomatal movements, increased root growth, proline accumulation, and the inhibition of shoot growth (10, 15, 29). In addition, many observations have indicated that salt stress leads to the rise in the level of endogenous ABA (42). During high salinity, drought, low temperature, and seed development (dehydration stress), elevated ABA levels appear to provoke discrete changes in gene expression (7, 14, 34).

Cytokinins, another class of plant growth regulators, are considered antagonists of ABA. ABA inhibits and cytokinin promotes the opening of leaf stomata and cotyledon expansion (4, 20). The increased accumulation of Chl caused by cytokinin treatment is an opposite effect to the senescence resulting from ABA treatment (2, 42). Total RNA synthesis is stimulated by the exogenous addition of cytokinin, whereas the exogenous addition of ABA reverses this response (37). Cytokinin levels themselves may also be directly affected by salt stress. Roots are the first tissues exposed to salt. They are also the primary sites of cytokinin synthesis (8). Drought and salt stress could also affect the transport of cytokinin from the root to the leaf (33), thereby directly influencing cytokinin-induced gene expression (1).

Mesembryanthemum crystallinum (common ice plant) provides one model system for studying whether responses to salt stress involve PGRs. After exposure to NaCl, this facultative halophyte responds by rapidly increasing the endogenous concentrations of the putative osmoprotectant proline (13). This is strictly a cellular response to stress (38). As the salt challenge continues, changes in transcript levels of diverse genes have been observed (22), some of which contribute to the establishment of CAM. One enzyme indicative of the switch from C_3 photosynthesis to CAM is PEPCase (EC 4.1.1.31). PEPCase enzyme and mRNA amounts are increased by salt treatment (17, 26). A single PEPCase isogene, *Ppc1*, is transcriptionally induced and is primarily responsible for the observed increase in enzymic activity during salt stress (11, 12). Although the responses to NaCl are well described in this species, the mechanism(s) responsible for initiating these changes are unknown.

To determine whether NaCl stress influences plant metabolism by altering the levels of the PGRs ABA and cytokinins, we have used two indicators of salt stress in *M. crystallinum*: PEPCase induction and proline accumulation. Because PEPCase accumulation is not induced by salt in suspension cells of this species (38), we used hydroponically grown plants to measure ABA and cytokinin levels in vivo during salt stress, as well as the accumulation of PEPCase and proline. ABA

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[2] Abbreviations: PGR, plant growth regulator; 6-BAP, 6-benzylaminopurine; 2iP, 2-isopentyladenine; PEPCase, phospho*enol*pyruvate carboxylase.

levels in leaves increase drastically during salt treatment, but PEPCase induction is not influenced either by the inhibition of ABA synthesis during salt stress or by the exogenous addition of ABA. We conclude that the salt-induced rise in ABA is not required for the induction of PEPCase or proline.

On the contrary, the exogenous addition of the cytokinin 6-BAP mimics salt treatment, inducing both PEPCase and proline accumulation in the absence of NaCl. Endogenous cytokinin (zeatin-type) levels, however, remain unchanged during salt stress. We suggest that exogenously added NaCl and cytokinin provide signals that traverse a common pathway, resulting in PEPCase and proline accumulation. Large-scale fluctuations in endogenous cytokinin and ABA concentrations cannot account for the changes in gene expression induced by NaCl.

MATERIALS AND METHODS

Plant Material

Mesembryanthemum crystallinum seeds were germinated in vermiculite soaked in 1× Hoagland solution. After 3 weeks of growth at 300 $\mu E\ m^{-2}\ s^{-1}$ (12-h day; 22°C day/18°C night) seedlings were placed in 1× Hoagland solution with double the iron concentration (supplied as Fe-Sequestrene 330) and were grown with constant aeration to the hydroponic solution. Growing conditions utilized fluorescent cool white lights (350 $\mu E\ m^{-2}\ s^{-1}$ maximum) on a 12-h day cycle, with day and night temperatures of 22 and 18°C, respectively (measured at the canopy level). Under these conditions, wilting at the end of the light cycle (in the absence of NaCl treatment) was minimized. Plants were grown either in 25- or 5-L black tubs or light-tight buckets. At least three plants per treatment were combined for individual experiments, and most experiments were repeated up to three times. Growth regulators and drugs were dissolved in DMSO or ethanol and added to fresh Hoagland solution. For all preincubation and time course experiments, fresh Hoagland solution was replaced together with appropriate additives or DMSO alone (0.1 mL/L) every 2 d. A standard salt stress treatment constituted the addition of NaCl to a final concentration of 400 mM. Plant material was immediately frozen in liquid N_2 and stored at −80°C.

Protein, RNA, and Proline Analysis

RNA was extracted from N_2 frozen leaf and root material, and northern blot and slot blot analysis were performed as described (26). Total soluble protein, western blots, and proline determinations were as described (38).

ABA and Cytokinin Determination

Total unbound ABA and cytokinins were extracted in the presence of 10^3 cpm/mL·50 mCi/mmol of either [^{14}C]ABA or [^{14}C]6-BAP in 10 mL/g fresh weight 80% (v/v) methanol/H_2O with 1 mg/L butylated hydroxytoluene overnight with shaking. During ABA extraction, tubes were light tight. Samples were then centrifuged (2000*g*, 10 min), the insoluble material re-extracted (in 10 mL/g fresh weight as before) for 1 h and centrifuged, and the supernatants combined and diluted to 70% methanol/H_2O. All samples were passed over a C_{18} Extract-Clean column (Alltech Associates, Deerfield, IL) to remove organic acids. Extraction efficiencies, cpm of either [^{14}C]ABA or [^{14}C]6-BAP remaining from the original 10^3 cpm added, were typically 90% (ABA) and 40% (cytokinin). Growth regulator estimations were done using antibody-linked indirect ELISA test kits directed against ABA or zeatin-type cytokinins (Idetek Inc., San Bruno, CA). The zeatin riboside kit cross-reacted with zeatin riboside, zeatin, and zeatin monophosphate.

RESULTS

Markers for Salt Stress: PEPCase Induction and Proline Accumulation

When 6-week-old, hydroponically grown plants were treated with 400 mM NaCl for 5 to 6 d, the extent of PEPCase mRNA and protein induction was similar to that reported earlier in soil-grown salt-treated plants of the same age (11, 26). A northern blot illustrating the stress-induced accumulation of PEPCase mRNA under these conditions is shown (Fig. 1). Slightly elevated levels of *Ppc1* transcripts were detected as early as 6 h after NaCl stress. After 30 h, PEPCase mRNA was abundant. In subsequent experiments, the 30-h time point was used to compare PEPCase induction under different PGR treatments. Western blot analysis with PEPCase-specific polyclonal antibodies showed similar increases in PEPCase protein levels in the NaCl-treated plants versus unstressed plants, as observed in soil-grown plants (11). Proline levels also increased dramatically upon salt stress (approximately 10-fold), consistent with previous findings in *M. crystallinum* plants and suspension cultures (13, 38). Induction of PEPCase mRNA and protein amount and the accumulation of proline are reliable markers of the response to salt stress in leaves of the ice plant.

ABA Levels Are Elevated after Salt Exposure

The increase in PEPCase enzyme and proline during salt stress coincided with a rise in the level of endogenous ABA,

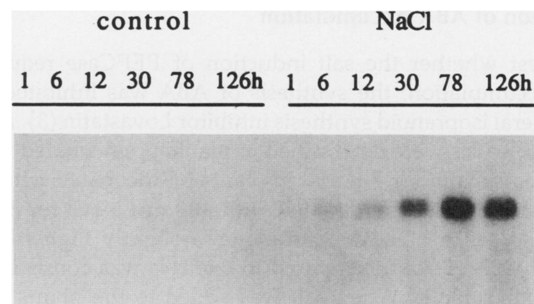

Figure 1. The induction of PEPCase mRNA in 6-week-old hydroponically grown plants by NaCl stress. Numbers correspond to hours after fresh nutrient solution (control) or addition of fresh nutrient solution containing 400 mM NaCl. Total RNA was prepared and 20 μg total RNA/lane were separated with electrophoresis in a 1% formaldehyde agarose gel. Gels were blotted onto NYTRAN filters and probed with random-primed PEPCase (*Ppc1*) probe.

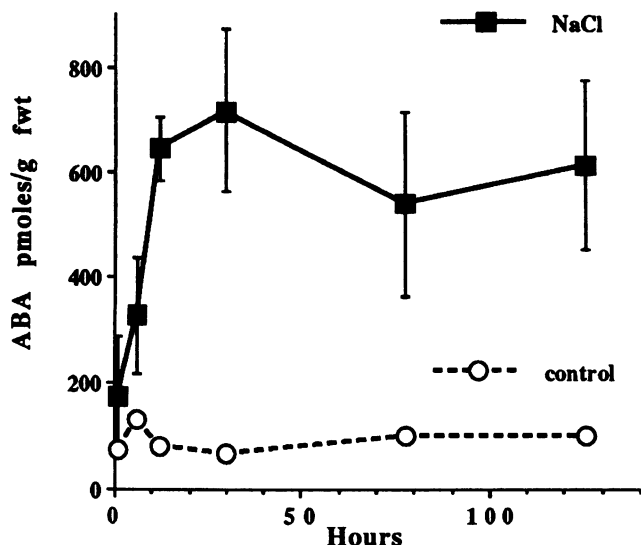

Figure 2. Analysis of endogenous ABA. Plants were grown, and the time of treatment with 400 mM NaCl (—■—) and without NaCl (-O-) was as in Figure 1. Each point represents the mean and SE (bars) of four to five plants pooled (all leaves) for each time point from at least three separate experiments. The SE in unstressed plants remained within the limits of the symbol used to depict the mean ABA concentration.

as determined during salt stress. In leaves, the endogenous level of ABA rose as early as 6 h after the addition of salt (Fig. 2). Within 30 h, an 8- to 10-fold increase in ABA was observed in salt-treated versus untreated plants (728 ± 168 and 82 ± 27 pmol/g fresh weight, respectively), and this high level persisted. The salt-triggered rise in ABA concentration paralleled the increase in PEPCase mRNA after salt treatment (see Fig. 1). In response to NaCl, ABA levels in roots also increased. From 12 to 120 h after the addition of salt, ABA concentrations in roots were 130 ± 19 pmol/g fresh weight in salt-stressed plants as compared to 67 ± 17 pmol/g fresh weight in roots of unstressed plants.

Inhibition of ABA Accumulation

To test whether the salt induction of PEPCase required ABA accumulation, the synthesis of ABA was inhibited by the general isoprenoid synthesis inhibitor Lovastatin (3). ABA levels in leaves were determined in plants preincubated with 10 μM Lovastatin for 7 d and in plants preincubated with 10 μM Lovastatin and then treated with 400 mM NaCl for 30 h. In the presence of Lovastatin alone, a slightly higher base level of ABA (2-fold) compared to controls was consistently observed (Table I). When salt was added to the plants pretreated with Lovastatin, a small increase in ABA levels was observed (1.3-fold). The accumulation of ABA observed in salt-stressed leaves was inhibited 75% by a pretreatment with 10 μM Lovastatin followed by salt (Table I). This effect was also observed in roots, where ABA concentrations were not significantly different in plants treated with Lovastatin or Lovastatin plus NaCl (Table I). Because PEPCase is only

Table I. *Decreased ABA Levels Following a Lovastatin Pretreatment*

Six-week-old leaf and root tissues were grown for 7 d in 10 μM Lovastatin. Four hundred millimolar NaCl (salt) was introduced into some hydroponic containers at day 6 and the plants were incubated for 30 h prior to harvest. Values are the mean and SE calculated from three pooled plants per experiment from three separate experiments.

Tissue Source	Treatment	ABA (SE)
		pmol/g fresh weight
Leaves	None	71 (2)
	Salt	868 (55)
	10 μM Lovastatin	148 (12)
	10 μM Lovastatin + Salt	196 (11)
Roots	None	77 (11)
	Salt	146 (5)
	10 μM Lovastatin	127 (2)
	10 μM Lovastatin + Salt	130 (4)

induced by NaCl in leaves, no further studies on roots have been included.

The extent of NaCl-induced accumulation of PEPCase mRNA, protein, and proline amounts was determined after preincubation with Lovastatin. Total RNA was isolated from leaves from the above-mentioned treatments, 5 to 0.31 μg of total RNA were slot-blotted, and the blot was probed with the salt-inducible PEPCase gene, *Ppc1*. Regardless of preculture with or without the inhibitor, PEPCase transcripts were

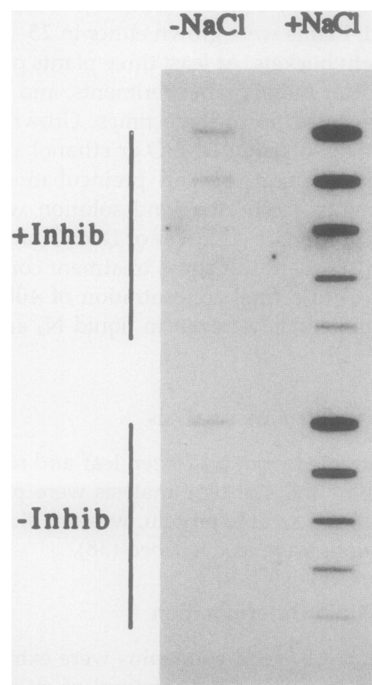

Figure 3. PEPCase mRNA status in the presence and absence of Lovastatin during salt stress. RNA slot blot of total RNA from leaves of plants pretreated with or without (±) the inhibitor (Inhib) Lovastatin for 7 d and ±NaCl (400 mM) for 30 h. Total RNA (5, 2.5, 1.25, 0.63, and 0.31 μg) was blotted and hybridized with the PEPCase (*Ppc1*) probe.

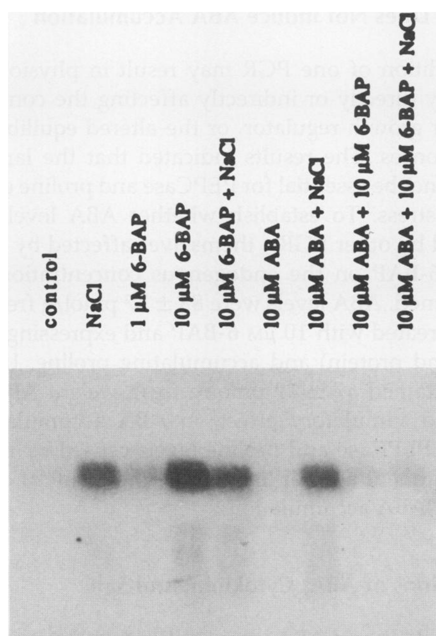

Figure 4. PEPCase mRNA accumulation induced by NaCl, 6-BAP, and ABA. Hydroponically grown 5-week-old plants were grown for 7 d (control), or were treated for 7 d with 1 μM 6-BAP, 10 μM 6-BAP, 10 μM ABA, or 10 μM 6-BAP + 10 μM ABA. At week 6, some plants received 400 mM NaCl for 30 h in addition to continuing with the above-mentioned growth regulator treatments. As described earlier, total RNA was prepared and subjected to northern analysis using the *Ppc1* probe.

8 to 16 times more abundant after a 30-h salt stress than in the absence of salt (Fig. 3). Western analysis indicated similar increases in PEPCase enzyme amounts (data not shown). Proline estimates from these samples were also performed. In the absence of salt stress, control and 10 μM Lovastatin-preincubated plants contained similar levels of proline (60 ± 21 and 77 ± 10 μg/g fresh weight, respectively). When challenged with 400 mM NaCl, plants had 802 ± 120 μg/g fresh weight proline, whereas the 10 μM Lovastatin-preincubated plants had slightly higher amounts, 1180 ± 58 μg/g fresh weight.

Exogenous ABA Treatment: PEPCase mRNA and Proline Levels

To supplement the inhibitor studies, we examined the influence of exogenous ABA on PEPCase mRNA and proline levels. When treated for 7 d with 10 μM ABA, the plants contained 275 ± 48 pmol/g fresh weight ABA in their leaves. This is 4 to 5 times higher than the ABA levels in leaves of untreated plants (see Fig. 2). The influence of added ABA on PEPCase and proline was then determined.

Exogenously applied 10 μM ABA (alone) did not induce PEPCase mRNA, as determined by northern analysis (Fig. 4). NaCl stress applied at the end of the 7-d ABA treatment resulted in an induction of PEPCase mRNA to the same extent as in the NaCl stress controls after a 30-h salt stress, indicating that prolonged ABA treatment did not inhibit the induction process (Fig. 4). Similar results were observed at the protein level by western blot (data not shown). Proline concentrations did not significantly differ between the untreated and ABA-treated plants (54 ± 23 pmol/g fresh weight versus 35 ± 15 μg/g fresh weight). When ABA treatment was combined with a 30-h NaCl stress, proline concentrations were induced to levels identical to NaCl alone (804 ± 163 versus 813 ± 206 μg/g fresh weight, respectively). Exogenously added ABA did not elicit either PEPCase or proline accumulation under our growth conditions.

Cytokinin Mimics the Effects of NaCl Stress

Because ABA was ineffective in the induction of PEPCase mRNA and protein and in proline accumulation, the influence of cytokinins was examined either alone or in combination with NaCl stress. The synthetic cytokinin 6-BAP was chosen for exogenous cytokinin treatments because it is considerably more stable than natural cytokinins (20). The addition of 6-BAP (1 and 10 μM) to the nutrient solution of hydroponically grown plants strongly induced the *Ppc1* transcript. The extent of transcript accumulation was greater in the 10 μM 6-BAP treatment than in the 1 μM treatment (Fig. 4). The magnitude of PEPCase mRNA accumulation in response to 10 μM 6-BAP was similar to that of the 30-h NaCl stress (alone) and also similar to that of the 400 mM NaCl plus 10 μM 6-BAP treatment (Fig. 4). Western blots showed similar results with respect to PEPCase enzyme accumulation (data not shown).

To delineate the time necessary for 6-BAP induction of PEPCase, mRNA and protein levels were analyzed from plants treated with 10 μM 6-BAP for 30 h, 78 h, and 7 d. The salt-induced M_r 110,000 protein PEPCase (encoded by *Ppc1*) was detectable after 78 h of BAP incubation. A large increase in the concentration of this protein was observed 7 d after

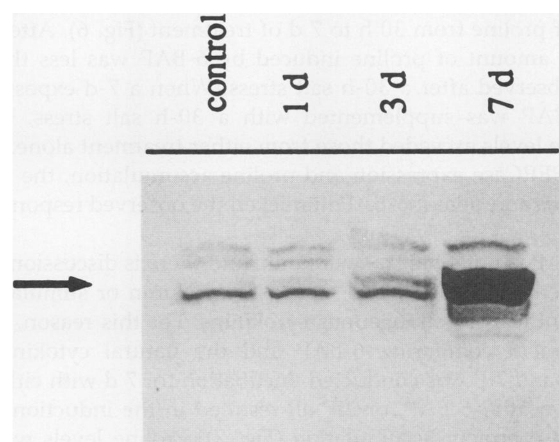

Figure 5. Time course of 6-BAP treatment of 5-week-old plants. Leaf material from three to four plants was harvested and pooled after 1, 3, and 7 d of cytokinin treatment. Control samples are from untreated plants that were grown alongside the 6-BAP-treated plants. Total proteins were extracted, and 30 μg of total protein (per treatment) were separated using 12.5% SDS-PAGE, western blotted, and probed with an anti-PEPCase antibody. The arrow indicates the NaCl-induced 110-kD *Ppc1*-encoded protein.

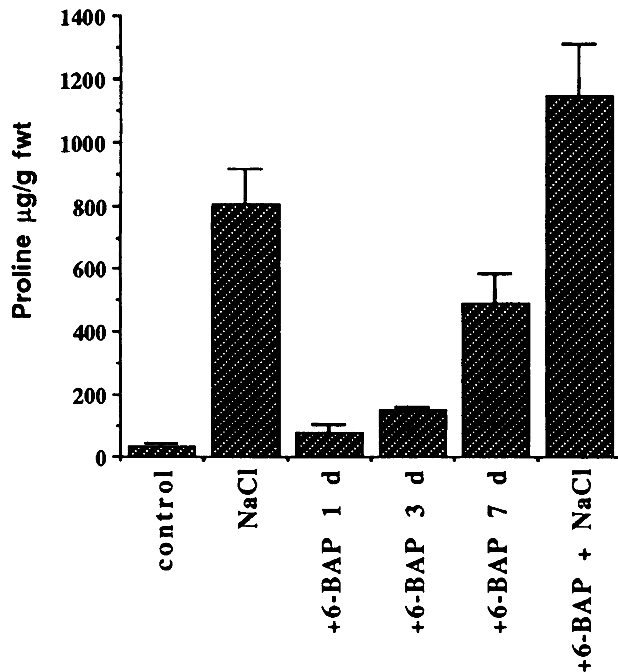

Figure 6. Exogenous 6-BAP treatment and proline amounts. Data represent the mean and SE (bars) of three pooled plants/experiment and three experiments. Treatments: control (control), 30 h 400 mM NaCl (NaCl), 10 µM 6-BAP for 1 d (1 d), 3 d (3 d), and 7 d (7 d), and 10 µM 6-BAP (7 d) plus salt for 30 h (+ 10 µM 6-BAP + NaCl). Plants were 6 weeks old at the end of these experiments.

6-BAP exposure (Fig. 5). A cross-reacting protein of M_r 109,000, a PEPCase isoform encoded by the *Ppc2* isogene (11), was expressed at low levels in all tissues regardless of the NaCl status (38).

The addition of 10 µM 6-BAP also induced the accumulation of proline from 30 h to 7 d of treatment (Fig. 6). After 7 d, the amount of proline induced by 6-BAP was less than that observed after a 30-h salt stress. When a 7-d exposure to 6-BAP was supplemented with a 30-h salt stress, the proline levels exceeded those from either treatment alone. In both PEPCase expression and proline accumulation, the exogenous addition of 6-BAP mimicked the observed responses to NaCl.

6-BAP is not a natural cytokinin, and there is discussion as to whether applied 6-BAP acts as a cytokinin or stimulates the synthesis of endogenous cytokinins. For this reason, an experiment comparing 6-BAP and the natural cytokinins zeatin and 2iP was conducted. Incubation for 7 d with either 10 µM zeatin, 6-BAP, or 2iP all resulted in the induction of PEPCase protein accumulation (Fig. 7). Proline levels were also increased by these cytokinin treatments. Control (untreated) plants contained 40 µg/g fresh weight proline, whereas zeatin, 2iP, 6-BAP, and NaCl treatment increased proline amounts to 1114, 268, 230, and 480 µg/g fresh weight, respectively. The slight wilting observed with 6-BAP and 2iP treatment was exaggerated by zeatin treatment. Cytokinins appeared to mimic the observed responses to NaCl in this facultative halophyte.

Cytokinin Does Not Induce ABA Accumulation

The addition of one PGR may result in physiological responses by directly or indirectly affecting the concentration of another growth regulator, or the altered equilibrium may elicit responses. The results indicated that the large rise in ABA may not be essential for PEPCase and proline expression after salt stress. To establish whether ABA levels may be influenced by other PGRs, themselves affected by NaCl, the effect of 6-BAP on the endogenous concentration of ABA was examined. ABA levels were 83 ± 27 pmol/g fresh weight in plants treated with 10 µM 6-BAP and expressing PEPCase (mRNA and protein) and accumulating proline. Unstressed plants contained 67 ± 19 pmol/g fresh weight ABA. 6-BAP showed no stimulatory effect on ABA accumulation. The effects on PEPCase and proline levels caused by exogenous 6-BAP treatment are not likely to be the result of cytokinin-stimulated ABA accumulation.

Combinations of ABA, Cytokinin, and Salt

NaCl, 6-BAP, and ABA were applied together to determine their combined effects on PEPCase induction and proline accumulation. When ABA was added together with either 6-BAP (Fig. 4, lane 8) or 6-BAP and salt (Fig. 4, lane 9), PEPCase mRNA levels were less than with cytokinin alone or with cytokinin plus salt-treated plants (compare lanes 4 and 5, Fig. 4). When compared to the unstressed control, proline concentration was not increased by ABA in these treatments (data not shown). The addition of both ABA and 6-BAP resulted in (a) proline levels comparable to the treatment with 6-BAP alone, (b) extensive wilting of the plants, and (c) a reduction in plant growth when compared to the untreated controls (data not shown). In the ABA + 6-BAP and ABA + 6-BAP + NaCl applications, PEPCase mRNA was induced to a lesser extent than by the 6-BAP treatment alone. This result may reflect the retarded growth of these plants.

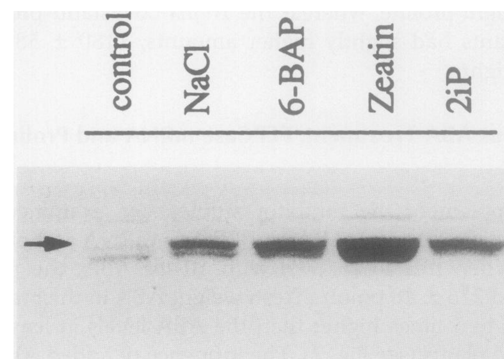

Figure 7. Natural cytokinins induce PEPCase. Five-week-old hydroponically grown plants (three per treatment) were incubated with either 10 µM of 6-BAP, zeatin, or 2iP; protein extracts were prepared and separated using SDS-PAGE; and the proteins were electroblotted onto nitrocellulose and probed with an anti-PEPCase antibody. The induced 110-kD *Ppc1*-encoded protein band is indicated by an arrow.

Endogenous Cytokinins and Salt Stress

To test whether cytokinin plays a physiological role during NaCl stress in the ice plant, the endogenous zeatin-type cytokinin levels were measured after salt stress in both roots and leaves. When compared to control levels, no significant differences in the levels of this cytokinin were observed in roots or leaves of plants treated from 6 to 78 h with NaCl (Fig. 8). After 78 h in fresh Hoagland solution, the cytokinin concentrations increased insignificantly in unstressed plants when compared to the earlier time points. In addition, leaf material contained higher levels of zeatin-type cytokinins than root tissue from the same plants.

DISCUSSION

Significance of Elevated ABA Levels and the Markers of Salt Stress

The age and growth environment of the ice plant undoubtedly affect its responsiveness to diverse biochemical cues (salt, PGRs). For these reasons, we haved used established growth conditions for *M. crystallinum* (26) with a hydroponic plant culture system to compare the influence of NaCl and the PGRs ABA and cytokinin upon osmotic stress-induced PEPCase and proline accumulation. Under this regime, NaCl induces ABA, PEPCase, and proline accumulation in 6-week-old plants. Even though ABA accumulates in the leaves when ABA (alone) is supplied to the hydroponic solution, neither proline nor PEPCase levels are increased. Lovastatin interruption of ABA synthesis does not affect the NaCl-elicited PEPCase and proline responses. Under much different growth conditions than the standard conditions employed here (higher temperature, light intensity, and longer day length), exogenous addition of ABA does stimulate PEPCase mRNA and protein accumulation (ref. 9; E.F. McElwain, in preparation). The fact that *M. crystallinum* always responds

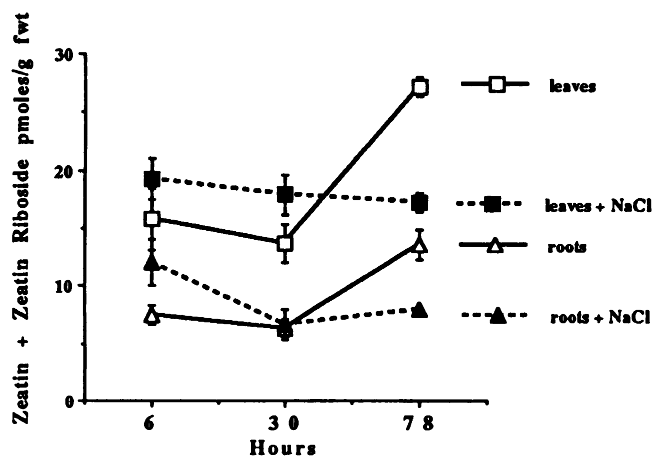

Figure 8. Endogenous zeatin-type cytokinin levels in the presence or absence of NaCl. Samples from roots (triangles) or leaves (squares) were from 6-week-old plants treated with (filled symbols) or without (open symbols) 400 mM NaCl for 6, 30, or 78 h. These data represent the mean and SE (bars) from two or three independent time course experiments.

to NaCl and is not always to added ABA, and that internal ABA accumulation is not necessary for PEPCase and proline accumulation suggests that a large increase in the ABA amount after NaCl treatment is not likely to be the primary inducer of global salt stress responses. Similarly, osmotic stress-induced proline accumulation is not dependent on an increase in endogenous ABA in barley (36). The elevation of ABA, PEPCase, and proline titers may be convergent consequences of salt stress.

Our results are consistent with information concerning the regulation of the stress-inducible genes, not all of which are ABA inducible (7). The best-characterized example of an ABA-inducible gene is E_m from wheat. Exogenous application of ABA to immature excised embryos results in increased transcription of E_m. However, E_m mRNA synthesis is not induced by ABA when mature embryos are used, suggesting that a signal other than ABA is responsible for embryo-specific expression of E_m (41). When immature embryos are cultured in high osmoticum, endogenous ABA titers do not change significantly, yet E_m mRNA accumulates to higher levels than when embryos are cultured in low osmoticum supplemented with ABA (23). Thus, E_m expression is not exclusively governed by alterations in endogenous ABA levels. In rice suspension cells, NaCl and exogenous ABA act synergistically, inducing the rice E_m homolog in an additive fashion (6). It has been suggested that more than one mechanism (and molecule) may be responsible for E_m induction and other "ABA-responsive" genes in vivo during environmental challenges (34).

Salt Stress and Cytokinin

In *M. crystallinum*, exogenous cytokinin triggers PEPCase and proline accumulation. To our knowledge, this is the first report where salt or cytokinin treatment results in identical effects. However, cytokinin and salt may differ in their mechanism of induction. There is a large difference in the response time between 6-BAP and NaCl induction of the salt stress markers. A 7-d incubation with 6-BAP increases the level of *Ppc1* transcript to a degree similar to a 30-h salt stress. When compared to a 30-h salt stress, zeatin is more efficient and 2iP and 6-BAP are less able to stimulate the accumulation of PEPCase and proline. When added together, NaCl and 6-BAP stimulate the accumulation of proline in an additive fashion. The activity elicited by exogenous cytokinin is not due to augmented ABA levels, as 6-BAP application has no effect on endogenous ABA titers. Both inducers (NaCl and cytokinins) participate in a related response pathway giving rise to PEPCase and proline accumulation.

Conceivably, salt stress could augment cytokinin concentrations and thereby induce expression of PEPCase and proline production soon after NaCl treatment. In many species, the zeatin-type cytokinins are the most active and most prevalent forms of cytokinin (20). Upon the induction of cytokinin synthesis, the zeatin-type cytokinins accumulate to a greater extent than other cytokinin classes, over 200-fold when compared to the uninduced state (35). For these reasons, zeatin-type cytokinins were characterized during salt stress. Unexpectedly, zeatin-type cytokinin levels did not change greatly in 400 mM NaCl (Fig. 8). Uniform or even

decreasing endogenous cytokinin concentrations during salt stress have been reported in other species (19, 39). Even though exogenous cytokinins (zeatin, 2iP, and 6-BAP) emulate responses characteristic of salt stress, it is doubtful that exposure to NaCl increases the de novo accumulation of cytokinins, thereby inducing the diagnostic NaCl responses in *M. crystallinum*. The stress (pathogenesis)-related proteins osmotin, chitinase, β-glucanase, and extensin have similar complex mechanisms of gene expression. ABA, cytokinin, auxin, wounding, and osmotic stress all induce transcription of some, but not all of these genes (25, 32).

Transmitters Triggered by Salt and Cytokinin

Large-scale fluctuations in the PGRs ABA and cytokinin are not causally involved in the initiation of NaCl responses in this halophyte. Conversely, cytokinins (and under some conditions ABA) mimic the effects of NaCl on PEPCase and proline expression. Our results indicate that cytokinins and NaCl, when sensed by the roots, elicit the transport of a signal to aerial leaves, where the signal initiates the two stress responses we have measured. Root to leaf communication has also been reported to modulate the control of stomatal movement (4). However, the identity of the secondary messenger(s) influenced by cytokinin and NaCl is presently unknown.

Any substance able to transmit information concerning the status of the plasmalemma to the cytoplasm broadly defines a secondary messenger (5). Putative secondary messengers in plants include: carbohydrates, oligosaccharides, and/or peptides (27). PGRs themselves may act directly as second messengers (30) or indirectly via PGR permeability during an episode of osmotic stress (16). One transmitter substance proposed to mediate signal transduction systems in plant cells is the fluctuation of cytosolic calcium (Ca^{2+}) (28). Ca^{2+} fluctuations play a central role in the stimulus response coupling of osmotic stress, plant cell turgor regulation, sucrose metabolism, pollen tube growth, the influence of ABA on protein synthesis and stomatal movement, and cytokinin-induced bud formation (18, 24, 31). Ca^{2+}-calmodulin effects have also been implicated in the regulation of Ca^{2+}-Mg^{2+}ATPases and NAD and protein kinase activities, the latter of which may act as second messengers themselves (5, 21). The parallel effects exerted by the primary messengers (here cytokinin or NaCl treatment) may result in altered intracellular calcium status in roots, influencing a sensing pathway to leaves and resulting in the observed biological responses in leaves.

PGR concentrations are well known to alter the developmental fate of plant tissues. Besides concentration, plant responsiveness to diverse environmental and biochemical cues include factors related to PGR: uptake (or receptor status), transport, antagonist levels, environmental conditions, and the heterogeneous nature of the plant cells being tested (40). As two distinct inducers (NaCl and cytokinin) both increase proline and PEPCase (mRNA and protein), a similar scenario may exist in *M. crystallinum*. Rather than a single inducer molecule, a variety of factors determine the responsiveness of this halophyte to the commencement of the salt stress responses described. Stimulation of gene expression by NaCl may be a direct result of NaCl (osmotic or ionic) and not indirectly through large changes in endogenous ABA or cytokinin levels. Although cytokinin and NaCl may act on the same secondary messenger (possibly Ca^{2+}), exactly how this transduction system may be recruited to affect gene expression remains unknown. In a whole plant context, a concerted effort to identify the signaling mechanism(s) triggered by cytokinins and environmental stress will enable us to address this question.

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