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Regulation of *Em* Gene Expression in Rice[1]

Interaction between Osmotic Stress and Abscisic Acid

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ABSTRACT

Expression of the *Em* gene was characterized in rice (*Oryza sativa* L.) suspension cultures following exposure of the cultures to various combinations of abscisic acid (ABA) and salt. Response-saturating concentrations of either ABA (50 micromolar) or NaCl (0.4 molar) rapidly induced (by 60 minutes) the accumulation of *Em* mRNA, with a maximum accumulation occurring 12 to 24 hours after treatment. NaCl-induced *Em* expression was accompanied by a doubling of endogenous ABA levels as determined by immunoassay. Inhibition of ABA biosynthesis by fluridone during NaCl treatment reduced the levels of endogenous ABA by fourfold and *Em* expression by 50%. Desiccation of the cultures to 12 to 15% of their initial fresh weight increased endogenous ABA more than twofold and was accompanied by an increase in *Em* mRNA levels. Exposure of the cultures to heat shock temperatures, chilling, or ultraviolet light neither increased endogenous ABA levels nor induced *Em* expression. When a subthreshold or saturating level of NaCl was added in combination with increasing levels of ABA, *Em* transcripts were detected at ABA concentrations that alone did not induce expression of *Em*. Treatment with saturating levels of both NaCl and ABA resulted in a doubling of *Em* transcript levels over the maximum signal for each treatment alone. Hence, our data suggested that salt interacted synergistically with ABA, in part because of the increased sensitivity of rice cells to ABA. The effect of salt stress on *Em* gene expression in rice suspension cells appeared to operate through two pathways: one is mediated through increases in the level of ABA; the other is via a unique salt response pathway that includes an intermediate that is common to both the salt and ABA response chains.

Physiological and molecular responses to ABA are controlled by the levels of ABA as well as by the sensitivity of tissues that are competent to respond (16, 17, 35). One factor that appears to alter the response of plant cells to ABA is Ψ[2]. The interaction between reduced Ψ and ABA has been experimentally well illustrated in rapeseed germination (29) and further elaborated in a mathematical model for tomato seed germination (26). Schopfer and Plachy (29) showed that reduced Ψ imposed by osmotic stress and ABA can quantitatively substitute for each other to inhibit rapeseed germination. For example, 50% inhibition of rapeseed germination is achieved with either 22 μM ABA, 10 μM ABA + 6 bars osmoticum, or 11 bars osmoticum. This two-factor interaction results in a shift in the concentration-response curve for ABA inhibition of germination toward lower ABA concentrations in the presence of an osmoticum, *i.e.* an osmotically induced change in the sensitivity of the tissue to ABA.

The capacity of exogenous ABA or osmoticum to quantitatively substitute for each other suggests a common intermediate in the osmotic stress and ABA response pathways. It is unclear, however, whether osmotic stress operates solely through changes in ABA levels to effect the response. Evidence for osmoticum operating through ABA has been suggested by studies in which endogenous levels of ABA increase in tandem with increasing levels of a particular mRNA after treatment with an osmoticum (12). However, a number of recent studies indicate that changes in ABA levels do not always accompany osmoticum induced physiological responses and strongly suggest that responses to osmotic stress cannot be explained solely by changes in ABA levels (3, 9, 24, 36). Furthermore, direct evidence for a synergistic interaction between ABA and osmotic stress at the level of gene expression has not been as clearly elucidated as the interaction on seed germination.

To identify intermediates in the pathway(s) that result in changes in the abundance of specific mRNAs in response to both an osmoticum and ABA, we chose to examine the expression of the ABA- and salt-responsive *Em* gene. *Em* encodes a hydrophilic protein of the late-embryo abundant class (8) that is one of the most abundant proteins in embryos of dry cereals such as wheat (*Triticum aestivum*) (7, 13, 27, 34). Levels of *Em* mRNA normally increase dramatically during maturation of wheat (27) and maize embryos (23), but expression of the *Em* gene is also observed in vegetative tissue exposed to ABA or osmotic stress (16, 27). The *Em* gene is well characterized (7, 18), and functional regions within its promoter involved in the ABA response have been identified (15, 20, 21, 27). Regulation of *Em* expression by ABA has been shown to occur at the transcriptional level as well as at a posttranscriptional step (34).

The objectives of this study were to characterize *Em* expression in rice suspension cultures derived from immature rice embryos when exposed to varying levels of ABA and an

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[2] Abbreviations: Ψ, water potential; *Em*, early methionine-labeled polypeptide gene; fluridone, 1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4-(1H)-pyridone; SSC, standard sodium citrate; bp, base pair.

osmoticum, NaCl. We demonstrate in this study that NaCl and ABA are strongly synergistic in controlling *Em* mRNA abundance. Furthermore, we show that NaCl operates not only through changes in ABA levels but also through an independent pathway that changes the sensitivity of rice (*Oryza sativa* L.) cells to ABA.

MATERIALS AND METHODS

Rice Cell Suspensions and Treatments

Rice (*Oryza sativa* L.) cell suspensions were initiated from callus and were maintained on liquid N6 medium containing 2 mg L^{-1} 2,4-D and 3% (w/v) sucrose, pH 5.8, at 22°C in a dark incubator with shaking (110 rpm) (5, 20). Suspensions were subcultured weekly into fresh medium and were used for experiments 5 to 7 d after transfer. For experimental treatments, (±)2-*cis*-4-*trans*-ABA (Sigma) was added to cultures from a 100 mM stock solution in ethanol, NaCl was from a 5 M aqueous stock solution, and mannitol was from a 50% stock solution in 0.5× N6 medium. Cultures generally were incubated following treatment for 24 h before extraction unless indicated otherwise. Fluridone (98.9% purity; Elanco) was added from a 30.4 mM stock solution in ethanol. Cultures were incubated in the presence of 100 or 200 μM fluridone for 36 h, then treated with 50 μM ABA or 0.4 M NaCl, and extracted 24 h later. In other experiments, cultures were subjected to low temperature (4°C for 1, 2, or 4 d), high temperature (37 or 42°C for 2 h or 50°C for 3 h followed by overnight incubation at 22°C), or UV light (350 nm, approximately 10 mW cm^{-2} for 5–60 min). Cultures were then returned to the incubator overnight before extraction. To determine whether desiccation of rice cells could induce *Em* mRNA accumulation, cultures were dehydrated at room temperature to approximately 90, 50, or 12 to 15% of their initial fresh weight by placing them on filter paper for various periods.

RNA Extraction

After appropriate incubation times, the medium was removed from treated cultures, and the cells were transferred to a polypropylene tube and immediately frozen in liquid N_2. The frozen cells were stored at −80°C until extraction. The rapid RNA extraction procedure, slightly modified from that described by Chomczynski and Sacchi (4), was used throughout this study. Routinely, 0.5 to 1 g fresh weight of rice suspension cells per sample were ground under liquid N_2 with a chilled mortar and pestle. The contents of the mortar were transferred with a chilled spatula to a chilled 30-mL Corex tube. After the liquid N_2 evaporated, 5 mL of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7], 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol) was added to the tube and vortexed. After the tube was warmed to 65°C for a few seconds, it was vortexed, and the published procedure (4) was followed using volumes of the reagents that were adjusted proportionally. The final RNA pellet was washed twice with 3 mL of 75% ethanol and the washed pellet dried under vacuum. The pellet was resuspended in diethylpyrocarbonate-treated water (300–500 μL), and this solution was centrifuged at 10,000*g* for 5 min to remove any residual insoluble material.

Quantitation of *Em* Transcript Abundance

Total RNA (10 μg per sample) was separated on 1.2% agarose gels containing formaldehyde (1). Each sample was applied in duplicate lanes. After electrophoresis, the portion of the gel containing one set of samples was stained with ethidium bromide and examined under UV light, and the portion of the gel containing the duplicate set was transferred to nylon membranes (Hybond-N, Amersham) using 10× SSC and the RNA UV cross-linked to the membrane.

The RNA was hybridized with a gel-purified fragment from the wheat *Em* gene (p1015) which has a 608-bp *Pst*I insert containing the entire coding region, 5′ and 3′ flanks, and a polyadenylated tail (18). DNA was labeled with [^{32}P]CTP by the random prime method according to the manufacturer's directions (U.S. Biochemical, Cleveland, OH). The membranes were hybridized in a solution containing 5× SSC, 5× Denhardt's solution, 0.5 mg mL^{-1} single-stranded salmon sperm DNA, 0.5% SDS, and the ^{32}P-labeled *Em* probe. Membranes were hybridized with the *Em* probe overnight at 65°C and washed at the same temperature with 2× SSC-0.1% SDS, and then 0.1× SSC-0.1% SDS.

The radioactivity on the washed membranes was counted directly using a radioanalytic imaging system (Ambis Systems, San Diego, CA) or indirectly by scanning densitometry (Molecular Dynamics, Sunnyvale, CA) of autoradiograms. A comparison of a series of blots using both quantitation methods under our experimental conditions and autoradiographic exposures indicated that the two methods yielded the same results. Uniformity of loading and integrity of the samples were estimated by visual inspection of total RNA and ribosomal bands in the ethidium bromide-stained portion of gels under UV light. For analyses in which a set of samples on one blot were to be compared with a set on another blot, an internal standard containing *Em* mRNA from a rice culture that had been treated with 50 μM ABA was run in duplicate with each set of samples on a gel. *Em* transcript abundance values for the samples were then expressed relative to the internal standard to correct for blot-to-blot variation. In the experiments concerning the ABA-osmoticum interaction, the corrected values within an experiment were then expressed relative to the 100 μM ABA treatment value within that experiment. Two experiments were performed, the average of the two was corrected, relative values for each data point were computed, and the SD was determined.

ABA Analyses

Endogenous ABA was determined by an indirect, competitive immunoassay adapted from that described by Walker-Simmons (33). After treatment, the rice cells were washed several times on filter paper, frozen in liquid N_2, and then lyophilized. The lyophilized cells (50 mg) were transferred to a microfuge tube, and 1.2 mL of cold 80% methanol containing 10 μg mL^{-1} butylated hydroxytoluene was added. The tube was vortexed and placed at 4°C with occasional agitation for 30 min. The tube was then centrifuged for 5 min at

16,000*g* and the supernatant collected. The pellet was reextracted twice, each time with 0.5 mL of cold 80% methanol, and centrifuged each time as above. After the supernatants were combined, the extract was adjusted to 70% methanol and then passed over a Sep-pak C_{18} column (Waters Associates, Milford, MA). ABA was eluted from the column with 5 mL of 70% methanol. The eluate (1 mL) was concentrated to just barely dry with a Speed-vac system (Savant) and the residue redissolved in 150 μL of distilled H_2O. The sample was then stored at −20°C until assay.

A monoclonal antibody (Idetek Inc., San Bruno, CA) that specifically recognizes the (+)-ABA isomer was used. The antibody was used at a working concentration of 1.23 μg mL^{-1}. Wells in Immulon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with an ABA-BSA conjugate (33). An aliquot (25 μL) of sample or a dilution thereof was added to each well followed by 175 μL of assay buffer containing the diluted antibody. The plates were incubated at room temperature for 3 h and washed. To each well, 200 μL of a 1:1000 dilution of anti-mouse IgG coupled to alkaline phosphatase (Sigma) was added, and the plates were incubated for 16 h at 4°C. The plates were then washed, and 200 μL of a 1 mg mL^{-1} solution of ρ-nitrophenyl phosphate (Sigma) in substrate buffer was added to each well. The plates were incubated at 37°C for 1 h and read at A_{405} on a plate reader. All samples were assayed in duplicate at each of two dilutions. A dilution series of pure ABA from 0.02 to 100 pmol/25 μL was included with each plate to derive a standard curve with triplicate readings for each ABA dilution.

RESULTS

Induction of *Em* Expression following ABA or NaCl Treatment

ABA and NaCl rapidly and strongly induced the accumulation of *Em* mRNA (Figs. 1 and 2). The *Em* probe from wheat (p1015) detected a single transcript (820 bp) in total rice RNA, approximately 40 bp larger than the *Em* transcript from wheat embryos (18). In the absence of NaCl or ABA, *Em* transcripts were not detected in either total RNA or polyadenylated RNA from rice suspension cultures.

The dose-response curve for *Em* mRNA was between 1 and 50 μM for ABA (Fig. 1A) and between 0.25 and 0.5 M for NaCl (Fig. 1B). No additional increase or decrease in *Em* levels was observed at concentrations of ABA between 50 and 200 μM. Concentrations of NaCl >0.5 M resulted in growth inhibition and cell death, which is reflected in a drastic reduction in *Em* expression at these concentrations (Fig. 1B). Although expression was clearly apparent by 1 h after addition of ABA or NaCl (Fig. 2A), samples taken over a shorter time span suggest that the response to ABA is faster than the response to NaCl (Fig. 2B).

Effect of Other Stresses on *Em* Expression

We examined the specificity of the ABA response in rice cells by subjecting the cultures to other stresses, some of which are known to induce expression of ABA-responsive genes in other systems (6, 16, 27). *Em* transcripts were not detected in rice cells dehydrated to 90 or 50% of their initial fresh weight.

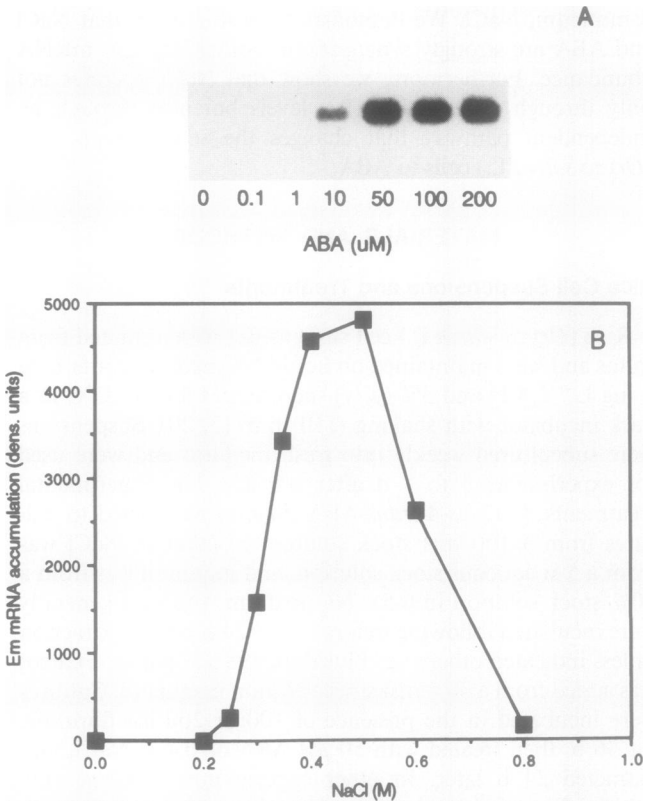

Figure 1. A, *Em* mRNA accumulation in rice suspension culture cells after treatment with various concentrations of ABA. *Em* mRNA is visualized in this autoradiogram after hybridization of total RNA with p1015 (see "Materials and Methods"). B, Concentration-response curve of NaCl induction of *Em* mRNA accumulation. The experiment was performed twice with similar results. Transcript abundance was estimated by scanning densitometry of autoradiograms prepared as in A.

However, dehydration to 12 to 15% of their initial fresh weight resulted in an increase in both endogenous ABA levels and *Em* expression (Table I). Neither low temperature, high temperature, nor UV light resulted in *Em* message accumulation or changes in endogenous levels of ABA (data not shown).

Interaction between ABA and Salt Stress

To begin to understand the interaction between salt and ABA, we determined whether the response to NaCl was dependent upon increases in endogenous ABA. Treatments with 0.25 and 0.4 M NaCl resulted in the doubling of endogenous ABA content 24 h later (Table I), similarly to the response of rice cells at a level of dehydration equivalent to 12 to 15% of their fresh weight (compare treatment 2 with 3, 5, and 6 in Table I). The kinetics of ABA accumulation indicated that ABA increased steadily during the first 6 h of treatment with 0.4 M NaCl and then maintained that level until 12 h (Fig. 3). After 12 h, the concentration of ABA decreased to about double the initial content by 24 h (data not shown). The increase in ABA levels in response to salt

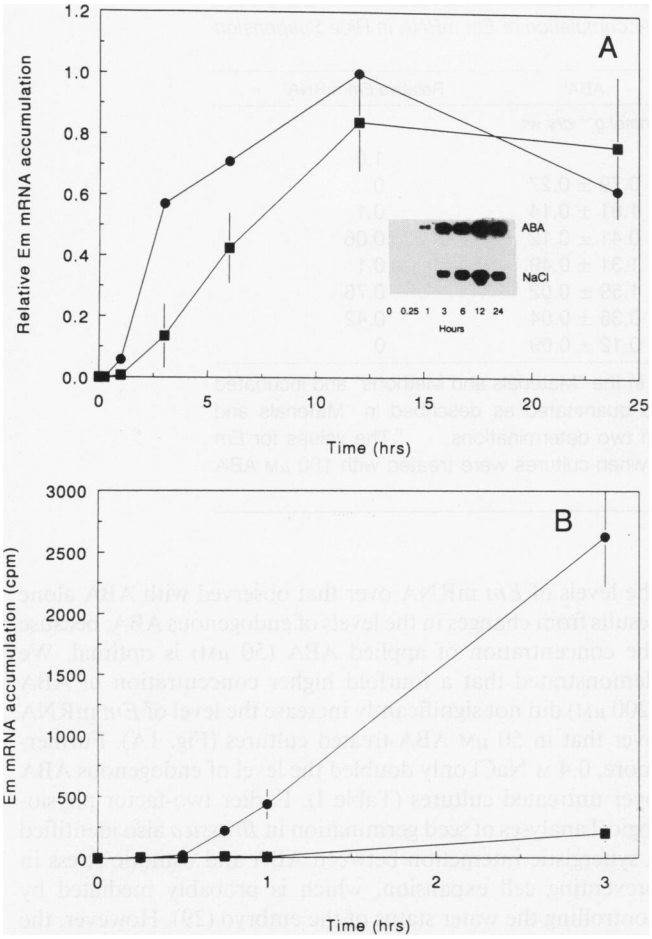

Figure 2. A, Time courses of *Em* mRNA accumulation in rice suspension culture cells following treatment with 200 μM ABA or 0.4 M NaCl. Quantitation of *Em* mRNA was by scanning densitometry of autoradiograms. Values within each treatment are expressed relative to the treatment value at 12 h. Values for the NaCl treatment are the means and SE from two experiments. ●, ABA; ■, NaCl. B, *Em* mRNA in a similar experiment except cultures were sampled at more time points within a shorter period. Transcript abundance was estimated by direct counting of radioactivity using an AMBIS radioanalytic imaging system. Values are the means and SE from three experiments. ●, ABA; ■, NaCl.

was accompanied by a proportional increase in *Em* message accumulation until 12 h (Fig. 3). However, at 24 h, there did not always appear to be a direct correlation between absolute levels of endogenous ABA levels and *Em* expression (compare treatments 2, 5, and 6 in Table I). At concentrations of NaCl <0.2 M, endogenous ABA levels did not increase and *Em* transcripts were not detected.

To determine whether the increase in ABA level associated with NaCl treatments was required for *Em* expression and was dependent on new ABA biosynthesis, we treated rice cultures with fluridone, an inhibitor of ABA biosynthesis (37). An 85% decrease in ABA concentration was observed when cultures were treated with 200 μM fluridone (Table I). When cultures were treated with fluridone + 0.4 M NaCl, a reduction of 78% in ABA levels (Table I) and 58% in *Em* expression (Fig. 4) was observed when compared to cultures treated with 0.4 M NaCl alone. Fluridone, however, did not inhibit the accumulation of *Em* mRNA induced by exogenously applied ABA (Fig. 4), indicating that the capacity for *Em* expression was not impaired by the fluridone treatment. Hence, about 50% of the NaCl-induced expression could be explained by increases in endogenous ABA levels. What accounts for the remainder? The level of ABA in NaCl + fluridone-treated cultures (0.36 nmol g^{-1} dry weight) is equal to or less than that in untreated cultures or cultures treated with 0.1 M NaCl, conditions under which little or no expression of *Em* occurs (compare treatments 2, 4, and 7 in Table I). The expression of *Em* at these normally subthreshold levels of endogenous ABA could be explained by a NaCl-induced change in the sensitivity of the tissue to endogenous levels of ABA and/or a response pathway that is, in part, independent of the ABA pathway but linked to *Em* expression.

To investigate further the interaction between ABA and NaCl, we treated cells with NaCl (0.1–0.4 M) in the presence of ABA (0–50 μM). Treatment with saturating levels of both NaCl (0.4 M) and ABA (50 μM) resulted in an approximate doubling in *Em* transcript levels over the maximum signal for each treatment alone (Fig. 5). *Em* transcripts were also detected when subthreshold levels of both NaCl (0.1 M) and ABA (1 μM) were present in the same treatment (Fig. 5). This effect could not be due to NaCl changes in ABA concentration, because 0.1 M NaCl did not increase endogenous levels of ABA (treatment 4 in Table I). Furthermore, when a saturating level of NaCl (0.4 M) was given together with increasing levels of ABA (0.1–50 μM), *Em* transcript levels increased proportionately between 0.1 and 1.0 μM ABA. In the absence of NaCl, these same concentrations of ABA (0.1–1.0 μM) did not induce *Em* expression (Fig. 1A). A proportionately similar effect was seen with 0.1 M and 0.25 M NaCl. This synergistic interaction between ABA and NaCl appears to be due to osmotic stress, because the same synergy and interaction was observed when 15% mannitol was used as an osmoticum (data not shown).

Hence, the data from the above experiments demonstrated that salt levels interacted synergistically with ABA, a part of which is through an effect of NaCl on altering the sensitivity of rice suspension cells to ABA. The data are not consistent with ABA and salt triggering an increase in *Em* transcript abundance by two totally independent response pathways or only through changes in ABA concentration. The proposed effect of NaCl on increasing the abundance of *Em* transcripts through two pathways, one by changes in ABA levels (1) and the second by altering the sensitivity of cells to ABA (2), is diagramed in Figure 6.

DISCUSSION

Em gene expression in rice suspension cultures is clearly regulated by ABA, NaCl, and water loss. When ABA and NaCl were added separately at their optimal concentrations, approximately the same level of *Em* mRNA accumulated after 24 h of each treatment. This result is similar to those reported for other ABA-responsive genes from rice (25), maize (*Zea mays*) (6, 12), tomato (*Lycopersicon esculentum*) (11), and others that were reviewed by Skriver and Mundy (31). A significant component associated with the NaCl- and dehy-

Table I. *Endogenous ABA Concentrations and Relative Accumulation of Em mRNA in Rice Suspension Culture Cells after Various Treatments*

Treatment[a]	ABA[b]	Relative *Em* mRNA[c]
	nmol g^{-1} dry wt.	
1. Control + ABA (100 μM)		1.0
2. Control (no ABA)	0.79 ± 0.27	0
3. Dehydration (12–15% initial fresh wt.)	1.81 ± 0.14	0.1
4. 0.1 M NaCl	0.41 ± 0.12	0.06
5. 0.25 M NaCl	1.31 ± 0.49	0.1
6. 0.4 M NaCl	1.59 ± 0.02	0.76
7. 0.4 M NaCl + Fluridone (200 μM)	0.36 ± 0.04	0.42
8. Fluridone (200 μM)	0.12 ± 0.09	0

[a] Rice suspension cultures were treated as described in the "Materials and Methods" and incubated at 22°C for 24 h. RNA and ABA were extracted and quantitated as described in "Materials and Methods." [b] ABA values are the means and SE from two determinations. [c] The values for *Em* transcript abundance are relative to the levels detected when cultures were treated with 100 μM ABA for 24 h.

dration-induced increase in *Em* mRNA levels was an approximate doubling of endogenous ABA levels (Table I). Addition of fluridone to NaCl-treated cultures resulted in a reduction in ABA levels by 78% to noninductive levels, with a corresponding decrease (by 45% relative to the 0.4 M NaCl control) in the abundance of *Em* mRNA. These data, plus a direct parallel between the time course of *Em* mRNA accumulation and an increase in endogenous ABA levels in NaCl-treated cultures (Fig. 3), suggest that the NaCl effect is likely to be mediated, at least in part, through changes in endogenous ABA levels.

When rice suspension cultures were treated with optimal concentrations of NaCl and ABA together, a doubling of the *Em* mRNA levels was detected (Fig. 5). It is unlikely under these conditions, however, that the NaCl effect in doubling the levels of *Em* mRNA over that observed with ABA alone results from changes in the levels of endogenous ABA, because the concentration of applied ABA (50 μM) is optimal. We demonstrated that a fourfold higher concentration of ABA (200 μM) did not significantly increase the level of *Em* mRNA over that in 50 μM ABA-treated cultures (Fig. 1A). Furthermore, 0.4 M NaCl only doubled the level of endogenous ABA over untreated cultures (Table I). Earlier two-factor physiological analyses of seed germination in *Brassica* also identified a synergistic interaction between ABA and osmotic stress in preventing cell expansion, which is probably mediated by controlling the water status of the embryo (29). However, the additive effect of ABA and NaCl on *Em* gene expression may not be indicative for all ABA- and salt-responsive genes.

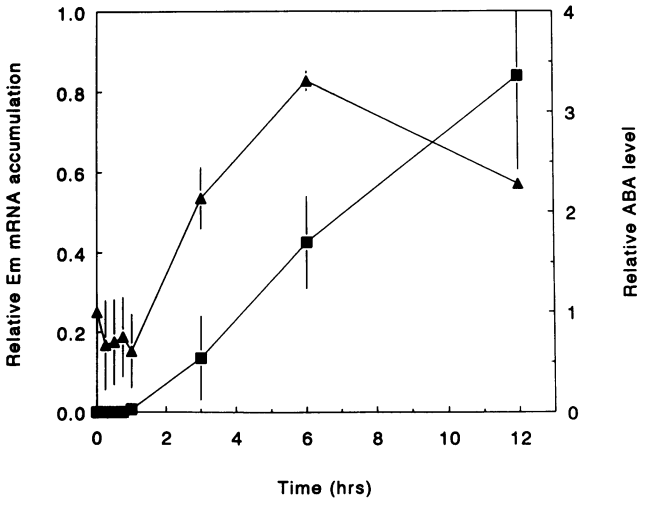

Figure 3. Relationship between the accumulation of endogenous ABA and *Em* transcript abundance following exposure of rice cells to 0.4 M NaCl. *Em* transcript abundance is expressed relative to the maximum value within an experiment. ABA levels are expressed relative to the 0 h value (0.79 ± 0.27 nmol/g dry weight). All values are the means and SE from two experiments. ▲, ABA; ■, *Em* mRNA.

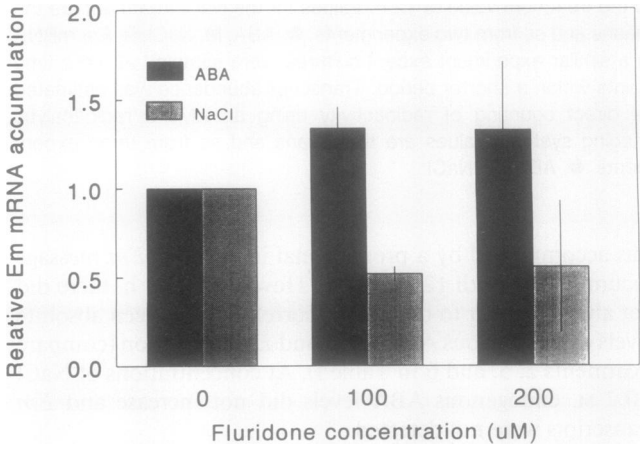

Figure 4. Effect of fluridone on ABA- and NaCl-induced *Em* mRNA accumulation. Values are the average of two experiments and are expressed relative to the corresponding treatment value for message abundance in the nonfluridone-treated cultures (0 μM fluridone). Bars, SD. Endogenous ABA concentrations in suspension culture cells treated with 0.4 M NaCl, 200 μM fluridone, and 200 μM fluridone + 0.4 M NaCl were, respectively, 1.59, 0.12, and 0.36 nmol g^{-1} dry weight. The ABA level in untreated cultures was 0.79 nmol g^{-1} dry weight.

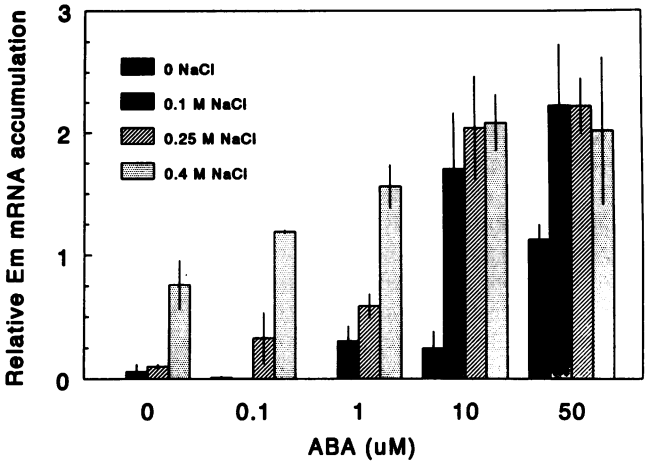

Figure 5. Interaction between ABA and NaCl for induction of *Em* mRNA accumulation. ABA and NaCl were added to rice cell cultures and extracted 24 h later. Corrected values for *Em* transcript abundance are expressed relative to the 100 μM ABA value. Values are the average of two experiments. Bars, SE.

Mundy and Chua (25) reported that the NaCl effect on *Rab* mRNA levels in rice suspension cultures is mediated entirely through ABA, because an additive effect of a combined NaCl and ABA treatment was not observed.

How then does the treatment with optimal amounts of both ABA and NaCl result in an approximate doubling of *Em* mRNA levels in the population of rice cells in the suspension culture? One possibility is that the suspension cultures are composed of at least two functional subpopulations of cells; one set could respond to ABA, another could respond to NaCl, or one previously unresponsive subpopulation of suspension cells could be made competent to respond to ABA when NaCl is present. In the latter case, a recruitment of more ABA-responding cells in the population might be the mechanism that results in an increase in the *Em* mRNA levels for the total population of cells. Our data and approach in this study do not eliminate these possible explanations of the NaCl effect in rice suspension cultures. However, we will assume for the remainder of this discussion that the cell suspension in our study is a single population of responding cells that do not change in their inherent competency to respond to ABA or NaCl.

A second alternative explanation is that different members of a *Em* gene family are responding differently to ABA and NaCl. Although the *Em* gene is represented in hexaploid wheat as a family of about five copies/diploid genome (18), the differential expression of gene family members is an unlikely explanation of the NaCl effect in rice suspension cultures because the *Em* gene in rice appears to be represented by a single copy in the rice genome (28).

Another explanation is that NaCl triggers the activation of a separate and distinct response pathway from ABA in this population. This NaCl pathway is linked to *Em* gene expression but would share no other common component with ABA or the ABA-response pathway. However, this is not likely given the synergistic effect of NaCl and ABA at subthreshold levels (discussed later). A more likely explanation of our results is that NaCl is altering a component(s) in some part of the ABA response pathway which is limiting the level of *Em* mRNA that is accumulated at saturating amounts of ABA. The result of an alteration in any one or combination of these intermediates would be a change in the effectiveness in which a given concentration of ABA alters the levels of *Em* mRNA, *i.e.* a change in "sensitivity" as reflected in an altered dose-response curve. For purposes of our discussion here, we will use the dose-response curve as the basis for our definition of sensitivity (10) and not the more restrictive, specific sensitivity measurement referred to as the control strength (32).

A change in sensitivity of cells to ABA was observed when optimal amounts of NaCl (0.4 M) were added together with subthreshold levels of ABA (0.1 or 1.0 μM). If these subthreshold concentrations of ABA are added alone, no *Em* mRNA is detected. However, when either is added together with 0.4 M NaCl, an increase in *Em* mRNA is observed that is proportional to the ABA concentration (Fig. 5). In addition, we were able to detect *Em* mRNA when both ABA and NaCl were present together at subthreshold levels (0.1 M NaCl + 1.0 μM ABA) (Fig. 5). In this latter example, it is especially important to note that treatment with 0.1 M NaCl alone did not increase endogenous ABA (Table I). Hence, the effect of 0.1 M NaCl, when applied together with normally noninducing levels of 0.1 to 1.0 μM ABA (Fig. 1A), is likely to be on increasing the sensitivity of cells to the applied ABA rather than to an increase in the endogenous amounts of ABA (Fig. 5). In a related result, Singh *et al.* (30) reported that osmotin, a protein associated with plant cells that have adapted to growing at low Ψ values, had a 15-fold higher mRNA level in salt-adapted tobacco (*Nicotiana tabacum*) suspension cells than in nonadapted cells treated with ABA.

What components of the ABA response pathway are limiting the accumulation in *Em* mRNA when stimulated with ABA alone that NaCl can relieve and result in an increased ABA effectiveness or sensitivity? Likely candidates in the stimulus-response coupling box (Fig. 6) might include the number of receptors, ion/turgor changes, second messenger

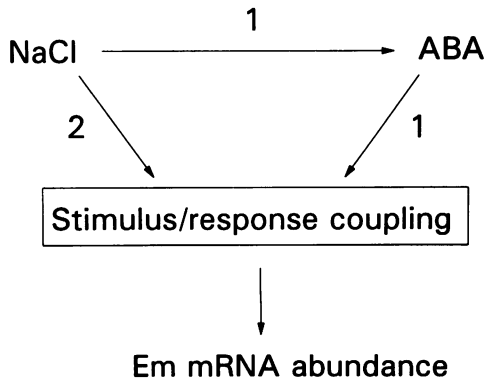

Figure 6. Model for ABA and NaCl induction of *Em* gene expression in rice suspension culture cells. NaCl may operate through ABA (path 1) or through an ABA-independent pathway (path 2) to increase the abundance of *Em* mRNA. Both pathways converge at a common intermediate step(s) to interact synergistically to induce *Em* gene expression.

intermediates, turnover rates of *Em* mRNA, etc. For example, changes in the dose-response curve of an auxin-induced response in tobacco protoplasts (*i.e.* plasma membrane hyperpolarization) can be mimicked by altering the number of putative auxin receptors (2). In this auxin-inducible system, when the sensitivity to auxin is increased, evidence has been presented that suggests a larger number of receptors are responsible. The characteristic of the resulting dose-response curve for increasing the sensitivity of tobacco protoplasts to auxin is very similar to the effect that we observed in this study with NaCl shifting the ABA dose-response curve toward a lower effective ABA concentration. Also, evidence by Lynch *et al.* (19) indicated that salt could increase the level of endogenous free Ca in maize root protoplasts. Given the recent studies that demonstrate changes in free Ca in the ABA response pathway (16, 22), perhaps salt increases the sensitivity of cells to ABA by increasing the level of free Ca. However, we have no evidence of NaCl altering any one of the intermediates (*e.g.* the receptor, free Ca, *Em* mRNA stability) in the ABA stimulus-response pathway.

Hence, our data strongly suggest that a significant part of the NaCl effect on the accumulation of *Em* mRNA in rice suspension cells appears to affect the sensitivity of cells to ABA. This as-yet unreported effect of an osmoticum such as NaCl on ABA-modulated gene expression may further clarify the role of ABA in the responses of plants to osmotic stress. Previous work concerning osmotic stress (and other environmental perturbations) has indicated a close interaction with ABA, primarily through changes in the concentration of ABA. However, transduction of the osmotic signal through changes only in ABA levels cannot be the sole mechanism, because several recent reports indicate that some osmotic stresses alter gene expression without changing ABA levels. For example, Morris *et al.* (24) demonstrated in wheat embryos that ABA or mannitol alone triggered *Em* transcript accumulation. Furthermore, unlike the effect we reported using NaCl as the osmoticum, the mannitol addition did not increase the level of endogenous ABA; yet, it triggered the accumulation of high levels of *Em* mRNA. The mannitol effect, however, was dependent on the presence of ABA. When endogenous ABA levels in wheat embryos were reduced to undetectable levels with the carotenoid inhibitor norflurazon, only very low levels of *Em* mRNA were observed when mannitol was added. They concluded that, for high levels of *Em* expression induced by the osmoticum, ABA was required, but in some unknown manner, mannitol increased the abundance of *Em* mRNA without a change in endogenous levels of ABA. Similarly, osmotic stress was shown to increase the abundance of other ABA-inducible mRNA from wheat (24) and rapeseed (9) without detectable changes in the endogenous ABA levels. Our results with NaCl in this study would predict that the osmoticum mannitol might increase the sensitivity of wheat embryos to ABA.

Our results showing that NaCl alters the sensitivity of rice suspension cells to ABA as measured by the accumulation of *Em* mRNA would predict that other molecular/cell/tissue responses of ABA might have altered dose-response curves (*i.e.* sensitivity) after treatment with an osmoticum. Our conclusion would suggest that the response of a plant cell to osmotic stress may include an altering of the sensitivity to ABA as well as the concentration of ABA. Alternatively, the basis of measured changes in ABA sensitivity associated with a given physiological process (*e.g.* germinating wheat grains in dormant and nondormant varieties [33]) may be explained by differences in the endogenous osmoticum. It is important to note that molecular responses of plant cells to osmotic stress are not all mediated by ABA, because Guerrero and Mullet (14) identified mRNA transcripts that are accumulated in response to a reduction in turgor and are not responsive to ABA. However, other ABA-responsive genes that respond to environmental stresses in other systems should now be tested to determine the general applicability of our results. Finally, the target in the ABA stimulus-response coupling pathway that is altered by NaCl, resulting in a greater accumulation of *Em* mRNA, is now being sought by genetic, biochemical, and molecular approaches. These approaches should lead to the identification of the intermediates in an ABA-mediated physiological response.

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