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Effects of Osmoprotectants upon NaCl Stress in Rice[1]

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Plants accumulate a number of osmoprotective substances in response to NaCl stress, one of them being proline (Pro). While characterizing some of the changes in solute accumulation in NaCl-stressed rice (*Oryza sativa* L.), we identified several other potential osmoprotectants. One such substance, trehalose, begins to accumulate in small amounts in roots after 3 d. We performed a series of experiments to compare the effects of Pro and trehalose on ion accumulation to determine whether the two chemicals protect the same physiological processes. We found that Pro either has no effect or, in some cases, exasperates the effect of NaCl on growth inhibition, chlorophyll loss, and induction of a highly sensitive marker for plant stress, the osmotically regulated *salT* gene. By contrast, low to moderate concentrations of trehalose reduce Na^+ accumulation, *salT* expression, and growth inhibition. Somewhat higher concentrations (10 mM) prevent NaCl-induced loss of chlorophyll in blades, preserve root integrity, and enhance growth. The results of this study indicate that during osmotic stress trehalose or carbohydrates might be more important for rice than Pro.

Adverse environmental conditions such as drought and saline soils can reduce a variety of activities essential for respiration (Criddle et al., 1989) and photosynthesis (Yeo and Flowers, 1982) in NaCl-sensitive plants. Unlike most toxins and herbicides, excess NaCl or insufficient water has no single cellular target. The deleterious effects of these stresses result from both dehydration, which can denature many proteins or membranes, and ion displacement, in which the accumulating chemical compound places inorganic cofactors needed for some enzymes to work efficiently. Plants exposed to these stresses induce many different types of genes. The expression of some may be merely symptomatic of the type of damage that ultimately leads to death. Nevertheless, based on work with stresses such as heat (Sanchez et al., 1992) or exposure to physiological conditions that generate free radicals (Tsang et al., 1991), the majority of the new gene products that are made initially are most probably produced to compensate for denatured proteins or to repair injuries to the organism.

Some of the proteins made during stress synthesize substances believed to serve as osmoprotectants (Delauney and Verma, 1990; Bartels et al., 1991; Vernon and Bohnert, 1992). NaCl-stressed rice (*Oryza sativa* L.), for example, accumulates polyamines (Krishnamurthy and Bhagwat, 1989) and Pro (Chou et al., 1991; Alia and Saradhi, 1993). When administered externally, these molecules have been found to protect plants from some of the damage that is caused by drought or excess salinity (Kavi Kishor, 1989; Génard et al., 1991; Krishnamurthy, 1991). Other plants have been found to accumulate common sugars (Kameli and Lösel, 1993), polyols (Loescher et al., 1992; Alexander et al., 1994), or in some cases less common sugars such as trehalose (Fougère et al., 1991; Drennan et al., 1993). It is generally assumed that Pro, polyols, and sugars serve as physiologically compatible solutes that increase as needed to maintain a favorable osmotic potential between the cell and its surroundings (Pollard and Wyn Jones, 1979). There is additional evidence that high concentrations of these substances stabilize some macromolecules or molecular assemblies, thus decreasing the loss of either enzyme activity or membrane integrity that occurs when water is limiting (Schwab and Gaff, 1990; Génard et al., 1991). Each of the structurally distinct osmoprotectants could, based on its size, shape, and charge, be expected to benefit different osmotically sensitive classes of molecules or structures within the cell.

During our investigation of osmodefense processes in rice, we noted that one 15-kD protein was induced reproducibly in double-strength MS medium or medium supplemented with 1% NaCl (approximately 170 mM), 1% KCl (approximately 135 mM), or several other salts. This protein was isolated and partially sequenced so that oligonucleotide probes could be made and used subsequently to isolate cDNA and genomic copies of this gene, which we have called *salT* (Claes et al., 1990; A.B. Garcia, unpublished results). Its messenger is normally expressed very weakly in roots of hydroponically grown rice plants but begins to accumulate within 2 to 6 h after the plants are transferred to 1% MS salt (approximating an ion concentration of 200 mM). Slower rates of accumulation occur when plants are

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Abbreviation: MS, Murashige-Skoog.

grown for 7 d or more with lower levels of NaCl (0.45–0.7%; Claes et al., 1990; S. Iyer and A. Caplan, unpublished results). Initial expression was predominantly in the leaf sheaths and appeared in the leaf lamina only after many days or when higher concentrations of salts were used. Uniform dehydration, growth in high concentrations of PEG, or treatment with ABA also elicited the same organ-dependent pattern of *sal*T expression. More recent studies have shown that this pattern is characteristic of plants approximately 6 weeks old. Seedlings, by comparison, show highest expression within the newly forming leaf and vascular tissue (A.B. Garcia, T. Gerats, J. de Almeida Engler, B. Claes, R. Villarroel, M. Van Montagu, and A. Caplan, unpublished results).

Nothing is known currently about the function of *sal*T in the plant. If, however, it has a protective role, then one expects that the magnitude of the inductive response correlates with the amount of damage inflicted by the stress. Following this hypothesis, we have investigated some of the cellular and subcellular changes in NaCl-stressed rice treated with different osmoprotectants and correlated these with *sal*T expression. In this paper we show that two osmoprotectants, Pro and trehalose, have very different effects on the growth, appearance, and ion concentrations in different parts of the plants experiencing NaCl stress. First, only exogenous trehalose significantly reduced the various forms of damage we have identified in this paper and permitted rice roots to develop aerenchyma-like structures. Second, trehalose suppressed *sal*T induction by NaCl. Furthermore, *sal*T expression correlated with Na^+ accumulation in sheaths of stressed plants, whereas it did not in the laminae. The fact that this initial pattern of expression was developmentally restricted may indicate that *sal*T contributes to an organ-mediated mechanism for adaptation rather than one used in every cell. Third, Pro, one of the most commonly investigated osmoprotectants (Kiyosue et al., 1996; Verbruggen et al., 1996), induced *sal*T even in the absence of NaCl and had a synergistic effect in its presence. Based on this last observation, it is possible that stress-induced Pro production naturally contributes to the induction of the *sal*T gene.

MATERIALS AND METHODS

Rice (*Oryza sativa* [L.] var Taichung Native 1) was obtained from the International Rice Research Institute (Los Baños, Philippines). *O. sativa* var Cypress was obtained from Dr. Steve Linscomb (Louisiana University, Baton Rouge). Seeds were sown on vermiculite impregnated with nutrient solution (Yoshida et al., 1976) and grown at 27°C for 14 d. Plants were transferred to hydroponic culture for 2 weeks, and inductions were performed by adding the appropriate concentrations of the chemical to the nutrient solution.

The experiments on seedlings were carried out in vitro. Seeds were dehusked, surface-sterilized with 30% (v/v) Domestos solution (Domestos solution is prepared as 100% stock and contains 10.5% sodium hypochlorite, 0.3% Na_2CO_3, 10% NaCl, and 0.5% NaOH) for 40 min, rinsed with sterile water, and transferred to one-half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1% Suc and 0.8% agar. Four-day-old seedlings were transferred to fresh medium containing 0, 1, 5, or 10 mM Pro or trehalose supplemented or not with 1% NaCl. After 10 d plantlets were harvested, measurements were taken of sheaths and laminae, and plant material was kept at −70°C until further analysis. All percentages in this paper are in weight per volume unless indicated otherwise.

RNA Gel-Blot Analysis

Total RNA was extracted by the method of Jones et al. (1985). Ten micrograms of RNA was separated on formaldehyde-denaturing gels and transferred to Hybond-N membranes (Amersham) as described in the manufacturer's protocol. Prehybridization and hybridization were carried out according to standard protocols (Sambrook et al., 1989). The *Eco*RI fragments of the *sal*T cDNA were radiolabeled by incorporation of [α-^{32}P]dCTP and the random primer-labeling method, according to the manufacturer's protocol (Boehringer Mannheim). Filters were washed using standard procedures. RNA extraction and RNA-blot analysis were repeated three times in independent experiments for each different treatment. Approximately 10 plants were used for each replicate in the experiment using 4-week-old plants and approximately 20 seedlings per replicate in the experiments with 14-d-old plants. Concentrations of RNA were verified spectrophotometrically and by subsequent hybridization to a uniformly expressed copy of *S*-adenosyl-L-Met synthase (Van Breusegem et al., 1994).

Ion Measurements

Plant material was dried for 24 h at 80°C, weighed, and extracted for 2 h in 100 mM acetic acid at 90°C (20 mL of extraction solution/g dried material), according to the method of Yeo and Flowers (1983). Measurements were done in a flame-emission photometer (Eppendorf) and concentrations were estimated by comparison with standard curves using Na^+.

Chlorophyll Determination

Chlorophyll was extracted from fresh tissue in 80% (v/v) ethanol by heating samples for 10 min at 80°C. Chlorophyll content was calculated according to the method of Arnon (1949). Three independent experiments were carried out for each treatment, using three 4-week-old plants. Two measurements were taken for each replicate. Variation between different experiments was not higher than 10%.

Light Microscopy

For each treatment, root segments of five plants were prepared for light microscopy as follows: fixation was done for 4 h at room temperature in 3% (v/v) glutaraldehyde in 0.1 mM cacodylate buffer at pH 7.2. Afterward, samples were washed three times for 2 h in cacodylate buffer and postfixed for 4 h in 1% OsO_4. Root segments were exten-

sively washed three times for 2 h in cacodylate buffer and gradually dehydrated for 2 h in 30% (v/v) ethanol, for 2 h in 50% (v/v) ethanol, overnight in 70% (v/v) ethanol at 4°C, and for 2 h in 95% (v/v) ethanol. To embed them, the samples were infiltrated overnight at 4°C in one-half 95% (v/v) ethanol and one-half London Resin white. Samples were infiltrated twice more overnight in 100% London Resin white. The samples were finally placed in gelatin capsules filled with fresh London Resin white and polymerized overnight at 60°C. Tissue sections were made using a diamond knife (Diatome, Biel, Switzerland), mounted on glass slides, and observed using phase-contrast microscopy.

Carbohydrate Analysis

Rice seedlings (var Cypress) were cultured hydroponically for 7 weeks and then transferred to fresh solution supplemented with 75 mM NaCl. Samples were harvested after 0 to 3 d and ground to a fine powder in liquid nitrogen with a precooled mortar and pestle. One gram of powered material was transferred to Corex tubes (DuPont) containing 10 μg mL^{-1} phenyl β-D-galactoside, an internal standard, and placed in an 80°C water bath for 10 min. Insoluble material was removed by centrifugation at 12,000*g* for 10 min. The supernatants were collected in fresh tubes and the pellets were washed twice in 80% ethanol and centrifuged as before, and each wash and the supernatants were pooled with the first supernatant. The extracts were then concentrated to a volume of 0.5 mL, using a rotary evaporator (Büchi Labortechnik, Konstanz, Germany), transferred to crimp-top vials, and dried to a residue at 60°C.

Trimethylsilyl derivatives of sugars, polyols, and acids were prepared according to a procedure modified from Sweeley et al. (1963). Typically, 0.015 mL of 2-dimethylaminoethanol and 0.4 mL of pyridine containing 30 mg mL^{-1} methoxyamine HCl were added to the crimp-top vials containing the dried extracts. Vials were capped and placed in an 80°C water bath and incubated for 1 h. After the reactions were cooled to room temperature (26–27°C), 0.4 mL of hexamethyl disilazane and 0.02 mL of trifluoroacetic acid were added and the vials were capped and incubated at room temperature for 1 h. The insoluble debris was removed by centrifugation; the supernatant from each vial was carefully transferred to fresh crimp-top vials and sealed.

A gas chromatograph (model 5890, series II, Hewlett-Packard) equipped with a flame-ionization detector and a 30-m methylpolysiloxane column (0.32-mm i.d., 1.0-μm film, DB-1, J&W, Folsom, CA) was used for analyses. The operating conditions were as follows: injector 200°C, detector 290°C, oven temperature 200°C for 3 min, ramped 5°C min^{-1} to 250°C and held for 1 min, ramped 10°C min^{-1} to 290°C and held for 13 min; flow 1.4 mL min^{-1}; and a split ratio of 30:1. Trimethylsilyl-derivatized compounds were identified by a gas chromatograph (model 5890, series II) equipped with a 5972 quadrupole mass selective detector (MS Chemstation software; National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health mass spectral database). Based on the identification of the most abundant solutes, mixed standards were prepared and run each time the machine was used. These standards were used to verify the retention times and derivatization efficiencies of all major sugars, polyols, and acids under investigation. Reconstruction experiments showed slight differences between the efficiencies of derivatization and/or detection of individual molecular species. As a consequence, the crude percentages obtained from each plant analysis were corrected by recovery coefficients derived from analysis of the corresponding standards.

RESULTS

NaCl Stress Induces Organ-Specific Changes in the Accumulation of Some Organic Solutes

Plant cells change the steady-state pool of many of their organic constituents, especially organic acids, sugars, and polyols, in response to changing osmotic conditions (Handa et al., 1983; Binzel et al., 1987). To characterize the osmotic defense processes in rice, we measured the amounts of some of these chemicals in the leaf blades and roots of hydroponically grown plants over the course of a 3-d treatment with NaCl. Figure 1 shows that mild NaCl stress had a very significant effect on the pools of most of the substances that we monitored. Citrate, malate, and inositol increased to detectable levels in leaf blades within 1 d and accumulated steadily thereafter. By contrast, Glc and Fru increased only slightly. Ascorbate, Suc, and salicylate concentrations changed little if at all for the 1st d but increased quickly after d 1 and 2. None of these changes occurred to any great extent in the roots. In addition to these basic components of metabolism, we detected several exotic compounds often thought to contribute in some special way to osmotic protection. Figure 2 shows that the polyols adonitol and sorbitol accumulated rapidly in blades and then declined, whereas arabitol and mannitol became abundant only after d 2. All of these solutes accumulated exclusively in the leaf blades. The only well-characterized osmoprotectant we identified was the disaccharide trehalose, which accumulated to detectable levels after d 2 in the roots.

Trehalose Protects Rice Seedlings Better than Pro

We were surprised that osmoprotective substances did not accumulate throughout the plant. One possibility is that each is needed to protect different types of molecules or cellular components. Therefore, we chose two structurally different osmoprotectants, Pro and trehalose, and investigated their effect on a number of NaCl-sensitive physiological processes in rice. Rice was treated with Pro or trehalose alone or together with 1% (approximately 170 mM) NaCl to measure their effect on NaCl-mediated inhibition of seedling growth.

Figure 3 shows that Pro and trehalose gave very different degrees of osmotic protection. NaCl reduced laminae growth by 45% and Pro did not reduce growth inhibition at all. In fact, Pro inhibited growth approximately 15% by itself. Trehalose, by contrast, produced no growth inhibition or visible changes in the appearance of the plants.

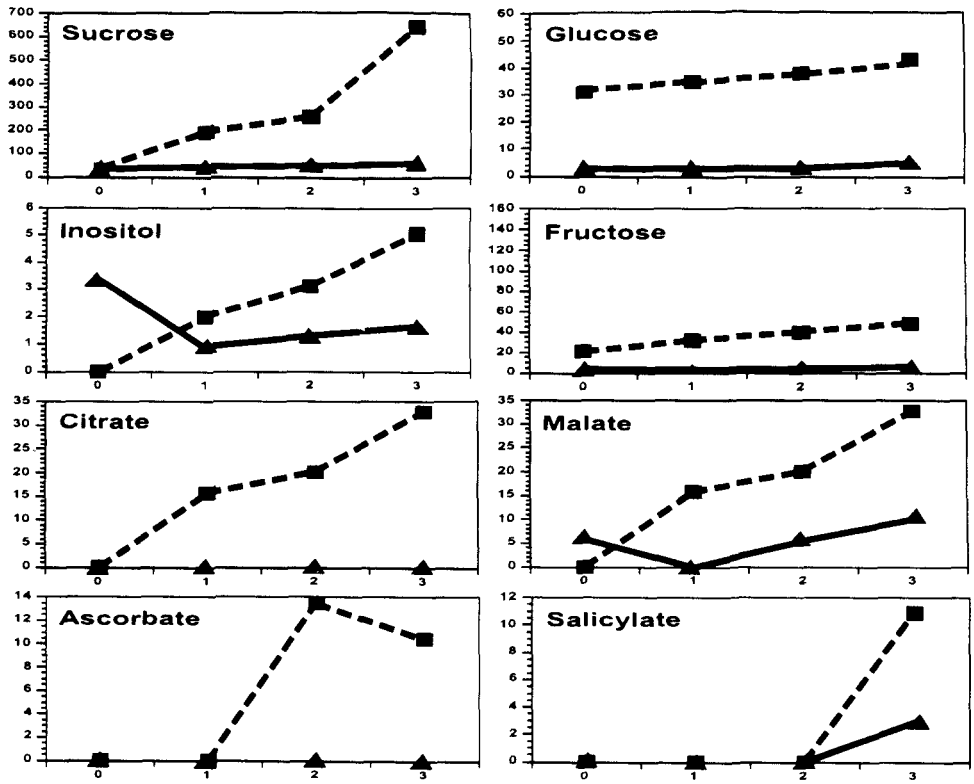

Figure 1. Accumulation of organic solutes during NaCl stress. Seven-week-old rice plants (cv Cypress) were transferred to hydroponic solution containing 75 mM NaCl. Samples were harvested at 0 to 3 d, split into leaf blades (dotted lines) and roots (solid lines), and prepared as described in "Materials and Methods." The solutes were derivatized and separated by GC. Specific solute peaks were quantitated, and the values were corrected to account for differences in the efficiency of detection (S. Iyer and A. Caplan, unpublished data). Ordinate, Concentration in μg 100 mg^{-1} fresh weight of each solute; abscissa, days after NaCl addition (start of NaCl stress).

More significantly, trehalose reduced the inhibitory effects of NaCl and did so over the range of the concentrations that were tested.

Trehalose Prevents Chlorophyll Loss

One visible symptom of ion accumulation in leaves is a concomitant loss of chlorophyll (Yeo and Flowers, 1983), indicating some form of disruption of the chloroplasts. NaCl-tolerant rice varieties vary in chlorophyll deterioration (Yeo and Flowers, 1983). To determine whether any of the osmoprotectants under study protected this sensitive cell compartment, we measured chlorophyll loss in 4-week-old plants after 3 d of growth in hydroponic medium containing 1% NaCl. Table I shows that chlorophyll levels decreased by 48% during this period. If, however, plants were simultaneously grown with 10 mM trehalose, no loss was seen. Pro, by contrast, accelerated this loss considerably at low concentrations, although the leaves continued to look healthy.

Osmoprotectants Alter Ion Partitioning between Mature Laminae and Sheaths

Osmoprotectants may preserve cell integrity in various ways. The simplest way would be to prevent ion entry into sensitive parts of the plant or to enhance ion excretion from them. To test for differences in ion accumulation, we determined the internal concentration of Na^+ in leaves of plants treated with NaCl, or NaCl and either Pro or trehalose. Table II shows that trehalose and Pro had little effect on sodium levels in laminae and sheaths of unstressed plants. A more striking effect was seen when stressed plants were surveyed. Sodium levels increased in NaCl-grown plants 8.5-fold in the sheaths and 94-fold in the laminae (Table II). Whereas Pro treatment hardly changed the sodium accumulation profile in sheaths and laminae, trehalose treatment led to a marked decrease of sodium accumulation in laminae, possibly accompanied by a (small) increase in sheaths.

Trehalose Facilitates Aerenchyma Formation in Seedling Roots

All studies discussed so far have shown that morphology and physiology of sheath and blade change when plants are grown with different osmoprotectants. Because the ions and the organic molecules added are absorbed through the roots, we investigated the morphology of the roots of treated seedlings. All roots of NaCl-treated plants are thicker than those of control plants, and neither of the osmoprotectants changed this. Figure 4 shows that root

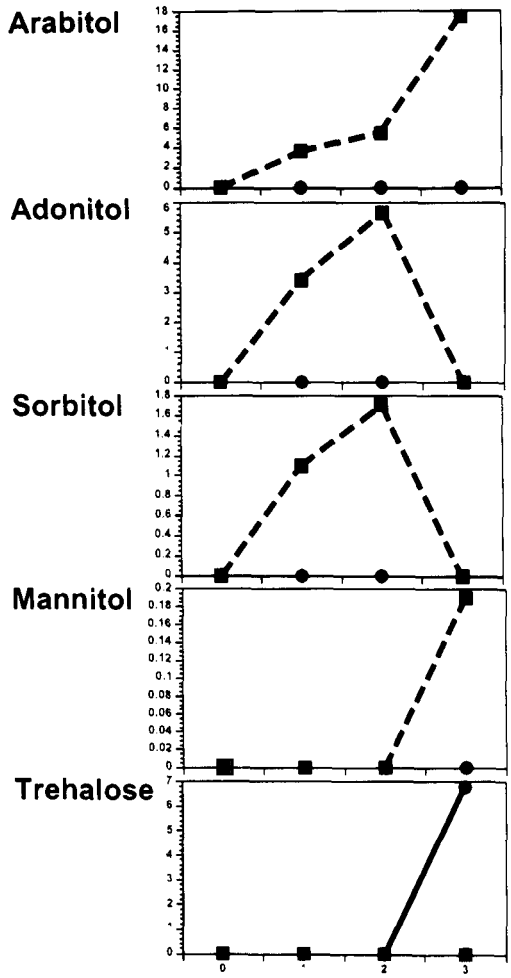

Figure 2. Accumulation of possible osmoprotectants during NaCl stress. Samples described in Figure 1 were analyzed for polyols and trehalose concentrations. Ordinate, Concentration in μg 100 mg^{-1} fresh weight of each solute; abscissa, days after NaCl addition (start of NaCl stress).

hairs disappear from NaCl-treated plants, and large portions of the epidermis are sloughed off. The two next layers making up the exodermis are also highly disorganized, as is the innermost part of the root consisting of the endodermis and vascular cylinder. The cortex between these two regions contains large, irregularly shaped air spaces.

Almost all cell layers of NaCl plus Pro-treated plants look somewhat similar to their respective controls grown with NaCl alone, indicating that Pro affords little protection to this organ. Roots of trehalose-treated plants exhibit the most orderly cell layers and most regular air spaces. These structures correspond to aerenchyma normally formed in mature roots of many cereals. They apparently are induced prematurely by osmotic stress. Aerenchyma allow oxygen to pass from leaves to roots. Several studies have shown that the rates of oxygen diffusion to the roots correlates with the ability to tolerate a variety of adverse soil conditions (Kawase, 1981). Under UV light the epidermis of control roots fluoresced green (lignin deposition), whereas the exodermis fluoresced blue (suberin deposition). The first layer of the exodermis from NaCl and trehalose plus NaCl fluoresced green. The bundle sheaths in all of the treatments, except of the NaCl-stressed control, fluoresced blue. These changes might indicate that the plants are increasing lignin synthesis during NaCl stress to make cell walls less permeable to water loss.

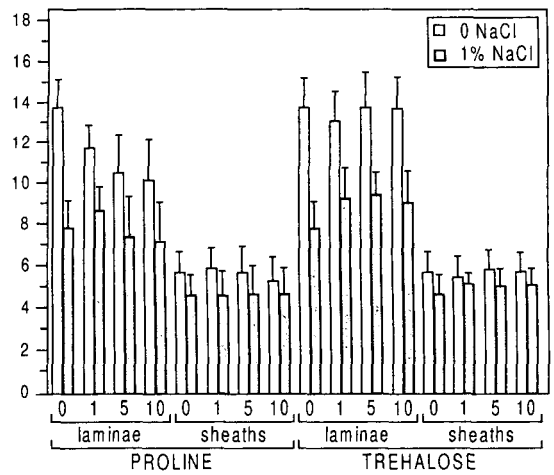

Figure 3. Length of seedling sheaths (in centimeters) and laminae after 10 d of growth with or without 1% (w/v) NaCl and additional supplements. Four-day-old rice seedlings were transferred to fresh medium containing 0, 1, 5, or 10 mM Pro or trehalose, as indicated. After 10 d of growth, organ length was measured. Error bars denote SDs for 100 seedlings.

We have also obtained sections from aerial portions of the treated plants (not shown). Cells of sheaths of NaCl-stressed plants, or of plants grown with NaCl plus Pro, were heavily swollen. These distortions left large intercellular spaces within the tissues. Plants treated with trehalose, however, had more regular cell layers and normal-sized cells (data not shown), even though, as discussed below, the sheaths still accumulated considerable amounts of NaCl.

salT Expression Is Not Determined Solely by Ion Accumulation

Osmotic stress changes the expression of many genes in rice. We presume gene expression is responding either to NaCl itself or to something produced because of the damage caused by NaCl. If either Pro or trehalose reduces cell

Table I. *Percentage of chlorophyll remaining in leaves of rice plants treated with Pro and trehalose in the presence of 1% NaCl for 3 d*

NaCl	Concentration Osmoprotectant	Remaining Chlorophyll	
		Pro	Trehalose
	mM	%	
− (Control)		100	100
1%	0	52	52
1%	1	22	67
1%	5	25	67
1%	10	41	114

Table II. *Amount of sodium ($\mu g\ g^{-1}$ dried tissue) in rice plants treated with different concentrations of Pro and trehalose in the presence (+) or absence (−) of 1% NaCl after 3 d of treatment and ratios (±) between NaCl and osmoprotectant-treated plants*

Concentration	Pro			Trehalose		
	−	+	±	−	+	±
mM						
Sheaths						
0	0.80	6.8	8.5	0.80	6.80	8.5
1	0.30	5.4	18.0	0.80	4.60	5.8
5	0.15	8.7	58.0	0.40	8.00	20.0
10	0.40	5.4	13.5	0.30	8.40	28.0
Laminae						
0	0.07	6.6	94.0	0.07	6.60	94.0
1	0.10	8.9	89.0	0.06	0.96	16.0
5	0.05	7.4	148.0	0.05	0.50	10.0
10	0.06	5.5	92.0	0.04	0.60	15.0

damage, then we should see expression of stress-induced genes decline proportionally. To test this, we selected an osmotically regulated gene, *sal*T (Claes et al., 1990), and correlated its expression first with the accumulation of sodium and then with the countereffects of the two osmoprotectants.

Based on published reports (Yeo and Flowers, 1982; Drew and Läuchli, 1985), we have proposed previously that the discontinuous pattern of *sal*T expression from roots to blades might correspond to a discontinuous ion gradient (Claes et al., 1990). To test how this gradient compared with that of *sal*T expression, we dissected mature rice plants from innermost (youngest) to outermost (oldest) leaves and measured both ion accumulation and gene expression. Figure 5 shows that after 1 d sodium levels had increased to the highest levels in the oldest sheaths of 4-week-old plants and to a lesser extent in younger organs. A similar pattern was seen in the laminae. The figure also shows that expression of *sal*T was generally highest in the older sheaths 1 to 3 and barely detectable elsewhere, even though lamina 1 contained almost as much sodium as sheath 3. These results indicate that, although *sal*T induction is correlated with an internal ion gradient, there might be developmental and physiological constraints restricting its accumulation in sheaths.

Pro Enhances *sal*T Expression, whereas Trehalose Suppresses It in Sheaths

Figure 4 shows how treatments affect the accumulation of *sal*T mRNA in roots. Control roots contain very little of the messenger until induced with 170 mM NaCl. Ten mM trehalose treatments had little or no effect on this. Unexpectedly, 10 mM Pro induced this gene by itself, despite the fact that it reduced basal levels of sodium in the sheath (Table II). Further studies were performed to determine how Pro and trehalose influenced gene expression elsewhere in the plant. Figure 6 shows representative examples of replicated experiments indicating *sal*T mRNA accumulation in sheaths and laminae after treatment of seedlings for 10 d or 4-week-old plants for 3 d with a range of concentrations of Pro. Despite differences in time of treatments or age of plants, both experiments led to the same conclusion. We found that low concentrations of Pro (5 and 10 mM) increased the levels of *sal*T expression in the lamina, and possibly the sheaths, of NaCl-stressed plants. More interestingly, these levels of Pro also induced *sal*T to a low degree in the sheaths of unstressed seedlings. This led us to test the effect of higher levels of Pro. Pro (50–200 mM) inhibited sheath growth of seedlings 45 to 60% (Fig. 7). At the same time, 50 mM Pro alone induced a 2-fold higher *sal*T expression level than 170 mM NaCl alone, whereas higher levels had greater effects. More importantly, the combination of NaCl and Pro gave higher expression than the sum of either treatment alone, as shown graphically in Figure 7c. This synergism may be an indication that the two stimuli act independently at some point in the signal transduction pathway regulating *sal*T.

As seen in Figure 8, trehalose had very different effects. Whereas low levels did enhance the effects of NaCl in adult laminae and seedling sheaths, higher levels suppressed *sal*T induction in adult laminae and sheaths. The variation between seedlings and adults may reflect differences in the rates of metabolism in the two populations.

DISCUSSION

This report confirms that rice does not accumulate ions uniformly (Yeo and Flowers, 1982). Untreated plants contained as much as 11-fold more sodium in their sheaths than in their laminae (Table II) and 2-fold more in their oldest organs (lamina 1; Fig. 5) than in their youngest (lamina 4; Fig. 5). When plants were stressed, sodium accumulated differentially in sheaths and laminae, increasing the levels 8.5- and 94-fold, respectively, after 3 d.

Growth with the high concentrations of NaCl affected the plants quite quickly. First, free sugars and organic acids potentially derived from them increased within 1 to 2 d of treatment (Fig. 1). Second, leaves began to lose chlorophyll (Table I), indicating that NaCl disrupts chloroplast integrity. Third, prolonged exposure reduced growth (Fig. 3) and interfered with normal root development (Fig. 4). Many root cells collapsed or failed to divide correctly so that the cell layers became disorganized. The exodermis

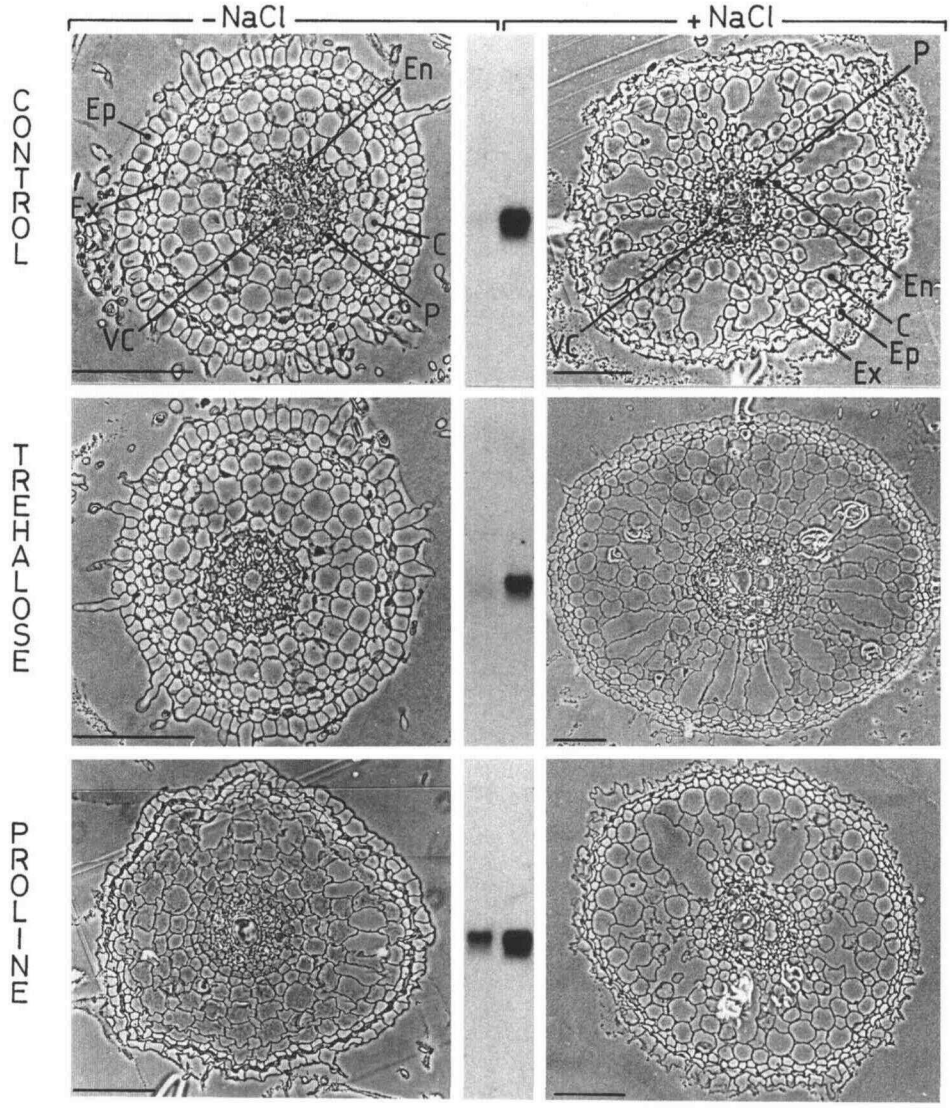

Figure 4. Root morphology and *salT* expression after growth with osmoprotectants and NaCl. Four-day-old rice seedlings were transferred to fresh medium containing 0 (−NaCl) or 1% (+NaCl) NaCl and 10 mM trehalose and Pro, as indicated. After 10 d of growth roots were examined or used for RNA preparations. The results of hybridization of a *salT* probe to RNA from stressed (right) and unstressed (left) plants are shown in the center. Note aerenchyma formation in trehalose plus NaCl-treated roots. Alterations are seen in epidermis (Ep), exodermis (Ex), cortex (C), endodermis (En), procambium (P), and vascular cylinder (VC).

also failed to form a layer of fluorescent material (possibly suberin) that was found in untreated roots. The roots may have been trying to form aerenchyma. These passageways commonly form in older, larger plants and in ones grown in microaerobic conditions (Armstrong, 1971). Plants may have been trying to form aerenchyma during NaCl stress to compensate for a reduction in metabolic activity due to salinity (Criddle et al., 1989; Schwarz et al., 1991). If so, the effort was largely unsuccessful, except when plants were provided with trehalose. Finally, growth on NaCl induced the accumulation of *salT* messenger, primarily in roots (Fig. 4) and in outermost leaf sheaths (Fig. 5). These experiments also showed that expression of this gene is not simply determined by the amount of sodium in the organ: During stress, some blades accumulated as much Na^+ as some sheaths and yet did not express this gene.

What Osmoprotectants Are Being Made in Rice during Stress?

We presume the damage we see would be more severe if rice did not attempt to counter the effects of NaCl by accumulating some osmoprotectants. Most of the commonly held ideas of the roles of stress-induced osmoprotectants come from three types of observations. The first evidence that these small molecules play a special role in plants was found in studies showing they accumulate during drought or in saline conditions (Leigh et al., 1981;

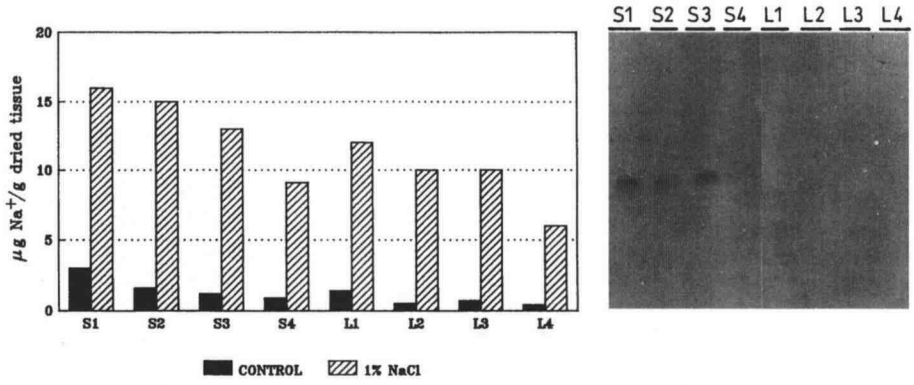

Figure 5. Correlation between *salT* expression and Na^+ accumulation. Four-week-old rice plants were transferred to media containing 1% (w/v) NaCl. After 1 d, RNA was extracted from sheath (S) and lamina (L) of leaves 1 (older) through 4 (younger) of 10 plants. Simultaneously, a portion of each pool of tissue was dried, weighed, and prepared for the determination of Na^+ content. Left, Na^+ in sheaths and laminae 1 to 4 from plants grown with and without NaCl. Right, *salT* expression in each part of each leaf after NaCl treatment.

Flores and Galston, 1982; Handa et al., 1983; Bhaskaran et al., 1985; Newton et al., 1986; Binzel et al., 1987). In addition, there is, in many cases, a correlation between the accumulation of specific chemicals and the degree of tolerance shown by different varieties within a given species (Grumet and Hanson, 1986; Erdei et al., 1990; Jain et al., 1991). The second indication that these molecules are important comes from demonstrations that exogenous application enhances plant tolerance (Lone et al., 1987; Kavi Kishor, 1989; Génard et al., 1991; Krishnamurthy, 1991). The newest evidence showing the importance of one of these molecules is that genetically elevated mannitol concentrations (Tarczynski et al., 1993) enhance plant growth under osmotically stressful conditions. Recently, Holmström et al. (1996) found that tobacco plants expressing a gene for trehalose synthesis lose water more slowly than untransformed plants. Because little trehalose was found in these transgenics, these authors propose that trehalose must prevent cell damage rather than alter osmotic potential. The third major line of evidence stems from in vitro studies showing that these molecules either reduce the loss of enzyme activity or preserve membrane structure during desiccation or in saline solutions (Pollard and Wyn Jones, 1979; Crowe et al., 1984a; Schwab and Gaff, 1990; Mamedov et al., 1991; Colaço et al., 1992). The concentrations of these exogenously added compounds needed to obtain a significant degree of protection are often quite high (200–500 mM Pro; Pollard and Wyn Jones, 1979; Crowe et al., 1984b; Schwab and Gaff, 1990) compared with what some plants produce. NaCl-stressed rice, for example, has been reported to accumulate 8 to 12 μmol Pro g^{-1} dry weight (Mali and Mehta, 1977), which might not produce the necessary molarity in the cell unless it is concentrated in a specific compartment.

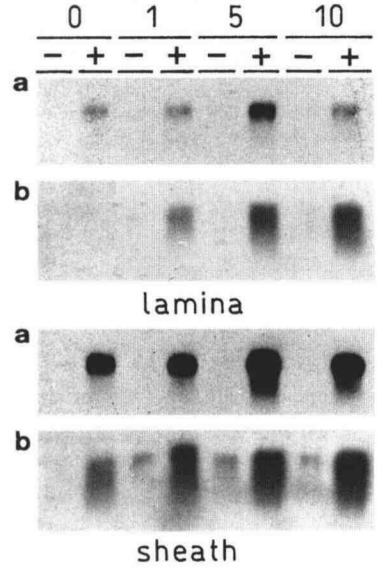

Figure 6. Induction of *salT* expression by Pro in the absence of NaCl. a, Four-week-old plants were transferred to media with the indicated concentration (mM) of Pro with (+) and without (−) 1% (w/v) NaCl. RNA was extracted from laminae and sheaths after 3 d and analyzed for *salT* expression. b, Four-day-old seedlings were transferred to sterile media containing the indicated concentration of Pro (mM) with (+) and without (−) 1% NaCl and grown for 10 d. RNA was then extracted and analyzed for *salT* expression.

Before we could begin to understand how exogenous applications of osmoprotectants might reduce stress-associated damage, we had to identify the types of molecules likely to be found in rice. Pro was known to accumulate in osmotically stressed rice (Mali and Mehta, 1977; Chou et al., 1991; Alia and Saradhi, 1993). Our survey of major solutes revealed that rice also accumulates various polyols and, more interestingly, trehalose. Each is found concentrated in a different part of the plant. This may be because each is used in different ways. For example, some of the solutes may protect organ-specific enzymes or structures, and others might balance the osmotic potential of a part of the plant against the accumulation of inorganic ions outside.

Trehalose Reduces Ion Concentration in the Laminae

Trehalose did not prevent plants from taking up excess NaCl, but it did reduce Na^+ accumulation in laminae

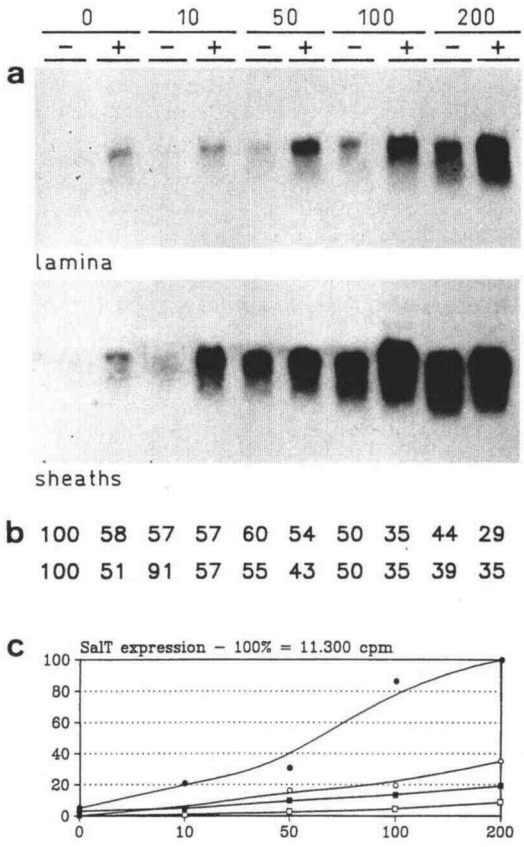

Figure 7. Synergistic action of Pro with NaCl to induce *salT*. a, Four-day-old seedlings were transferred for 10 d to medium with 0 to 200 mM Pro with (+) and without (−) 1% (w/v) NaCl. RNA gel-blot analysis of total RNA of laminae and sheaths hybridized to *salT* cDNA. b, After 10 d seedling length was measured and expressed relative to that of untreated plants (top row for laminae; bottom row for sheaths). c, The portion of the RNA gel blot corresponding to the *salT* mRNA was excised from the filter, dried, and counted in a scintillation counter with scintillation fluid. Graph shows percentage of maximum counts (11,300 cpm) corresponding to *salT* expression with increasing Pro concentration (abscissa). □, Lamina control; ■, lamina 1% NaCl; ○, sheath control; ●, sheath 1% NaCl.

10-fold. This alone may have allowed plants to continue growing (Table I) without loss of chlorophyll (Table II). Nevertheless, trehalose did not prevent plants from accumulating normal amounts of sodium under control conditions; nor did it prevent plants from accumulating excess NaCl in their sheaths during stress conditions (Table II). It is therefore unlikely that trehalose prevented the influx of ions into the plant or changed the osmotic pressure within the sheath so much that ions could not be transported upward. We can only speculate how trehalose exerts its effect on Na^+ pools. One possibility is that by preserving the integrity and native state of proteins and lipid bilayers (Crowe et al., 1984a, 1984b; Hincha, 1989; Colaço et al., 1992) it maintains the pumps specifically needed to exclude excess NaCl from the photosynthetic organelles. This could account for the greater accumulation of sodium in the blades. Preservation of other pumps or cellular structures in the roots may permit other adaptive processes, such as aerenchyma formation, to continue unimpeded. All of the plants analyzed had been cultured with 30 mM Suc (Fig. 4), and yet only trehalose-treated plants had well-developed aerenchyma and well-preserved root cells. One of the additional effects of trehalose was that high concentrations suppressed the induction of *salT* in several parts of the plant, although low concentrations of the sugar actually enhanced *salT* expression somewhat. For this, we offer the following interpretation. Trehalose has been found to be more effective than most sugars at increasing lipid bilayer fluidity (Crowe et al., 1984a, 1984b) and at preserving enzyme stability during drying (Colaço et al., 1992). If trehalose must initiate a phase change or intercalate into a target structure in the cell to prevent it from being denatured by NaCl, then suboptimal levels of the sugar would lead to parts of the target forming bonds with trehalose, whereas other parts would be making hydrophilic contacts with water or inorganic ions. The additional disorder that this asymmetry would introduce would add to the distortion of the molecule meant to be protected. By contrast, higher trehalose concentrations would provide sufficient sugar to produce a more regular covering for the osmotically sensitive target and so reduce the damage that in turn induces *salT*. The differences in response that we observed between lamina and sheath or between short and prolonged treatment might reflect differences in accumulation or catabolism of trehalose in different parts of the plant.

Pro Induces *salT* Messenger Accumulation

We were surprised that exogenous Pro had few positive effects on rice. Whereas the leaf laminae were not shriveled, growth was inhibited and chlorophyll loss was accelerated. Root cell layers of plants grown with NaCl and Pro

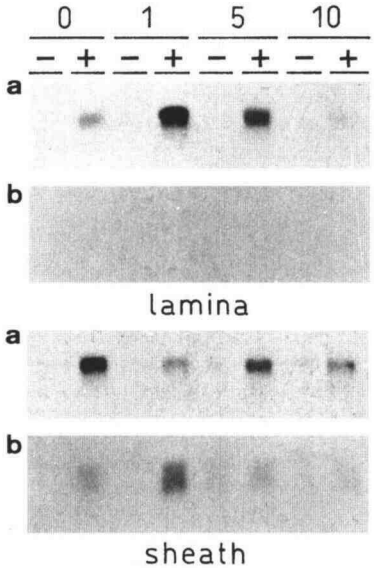

Figure 8. Suppression of *salT* expression by high concentrations of trehalose. Plants were grown as indicated in the legend of Figure 6, except with the indicated concentrations (mM) of trehalose instead of Pro. RNA was extracted from laminae and sheaths after 3 d of culture and analyzed for *salT* expression.

were less disordered than those grown with NaCl alone, but aerenchyma formation was less complete than in plants protected by exogenous trehalose. Pro also had only slight effects on sodium accumulation. In contrast to trehalose, Pro was a potent inducer of *sal*T, both by itself and synergistically with NaCl. This synergism and greater sensitivity to Pro than to NaCl raises the possibility that Pro accumulation during stress is a natural regulator of *sal*T. Further studies will be needed to clarify whether the gene is induced directly by Pro or by some other molecule associated with growth inhibition.

We do not fully understand how Pro, synthesized during osmotic stress, is being used. Whereas trehalose is beneficial to growth whether or not plants are stressed, added Pro seems to be deleterious to unstressed rice and only marginally useful to stressed plants. This by no means implies that Pro accumulation plays no adaptive role. It may be protecting a few essential targets that cannot be protected by trehalose or any of the other solutes that rice accumulates. Because rice accumulates Pro normally when stressed (Mali and Mehta, 1977; Chou et al., 1991; Alia and Sarahi, 1993), it is possible that the exogenous addition is not necessary and may have overtaxed other systems. Whereas trehalose seemed the most effective of the two osmoprotectants tested here, it is important to note that trehalose is not made throughout the plant nor does it accumulate quickly in roots. It is possible that one or more of the other solutes actually plays the greatest role in protecting rice plants. By the same token, it is possible that only one or two compounds are limiting for growth in a saline condition so that identifying and supplementing those might be sufficient to make rice significantly more osmotolerant.

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