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Alteration of Starch-Sucrose Transition in Germinating Wheat Seed under Sodium Chloride Salinity

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Alterations in starch-sucrose transition during germination were studied in wheat seeds under saline conditions. NaCl significantly reduced the speed of germination and resultant seedling growth, but delayed the degradation of seed storage components. The endogenous level of ABA increased while osmotic potential decreased. NaCl also inhibited the expression of α-amylase. Increasing the concentration of NaCl induced the expression of sucrose phosphate synthase, and sugars, including sucrose, were accumulated in the seedlings. This accumulation of sugar closely correlated with an increase in ABA. However, sugar accumulation was reversible when the salt stress was removed. Overall our results strongly suggest that the germinating wheat seeds alter the starch-to-sucrose conversion to adapt for salt stress. This is probably mediated by the increase in ABA.

Keywords: ABA, α-amylase, germination, NaCl stress, sucrose, wheat seed

Salinity stress is a major limit in agriculture throughout the world. Because a plant's ability to cope is critical when determining crop distribution and productivity, it is important to understand the adaptation mechanisms to saline environments. Salinity may affect seed germination in two ways: (a) osmotically, by decreasing the ease with which seeds take up water; and (b) ionically, by facilitating the uptake of ions that, in excess amounts, are toxic embryonic activity (Khan et al., 1997).

Plant responses to environmental stress are expressed through many processes; phytohormones probably play a central role in the integration of these responses (Saab et al., 1990; Roy et al., 1995; Bray, 1997). For example, ABA promotes the synthesis of a unique class of proteins that may have some functional, protective role (Robertson et al., 1989; Xu et al., 1996; Muramoto et al., 1999). ABA also prevents another group of proteins important for seed germination (Lovegrove and Hooley, 2000). Previously, we showed glucose uptake and its conversion to sucrose in the scutellar tissues. However, ABA inhibited the gibberellin-induced α-amylase expression in the aleurone layer of germinating rice seed (Kashem et al., 1998).

Water balance generally is conserved within the plant, so metabolic activities are sensitive to high salt concentrations. Therefore, osmotic potentials within the cytoplasm are maintained by 'compatible solutes', while inorganic salts accumulate inside the vacuole (Shannon et al., 1994). Plants under salinity stress accumulate a number of organic solutes which have little effect on plant metabolism (Bohnert et al., 1995; Gilbert et al., 1998). These metabolites include amino acids, polyamines, and carbohydrates, e.g., mannitol, sucrose and raffinose oligosaccharides. Experiments with model systems have demonstrated that sugars can protect the structural integrity of membranes during dehydration by preventing membrane fusion, phase transition, and phase separation (Crowe and Crowe, 1992).

Little is known about the mechanisms of seedling growth inhibition and the role of ABA in a salt-stressed plant system. Here, we report the alteration of

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Abbreviations: F-6-P, fructose 6-phosphate; sucrose-P, sucrose phosphate; SPS, sucrose phosphate synthase; UGPase, UDP-glucose pyrophosphorylase

starch-sucrose transition in germinating wheat seeds under NaCl salinity.

MATERIALS AND METHODS

Plant Materials

Wheat seeds (*Triticum aestivum* L. cv. Koyuki) were sterilized in 1% sodium hypochlorite for 5 min and thoroughly rinsed in sterile H_2O. Under standard experimental conditions, 25 seeds were incubated in the dark at 25°C for five days in Petri dishes, using two layers of filter paper.

Analysis of ABA

Five days after imbibition, 25 germinating whole wheat seeds (containing seedlings) were ground with 80% methanol (2 mL per seed). The supernatant was collected by centrifugation at 10,000*g* for 15 min at 4°C, and the residues were re-extracted. This combined supernatant was evaporated, volume reduced to 5 mL, then filtrated through a 0.2-μ cellulose acetate filter (Advantec, Tokyo), and adjusted with acetic acid to pH 2.8. The acidic content was extracted with ethyl acetate three times. This fraction was evaporated, volume reduced to 0.2 mL, and finally diluted with trizma buffered saline, which comprised 25 mM trizma (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$ and 3 mM sodium azide up to 6 mL. A 0.1 mL aliquot was used for the analysis of ABA, following the protocol for the Phytodetek ABA analysis kit (Agdia, Elkhart, IN, USA).

Measurement of Osmotic Potential

The procedure for osmotic potential measurement was essentially the same as described by Sultana et al. (1999). Seedlings (roots and shoots) were dissected from germinating wheat seeds five days after imbibition. The fresh samples were then pressed with a garlic squeezer to extract the cell sap. Osmotic potential was determined with a psychrometer (Wescor RH52, Logan, UT, USA).

Enzyme Assays

SPS assay: Seedlings were extracted with 50 mM Hepes-NaOH buffer (pH 7.5) that contained 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 2 mM DTT, and 0.5 mM PMSF, then centrifuged at 18,000*g* for 20 min at 4°C. Activity of the supernatant was assayed as described by Cheikh and Brenner (1992), measuring fructose-6-phosphate (F-6-P) dependent sucrose (+ Sucrose-P) formation from UDP-glucose. The assay mixture contained 28 mM UDP-glucose, 10 mM F-6-P, 15 mM $MgCl_2$, and 50 mM Hepes-NaOH (pH 7.5). An aliquot of the extract was added to the assay mixture to initiate the reaction. The mixture (70 μL) was incubated at 30°C for 10 min, and the reaction was terminated by the addition of 70 μL of 1N NaOH. The remaining hexose was then destroyed by placing the tubes in a boiling water bath for 10 min, and allowing them to cool. After the addition of 250 μL of 0.1% (w/v) resorcinol and 750 μL of 30% (v/v) HCl, the mixture was incubated at 80°C for 10 min. After the tubes cooled, absorbance at 520 nm was determined.

UGPase assay: Assay and extraction procedures of UDP-glucose pyrophosphate were essentially the same as reported by Kimura et al. (1992). The reaction mixture contained 5 mM glucose-1-P, 5 mM UTP, 5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 1.6 units of inorganic pyrophosphatase, and an aliquot of extract (20 μL) in a total volume of 125 μL. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 125 μL of 0.3 M perchloric acid. The reaction mixture was then placed in an ice-bath. Afterward, this mixture was centrifuged at 10,000*g* for 10 min at 4°C to remove denatured protein. The supernatant (50 μL) was added to the following mixture to measure Pi liberation: 0.3 M perchloric acid (250 μL), 0.2 mM $CuSO_4$ (50 μL), and 200 μL of Fiske-SubbaRow mixture [one volume of reducing reagent (0.2% 1-amino-2-naphthol-4-sulfonic acid, 1.2% Na_2CO_3 and 12% $NaHSO_3$) and two volumes of 2.5% ammonium molybdate] in a total volume of 1 mL with distilled water. Reduced phosphomolybdate color was measured at 700 nm.

α-Amylase assay: Preparation of the enzyme sample and assay were described by Kashem et al. (1998). One enzyme unit was defined according to the procedure detailed by Okamoto and Akazawa (1978).

Other Assays

Total sugar content was estimated by the method described by Avigad (1990); protein content by the dye binding procedure of Bradford (1976), with γ-globulin as a standard.

Sugar Analysis by HPLC

Seedlings (0.4 g) were ground in 1 mL of 50 mM

Tris-HCl buffer (pH 7.0) containing 3 mM $CaCl_2$ and 4 mM NaCl, then centrifuged at 18,000g for 20 min at 4°C. A 0.5-mL portion of the extract was added to an equal volume of organic solvent (chloroform: isoamylalcohol = 24:1) and vigorously mixed for 5 min. After centrifugation at 3,000g for 10 min, the aqueous phase was carefully collected. After H_2O (0.5 mL) was added, the residue was re-centrifuged, and the aqueous phase collected. The combined aqueous phase was lyophilized. A sugar sample was dissolved with H_2O and subjected to an automatic sugar analyzer (Hitachi L-6200 high-performance liquid chromatography system, Tokyo, Japan). Here, samples were applied to a sugar separation column (Hitachi #3013-N, 4 mm I.D. × 150 mm) at 55°C. They were eluted with 4 mL of 100 mM boric acid and 9 mM NaOH; 12 mL of a linear gradient of 100 mM boric acid and 9 mM NaOH to 300 mM boric acid and 15 mM NaOH; and 12 mL of 300 mM boric acid and 15 mM NaOH at a flow rate of 0.5 mL min^{-1}. The separated sugar was allowed to react with phenylhydrazine (phosphoric acid: acetic acid: phenylhydrazine = 0.543:0.442:0.015; v:v:v) and detected by a fluorescence spectrophotometer (Hitachi F-1050) at Ex 330 nm and Em 470 nm. Estimated sugar recovery in the extraction procedure was 75% from the experiment, using the standard sugar solution (10 mM glucose, 10 mM fructose, 10 mM maltose, and 10 mM sucrose).

Immunoblotting

The procedure for immunoblotting was essentially the same as reported by Kashem et al. (1998). Proteins in the SDS-gel were transferred to a nitrocellulose sheet (Advantec, Tokyo, Japan) in 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at 100 mA for 2 h at 4°C, using an electroblotter (Model AE-6675; Atto, Tokyo, Japan). The nitrocellulose sheet with blotted protein was soaked twice for 30 min in PBS-Tween containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3% (w/v) skimmed milk, and 0.05% (w/v) Tween 20. It was then incubated with polyclonal antibodies of anti-rice α-amylase I-1 (Mitsui et al., 1996), anti-rice UGPase (Kimura et al., 1992) or anti-maize SPS_{90} (Calgene, Davis, CA, USA) at 37°C for 30 min. The sheet was washed three times (5 min each) with PBS-Tween, then incubated with peroxidase-conjugated goat anti-rabbit IgG at 37°C for 30 min. The sheet was washed three times with PBS-Tween, followed by a single wash with 15 mM phosphate buffer (pH 6.8), and then incubated in a reaction mixture containing 0.03% (w/v) 3,3'-diaminobenzidine, and 0.003% (v/v) H_2O_2. After suitable color development, the nitrocellulose sheet was washed thoroughly in water.

RESULTS AND DISCUSSION

Plant productivity is greatly influenced by environmental stresses, such as salinity, drought, flooding, and freezing. Fundamental studies on the plant's responses to stress are necessary to find a strategy for improving stress tolerance in plants. The starch-sucrose transition is one of the most important metabolic processes in germinating cereal seeds (Akazawa et al., 1988). Amylolytic enzymes degrade storage starch to glucose, and glucose is converted to sucrose by a sucrose-synthesizing pathway that includes UGPase and SPS. We examined the responses of wheat seed to salt stress, particularly the alteration of the starch-sucrose transition during germination under NaCl salinity.

Although the speed of germination decreased with increasing salinity (Fig. 1A), the final % germination was not dependent on the level of salinity (data not shown). Elongation of roots and shoots was strongly prevented by NaCl treatment (Fig. 1B). Based on dry weights of seed and seedling (root and shoot), NaCl prevented the degradation and utilization of storage compounds in the seed (Fig. 1C).

We also examined the endogenous levels of ABA in germinating wheat seeds, as well as osmotic potential and sugar accumulation in the seedlings under salt stress. The ABA content increased 5-fold at a concentration of 300 mM NaCl compared with the control (Fig. 2A). NaCl caused a decrease in osmotic potential from -0.4 to -1.9 MPa at 300 mM (Fig. 2B). The sugar content in the seedlings markedly increased to approximately 10-fold under maximum salt stress (Fig. 2C). Thus, the NaCl-dependent changes in osmotic potential, ABA, and sugar contents were closely related to each other (Fig. 2). In addition, the 24 h short-term incubation with NaCl caused sugar to accumulate in the isolated seedlings (Table 1). This indicated that the sugar accumulation in the roots and shoots under salinity occurred without the carbon flow from the starchy endosperm. The accumulated sugars included glucose, fructose, and sucrose, but not maltose. In particular, the accumulation rate for sucrose was the most rapid (Table 1).

Furthermore, sucrose phosphate synthase (SPS) activities in the seedlings were enhanced by NaCl,

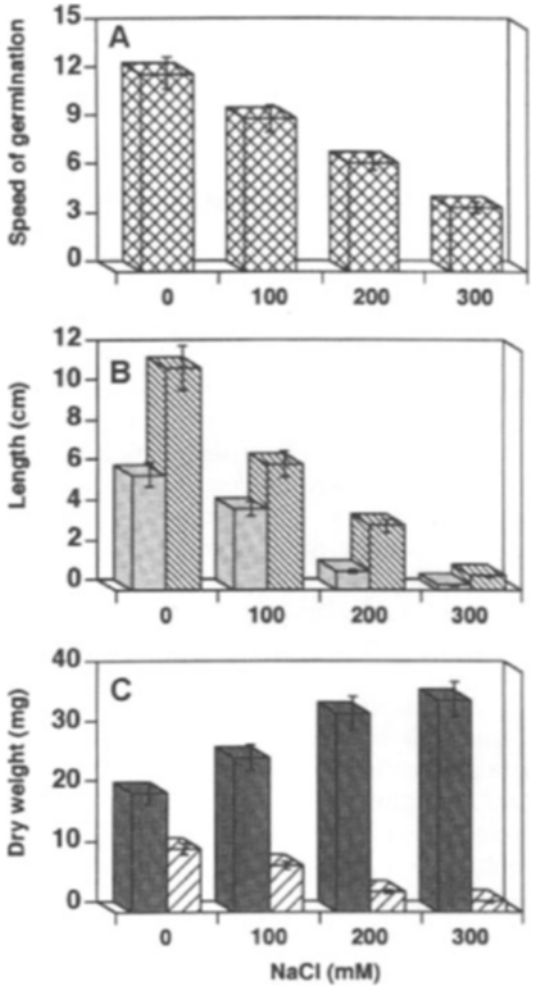

Figure 1. Effects of NaCl on (A) speed of germination; (B) root (▨) and shoot (▩) length and (C) seed (▩) and seedling (root and shoot, ▨) dry matter in germinating wheat seeds. Wheat seeds were incubated in the presence of different concentrations of NaCl (0-300 mM) at 25°C for five days. After germination, seeds and seedling parts were separated, and root and shoot length were measured. Seeds and seedlings were dried at 70°C for 48 h and their dry weights were determined. Speed of germination was calculated according to the formula: Speed of germination = $\Sigma G_n/D_n$, where G_n is number of seeds germinated on day n and D_n is days from initial sowing (Kashem et al., 2000). Each value is the mean ± S.D. of three experiments.

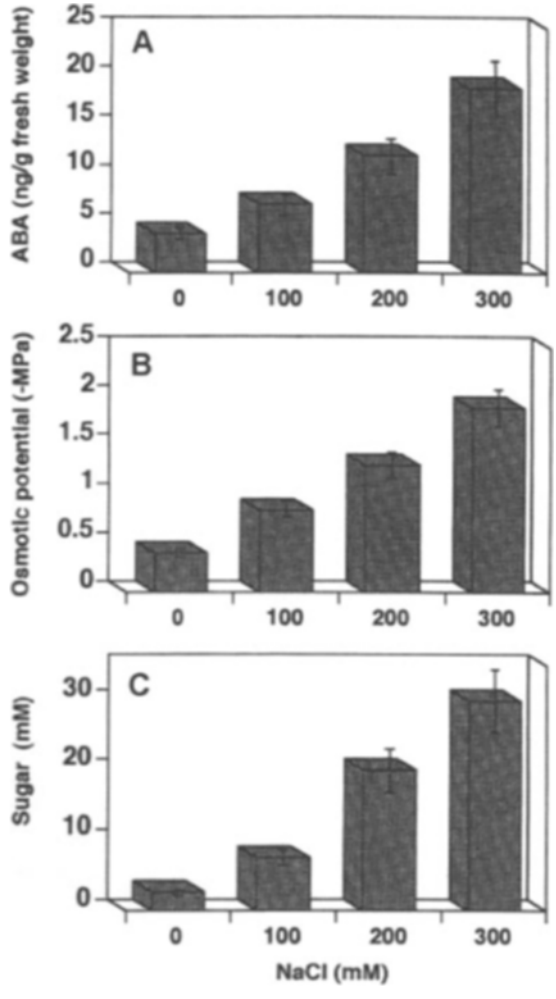

Figure 2. Effects of NaCl on (A) ABA content, (B) osmotic potential, and (C) sugar content in germinating seed. Wheat seeds were incubated in the presence of different concentrations of NaCl (0-300 mM) at 25°C for five days. ABA content was determined in the germinating whole seeds, and the dissected seedlings (roots and shoots) were analyzed for osmotic potential and sugar content. Assay procedures are described in the text. Each value is the mean ± S.D. of three experiments.

whereas UDP-glucose pyrophosphorylase (UGPase) activity was only weakly induced (Table 2). In contrast, NaCl significantly decreased the expression of α-amylase activity (Table 2). These results were reconfirmed by immunoblot analyses employing specific antibodies against SPS, UGPase and -amylase (Fig. 3). The apparent correlation between sucrose synthesis and SPS expression (Table 1 and 2) suggests that SPS plays a critical role in sucrose synthesis under salt stress. As shown in Figure 2 and Table 2, the level of UGPase was not significantly altered in response to increased NaCl, ABA, or sugar concentrations. The UGPase reaction may, perhaps, be channeled in vivo, either toward UDP-glucose pyrophosphorolysis or synthesis, due to a metabolic coupling to other reactions of the sugar pathway. However, UGPase is not considered a highly regulated step in the flux of carbons through sugar pathways via fine regulation of its catalytic activity (Kleczkowski, 1994; Sowokinos et

Table 1. Effect of NaCl on sugar accumulation in isolated wheat seedlings. Seedlings (roots and shoots), dissected from seeds that had germinated at 25°C for two days in water, were incubated with different concentrations of NaCl (0-300 mM) at 25°C for 24 h. The tissue extracts were analyzed for sugar contents by HPLC.

NaCl (mM)	Total sugar	Glucose	Fructose	Maltose	Sucrose
	mM (molar ratio)				
0	13.41	5.26 (0.39)	6.74 (0.50)	0.22 (0.016)	1.19 (0.09)
100	15.97	5.67 (0.36)	6.89 (0.43)	0.22 (0.013)	3.19 (0.20)
200	22.13	7.22 (0.39)	10.26 (0.46)	0.20 (0.009)	4.45 (0.20)
300	23.40	7.96 (0.34)	10.66 (0.46)	0.18 (0.007)	4.60 (0.19)

Table 2. Effects of NaCl on expression of SPS, UGPase, and -amylase activities in germinating wheat seeds. Seeds treated with NaCl (0-300 mM) at 25°C for five days were cut and separated into seed and seedling parts. The seedling (root and shoot) extracts were assayed for SPS and UGPase, and the seed extracts were subjected to amylase assay. Each value is the mean±S.D. of triplicate experiments.

NaCl (mM)	SPS (μmol h^{-1} (mg protein)$^{-1}$)	UGPase (μmol h^{-1} (mg protein)$^{-1}$)	Amylase (units $seed^{-1}$)
0	1.08±0.10	26.1±2.5	1825±103
100	2.06±0.15	29.7±2.0	1510±121
200	4.10±0.31	31.1±3.4	1086± 90
300	5.92±0.62	31.5±2.6	918± 63

al., 1997).

The primary action of osmotic inhibition is to retard the critical uptake of water during germination (Kahn, 1960). Presumably the effect of water stress on imbibition, due to salinity, is the main cause for delaying germination, as indicated by Khan et al. (1997). Likewise, NaCl also increased the ABA level and decreased the level of gibberellin in germinating rice seed (Roy et al., 1995). Our results indicated that salt stress stimulated the formation of ABA in the germinating seeds (Fig. 2A). Phytohormones apparently are involved in the inhibition of seedling growth under salinity, although we have no data for the effect of water stress on ABA-forming enzymes.

The change in ABA level in germinating wheat seeds under salt stress was well matched to those trends in sugar accumulation (Fig. 2) and SPS expression (Fig. 3, Table 2). Previously we demonstrated that ABA stimulated glucose uptake and conversion of glucose to sucrose in rice scutellar tissues (Kashem et al., 1998). Expression of α-amylase in germinating cereal seeds was induced by GA, but was prevented by ABA and sugar (Mitsui and Itoh, 1997). Robertson et al. (1989) have reported that α-amylase inhibitor synthesis in barley embryos and young seedlings is induced by ABA and dehydration stress. Here, we suggest that the expression of α-amylase activity in germinating wheat seeds is significantly reduced under salinity, mediated by ABA (Figs. 2 and 3; Table 2). Our findings imply that the conversion of starch to sucrose in the NaCl-treated seed is controlled by ABA level.

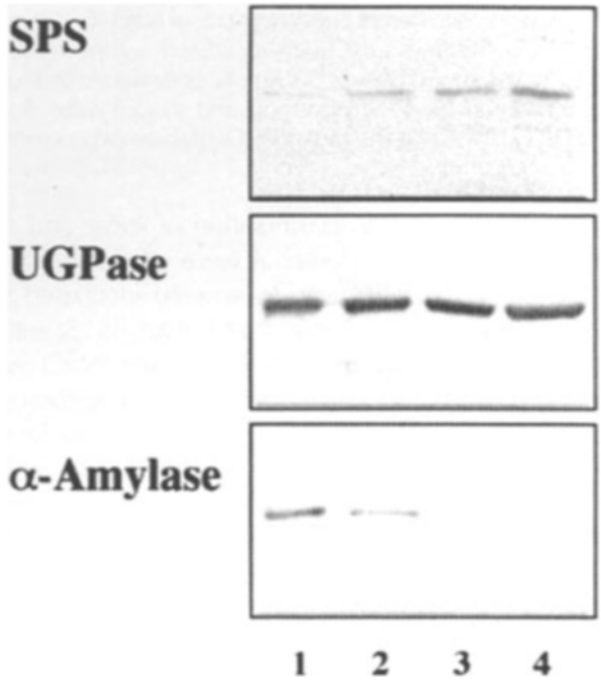

Figure 3. Effects of NaCl on expression of SPS, UGPase, and α-amylase molecules in germinating wheat seeds. Seeds treated with NaCl (0-300 mM) at 25°C for five days were cut and separated into seed and seedling (root and shoot) parts. Seedling extracts were subjected to immunoblot analyses with anti-SPS and anti-UGPase antibodies, while seed extracts were subjected to analysis of α-amylase with anti-α-amylase antibodies. Lane 1, 0 mM; Lane 2, 100 mM; Lane 3, 200 mM; Lane 4, 300 mM NaCl.

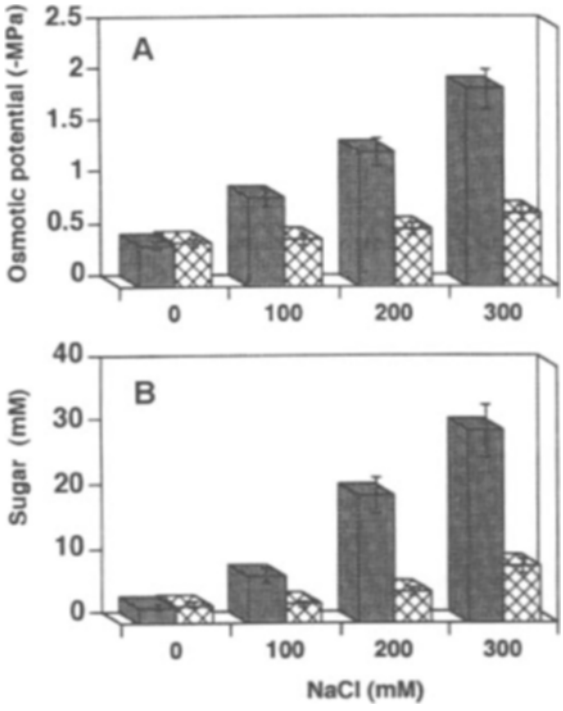

Figure 4. Reversibility of salt stress-induced accumulation of sugar in germinating seeds. Wheat seeds were incubated in the presence of different concentrations of NaCl (0-300 mM) at 25°C for five days (▩), then incubated without NaCl at 25°C for two more days (▨). Osmotic potential (A) and sugar content (B) in the seedlings (roots and shoots) were determined. Each value is the mean ± S.D. of three experiments.

The NaCl-induced accumulation of sugar and the decrease in osmotic potential were reversible when those treated germinating seeds were incubated for another two days without NaCl (Fig. 4). Seedling growth dramatically increased after the NaCl was removed (data not shown). Sugar, including sucrose, is an organic solutes thought to be responsible for maintaining the water balance in plant cells under salinity (Shannon et al., 1994; Bohnert et al., 1995). They protect the structural integrity of membranes during dehydration by preventing membrane fusion, phase transition, and phase separation (Crowe and Crowe, 1992), and by restoring cell volume and turgor, reducing cell damage induced by free radicals, and protecting and stabilizing enzymes and membrane structure (Timasheff and Arakawa, 1989). Based on these findings, we speculate that the plant tolerates the stress period by inducing the growth inhibitor ABA, which then activates sugar accumulation for adaptation to stress. The adaptive process includes the negative control of growth and production of α-amylase in the germinating wheat seed.

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