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Involvement of Abscisic Acid in Photosynthetic Process in *Hordeum vulgare* L. during Salinity Stress

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Abstract. In *Hordeum vulgare* L. plants, NaCl stress imposed through the root medium for a period of 8 days decreased the rate of CO_2 assimilation, the chlorophyll and protein leaf content, and the activity of ribulose-1,5-bisphosphate carboxylase. The activity of phosphoenolpyruvate carboxylase was twofold over the control. Pretreatment with abscisic acid (ABA) for 3 days before salinization diminished the inhibitory effect of NaCl on the rate of CO_2 fixation. The leaf Na^+ and Cl^- content decreased in ABA-pretreated plants. Both ABA and NaCl treatments led to an increase in the endogenous level of ABA in the plant leaves. Patterns of total proteins extracted from the leaves of control or ABA- and salt-treated plants were compared. Both ABA and NaCl induced marked quantitative and qualitative changes in the polypeptide profiles concerning mainly the proteins with approximately equal mobility. The results are discussed in terms of a possible role of ABA in increasing the salt tolerance when ABA is applied to the plants for a short period before exposure to salinity stress, thus improving the invulnerability to unfavorable conditions.

Among environmental stresses NaCl salinity is one of the main limitations to the growth and photosynthesis of nonhalophytic plants. The extent of this inhibition is correlated to the extent of NaCl salinity, the plant species sensitivity, and the environment.

Physiologic and biochemical responses to NaCl salinity include lowered leaf water potentials, increased stomatal resistance, altered ion relations, and slower net CO_2 assimilation rates (Bethke and Drew 1992, Greenway and Munns 1980, Poljakoff-Mayber and Gale 1975). Much of the reported reduction of CO_2 assimilation can be attributed to the direct effect of salinity on the biochemical reactions of photosynthesis, e.g. reduced activity of ribulose-1,5-bisphosphate carboxylase (RuBPC) (Miteva and Vaklinova 1991, Seemann and Sharkey 1986), inhibition of photosynthetic electron transport and PSII activity (Maslenkova et al. 1991, Mohanty and Saradhi 1992), deleterious effects on structural and functional integrity of cell walls, cellular and intercellular membranes (Kurth et al. 1986, Leopold and Willing 1984), alterations in polypeptide profiles (Hurkman et al. 1989; Miteva et al. 1992, Rani and Reddy 1994), and also in gene expression (Claes et al. 1990).

Moreover, unavoidable uptake of specific ions by the plants and accumulation of these in the leaves are widely assumed to result in inhibition of photosynthesis (Seemann and Critchley 1985). There are reports of a decline in photosynthesis in glycophytes caused by increased Na^+ and Cl^- ion concentration in leaves (Robinson et al. 1983).

To improve salt tolerance nonhalophytic species have developed different strategies that allow plants to withstand salinity stress (see Greenway and Munns 1980 for review).

The tolerance of certain plants to conditions of stress, particularly osmotic stress, appears to be related to their capacity to produce a high level of abscisic acid (ABA). Elevated levels of ABA have been reported as a result of water stress (Henson and Quarrie 1981) and salt stress (Downton and Loveys 1981, Walker and Dumbroff 1981).

Abbreviations: RuBPC, ribulose-1,5-bisphosphate carboxylase; PSII, photosystem II; ABA, abscisic acid; PEPC, phosphoenolpyruvate carboxylase; DTTr, dithiothreitol; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGEr, polyacrylamide gel electrophoresis.

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It has been shown that treating plants with ABA frequently hardens them against stress, thus ABA may play a role in stress tolerance (Amzallag et al. 1990, La Rosa et al. 1985). In fact it has been shown that ABA facilitates the adaptation of isolated cells to increased NaCl concentration (Eberhardt and Wegmann 1989). Our studies with salt-treated barley seedlings revealed significant changes in thylakoid membrane proteins which resemble to a great extent the specific changes in polypeptide profiles after exogenous ABA application to the root medium (Maslenkova et al. 1993). Moreover, ABA pretreatment of the seedlings before salinization greatly decreased the inhibitory effect of NaCl on oxygen evolving activity of isolated chloroplasts. Considering these results, we assumed that this phytohormone plays a specific role in membrane stabilization by inducing its own set of proteins as an adaptive response to salinity.

It is well known that photosynthetic capacity depends on the balance between the photosynthetic light utilization and CO_2 assimilation. The observed changes in the electron transport activity of the membranes may affect the generation of ATP and reducing equivalents, thus leading to alterations in CO_2 fixation and enzymes activity. In this respect, we were interested in studying the importance of nonstomatal (biochemical) factors in the observed decline in photosynthesis. Second, we examined whether the changes in Na^+ Cl^- ion accumulation in the roots and leaves of barley plants during different treatments resulted in differences in the photosynthetic process.

Our approach was to treat barley plants with ABA before salinization or to subject them to progressively increasing NaCl concentrations. The obtained responses could then be compared with those occurring in leaves that were treated with ABA or subjected to a high concentration of NaCl, thus allowing us to study the possible role of this phytohormone in the process of the stress adaptation.

Materials and Methods

Plant Material

Seeds of barley (*Hordeum vulgare* L., *var.* Alfa) were germinated for 2 days in two layers in moist filter paper in vermiculite at 25°C in the dark. Then they were transferred into Petri dishes containing 40 ml of distilled water or equal amounts of water solution at the required NaCl and ABA concentrations (100 mM NaCl and 10 μM ABA). During the experimental period, the seedlings grew in a growth chamber under white fluorescent lamps (35 Wm^{-2}), with 12-h light/dark periods. Day/night temperatures were 25/20°C; relative humidity was about 50%. To obtain NaCl stepwise-treated plants, the daily increase in NaCl concentration was 25 mM until 100 mM NaCl was reached. In the experiments with ABA-pretreated plants 10 μM phytohormone was added to the growth medium of the seedlings for 3 days prior to salinization with 100 mM NaCl for 4 days.

CO_2 fixation

Photosynthetic rates were measured using leaf slices by the method of Rathnam and Chollet (1980). One gram of leaf blade tissue was cut perpendicular to the veins into 1-mm slices. Slices were incubated in 5 ml of buffer in a 25-ml Erlenmeyer flask at 25°C for 5 min at 120 Wm^{-2} light intensity. The buffer contained: 0.33 M sorbitol, 0.05 M Hepes-NaOH, 0.002 M KNO_3, 0.002 M EDTA, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.0005 M K_2HPO_4, 0.02 M NaCl, and 0.2 M sodium isoascorbate (pH 7.6). At the end of the preincubation period, 20 mM $NaHCO_3$ containing 40 μCi of $NaH^{14}CO_3$ (14.3 μCi/μM) was added to each sample. They were allowed to fix CO_2 for 10 min. The reaction was stopped by adding boiling 80% ethanol. Tissues were subsequently extracted eight times with boiling ethanol of the same concentration. Combined extracts were reduced to dryness in vacuo at 40°C and were dissolved in 25 ml of distilled water. An aliquot was measured using a Packard Tri-Carb liquid scintilation counter.

Enzyme Extraction and Assays

Leaf tissue without the major veins was ground in a mortar on ice in a ratio of 1 g (fresh weight) to 5 ml of cold extraction medium containing the above buffer for CO_2 fixation. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000 × g for 15 min and the supernatant used directly for enzyme assay.

RuBPC (EC 4.1.1.39) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) activities were assayed from the activated crude preparation by following the incorporation of $NaH^{14}CO_3$ into acid-stable products.

The assay mixture for RuBPC contained in 50 mM Hepes-NaOH (pH 8.0): 20 μmol of $MgCl_2$, 1 μmol of DTT, 20 μmol of $NaHCO_3$ (containing 40 μCi of ^{14}C, specific radioactivity 14.3 μCi/μmol), and enzyme extract equivalent to 0.3–0.4 mg of protein. The reaction volume was 1 ml. Reactions, at 25°C, were initiated by the addition of 2 μmol of RuBP and stopped after 1 min with 6 N HCl.

The assay mixture for PEPC activity contained in 50 mM Hepes-NaOH (pH 8.0): 20 μmol of $MgCl_2$, 0.4 μmol of NADH, 20 μmol of $NaHCO_3$ (containing 40 μCi of ^{14}C, specific radioactivity 14.3 μCi/μmol), 1 μmol of DTT, and enzyme extract equivalent to 0.3–0.4 mg of protein. The reaction volume was 1 ml. Reactions, at 30°C, were initiated by the addition of 3 μmol of PEP. The reaction time was 1 min.

The amount of fixed $^{14}CO_2$ was measured in a liquid scintillation counter.

Protein was determined by the method of Lowry et al. (1951), with BSA as a standard. Chlorophyll content was measured according to Arnon (1949).

ABA Analysis

ABA was extracted from leaf tissue samples (200–250 μg, dry mass) with 80% (v/v) aqueous and then 100% methanol + 100 mg/L butylated hydroxy toluene (1 μg of tissue, dry weight, per 1 μl of extract) for 24 h and 6 h, respectively, at 4°C in darkness. The combined extracts were dried under N_2, and the residue was

Table 1. Effect of NaCl and ABA on photosynthetic CO_2 fixation and on the chlorophyll (Chl) content of barley leaves. The details are given under "Materials and Methods".[a]

Treatment	CO_2 fixation (μmol CO_2 (mg Chl h)$^{-1}$)	Chlorophyll (a + b) (mg Chl (g FW)$^{-1}$)
Control	110.40 ± 8.2	2.26 ± 0.064
100 mM NaCl	60.40 ± 2.8	1.75 ± 0.040
25–100 mM NaCl[b]	88.10 ± 4.1	1.73 ± 0.11
10 μM ABA	69.40 ± 7.1	2.03 ± 0.069
10 μM ABA + 100 mM NaCl	94.27 ± 6.9	1.67 ± 0.018

[a] The values are the mean from three separate experiments for all investigated parameters (±S.E.).
[b] Stepwise increased NaCl concentration from 25 to 100 mM within 8 days.

dissolved in 50 μl of methanol and diluted with 200 μl of Tris-buffered saline (50 mM Tris-Cl (pH 7.8) containing 150 mM NaCl and 1 mM $MgCl_2$).

ABA was estimated by ELISA as described by Harris et al. (1988) and modified by Zhang et al. (1991).

Ion Analysis

For the analysis of inorganic ions, plant material (roots and leaves) was dried at 80°C for 48 h and powdered in a mortar. The ashed samples were used to determine Na^+ and Cl^- ion content. The Na^+ concentration was determined by flame photometer. Cl^- concentration was measured by silver ion filtration according to Cotlove (1963).

Gel Electrophoresis

One-dimensional gel electrophoresis was performed according to the procedure of Laemmli (1970) on 15% (w/v) acrylamide slab gels (1.5 mm thick) containing 0.1% (w/v) SDS and 375 mM Tris-HCl (pH 7.8). Samples of fractions were solubilized in a sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 10% (v/v) glycerol. Samples of fractions containing 30 μg of protein were boiled for 3 min in sample buffer and loaded on the gels. After electrophoresis, gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 in methanol/acetic acid/water (4:1:5, v/v). Destaining was carried out in methanol/acetic acid/water (4:1:5, v/v. The dried gels were scanned at 560 nm using Shimadzu CS-930 TLC. Molecular weights were estimated from a standard plot using lysosyme (14,300), β-lactoglobulin (18,400), trypsinogen (24,000), CA (30,000), ovalbumin (43,000), BSA (66,000), and phosphorylase A (94,000).

Chemicals

(±)-ABA was purchased from Fluka AG. All other chemical were obtained from Sigma.

Results

CO_2 Fixation, Enzyme Activities, Chlorophyll and Protein Contents

Treatment of barley seedlings with 100 mM NaCl or 10 μM ABA for 8 days caused a significant decline in the rate of photosynthetic CO_2 assimilation (Table 1). The two treatments inhibited the growth of the seedlings (data not shown). In contrast, when the seedlings were exposed to stepwise changes in NaCl concentration or pretreated with 10 μM ABA for 3 days before salinization, plant growth was only slightly affected, and the CO_2 fixation rate was less inhibited.

The chlorophyll (a + b) content decreased in all variants treated with NaCl. By contrast, the long term treatment of barley seedlings with 10 μM ABA had an insignificant effect on the chlorophyll content (Table 1).

RuBPC activity was very strongly reduced when plants were treated with 100 mM NaCl or 10 μM ABA (Table 2). The activity of the enzyme was decreased by salinity stress to approximately 70%, by ABA to 50% of control. The pretreatment with ABA showed the same inhibition by either ABA or NaCl alone. It should be noted that stepwise treatment with NaCl fully eliminated the inhibition of the enzyme activity by the prolonged treatment of barley seedlings with 100 mM NaCl.

When barley plants were grown on 100 mM NaCl or 10 μM ABA the activity of PEPC increased more than twice (Table 2). Pretreatment with ABA or a progressive increase in NaCl concentration also caused an increase in the enzyme activity but to a lower extent.

The leaf protein content was decreased in all treatments with the greater decrease in 100 mM NaCl-treated plants (Table 2).

Ionic Distribution within Leaves and Roots

Sodium and chloride relations as influenced by external salinity are shown in Table 3. Root and leaf concentration of both Na^+ and Cl^- increased greatly with salinization. Leaf Cl^- accumulation was in excess of Na^+ for all treatments, except in ABA-treated plants. This disparity in ion concentration has also been shown by other authors (Bethke and Drew 1992, Lauchli and Wieneke

Table 2. Activity of RuBPC and PEPC of barley plants after treatment with NaCl and ABA. The data are averages from six separate experiments ± S.E.

Treatment	RuBPC (μM CO_2 (mg pt· min)$^{-1}$)	PEPC (μM CO_2 (mg pt· min)$^{-1}$)	Protein content (mg · (g FW)$^{-1}$)
Control	0.337 ± 0.08	0.053 ± 0.012	8.34 ± 1.29
100 mM NaCl	0.095 ± 0.04	0.139 ± 0.05	4.94 ± 0.64
25–100 mM NaCl	0.39 ± 0.02	0.077 ± 0.02	5.95 ± 1.15
10 μM ABA	0.173 ± 0.11	0.117 ± 0.06	6.64 ± 1.04
10 μM ABA + 100 mM NaCl	0.169 ± 0.08	0.098 ± 0.03	6.10 ± 0.59

Table 3. Content of Na^+ and Cl^- in barley plants treated with NaCl and ABA. The data are averages from two separate experiments ± S.E.

	Na^+ (mg/g DW)		Cl^- (mg/g DW)	
Treatment	Roots	Leaves	Roots	Leaves
Control	6.47 ± 0.9	3.13 ± 0.02	2.98 ± 0.20	5.98 ± 0.16
100 mM NaCl	72.0 ± 11.53	19.17 ± 0.29	85.20 ± 8.59	45.50 ± 9.15
25–100 mM NaCl	47.67 ± 17.00	26.33 ± 6.66	61.67 ± 0.43	42.60 ± 3.13
10 μM ABA	4.70 ± 3.11	2.63 ± 0.99	2.65 ± 0.21	2.90 ± 1.48
10 μM ABA + 100 mM NaCl	72.33 ± 12.42	13.66 ± 0.58	85.73 ± 0.18	36.18 ± 1.94

1979). Compared with the untreated plants, barley seedlings treated for 8 days with 100 mM NaCl showed an 11- and 28-fold increase in Na^+ and Cl^- concentrations in the roots and about 6.5- and 7.5-fold in the leaves. A stepwise salt increase to 100 mM NaCl resulted in an ion content increase of only sevenfold for Na^+ and 20-fold for Cl^- in the roots (the percentage values not presented). As a result of pretreatment with 10 μM ABA the leaf Na^+ and Cl^- content decreased in comparison with the salt-treated plants. The content of Na^+ and Cl^- in the roots of ABA-pretreated plants was similar to that in 100 mM NaCl-treated plants and higher than in the stepwise NaCl-treated plants.

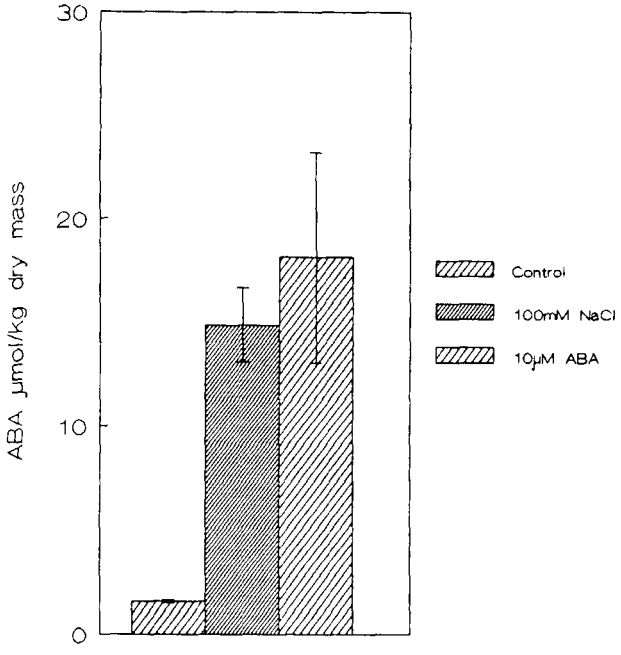

Fig. 1. ABA content in barley seedlings grown in the presence of control (H_2O), 100 mM NaCl, and 10 μM ABA. The data are averages from three independent experiments.

Endogenous ABA Level

The data presented in Fig. 1 show that the level of ABA in plants treated with 100 mM NaCl was about 15-fold higher than that of the control. Exogenous application of 10 μM ABA caused more than an 18-fold ABA accumulation in treated plants.

Changes in Polypeptide Patterns

Total proteins were extracted from the leaves and analyzed by SDS-PAGE (Fig. 2). The major polypeptide bands in the control had molecular masses of 60, 55, 47, 30, 27, 25, and 15 kDa. The main differences established between control and long term treated with NaCl plants were: (1) 60-, 37-, 27-, and 30-kDa bands are more intensive in NaCl treatment; (2) the level of 55- and 15-kDa polypeptides corresponding to LSU and SSU of RuBPC was strongly decreased. In ABA-treated plants the most

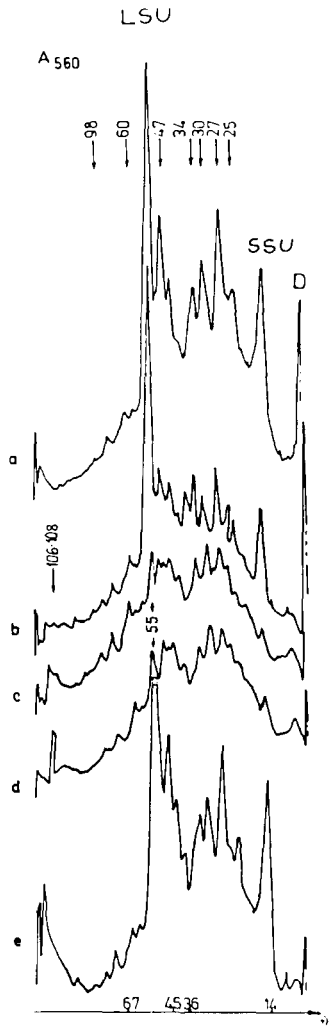

Fig. 2. Gel scans of soluble proteins from barley leaves after 15% SDS-PAGE. **a**, control (H_2O); **b**, 10 μM ABA; **c**, 100 mM NaCl; **d**, 10 μM ABA + 100 mM NaCl; **e**, 25–100 mM NaCl. Soluble protein was extracted from leaf tissue harvested on day 8 and prepared for electrophoresis as described under "Materials and Methods." All lanes were loaded with 30 μg of protein. The major protein differences among control and treated plants are indicated in kDa with **arrows.**

prominent were the polypeptides with molecular masses of 55, 47, 42, 30, 27, 25, and 15 kDa. The intensity of LSU and SSU of RuBPC was decreased compared with that of the control. Two bands with molecular masses of 98 and 76 kDa from the high molecular polypeptides increased in both ABA- and NaCl-treated plants. Changes in the polypeptide profiles of barley plants pretreated with ABA before salinization resemble the changes in polypeptides induced by exogenous application of ABA or prolonged treatment with 100 mM NaCl. In these treatments the level of LSU and SSU of RuBPC was much lower compared with the control but better expressed than in the NaCl-treated variants. It is obvious that ABA and 100 mM NaCl treatments led to changes in the level of some common soluble polypeptides. Polypeptide profiles of stepwise NaCl-treated plants showed similarity to the controls.

Discussion

Barley is one of the most salt-tolerant crop plants among glycophyta species. It has been shown that long term treatment (8 days) of barley seedlings with 25 and 50 mM NaCl had no effect on the seedlings' growth, dry matter accumulation of shoots and roots, or on the rate of photosynthesis and the activity of RuBPC (Miteva and Vaklinova 1989). However, after 8 days of continuous exposure of barley seedlings to 100 mM NaCl, there were visible changes in the growth rate, dry matter accumulation, and photosynthetic response (Miteva and Popova 1991, Miteva and Vaklinova 1991).

In this study a gradual increase in NaCl concentration as well as pretreatment of the seedlings with 10 μM ABA before salinization did not influence the growth of the plants; they looked like the control plants.

The reduction in leaf chlorophyll concentration is a general phenomenon in sensitive plants subjected to salt stress (Robinson et al. 1983, Stiborova et al. 1987). In our experiments, the content of chlorophyll was decreased by NaCl to an approximately equal extent in all variants treated with NaCl (Table 1). Treatment with 10 μM ABA for 8 days had no significant effect on the chlorophyll content; regardless of that, a decrease in the rate of photosynthesis was observed. We failed to show a correlation between the degree of inhibition of salt stress on the chlorophyll content and the intensity of photosynthesis. This indicates that the loss of chlorophyll is unlikely to be the primary cause of photosynthetic reduction for salinized barley plants. Similar conclusions have been drawn for other glycophytes exposed to NaCl salinity (Bethke and Drew 1992, Seemann and Chritchley 1985).

High salt concentration and 10 μM ABA, when applied separately to the growth media, have marked inhibitory effects on the rate of photosynthesis. The CO_2 fixation rate was reduced to 45 and 37%, respectively (Table 1). This inhibitory effect was overcome to a significant extent during the stepwise treatment and by the ABA pretreatment of the seedlings.

The effect of NaCl salinity on photosynthetic capacity is also reflected by the inhibition of RuBPC activity (Table 2).

Seemann and Sharkey (1986) interpreted the observed inhibition of photosynthesis as a result of a lowered pool of RuBP and a reduced efficiency of RuBPC, and they hypothesized that a probable reason for the reduced RuBP regeneration capacity could be the lowered capacity for ATP formation. Actually, this suggestion is consistent with our data, indicating that high salinity brings about a reduction in the rate of electron transport (Maslenkova et al. 1993). Another possible reason for the observed inhibition of NaCl salinity on photosynthetic ability and RuBPC activity could be the inhibiting effect of salinity stress on protein synthesis, including the synthesis of RuBPC (Miteva et al. 1992). We have made similar observations in this study (Fig. 2). When the seedlings were exposed to stepwise changes in NaCl concentration a certain stabilization of the photosynthetic parameters was observed. The electron transport activity of isolated thylakoids showed an insignificant inhibition (Maslenkova et al. 1993). The CO_2 fixation rate was slightly reduced, and the RuBPC activity was approximately equal to the one of the control unstressed plants (Table 2).

Decreases in photosynthetic ability observed in plants treated long term with 100 mM NaCl are unlikely to be solely a result of the salinity treatment. Plants treated with a daily increasing NaCl concentration up to 100 mM had little or no decline in photosynthetic ability and RuBPC activity, indicating that barley plant are capable of tolerating a relatively high level of salinity.

Data presented in Tables 1 and 2 show that prolonged ABA treatment of barley seedlings also reduced the CO_2 fixation rate and the RuBPC activity. These data are consistent with results reported previously by us (Popova et al. 1987). But when the seedlings were treated with 10 μM ABA for 3 days before salinization one clear improvement of the above mentioned photosynthetic parameters was established. It has been shown that this way of treatment leads also to a stabilization of the operative evolving centers. Judging from these data and from those reported previously for the stabilization of the oxygen-evolving centers under the same experimental conditions, it may be concluded that ABA is related to the process of adaptation to salinity, i.e. to the ability of barley plants to grow and tolerate 100 mM NaCl concentration, which is inhibitory for the plant without ABA pretreatment.

Our data show that exogenous application of ABA causes an accumulation of the hormone in the leaves to values comparable to those in stress-induced ABA accumulation (Fig. 1).

There are reports that the high level of endogenous ABA during salinization correlates with plant resistance to the stress (Downton and Loveys 1981, Walker and Dumbroff 1981). Class genes and different stress-specific proteins have been observed to accumulate in a wide range of plant species in response to environmental stresses and ABA treatment (Singh et al. 1984, Skiver and Mundy 1990). The possible role of these ABA- and stress-induced proteins is discussed in connection with their stabilizing and protecting functions against damage on plants caused by dehydration, heat, or high salt concentrations.

Data reported here as well as in our previous communications show that exogenous treatment of barley seedlings with ABA and NaCl salinity induce the appearance of some common soluble polypeptides (Maslenkova et l. 1992, Miteva et al. 1992, Popova et al. 1994). It is likely that ABA and salinity trigger one and the same genetic system and that some of these common polypeptides may play a definite role in adaptation to salinity. Between the soluble photosynthetic proteins we might suggest that the increased PEPC activity after treatment with NaCl and ABA was due to activation of the existing enzyme or to an increased quantity of this protein. It is quite possible that the observed increase in PEPC activity serves as an adaptive mechanism against the lowered CO_2 conductivity as a consequence of stomatal closure like that observed by us for barley seedlings subjected to drought stress (Popova et al. in press).

Better understanding of the physiologic and biochemical basis of the photosynthetic response to salinity requires a knowledge of the specific localization of ions within the plant cell. In this respect Behl and Raschke (1986) suggested that the protective effect of ABA might be due to its maintenance of membrane integrity by lowering the uptake rate of NaCl or inducing an enhanced extrusion of detrimental ions.

Our present experiments show that pretreatment of barley seedlings with ABA before salinization decreases Na^+ and Cl^- accumulation in leaves (Table 3) and could be one possible reason for the observed protective action of ABA on the investigated parameters.

In conclusion, using different experimental approaches we have provided new data indicating an involvement of endogenous ABA in increasing plant tolerance to a NaCl salinity stress. The present data and those of Maslenkova et al. (1993) support the view that the elevated level of ABA may play a role in the reaction of photosynthetic apparatus to salt stress. Part of this response involves stabilization of photochemical membrane function and CO_2 fixation ability, induction of spe-

cific proteins, and diminishing the content of Na^+ and Cl^- ions within the leaves.

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