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Differences in Photosynthetic Characterization of Salt Tolerance for Two Rice (*Oryza sativa*) Cultivars

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We photosynthetically characterized two rice cultivars - salt-sensitive 'Annapurna', and salt-tolerant 'Dongjin' - growing under NaCl stress. Both cultivars showed an increase in F_o/F_m (the ratio of initial to maximal chlorophyll fluorescence) and a decrease in F_v/F_m (an indicator of the photochemical efficiency of PS II). In particular, the F_v value for Annapurna significant declined while F_o/F_m was enhanced when plants were exposed to salt stress for 4 d. Annapurna also exhibited more rapid decreases in the coefficients for photochemical quenching (qQ) and non-photochemical quenching (qNP) than did Dongjin. In contrast, zeaxanthin formation was largely influenced by exposure to light rather than to high salinity, with Annapurna having a higher rate of production compared with Dongjin. When both cultivars were exposed to salt stress for 2 d, Annapurna had a much lower rate of photosynthetic oxygen evolution, corresponding to only 46% of the control; the rate for Dongjin was 90% of the control. Salt stress in both cultivars induced the accumulation of two osmoprotectants, glycinebetaine and proline, the rate being higher for the latter. These results indicate that Annapurna is more sensitive than Dongjin to salt stress, in terms of its deterioration in photosynthetic function.

Keywords: carotenoids, chlorophyll *a* fluorescence, glycinebetaine, proline, salt stress

Salinity is a major environmental factor that limits growth and productivity of cereal crops worldwide. High concentrations of NaCl trigger various interacting events, including a delay in shoot and root development, inhibition of enzyme activities, and decreases in photosynthetic rates and carbon-use efficiency. One approach to understanding the mechanism of salt stress in plants is to try to elucidate the biological characteristics associated with growth and survival under high-saline conditions, as well as the physiological and biochemical differences between halophytes and non-halophytes. Another approach is to develop genetically engineered plants with enhanced tolerance to salt stress (Epstein et al., 1980).

Plants may be able to acclimatize themselves to unfavorable environments, such as salt stress and water deficits, by accumulating organic, low-molecular-weight compounds within the cytoplasm to facilitate osmotic adjustment (Storey and Jones, 1977; Ostrem et al., 1987). In the chenopods, glycinebetaine, a quaternary ammonium compound, is synthesized in the chloroplast through the following pathway: choline → betaine aldehyde → glycinebetaine (Hanson et al., 1985). This pathway might be catalyzed by light. One of the major functions of glycinebetaine is to delay rapid senescence and to increase enzyme activity in the cells metabolic pathways. In some plants, this compound plays an important role in maintaining oxygen-evolving activity under high-saline conditions. This is accomplished through the decreased dissociation of the 23- and 33-kDa polypeptides - both components of oxygen-evolving complexes - from the thylakoids (Murata et al., 1994). Another compound, proline, also functions compatibly in stress adaptation (Serrano and Gaxiola, 1994). However, it has been suggested that proline accumulation is merely a consequence of stress and does not, in itself, lead to salt tolerance (Moftah and Michel, 1987).

The decline in photosynthetic rates because of salt stress induces an increase in excess excitation energy, a result that is potentially harmful to the photosynthetic system (i.e., photoinhibition; Brugnoli and Björkman, 1992). Carotenoids, which are known cardinal pigments for protecting the photosynthetic apparatus from photoinhibition, can quench triplet excited chlorophyll molecules and reactive singlet oxygen ($^1O_2$) molecules. When plants are exposed to excess light, the composition of carotenoids rapidly changes in light harvesting complexes (LHCs); violaxanthin (V) is

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quickly converted to zeaxanthin (Z), via the intermediate antheraxanthin (A), under the action of the violaxanthin de-epoxidase (Gilmore, 1997). Although this xanthophyll cycle has been extensively studied, its physiological role against salt stress is not yet completely understood.

The objective of this study was to investigate and compare the possible mechanisms responsible for photo-chemical and physiological responses in 'Annapurna', a salt-sensitive rice cultivar, and 'Dongjin', which is salt-tolerant. In particular, the effects of salt stress were studied to elucidate the process plants undergo in producing responses to high saline concentrations, as well as to analyze their recovery from NaCl stress. For these purposes, we studied the accumulation of osmoprotectants, i.e., glycinebetaine and proline; oxygen-evolving activity; the kinetics of chlorophyll fluorescence; and the composition of carotenoids during salt stress and subsequent recovery.

MATERIALS AND METHODS

Plant Material and Conditions for Growth and Stress Treatments

Seeds of rice (*Oryza sativa* L.) cultivars, 'Annapurna' (Indica type) and 'Dongjin' (Japonica type), were germinated in water for 5 d at 25°C under darkness. Afterward, they were planted in a pot containing moistened vermiculite, then cultivated in a growth chamber for 21 d. The environmental conditions in the chamber were 70% humidity, 25°C, and a light intensity of 200 $\mu molm^{-2}s^{-1}$ over a 16-h photoperiod. The second leaves of 21-d-old plants were used as the experimental materials. For our salt-stress experiments, the plants were grown under the previously described conditions for 3 to 4 d in vermiculite supplemented with 150 mM NaCl. These treated plants were then immediately irrigated with water, and their subsequent recovery was monitored for 2 d. The control material was defined as the second leaves of 21-d-old plants that were not exposed to salt stress. Plant tissues were collected at Days 1, 2, 3, and 4 of the stress period, and at Days 1 and 2 during recovery. All of the experiments were repeated at least three times.

Extraction and Measurement of Glycinebetaine and Proline

Leaf tissues (~2 g FW) were homogenized in 10 mL methanol-chloroform-water (12:5:3) on ice. The homogenate was then centrifuged at 1000g for 10 min, and the clear supernatant (methanol-water fraction) was removed and stored. After the pH of this fraction was adjusted to between 5 and 7, the sample was applied to Dowex 50W in a 1-cm-diameter glass column. Quaternary ammonium compounds together with amino acids were eluted with 4 M HCl. The acid eluant was dried in vacuo, then resolved in a suitable volume of dH_2O. The solution was analyzed by ^1H nuclear magnetic resonance (NMR) spectroscopy in a JEOL FX 90Q Fourier transform NMR Spectrometer (Jones et al., 1986).

Determination of O_2 Evolution and Chlorophyll Content

Photosynthetic O_2 evolution at 24°C was measured with a Clark-type oxygen electrode (YSI 5300, Yellow Springs Inst. Co., Ohio, USA). The initial rate was used to calculate oxygen-evolving activity. A Schott KL1500-T lamp provided a saturation-light intensity of 1200 $\mu molm^{-2}s^{-1}$. Chlorophyll content was measured according to Holden (1965).

Calculation of Chlorophyll Fluorescence

The emission of chlorophyll *a* fluorescence from the upper surfaces of the leaves was routinely monitored under light by using a Plant Efficiency Analyzer (PEA; Hansatech, UK) and a PAM Chlorophyll Fluorometer (Walz; Effeltrich, Germany). The initial level (F_o) of fluorescence was elicited by a weak red light (655 nm, 1 $\mu molm^{-2}s^{-1}$, modulated at 1.6 KHz), and was measured with a photodiode at a wavelength > 700 nm. Maximal fluorescence (F_m) was induced by a 1-s pulse of white light (4000 $\mu molm^{-2}s^{-1}$). The maximum variable fluorescence (F_v) was calculated as the difference between F_m and F_o at a specific time.

Analysis of Xanthophyll-Cycle Pigments

We used acetone to extract three pigments - violaxanthin, antheraxanthin, and zeaxanthin. HPLC analysis with a Zobax ODS column (4.6 × 250.0 mm; Mac-Mod Analytical, Chadds Ford, PA, USA) was performed as described by Thayer and Björkman (1990).

RESULTS

Changes in Chlorophyll Fluorescence

F_v/F_m (variable yield of fluorescence/maximum yield

of fluorescence) can be used as an indicator of the photochemical efficiency of PS II. When the two rice cultivars were exposed to NaCl stress for 4 d, the efficiency of treated Dongjin plants decreased slightly, to about 89% of that measured in the control (Fig. 1). In contrast, the level of F_v in Annapurna was dramatically lower after the salt treatment, indicating that this stress induced significant inhibition of photosynthetic efficiency. However, the value for F_o/F_m (the ratio of initial to maximal chlorophyll fluorescence) of this cultivar increased by 107% compared with the control. This was attributed to the rise in the calculated value for F_o, which can be interpreted as a reduction in the rate constant of energy-trapping by PS II centers (Havaux, 1993) that may result from a physical dissociation of the light-harvesting complex from the PS II core.

The oxidation state of Q_A (the primary quinone acceptor of PS II), as monitored in terms of qQ (photochemical quenching), markedly declined in Annapurna exposed to 3 d of salt stress, compared with Donjin (Fig. 2). The former showed an almost complete loss of qQ, as well as qNP (non-photochemical quenching) and qE (energy-dependent quenching), after 4 d. These results suggest that the two cultivars may differ in their levels of potential quantum yield of PS II as a response to salt stress.

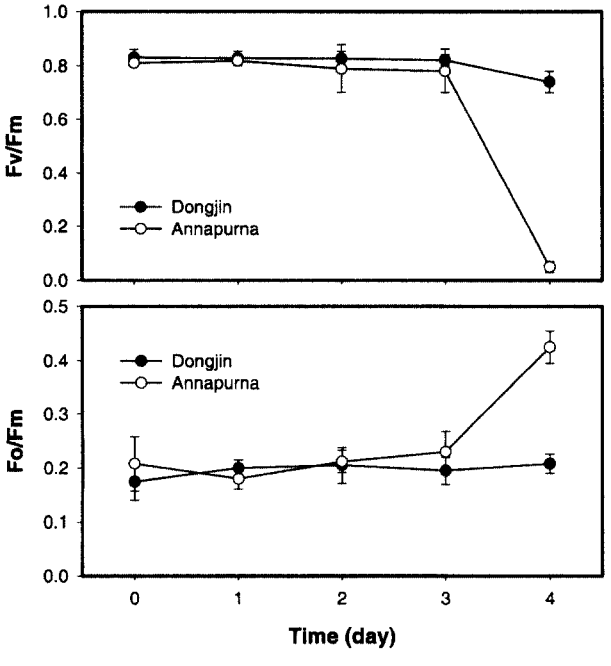

Figure 1. Changes in chlorophyll-fluorescence parameters F_v/F_m and F_o/F_m in the salt-sensitive cultivar Annapurna (– ● –) and the salt-tolerant cultivar Dongjin (– ○ –) after rice plants were subjected to NaCl stress for 4 d. The initial level (F_o) of chlorophyll fluorescence was elicited by a weak red light (655 nm, 1 μmol $m^{-2}s^{-1}$, modulated at 1.6 KHz). Maximum fluorescence (F_m) was achieved with a short (1-s) pulse of white light (4000 μmol $m^{-2}s^{-1}$). Variable fluorescence (F_v) is the difference between F_m and F_o.

Changes in Xanthophyll Cycle Pigments

Relative distributions were compared for violaxanthin, antheraxanthin, and zeaxanthin in the seedlings of rice cultivars grown under various conditions (Table 1). Exposure to light caused violaxanthin to be converted into zeaxanthin, with antheraxanthin as the intermediary. Afterward, the plants were treated with a sulfhydryl reagent, DTT, under dim light for 2 h. This step, leading to the inhibition of vio-laxanthin de-epoxidase, prompted a dramatic decline in the formation of light-induced zeaxanthin compared with that measured in the light treatment. In contrast, the amount of

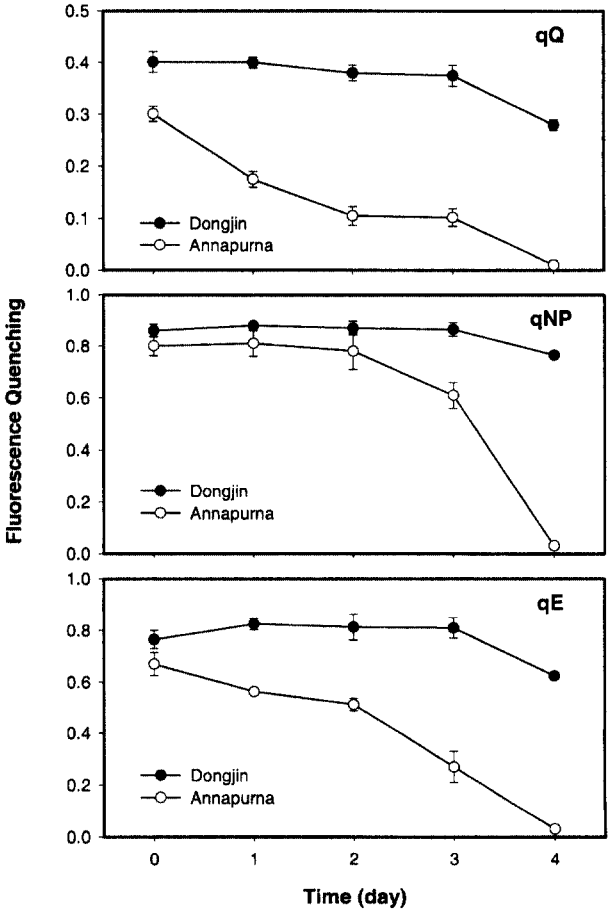

Figure 2. Changes in the fluorescence-quenching parameters qQ, qNP, and QE in the salt-sensitive cultivar Annapurna (– ● –) and the salt-tolerant cultivar Dongjin (– ○ –) for rice plants subjected to NaCl stress for 4 d.

Table 1. Comparison of relative distributions of violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) in leaves of rice cultivars (Dongjin and Annapurna) grown under various conditions. recov., recovery; DTT, dithiothreitol.

	Dongjin			Annapurna		
	V	A	Z	V	A	Z
	(% of V + A + Z)					
Control-Dark	100	0	0	92 ± 0.82	7 ± 0.76	1 ± 0.72
Control-Light	48 ± 0.46	2 ± 0.75	50 ± 0.29	34 ± 1.20	4 ± 0.46	62 ± 1.66
Control-Light-DTT	71 ± 0.64	21 ± 1.30	8 ± 2.88	37 ± 0.70	28 ± 0.72	35 ± 1.40
Control-DTT	97 ± 0.15	2 ± 0.31	1 ± 0.17	94 ± 0.80	5 ± 0.88	1 ± 0.40
Control-DTT-Light	55 ± 2.81	3 ± 0.44	42 ± 3.20	51 ± 1.86	5 ± 0.59	44 ± 2.40
Salt-Dark	98 ± 0.91	1 ± 0.86	1 ± 0.06	91 ± 1.51	7 ± 0.60	2 ± 1.60
Salt-Light	45 ± 1.96	3 ± 0.66	52 ± 2.58	30 ± 1.95	7 ± 0.45	63 ± 2.38
Salt-Light-DTT	48 ± 0.85	25 ± 2.02	27 ± 2.65	27 ± 1.02	25 ± 3.04	48 ± 2.21
Salt-DTT	94 ± 1.27	4 ± 0.83	2 ± 1.66	90 ± 1.15	8 ± 1.11	2 ± 1.88
Salt-DTT-Light	51 ± 3.77	5 ± 1.20	44 ± 2.57	38 ± 4.74	6 ± 0.78	56 ± 3.97
Recov.-Dark	96 ± 1.31	3 ± 0.95	1 ± 0.40	88 ± 1.16	9 ± 0.67	3 ± 0.78
Recov.-Light	43 ± 1.61	3 ± 0.38	54 ± 1.74	24 ± 0.79	9 ± 0.68	67 ± 1.27
Recov.-Light-DTT	56 ± 1.47	23 ± 1.40	21 ± 2.80	37 ± 1.41	30 ± 2.40	33 ± 3.80
Recov.-DTT	95 ± 0.85	3 ± 0.93	2 ± 0.27	93 ± 0.95	5 ± 0.66	2 ± 0.95
Recov.-DTT-Light	58 ± 3.50	6 ± 0.99	36 ± 2.51	33 ± 2.86	8 ± 0.56	59 ± 2.59

intermediate antheraxanthin that evolved was significantly increased. When xanthophyll cycles were compared between the two cultivars, the Anna-purna showed a higher level in the light-induced formation of zeaxanthin.

Changes in Oxygen-Evolving Activity

After 2 d of treatment with NaCl, the oxygen-evolving activity in Dongjin was slightly decreased, to 90% of the control, while that of Annapurna significantly declined, reaching 46% of the control (Fig. 3).

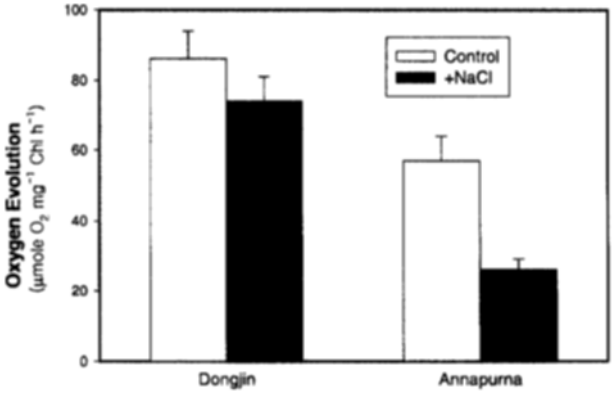

Figure 3. Changes in oxygen-evolving activity of chloroplasts isolated from rice plants subjected to NaCl stress for 2 d in the salt-sensitive cultivar Annapurna and the salt-tolerant cultivar Dongjin. Photosynthetic O_2 evolution was measured with a Clark-type oxygen electrode at 24°C. The intensity of light source was 1200 μmol $m^{-2}s^{-1}$. □, control; ■, treatment with 150 mM NaCl.

Changes in Glycinebetaine and Proline Contents

To clarify the role of compatible solutes in rice cultivars during salt stress, we examined the changes in glycinebetaine and proline contents. Our plants were grown for 3 d in vermiculite that had been supplemented with 150 mM NaCl. Compared with the controls, treated plants had increased concentrations of both compounds (Table 2), with the rate of proline accumulation being higher than that of glycinebetaine. Moreover, salt-stressed Annapurna plants showed higher rates of glycinebetaine and proline accumulation than did Dongjin.

DISCUSSION

We treated plants of two rice cultivars with 150 mM NaCl to determine their photosynthetic responses to salt stress as well as their ability to recover after refreshing. The decreases in F_v/F_m (Fig. 1) measured here are considered to be a reflection of the depression in the potential quantum yield of Photosystem II, which results from an increase in F_o and/or a decrease in F_v. Higher calculations for F_o may possibly be a result of a decrease in the probability of energy transfer from the antenna chlorophyll to the reaction center (Chun et al., 1996). In contrast, measured decreases in F_v seem to be attributable either to the limitation on electron donation to PS II, which is induced by the accumulation of the PS II reaction centers with stable

Table 2. Comparison of glycinebetaine and proline contents measured in Dongjin and Annapurna rice cultivars treated with 150 mM NaCl.

	Dongjin		Annapurna	
	Glycinebetaine	Proline	Glycinebetaine	Proline
	$\mu g\ g^{-1}$ fresh weight			
Control	375 ± 4.04	368 ± 7.93	169 ± 5.86	331 ± 22.68
Salt Treatment	450 ± 14.05	599 ± 8.74	375 ± 15.31	1013 ± 26.46
Recovery	248 ± 6.11	331 ± 2.52	248 ± 9.02	1013 ± 22.50

reduced Q_A, or to a partial dissociation of the antennae from the centers (Laasch, 1987; Vass et al., 1992). Inhibited CO_2 assimilation under salt stress could be expected to affect the redox state of the PS II electron acceptor and, thus, photochemical quenching. The decrease in the qQ component may result in a limitation of the dark reaction because lowered levels of activity in the Calvin cycle will induce NADPH accumulation as well as eventual feedback limitations of electron transport. Therefore, the severe decrease in qQ (Fig. 2) from Annapurna indicates that this cultivar may be more sensitive than Dongjin to salt stress.

Salt stress induced a significant decrease in the coefficients of non-photochemical quenching in Annapurna as compared to Dongjin (Fig. 2). The coefficients for energy-dependent quenching seemed to occupy most portions of qNP, changing in a parallel fashion with those of qNP following the salt treatment. These results indicate that, for qNP formation, qE is dominant among factors such as state transitions and photoinhibition (Krause and Weis, 1991). Playing an important role in the dissipation of energy at PS II, qE is triggered by the enhancement of proton concentrations in the lumen of the thylakoid. It has also been proposed that qE is critical in protecting the photosynthetic apparatus that is exposed to excess light (Krause and Behrend, 1986). Therefore, the rapid decline in qE and qNP observed in stressed Annapurna plants may have resulted from a collapse in the buildup of protons because of either limited electron transport or the salt stress-stimulated disassembly of the thylakoid membrane components. Demming-Adams et al. (1989) have suggested that qE depends on the conversion of violaxanthin into zeaxanthin in the xanthophyll cycle. Our findings here also indicated that Annapurna was more sensitive than Dongjin to salt in terms of its weakening in photosynthetic functions.

We also found that Annapurna was more sensitive to salt stress based on observations of its deterioration in oxygen-evolving activities. This may have been caused by a dissociation of the 23- and 33-kDa proteins located on the lumenal surface of the thylakoid membranes. Our hypothesis is supported by the fact, when salt stress induces the inhibition of photochemical reactions, a primary reaction is this previously described dissociation of those proteins that are extrinsically bound to the reaction-center proteins of PS II on the lumenal surfaces, an important component in the oxygen-evolving complex of PS II (Murata et al., 1992).

It has been suggested that glycinebetaine, which is suitable for maintaining a cell's osmotic balance, can prompt the evolution of oxygen from the original state of inactivation triggered by salt stress (Miyao and Murata, 1986). The two cultivars treated in this study had higher levels of glycinebetaine than were measured in the control (Table 2). Therefore, its accumulation in the chloroplasts may be a key factor in protecting oxygen-evolving activities and preventing the inactivation of enzymes when plants are exposed to higher amounts of NaCl. Proline also may be an important compatible solute in this mechanism of tolerance or regulation against salt stress.

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