Regulation of Ammonium Accumulation during Salt Stress in Rice (Oryza sativa L.) Seedlings

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Abstract: Metabolic processes related to ammonium release and assimilation were investigated in a salt-sensitive rice (Oryza sativa L.) cultivar Anapurna. Ammonium content of the 3rd leaves increased 3-4 times when seedlings were treated with 100 mM NaCl for 6 days under both growth chamber light condition and in darkness (non-photorespiration). An in vitro experiment revealed strong inhibition of protein synthesis as an effect of NaCl on the incorporation of ¹⁴C-leucine into protein. Exposure to salt stress slightly increased leaf proteolytic activity. The increase of proteolytic activity and decrease of protein synthesis, which directly causes accumulation of free amino acids, might lower the need for ammonium incorporation to form amino acids and indirectly cause the excessive accumulation of ammonium. No significant changes in the assimilatory activities of glutamine synthetase (GS; EC 6.3.1.2) and ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1) were found under salt stress. Salt treatment changed the balance of the direction of glutamate dehydrogenase (NAD(H); EC 1.4.1.2): the aminating (NADH-GDH) activity increased while deaminating (NAD-GDH) activity decreased. We conclude that the accumulation of ammonium under salt stress was not due to inhibition of assimilatory activity of GS/GOGAT cycle or aminating GDH. Since these enzymes require supply of C-skeleton in the form of 2-oxoglutarate, reductant and energy to function, the reduction of photosynthetic capacity and the decrease of 2-oxoglutarate might be responsible for the excess accumulation of ammonium in salt-stressed seedlings.

Key words: Glutamate dehydrogenase, Glutamate synthase, Glutamine synthetase, 2-Oxoglutarate, Protease, Protein synthesis.

Salt stress is reported to affect nitrogen metabolism - one of the most important biochemical processes in plants (Levitt, 1980). Besides the massive accumulation of free amino acids, ammonium accumulation under salt stress has been reported (Lutts et al., 1999; Hoai et al., 2003). In plants, ammonium is the key metabolite situated at the central crossroad between C and N assimilation pathways (Inokuchi et al., 2002). Many biochemical and molecular biological studies have advanced our knowledge on ammonium sources and assimilation (Lam et al., 1996; Kronzucker et al., 1999; Lancien et al., 2000). However, there are few reports on the changes of ammonium production and assimilation in plant growing under salt stress.

In our previous study, the disturbed nitrogen metabolism under salt stress led to a high accumulation of ammonium in shoots; this was highly correlated with the salt sensitivity of rice seedlings (Hoai et al., 2003). We suggested that the increase of ammonium may aggravate the detrimental effect of salt stress, and the regulation of ammonium accumulation and its assimilation mechanisms might play an important role in the salt tolerance of rice. In this study, several metabolic processes that might contribute to ammonium accumulation were investigated using a sensitive rice cultivar.

In C3 plants, photorespiration is a large source of ammonium release (Lea, 1999). Increase of photorespiration under various stresses has been reported (Wingler et al., 1999; Noctor, 2002). Thus it is impossible to neglect the importance of photorespiration in stress-induced ammonium accumulation. However, the fact that accumulation of ammonium was observed even in C4 grass species (Yamamoto et al., 2003) suggests the involvement of other metabolic processes in the stress-induced ammonium accumulation. Furthermore, accumulation of ammonium was often found together with the accumulation of free amino acids (Lutts et al., 1999; Hoai et al., 2003). Therefore, it is necessary

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Abbreviations: Fd-GOGAT, ferredoxin-dependent glutamate synthase; GDH, glutamate dehydrogenase; GOGAT, glutamine-2-oxoglutarate amidotransferase, glutamate synthase; GS, glutamine synthetase; 2-OG, 2-oxoglutarate; TCA, trichloroacetic acid; Fv/Fm, ratio of variable to maximum fluorescence, quantum yield of photosystem II; PSII, photosystem II; Φmol, quantum yield of photosystem II photochemistry.
to investigate the effect of salt stress on protein synthesis and proteolysis in relation to ammonium accumulation.

In higher plants, ammonium is assimilated via two major pathways: glutamine synthetase/glutamate-2-oxoglutarate amidotransferase (GS/GOGAT) and glutamate dehydrogenase (GDH) cycles (Lea, 1999). The GS/GOGAT cycle is comprised of the two enzymes: glutamine synthetase and glutamate synthase. Glutamine synthetase (GS; EC 6.3.1.2) catalyzes the formation of glutamine in an ATP-dependent reaction using ammonium and glutamate. Glutamate synthase (GOGAT) catalyzes the transfer of the amide group of glutamine formed by glutamine synthetase to 2-oxoglutarate (2-OG) to yield two molecules of glutamate. Two species of GOGAT are present; one requiring NADH as reductant (NADH-GOGAT; EC 1.4.1.14) and the other requiring ferredoxin (Fd-GOGAT; EC 1.4.7.1). This GS/GOGAT cycle represents the major pathway in the assimilation of ammonium under normal metabolic conditions of plants (Lea, 1999). There have been certain reports on changes of GS, GOGAT activities in plants exposed to salt stress (Berteli et al., 1995; Lutts et al., 1999). However, these reports were focused on changes of GS/GOGAT in relation to the production of glutamate which plays an important role in proline synthesis rather than the assimilation of ammonium. Another possible ammonium assimilation pathway is via the action of glutamate dehydrogenase (NAD(H)-GDH; EC 1.4.1.2). NADH-GDH catalyzes the amination of 2-oxoglutarate and NAD-GDH catalyzes the deamination of glutamate (Lam et al., 1996). Although GDH has low affinity to ammonium, the implication of GDH in the response to stress conditions has been suggested (Balestrasse et al., 2003; Dubois et al., 2003; Restivo, 2004). In this study, changes in the activity of GS/GOGAT and GDH in salt-stressed seedlings were measured. Moreover, since the operation of these enzymes require a steady supply of carbon skeleton (in the form of 2-OG), reductants and energy derived from photosynthesis, the changes in photosynthetic capacity and 2-OG content under salt stress were also studied.

1. Plant growth and salt treatment

Seeds of rice (Oryza sativa L.) cultivar Anapurna were used. Anapurna was previously evaluated as a salt-sensitive cultivar which accumulates relatively high level of Na’ in the shoots (Hoai et al., 2003). The rice seeds were surface sterilized with 70% ethanol for 5 min and with 10% H2O2 for 10 min. They were then washed thoroughly, soaked in distilled water for 48 h then incubated for 24 h at 30°C under moist, dark conditions. The germinated seeds were sown in a stainless net and floated on deionized water in the dark. After 24 h, the net was transferred to a plastic container containing 5 L of Yoshida’s nutrient solution. Seedlings were grown in a growth chamber under a 12h-photoperiod, 25/20°C day/night, 250 μEm−2s−1 light intensity and relative humidity of 70 to 80%. The nutrient solution was changed every 2 days and pH was adjusted daily to between 5.6 and 5.8 by adding either 1 N NaOH or HCl. Salt treatment was started 20 days after germination. Uniform seedlings that had complete 3rd leaves and 4th leaves of 1-2 cm length (leaf number 3.2-3.5) were transferred to a plastic bottle containing 500 mL of the above nutrient solution with or without 100 mM NaCl. Salt treatment was carried out for 6 days in the normal light condition of the growth chamber or in the dark when indicated.

2. Measurement of growth parameters and chlorophyll contents

Total fresh weight (FW) and dry weight (DW) of five seedlings were measured 6 days after the start of treatment with NaCl. Chlorophyll fluorescence was measured on the same position of the 3rd leaf blades in the control and salt-treated seedlings at 2, 4 and 6 days after the start of treatment. Measurement was conducted at between 11:00 and 12:00 using a chlorophyll fluorometer (Open-fluorcam, photon systems instrument, Czech). The potential quantum efficiency of photosystem II (PSII) (Fv/Fm) and the efficiency of PSII photochemistry (ΦPSII) were calculated according to Maxwell and Johnson (2000).

The 3rd leaf blades were harvested at 2, 4 and 6 days after the start of treatment. Chlorophyll and carotenoid contents were determined using the method of Chappelle et al. (1992). Chlorophyll was extracted by soaking excised leaves in dimethyl sulfoxide at 50°C for 24 h in the dark. The concentrations of the extracted pigments were calculated from the absorbance values at 664, 648, and 470 nm.

3. Determination of ammonium and 2-oxoglutarate contents

The 3rd leaf blades of control and salt-treated seedlings were harvested at 0, 2, 4 and 6 days after the start of treatment with NaCl. Ammonium content was determined according to desmaison et al. (1984). Five hundred milligrams of frozen leaves were ground in liquid nitrogen and homogenised with 4 mL of 15 mM HCl. The homogenate was centrifuged at 2000 × g for 5 min at 4°C. Five hundred microliters of the supernatant was deproteinised with 100 μL 5-sulfosalicylic acid (10% w/v) and kept on ice for 15 min. The mixture was again centrifuged at 2000 × g for 15 min at 4°C. Five hundred microliters of the supernatant was collected and adjusted to pH 2.2-2.3. Ammonium content was measured with an automatic amino acid analyzer (JEOL, JLC-300,
Japan). 2-Oxoglutarate was extracted using absolute ethanol and determined enzymatically with glutamate dehydrogenase (Novitskaya et al., 2002).

4. Protein synthesis

To study the effect of NaCl on protein synthesis, we collected the immature part from the 4th leaves rolling inside the 3rd leaf sheaths of 20-day-old seedlings (leaf segment). Protein synthesis was determined according to the method described by Matsumoto et al. (1991) by measuring the incorporation of 14C-leucine into protein. Leaf segments (30 mg) were in a glass vial containing 4.9 mL of incubation buffer containing 0.01 M K-P buffer (pH 6.0) and 1% (w/v) of sucrose. NaCl was added to the medium to make the final concentration 0, 50, or 100 mM. A protein synthesis inhibitor cycloheximide (10 μM) was also added for comparison. The vials were shaken at 60 rpm, at 25°C. After 30 and 60 min (treatment with NaCl and cycloheximide), 0.1 mL of 14C (U)-leucine (18.5 kBq) was added and the vial was shaken at 60 rpm, for 120 min, at 25°C. Then the samples were collected and rinsed with non-labelled leucine and distilled water. The samples were then homogenized with 5% (w/v) cold trichloroacetic acid (TCA) and incubated on ice for 45 min for protein precipitation. After that, the homogenate was filtered with a Whatman GF/C glass micro fibre filter. The TCA-precipitable protein on the filter was collected and the radio activity (dpm; disintegration per min) was determined with a scintillation counter (Beckman LS 5000 TA).

5. Protease assay

The total proteolytic activity in leaves of control and salt-treated seedlings were determined using the casein hydrolysis assay of Kunitz (1947) but optimized according to the plant materials. The 3rd leaf blades were harvested at 0, 2, 4, and 6 days after the start of treatment with NaCl. Leaves were ground in liquid nitrogen and homogenized in 0.1 M K-P buffer (pH 7.6). The homogenate was then centrifuged at 15000 × g for 20 min at 4°C. The supernatant was filtered through Miracloth and used as the enzyme extract.

The assay was carried out with an enzyme-substrate mixture consisting of 1 mL of 1% (w/v) purified casein in 0.1 M K-P buffer (pH 7.6) and 1 mL of the enzyme extract. After incubation at 35°C for 1 h, the reaction was terminated by the addition of 3 mL of 5% (w/v) TCA. After standing for 30 min at room temperature, contents were centrifuged at 10000 × g for 20 min. Absorbance of the supernatant was recorded at 280 nm to measure the amount of tyrosine produced. A blank was prepared by pre-incubating a mixture of TCA and casein for 30 min at 35°C to prevent any hydrolysis activities before the addition of enzyme extract. This allows for subtraction of absorbance caused by reagents and especially enzyme extract due to the possible presence of materials that have absorption at a wavelength similar to the product from hydrolysis. Soluble protein content in the extract was determined according to Bradford (1976) using bovine serum albumin as the standard. Enzyme activity was expressed as U mg−1 protein. One unit of enzyme activity was defined as the amount of enzyme needed to produce 1 mg tyrosine per h.

6. Glutamine synthetase assay

The total GS activity in the 3rd leaves was measured according to the method of O’Neal and Joy (1973). Leaves were ground into fine powder in liquid nitrogen. The samples were then homogenized in the extraction buffer containing 50 mM tris-HCl buffer (pH 7.2), 0.01% mercaptoethanol and 0.5 mM Na2EDTA. The homogenate was centrifuged at 15000 × g for 20 min at 4°C. The supernatant was filtered through Miracloth and the extract was used for the enzyme assay. GS activity was determined by measuring the formation of γ-glutamyl hydroxamate. The reaction mixture contained 50 mM imidazole-HCl (pH 8), 80 mM sodium glutamate, 16 mM ATP, 50 mM MgSO4, and the enzyme extract. The reaction mixture was pre-incubated at 28°C for 5 min. The reaction was initiated by the addition of 0.1 mL of hydroxylamine solution (1 M NH2OH.HCl : 1 M NaOH : H2O 1:1:2 v:v:v). The mixture was then incubated at 28°C for 20 min and terminated by the addition of 0.4 mL of FeCl3 solution, a 1:1:1 (v:v:v) mixture of 10% FeCl3 dissolved in 0.2 M HCl : 24% TCA : 0.5 M HCl. The reaction mixture was then centrifuged at 5000 × g for 10 min, at 4°C. The appearance of γ-glutamyl hydroxamate in the supernatant was assessed at 540 nm with a spectrophotometer (UV 2450 Shimadzu, Kyoto, Japan). Enzyme activity was expressed as U mg−1 protein. One unit of enzyme activity represents 1 nmol of γ-glutamyl hydroxamate formed per min.

7. Glutamate synthase and glutamate dehydrogenase assays

GOGAT and GDH were measured according to the method of Groat and Vance (1981). Leaves were ground in liquid nitrogen and homogenized in the extraction buffer consisting of 100 mM Mes-NaOH pH 6.8, 100 mM sucrose, 2% 2-mercaptoethanol, 15% ethylene glycol and 0.1 mM of phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 30000 × g for 20 min at 4°C. The supernatant was filtered and used for GOGAT and GDH assays.

Ferredoxin-dependent glutamate synthase (Fd-GOGAT) was measured by quantification of glutamate produced (Matoh and Takahashi, 1981). The reaction mixture contained 125 mM K-P buffer (pH 7.5), 5 mM 2-oxoglutarate, 5 mM glutamine, 1
mM aminooxyacetate, 1 mM methylviologen, and the enzyme extract. The reaction was started by adding 14 mM sodium dithionite dissolved in 0.2 M NaHCO₃. After incubating for 45 min at 30°C, the reaction was stopped in a boiling water bath for 5 min. After centrifugation, glutamate produced in the reaction mixture was quantified with an amino acid analyser (JEOL, JLC-300, Japan).

GDH was measured spectrophotometrically monitoring absorbance due to NADH at 340 nm (Groat and Vance, 1981). NAD-GDH (oxidative deaminating) was determined in 100 mM Tris-HCl, pH 7.6 containing 0.1% 2-mercaptoethanol, 2.5 mM 2-oxoglutarate and 20 mM NH₄⁺. NADH-GDH was measured spectrophotometrically monitoring absorbance due to NADH at 340 nm (Groat and Vance, 1981). NAD-GDH (oxidative deaminating) was assayed in 100 mM K-P, 0.5 mM NAD and 50 mM L-glutamate. NADH-GDH was determined in 100 mM Tris-HCl, pH 8.0 with 14 mM aminoxyacetate, 1 mM methylviologen, and the reductive aminating activity was assayed in 100 mM K-P, pH 7.6 containing 0.1% 2-mercaptoethanol, 2.5 mM 2-oxoglutarate and 20 mM NH₄⁺. Enzymes activities were corrected for endogenous NADH reduction in the absence of the specific substrate. Enzyme activity was expressed as U mg⁻¹ protein. One unit of enzyme activity is defined as 1 nmol of NAD/NADH oxidized/reduced per min.

### Results

1. **Effect of salt stress on growth of rice seedlings**

   Anapurna has been evaluated previously as a salt-sensitive cultivar (Hoai et al., 2003). In this experiment, the growth of seedlings was strongly inhibited by a 6-day treatment with 100 mM NaCl (Table 1). Fresh weight and dry weight of seedlings dropped to 51% and 69% of the controls, respectively. Elongation of the 4th leaf blades was also strongly inhibited: approximately 60% inhibition (Table 1).

   Chlorophyll contents of the 3rd leaves in salt-treated seedlings were consistently lower than that in the controls (Table 2). At 6 days after the start of treatment, chlorophyll a, b and carotenoid contents were 64, 61 and 58% of controls, respectively.

   Salt treatment also induced changes in the chlorophyll fluorescence of the leaves. The quantum yield of PSII, as indicated by Fv/Fm, was not affected by salt treatment for 2 and 4 days, but that in the 3rd leaves was significantly lower in salt-treated seedlings than in the controls after the 6-day treatment with salt (Table 2). Fv/Fm was slightly decreased by the salt treatment in both control and treated seedlings. However, the efficiency of PSII photochemistry (Φpsii) was greatly affected by the salt stress: 64, 32 and 26% of the control value at 2, 4 and 6 days after the start of treatment, respectively.

   2-Oxoglutarate content of the 3rd leaves in the control and salt-treated seedlings was not affected by salt treatment for 2 and 4 days, but was slightly decreased by the salt treatment for 6 days (Table 2).

2. **Accumulation of ammonium under salt stress in rice seedlings kept in darkness**

   In this experiment, during 6 days of salt treatment, seedlings were kept in the normal light condition of the growth chamber (light) and in darkness (dark) to examine the effect of photorespiration on ammonium accumulation. Ammonium content of the 3rd leaves in the control and salt-treated seedlings was measured at 2, 4, and 6 days after the start of salt treatment (Fig. 1). In leaves of the seedlings incubated in light, ammonium accumulation was observed after a 4-day exposure to salt stress. Ammonium contents after a 4- and 6-day exposure to the stress were 3 and 4 times higher, respectively, than that of the controls.

   In the dark-incubated seedlings, high concentrations of ammonium were detected. Ammonium content increased with the lapse of time in the dark in both control and salt-treated seedlings. Salt induced

### Table 1. Effect of salt stress (100 mM, 6 days) on growth of rice seedlings, T/C: treated/control.

| Parameter                  | Control | Treated | T/C (%) |
|----------------------------|---------|---------|---------|
| Fresh weight of 5 seedlings (g) | 2.37 ± 0.16 | 1.22 ± 0.04 | 51      |
| Dry weight of 5 seedlings (g)   | 0.32 ± 0.03 | 0.22 ± 0.02 | 69      |
| 4th leaf length (cm)            | 17.6 ± 2.0 | 7.8 ± 2.5 | 44      |

| Chlorophyll a C | 0.94 ± 0.03 | 1.10 ± 0.01 | 1.17 ± 0.05 | 1.27 ± 0.04 |
| T              | 0.95 ± 0.01 | 0.95 ± 0.03 | 0.81 ± 0.03 |           |
|                | (86)       | (81)       |           | (64)       |
| Chlorophyll b C | 0.17 ± 0.01 | 0.22 ± 0.01 | 0.24 ± 0.01 | 0.28 ± 0.01 |
| T              | 0.19 ± 0.01 | 0.20 ± 0.01 | 0.17 ± 0.01 |           |
|                | (86)       | (83)       |           | (61)       |
| Carotenoids C  | 0.23 ± 0.01 | 0.27 ± 0.00 | 0.28 ± 0.01 | 0.24 ± 0.01 |
| T              | 0.23 ± 0.00 | 0.22 ± 0.00 | 0.14 ± 0.01 |           |
|                | (85)       | (79)       |           | (58)       |
| Fv/Fm C        | 0.82 ± 0.02 | 0.80 ± 0.01 | 0.78 ± 0.04 | 0.78 ± 0.01 |
| T              | 0.80 ± 0.02 | 0.75 ± 0.04 | 0.64 ± 0.05 |           |
|                | ns         | ns         |           | (82)       |
| Φpsii C        | 0.53 ± 0.01 | 0.42 ± 0.04 | 0.46 ± 0.03 | 0.38 ± 0.04 |
| T              | 0.27 ± 0.01 | 0.15 ± 0.03 | 0.10 ± 0.03 |           |
|                | (64)       | (32)       |           | (26)       |
| 2-OG C         | 179.6 ± 27 | 167.9 ± 5.3 | 152.6 ± 10 | 180.8 ± 16.0 |
| T              | 154.9 ± 5.8 | 132.6 ± 7.9 | 136.1 ± 12.6 |           |

### Table 2. Effect of salt stress (100 mM NaCl) on chlorophyll fluorescence, chlorophyll and 2-oxoglutarate contents (2-OG) in the 3rd leaves of rice seedlings, C: control, T: treated. Data are means ± standard error of 3 replications. Chlorophyll and carotenoids contents are presented as mg g⁻¹ FW. 2-OG contents presented as nmol g⁻¹ FW. Data in parentheses are percentage of treated/control given when significantly different with controls by one way ANOVA, P < 0.05. ns: not significant.
ammonium accumulation was already observed after a 2-day exposure to stress, and ammonium content of salt-treated seedlings was consistently 3 times higher than that of the controls.

3. Protein synthesis and proteolytic activity under salt stress

Protein synthesis was strongly inhibited by NaCl and cycloheximide treatments (Fig. 2). The effect of cycloheximide and NaCl on the incorporation of $^{14}$C-leucine into protein in the segments of the immature 4th leaf varied with the duration of treatment and the concentration of NaCl. Treatment with 50 mM NaCl reduced protein synthesis activity by 10 and 30% after a 30- and 60-min treatment, respectively. The treatment with 100 mM NaCl reduced it by 40 and 50% after a 30- and 60-min treatment, respectively. Cycloheximide (10 µM) strongly inhibited protein synthesis: approximately 80%.

The protease activity in the leaves of salt-treated seedlings was slightly but significantly higher than that of controls after a 4- and 6-day treatment (Table 3). Soluble protein content after a 6-day treatment was approximately 86% of the control (Table 3).

4. Activities of glutamine synthetase and glutamate synthase under salt stress

In leaf extract, activities of both GS and Fd-GOGAT slightly increased with leaf maturation in both control and salt-treated seedlings, but no significant difference between control and treated seedlings was found (Table 3). NADH-dependent glutamate synthase (NADH-GOGAT) activity was low or not detected (data not shown).

5. Glutamate dehydrogenase activity under salt stress

GDH activity in the aminating (NADH) and deaminating (NAD) directions was determined in the leaves of control and salt stressed seedlings. NADH-
GDH was increased by salt treatment. NADH-GDH activity after a 4- and 6-day treatment with NaCl was approximately 1.3-1.5 times higher than that of the controls (Fig. 3A). The GDH deaminating activity (NAD-GDH) was lower than aminating activity (NADH-GDH) (Fig. 3B). NAD-GDH activity decreased with time of treatment in both control and salt-stressed seedlings. NAD-GDH activity of salt-stressed seedlings was slightly lower than that of the controls. The ratio of NADH/NAD-GDH activity increased with time after the start of treatment (Fig. 3C). NADH/NAD-GDH ratio of salt stressed seedlings was consistently higher than that of controls.

**Discussion**

Rice is a moderately salt-sensitive crop species which is relatively ineffective in controlling the influx of salt ions (Na⁺ and Cl⁻) to the shoots (Yeo and Flowers, 1982). A comparative study using rice cultivars differing in salt sensitivity revealed that accumulation of ammonium was correlated with Na⁺ absorption and the sensitivity to salt (Hoai et al., 2003). Therefore we used the salt-sensitive cultivar Anapurna in the present study. Our primary experiments demonstrated that 100 mM NaCl did not cause severe damages during treatment, but had a reproducible effect on growth, photosynthesis and other biochemical pathways in rice seedlings.

Reduction of photosynthesis under salt and various environmental stresses has been documented (Delfine et al., 1999; Ghosh et al., 2001). In the present study, salt stress induced changes in chlorophyll contents and chlorophyll fluorescence in the 3rd leaves of seedlings (Table 2). The reduction of chlorophyll induced a relevant reduction in light absorbance of leaves. The low chlorophyll and carotenoid contents of leaves in stressed seedlings indicate a reduction of photosynthetic capacity. Furthermore, the quantum yield of PSII (Fv/Fm) was decreased by salt stress. The decrease of Fv/Fm, a sign of photoinhibition, is commonly observed in plants exposed to environmental stresses (Maxwell and Johnson, 2000). ΦPSII which indicates the proportion of the light absorbed by chlorophylls to that used in photochemistry is the most useful parameter in chlorophyll fluorescence study. There is a strong relationship between ΦPSII and the efficiency of carbon fixation and therefore changes in the efficiency of PSII can reflect the overall photosynthetic performance of plants (Maxwell and Johnson, 2000). In the present study, the drastic reduction of ΦPSII together with the reduction of light absorbance due to the decrease of chlorophyll and carotenoid contents indicated that the photosynthetic capacity of the seedlings was reduced by salt stress.

In C3 plants, ammonium is liberated at a very high rate by the conversion of glycine to serine during
photorespiration (Lea, 1999; Novitskaya et al., 2002). Because of the difficulties in estimating the rate of photorespiration, we quantitated the ammonium accumulation under salt stress in complete darkness when photorespiration is absent. We found that a large amount of ammonium accumulated in the dark-incubated rice seedlings (Fig. 1). A pronounced increase of ammonium in the dark-incubated wheat has also been observed by Peeters and Van Laere (1992). They found that the accumulation of ammonium coincided with the disappearance of GS and GOGAT activities in the dark. It was also described previously that GS and GOGAT are light-dependent enzymes, which require energy and C skeletons produced via photosynthesis (Lea, 1999). The high concentration of ammonium found in the dark-incubated seedlings might be a consequence of the reduction in the assimilation capacity and the decrease of 2-OG content in salt-treated seedlings (Table 3) might also be related to the oxidative stress. The decrease of protein synthesis and increase of protein degradation in salt-treated seedlings (Table 2) supported this hypothesis. These results indicate that photorespiration contributed to the increase of ammonium content but was not the major reason for the massive accumulation of ammonium under salt stress.

Chlorophyll loss is commonly used as an indicator of senescence which is accelerated under many environmental stresses (Murchie et al., 2002; Buchanan et al., 2003). During senescence under oxidative stress, the production of active oxygen species that damage lipids and DNA is enhanced and these active oxygen species can lead to oxidation of amino acids resulting in protein fragmentation. These fragmented proteins are more susceptible to degradation by proteases (McKersie, 1996). The increase of protease activity and decrease of soluble protein content in salt-treated seedlings (Table 3) might also be related to the oxidative stress. The decrease of protein synthesis and increase of protein degradation in salt-treated seedlings might directly relate to the increase of free amino acids reported previously (Hoai et al., 2003); this in return might lower the need of ammonium for amino acids synthesis and cause the excessive accumulation of free ammonium.

In higher plants, the GS/GOGAT cycle is considered as the main route of ammonium assimilation, and GS with its high affinity for ammonium is viewed as a scavenger of ammonium (Lea, 1999). Although there are certain reports on GS inactivation due to oxidative stress (Humanes et al., 1995; Ishida et al., 2002), which is an important component of salt stress (Hernandez et al., 2001), in the present study, leaf GS activity was not affected by NaCl (Table 3). Co-ordinate functioning with GS, GOGAT received more attention in studies on salt stress, mostly in view of glutamate supply for proline biosynthesis (Berteli et al., 1995; Lutts et al., 1999). In leaves, Fd-GOGAT has a major role in the assimilation of ammonium (Lea, 1999). In our experiment, Fd-GOGAT, which contributed to almost all leaf GOGAT activity, was unaffected by NaCl treatment (Table 3). Our data indicate that GS/GOGAT functioned properly under salt stress, and the excessive accumulation of ammonium was not due to alteration of these enzymes activities.

GDH, has been considered as a key enzyme in ammonium assimilation before the discovery of GS and GOGAT, but there are few reports on its role in salt stressed plants. It has been suggested that GDH would be active in deamination rather than amination, providing 2-OG for tricarboxylic acid cycle, and its role in plant ammonium assimilation is still a matter of debate (Inokuchi et al., 2002; Restivo, 2004). However, there are some reports that in the presence of high concentrations of ammonium GDH plays an important role in ammonium assimilation and detoxification (Lam et al., 1996). In our experiment, salt stress increased the aminating activity and aminating/deaminating ratio of GDH (Fig. 3C): this indicates the higher need of GDH for ammonium assimilation/detoxification. Besides, although GDH has a low affinity for ammonium, the enzyme is less energy-demanding than GS and therefore it could be more effective in ammonium assimilation under stress.

Studies on the operations of enzymes taking part in ammonium assimilation we conclude that excessive accumulation of ammonium was not due to reduction of the assimilatory activity of GS/GOGAT or aminating-GDH. However, the reduction of photosynthesis might result in shortage of carbon skeleton, redundant and energy supply for GS/GOGAT and GDH; this might be responsible for the excessive accumulation of ammonium. The observed reduction of photosynthetic capacity and the decrease of 2-OG content in salt-treated seedlings (Table 2) supported this hypothesis. It should, therefore, be interesting in the future to test the effect of exogenous supply of carbon skeleton on the assimilation of the stress-induced ammonium.

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