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Author(s)	Nakamura, Ichiro; Murayama, Seiichi; Tobita, Satoshi; Ba Bong, Bui; Yanagihara, Seiji; Ishimine, Yukio; Kawamitsu, Yoshinobu
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## Effect of NaCl on the Photosynthesis, Water Relations and Free Proline Accumulation in the Wild *Oryza* Species

Ichiro Nakamura, Seiichi Murayama\*, Satoshi Tobita\*\*, Bui Ba Bong\*\*\*, Seiji Yanagihara\*\*\*\*, Yukio Ishimine\* and Yoshinobu Kawamitsu\*

(United Graduate School of Agricultural Sciences, Kagoshima University, Kagoshima 890-0065, Japan; \*Fac. of Agri., Univ. of the Ryukyus, Nishihara, Okinawa 903-0213, Japan; \*\*Japan International Research Center for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan; \*\*\*Cuu Long Delta Rice Research Institute, Omon, Cantho, Vietnam; \*\*\*\*International Rice Research Institute (IRRI) DAPO Box 7777, Metro Manila, The Philippines)

**Abstract:** The physiological responses of wild *Oryza* species (*Oryza latifolia* Desv., a salt-tolerant species and *O. rufipogon* Griff., a salt-susceptible species) to salinity stress were investigated by comparing with check varieties of cultivated rice (*O. sativa* L.), SR26B (salt-tolerant) and IR28 (salt-susceptible). As the NaCl concentration of water culture solution was raised to 12 dS m<sup>-1</sup> (about 113mM), leaf Na<sup>+</sup> content per dry matter in wild *Oryza* species increased from 4 to 17 times as compared with the control (no NaCl treatment), whereas the accumulation was lower in cultivated rice varieties. The increased concentration of leaf Na<sup>+</sup> resulted in the decrease in leaf water potential ( $\Psi_w$ ) in all rice species, although the degree of decline in photosynthetic rate was different among the varieties. It was notable that the photosynthetic rate was almost constant in *O. latifolia* though  $\Psi_w$  decreased to -2.0 MPa with the increased NaCl concentration in the water culture solution. A significant negative correlation between free-proline content and osmotic potential ( $\Psi_s$ ) of leaf blade was found in both salt tolerant entries, SR26B and *O. latifolia*. In spite of higher leaf Na<sup>+</sup> storage, the survival rate of *O. latifolia* was higher than that of SR26B, indicating that the mechanism of salt tolerance in *O. latifolia* may be different from that in SR26B.

**Key words:** *Oryza latifolia*, *O. rufipogon*, Osmotic potential, Proline, Salt tolerance, Water potential.

For more than half a century, increasing salt accumulation in soil is one of the greatest problems in arid and semi-arid agricultural zones in the world (Munns et al., 1999). Accumulation of excess salt in soil induces ionic and osmotic stresses and it decreases plant growth and yield (Boyer, 1982). Cultivated rice (*Oryza sativa* L.), a staple food for billions of people in the world, not being a halophyte is especially sensitive to salt (Gregorio et al., 1997; Munns et al., 1999). Grain yield of rice plants is reduced by 70% to 100% of its maximal yield performance by salinity (Heenan et al., 1988). Although the degree of salt tolerance of rice plants differs among species and varieties, some of them show high resistance to salinity (Yeo and Flowers, 1986). Understanding of the physiological mechanisms of the tolerance in rice species should provide important information for setting rice plant-breeding strategies.

Higher plants have developed a sophisticated and complex set of adaptive responses allowing them to withstand salt stress. For example, 1) selective absorption of ions in roots (Badger and Ungar, 1990), 2) positive elimination of the absorbed salt (Matsushita and Matoh, 1991), and 3) storage of adaptation solutes in cytoplasm to maintain osmotic pressure (Munns and Termaat, 1986; Kalaji and Pietkiewicz, 1993). It has been proposed that rice acquires salt tolerance by excluding sodium ion from the roots (Akita and Cabuslay,

1990; Gregorio and Senadhira, 1993; Tsuchiya et al., 1994), and accumulation of proline and glycinebetaine in leaves (Harinasut et al., 1996) for osmotic adjustment. However, these studies were made using cultivated rice species. Some wild rice species showed superior physiological characteristics, such as in photosynthetic capacity. Yeo et al. (1994) found that some wild rice species showed extremely higher photosynthetic rate in comparison with cultivated species. They proposed that usage of wild rice species as genetic resources is an instructive strategy for improvement of the physiological traits of cultivated species. However, there have been few investigations elucidating the mechanisms to increasing stress tolerance in wild rice species. Bong et al. (1994) screened seedlings of 23 lines of 7 wild rice species in regard to survival ratio under high salt conditions, and they selected 7 lines as stress-tolerant, but they did not investigate the mechanism of salinity tolerance in the wild *Oryza* species.

In this study, we examined physiological responses of the wild rice species to salinity, i.e., photosynthetic rate, water potential, osmotic potential, and proline contents under salt-stress conditions. In addition, we compared these traits of wild *Oryza* species with cultivated rice different in salt tolerance; SR26B (salt-tolerant) and IR28 (salt-susceptible).

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**Abbreviations:** DAT, days after NaCl treatment;  $\Psi_s$ , osmotic potential;  $\Psi_w$ , leaf water potential; VPD, vapor pressure deficit.

## Materials and Methods

Wild *Oryza* species were screened for salt tolerance at JIRCAS Okinawa Branch, Japan in 1994. Seedlings of wild rice species were subjected to NaCl stress at 12 dS m<sup>-1</sup> (about 113 mM NaCl). Based on the survival rate (more than 80%) at 14 days after NaCl treatment (DAT), seven accessions from 4 wild rice species were screened. A salt-tolerant species, *Oryza latifolia* Desv. (IRGC Acc. No. 100965), and a susceptible species, *O. rufipogon* Griff. (IRGC Acc. No. 105390) were used. A follow-up experiment was conducted with these two wild species at the University of the Ryukyus, Okinawa, Japan in 1998. *O. sativa* L. cv. SR26B (salt-tolerant) and *O. sativa* L. cv. IR28 (salt susceptible) were used as the check varieties. Plants were grown in a glasshouse. The seeds of wild rice species were incubated at 45°C for three d in a forced draft oven to break the dormancy. The seeds of all rice plants were disinfected with a systemic fungicide (Benlate T) and were sown in nursery box (60×35×8 cm) containing red soil. Two seedlings at the 3–4 leaf stage of each entry were transplanted into plastic container (48×80×20 cm) containing Kimura B water-culture solution.

At 58 days after sowing, saturated NaCl solution was added to the culture medium up to the final EC of 6 dS m<sup>-1</sup> (about 56 mM NaCl) and 12 dS m<sup>-1</sup>. The culture solution was changed newly every week, and pH was adjusted to 5.0–5.5 every day. Each treatment consisted of three replications.

### 1. Survival rate under salt stress

Twenty seedlings of each species that had been grown in Kimura B solution for 4 weeks were transplanted into Styrofoam board (82×52×2 cm, with 7.07 cm<sup>2</sup> holes). At 40 days after transplanting (DAT), saturated NaCl solution was added to the culture medium up to 12 dS m<sup>-1</sup> of EC, and plants having tillers with more than three green leaves were counted. The survival rate was calculated as the percentage of the plants that have such tillers to total plants examined.

### 2. Measurement of CO<sub>2</sub> assimilation rate.

CO<sub>2</sub> assimilation rate was measured on the youngest fully expanded leaf of the main culm and adjacent tillers at 10 or 11 leaf stage. Plants were illuminated under a photon flux density of 1000 to 1200 μmol m<sup>-2</sup> s<sup>-1</sup> for one hour before the measurement.

The leaves were put in an assimilation chamber (Kawamitsu et al., 1999), which can control environmental conditions, viz., light intensity, leaf temperature, vapor pressure deficit, and CO<sub>2</sub> concentration, during the measurement.

### 3. Leaf Na<sup>+</sup> content

Leaf samples were taken for Na<sup>+</sup> analysis from the same leaves used for the measurement of CO<sub>2</sub> assimila-

tion. The leaves were washed with distilled water and dried at 80°C for 72 h. Dried samples (0.25 g) were crushed and soaked in 50 ml of 0.1% nitric acid. Na<sup>+</sup> was extracted by incubating for 24 h at 80°C. The extract was filtered with a membrane filter (0.45 μm), and was subjected to ion chromatograph (DX-AQ, Dionex) using a Guard column (Ionpac, CG12) and separation column (Ionpac, CS12). Methanesulfonic acid (20 mM) was used as an eluent.

### 4. Leaf water potential and osmotic potential

For measurement of leaf water potential, immediately after the measurement of CO<sub>2</sub> assimilation, leaf discs of about 3.1 cm<sup>2</sup> were punched out from the center of the leaf blade used for the measurement of CO<sub>2</sub> assimilation rate, and were put in the measurement chamber. The leaf water potential (Ψ<sub>w</sub>) was determined with an isopiestic thermocouple psychrometer according to the method of Boyer (1995). Sucrose solution of the designated concentration was used for calibration.

Osmotic potential (Ψ<sub>s</sub>) was measured on the same leaf used for CO<sub>2</sub> assimilation and Ψ<sub>w</sub> measurement. Leaf discs were punched out from the leaf blades used for the Ψ<sub>w</sub> measurement and immediately put into a 1-ml syringe. The syringe was kept in a freezer at -40°C for 7 days, the frozen sample was pressed firmly with a plunger, to squeeze out the sap. A drop of the extract was placed on the thermocouple spiral of the isopiestic psychrometer (Boyer, 1995).

### 5. Free-proline content

Leaves were frozen in liquid nitrogen immediately after measuring Ψ<sub>w</sub> and dried in a lyophilization apparatus (VD-41, Taitec). Free-proline was extracted in 75% ethanol from approximately 50 mg of each sample with an accelerated solvent extraction system (ASE) (Dionex, ASE-200). The extract solution was defatted by chloroform. The resultant solution was concentrated by a rotary evaporator and were diluted in 5 mL of sodium citrate buffer solution (pH 2.2) and filtered through a 0.45 μm membrane filter. Free-proline contents in the filtrate were measured by HPLC (Shimadzu, LC-10A).

## Results

Figure 1 shows the survival rate at 40 days after the NaCl treatment. In both salt sensitive entries, IR28 and *O. rufipogon*, almost all plants died by the treatment; however, the salt tolerant entries, SR26B and *O. latifolia* survived with survival rates of 15% and 65%, respectively. These results clearly showed that the wild rice species *O. latifolia* has stronger resistance to salt stress than that of the salt-tolerant cultivar, SR26B.

Figure 2 shows the Na<sup>+</sup> content in the leaf with or without NaCl treatment. In both the cultivated rice varieties, the Na<sup>+</sup> content was almost the same independent of the salt sensitivity. Whereas both of the wild rice species accumulate Na<sup>+</sup> in leaves more than in the

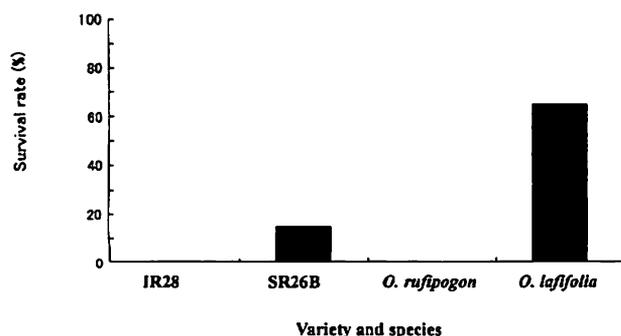


Fig. 1. Survival rate of rice plant under  $12 \text{ ds m}^{-1}$  NaCl treatment.

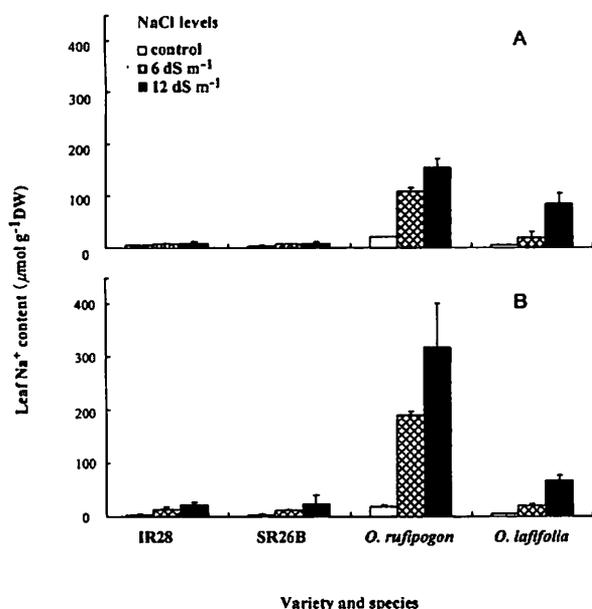


Fig. 2. Leaf  $\text{Na}^+$  content of rice plant measured at 7 d (A) and 14 d (B) after NaCl treatment. Data are means of 3 plants  $\pm$  SD.

cultivated rice. Especially the salt-sensitive wild rice, *O. rufipogon*, accumulated significant amount of  $\text{Na}^+$  in the leaves. The salt-tolerant species, *O. latifolia* and SR26B also accumulated  $\text{Na}^+$  in leaves but the level of  $\text{Na}^+$  was different between them. The amount of  $\text{Na}^+$  in *O. latifolia* leaves was about three to ten times higher than in SR26B.

Figure 3 shows the effect of the leaf  $\text{Na}^+$  content on  $\Psi_w$ . In cultivated rice, which accumulated  $\text{Na}^+$  in leaves at a low level, the  $\Psi_w$  was decreased up to  $-1.5 \text{ MPa}$ .  $\Psi_w$  of *O. rufipogon*, which accumulated  $\text{Na}^+$  in leaves at a high level, tended to decline with the increase of  $\text{Na}^+$  content. However, the relationship between leaf water potential and  $\text{Na}^+$  content was not clear in *O. latifolia*, which absorbed  $\text{Na}^+$  at a high level.

Figure 4 shows the relationship between photosynthetic rate and  $\Psi_w$  in leaves of cultivated and wild rice. The photosynthetic rate of IR28 and SR26B was declined down to about 40% of the maximal values in

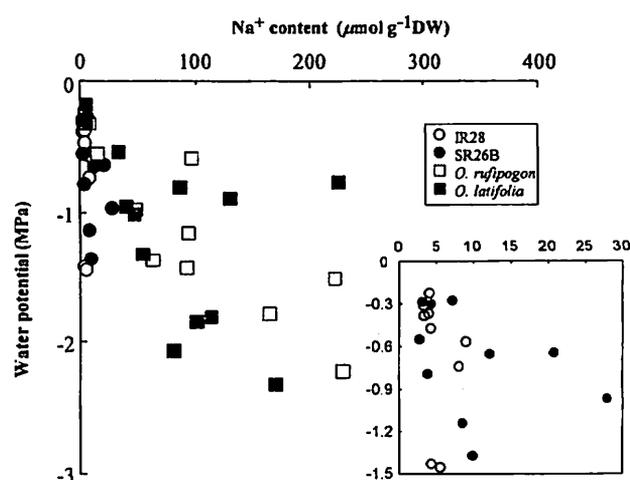


Fig. 3. Relationship between water potential and  $\text{Na}^+$  content in younger fully expanded leaf grown in different NaCl concentrations. Note: Data obtained at low values of  $\text{Na}^+$  content of IRR28 and SR26B are expanded in the inset for clarity.

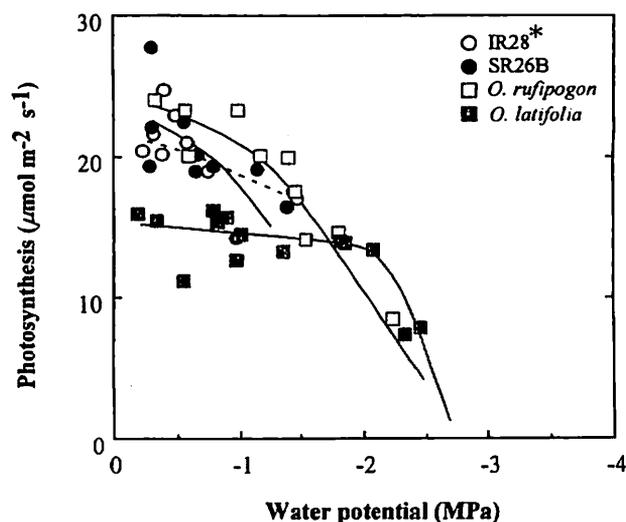


Fig. 4. Relationship between photosynthesis and water potential in rice plants grown in different NaCl concentrations. \*: IR28 was shown by the broken line.

response to the decrease in water potential. At about  $-1.5 \text{ MPa}$  of  $\Psi_w$ , leaves withered and photosynthetic rate could not be measured in those leaves. In *O. rufipogon* photosynthetic rate also decreased as  $\Psi_w$  declined to 35% of the maximal values. However, in *O. latifolia*, photosynthetic rate was 84% of the initial values even when the  $\Psi_w$  was around  $-2.0 \text{ MPa}$ .

The free-proline content in leaves was analyzed as an organic osmolyte (Fig. 5). With NaCl treatment, free-proline accumulation was higher than in the control in the all cultivars except *O. rufipogon*. SR26B accumulated free proline at the highest level, about two times higher than that in the control.

The relationship between the free-proline content and  $\Psi_s$  of the leaves was examined for each variety and

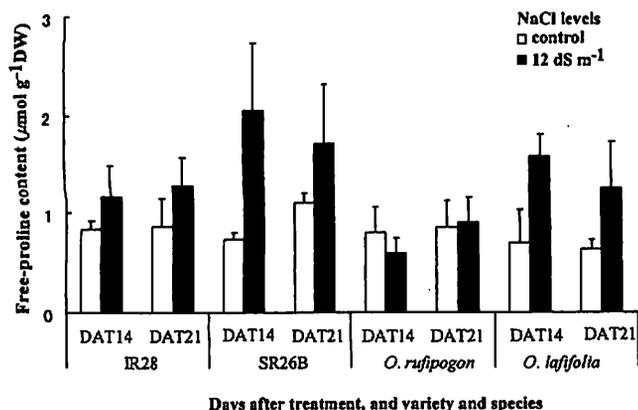


Fig. 5. Effects of NaCl on free-proline content in rice leaves. Data are means of 4 plants  $\pm$  SD. DAT: days after NaCl treatment.

species (Fig. 6). The negative correlations between them were found in the tolerant entries, SR26B and *O. latifolia*. In those species, the  $\Psi_s$  reduced with the increased free-proline content in leaves.

### Discussion

To date, many studies have been carried out to elucidate the physiological responses of rice plants to salinity. *Oryza sativa* L. is considered to be moderately sensitive to salinity (Munns et al., 1999). Although there is a wide range of variation in salt tolerance among the *O. sativa* varieties; SR26B is classified as one of the most tolerant varieties (Gregorio et al., 1997). In the present study the wild *Oryza* species, *O. latifolia*, showed higher resistance to salinity than the salt-tolerant cultivated variety. The survival rate of *O. latifolia* was 50% higher than that of the SR26B (Fig. 1).

Mechanism of salt tolerance in higher plant has been divided into two categories, avoidance and tolerance against salt. In rice, the ability of excluding the sodium ion at exodermis and endodermis (Tsuchiya et al., 1994) is one of the main salt tolerance mechanisms. In the present study, sodium ion contents per dry matter at 7 and 14 DAT were lower in the cultivated varieties (Fig. 2). This result was similar to the study of Tsuchiya et al. (1992) which showed that salt-resistant indica cultivars had a mechanism of sodium exclusion from roots. On the other hand, the wild *Oryza* species, *O. latifolia*, seemed to resist to salt by a mechanism other than  $\text{Na}^+$  exclusion. This wild rice species showed a higher survival rate than the salt-tolerant variety, but accumulated three times more  $\text{Na}^+$  than SR26B at 14 days after the salt stress. In other words, *O. latifolia* is inferior to SR26B in the  $\text{Na}^+$  exclusion.

The photosynthetic rate of rice plants is reduced by increasing  $\text{Na}^+$  content in leaves (Cho et al., 1995) due to stomatal closure followed by decrease in intercellular  $\text{CO}_2$  concentration (Kalaji and Nalbrczyk, 1991; Schwarz and Gale, 1981; Seemann and Sharkey, 1986). The photosynthetic rate in the cultivated varieties and *O. rufipogon* was lowered along with the decrease in  $\Psi_w$

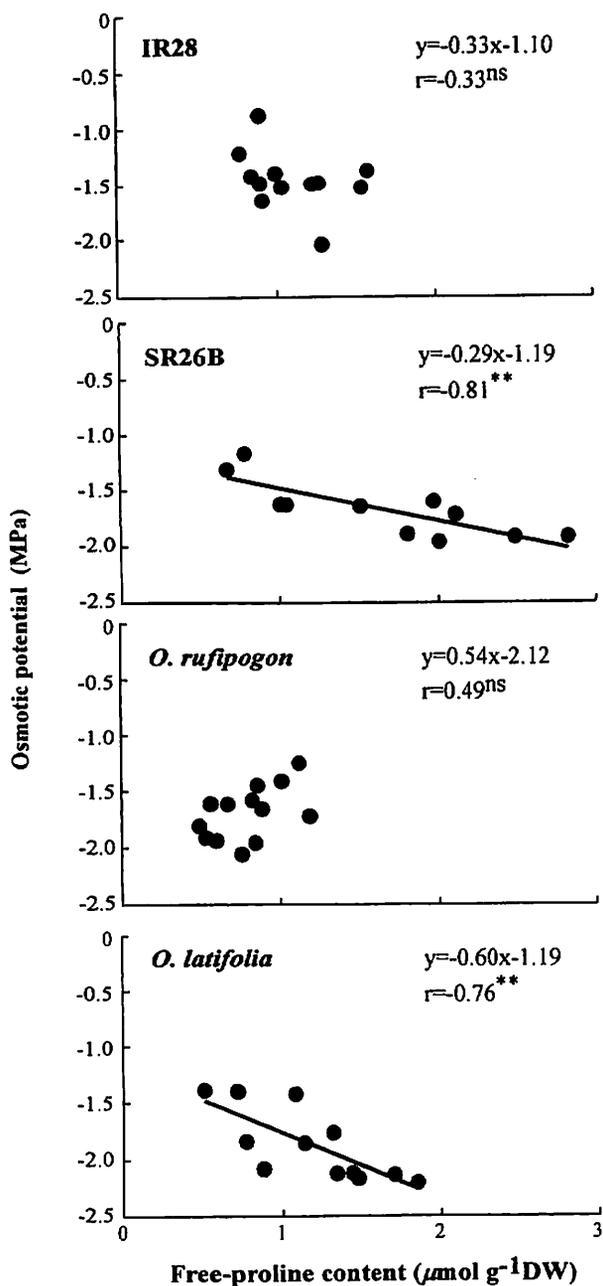


Fig. 6. Relationship between osmotic potential and free-proline content in younger fully expanded leaf. ns and \*\*: not significant and significant at 1% level, respectively.

(Fig. 4), but the rate in *O. latifolia* was stable (around  $12 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) until  $-2.0$  MPa in  $\Psi_w$ . This indicated that *O. latifolia* had mechanism for maintaining the photosynthesis, even if  $\Psi_w$  was decreased.

Plants were subjected to osmotic stress, induced by sodium accumulation inside leaf, peculiarly synthesize and accumulate various organic compounds (saccharides, glycinebetaine, proline etc.) for osmotic adjustment, in order to balance the osmotic gradient of the sodium that accumulates in the vacuole (Stewart et al., 1966; Storey and Wyn Jones, 1977; Greenway and Munns, 1980). However, compatible solutes that plants ac-

cumulated in their leaves differ among species. Moreover, the magnitude of the accumulation of compatible solutes was dependent on its ability for osmotic adjustment. However there are reports that salt tolerant cultivated rice accumulated less free-proline than salt sensitive ones (Lutts et al., 1996). Proline accumulation is one of the most frequently reported modifications induced by salt stress in plants, and cytoplasmic accumulation of proline is thought to be involved in osmotic adjustment of stressed tissues (Kavi Kishor et al., 1995). Thus, we measured the free-proline content in the four different species as a main compatible solute of rice.

In the present study, there was a positive significant correlation (SR26B is  $r=0.81$  and *O. latifolia* is  $r=0.76$ ) between free proline content and osmotic potential in the salt tolerant species. This shows that proline participates in decreasing osmotic potential, at least in these two tolerant species. However, there was a difference among the species in the degree of proline synthesis in response to  $\text{Na}^+$  accumulation. For instance, SR26B accumulates  $\text{Na}^+$  by  $19.6 \mu\text{mol} \cdot \text{gDW}^{-1}$  and synthesizes proline by  $1.4 \mu\text{mol} \cdot \text{gDW}^{-1}$  within 14 days. However, *O. latifolia* accumulates  $\text{Na}^+$  by  $61.9 \mu\text{mol} \cdot \text{gDW}^{-1}$  and synthesizes proline by  $0.9 \mu\text{mol} \cdot \text{gDW}^{-1}$  within the same time period in the leaf. In SR26B, the activity of free-proline synthesis per sodium content was five times faster than in *O. latifolia*. In short, the cultivated rice quickly and sensitively reacts to the lowering of water potential with the increase in the leaf  $\text{Na}^+$  content and seems to have a high ability to form proline. Although, the wild *Oryza* species (*O. latifolia*) contained a smaller amount of proline than cultivated species, the former accumulated larger amounts of  $\text{Na}^+$  in the leaves than the latter. The survival rate under salt stress was higher in *O. latifolia* than in the cultivated rice. This suggested that *O. latifolia* might have some functions of salt tolerance different from SR26B. *O. latifolia* may protect the photosynthetic apparatus, or make the plasmalemma strong against sodium or chloride ions by locally accumulating free-proline. Further investigation is necessary to examine the intriguing traits of salt tolerance in *O. latifolia*.

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