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Overexpression of chloroplast glutamine synthetase conferred salt tolerance in rice

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Introduction

Accumulation of salts in irrigated soil are primary factors depressing yield in crop production, because the major crops are almost universally non-halophytic. Organisms that thrive in hypersaline environments possess specific mechanisms to adjust their internal osmotic status. One such mechanism is the ability to accumulate low molecular weight organic compatible solutes such as sugars, some amino acids and quarternary ammonium compounds, which are believed to be essential for adaptability of plant cells to high salinity. Other mechanisms of adaptation to high salinity are the exclusion of Na^+ from the sodium sensitive sites which has been proposed as a function of an Na^+/H^+ antiporter and Na^+ ATPase. Expression of compatible solutes and heterologous sodium efflux transporters may be a useful approach to improve the salt tolerance of photosynthetic organisms. Indeed, the increase of salt tolerance or water stress tolerance of photosynthetic organisms transformed with genes for synthesis of compatible solutes was demonstrated . In addition to these toxic effects, salt stress also causes the induction of oxidative stress (Gossett *et al*., 1994; Hernandez *et al*., 1995; Burdon *et al*., 1996). Upon salt stress, stomatal closure triggered by abscisic acid (ABA) limits CO_2 supply to the leaf leading to overreduction of the photosynthetic electron transport (ET) chain (Osmond and Grace, 1995). The overreduction of ET chain causes the generation of active oxygen species such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical, and therefore, the enhancement of enzyme activity involved in active oxygen scavenging systems may be a potent strategy to increase salt tolerance (Asada, 1999).

An alternative strategy to cope with oxidative damage under salt stress might be the suppression of active oxygen production. Although the photoprotective role of photorespiration is controversial (Ogren, 1984), photorespiration may function as a possible route for the dissipation of excess light energy or reducing power (Osmond and Grace, 1995; Willekens *et al*., 1997; Brisson *et al*., 1998). Photorespiration is a metabolic pathway in which CO_2 is released by light and is linked to Calvin-Benson cycle through the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Although photorespiration includes many metabolic steps which are performed across chloroplasts, mitochondria and peroxisomes, several studies suggest that the rate-limiting step is the reassimilation of ammonia catalyzed by chloroplastic glutamine synthetase (GS2) (Wallsgrove *et al*., 1987; Hausler *et al*., 1994). Recently, Kozaki and Takeba (1996) have demonstrated that a transgenic tobacco plant overexpressing GS2 had increased

photorespiration capacity and increased tolerance to high light intensity. Therefore, we investigated whether the overexpression of GS2 in rice plants conferred salt resistance.

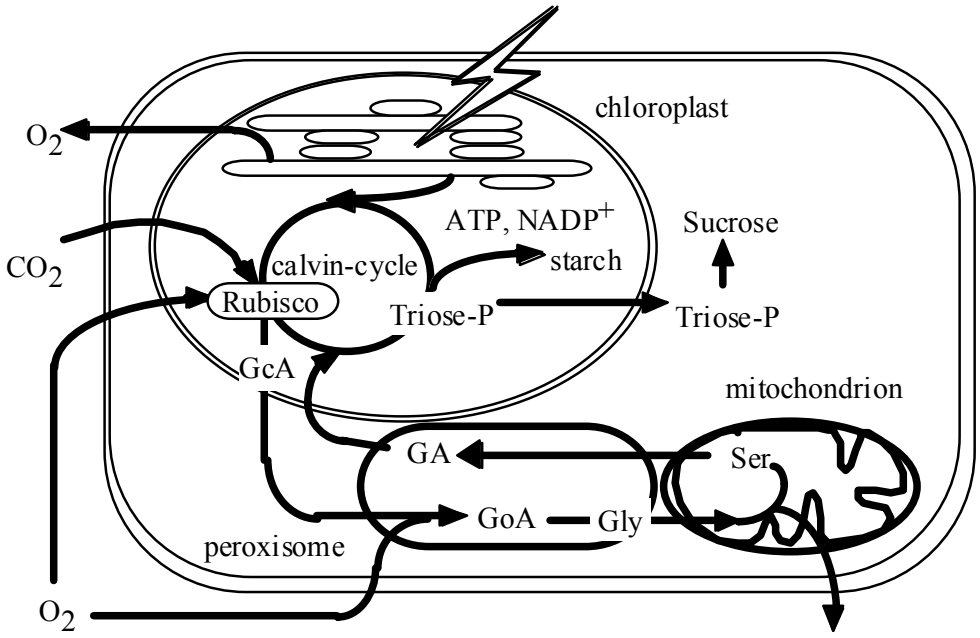

Fig. 1. A schematic model of photorespiration.

Materials and Methods

Rice seedlings were grown hydroponically in 10-fold-diluted Murashige and Skoog (MS) medium under cycles of 16-h light (180 μE m^{-2} s^{-1}) at 28°C and 8-h dark at 25°C in a plant growth chamber. For salt stress, 4-week-old mature seedlings were cultured in fresh 10-fold diluted MS medium containing 150 mM NaCl under the same day/night conditions. The medium was exchanged daily. Cold stress was given by exposing the plants to 18°C under continuous irradiation (150 μE m^{-2} s^{-1}). After an appropriate time, the growth temperature during the light period was changed to 40°C, but that of the dark period was kept at 25°C. Rice GS2 cDNA flanked by catalase first intron was ligated into the binary vector pBI221, and was placed between the cauliflower mosaic virus 35S promoter and nopaline synthetase terminator (Nos-ter) regions. The transformants were selected by using hygromysin and by screening for the presence of inserts using polymerase chain reaction (PCR). Plant homozygous for the introduced gene were selected from R2 seeds and used for subsequent experiments.

Photorespiration activity was estimated from the amount of CO_2 released from a rice leaf in CO_2-free air under continuous irradiation. CO_2 concentration were measured and recorded using an system HCM-1000 and a data acquisition software DA-1000 (Heinz Waltz, Germany). The intact leaf was held in a small climatized measuring cuvette 1010-M equipped with a light unit. CO_2-free air was supplied to the measuring cuvette at a flow rate of 600 m*l*/min which was kept at 28°C. The photorespiration activity was calculated from the difference in CO_2 concentrations after 10-min in darkness and after 30-min in light (500 μE m^{-2} s^{-1}). For the measurements of various photosynthetic activities, leaves approximately 10 cm long, 5 to 15 cm from the tip, were used. The apparent quantum yield in PSII in air and CO_2-free air were consecutively measured using MINI-PAM and HCM-1000. A glass fiber was connected to the upper adapter board. Before the measurements, rice leaves were dark

adapted for 30 min, and then the quantum yield in PSII was recorded under continuous light (200 μE m^{-2} s^{-1}). After 60-min, the quantum yield in PSII was calculated by the equation, ΔF/Fm'=(Fm'-F)/Fm', where, F and Fm' represent the fluorescence levels under irradiation before and after a saturating flash, respectively. Intensity and duration of a flash was 7000 μE m^{-2} s^{-1} and 800 ms, respectively. The maximum quantum yield in PSII was calculated by the equation, (Fm-Fo)/Fm, where Fo and Fm represent the fluorescence levels before and after a saturating flash, respectively.

For ion analysis, leaf extracts were obtained in the same way as soluble protein except the use of distilled water instead of extraction buffer. The ammonium ions were measured using the method of Bewthelot reaction (Hoshida et al., 2000). Equal amount (50 μl) of leaf extracts and phenol reagent (1% phenol, 0.005% sodium nitroprusside) were mixed. After addition of alkaline sodium hypochlorite reagent (in final concentrations of 0.5% NaOH, 0.042% sodium hypochlorite), the mixture was incubated at 37°C for 20 min and the absorbance at 625 nm was determined. Ammonium contents in the samples were calculated from the standard curve of $(NH_4)_2SO_4$. Na^+ ion contents in the leaf extracts were measured with a Shimadzu Personal Ion Analyzer PIA-1000. Extraction of proteins ,SDS-PAGE, and immunoblotting analysis was done as previously described (Hibino *et al*., 2001). An antiserum against the rice GS2 was a generous gift by Prof. G. Takeba (Kozaki and Takeba, 1996).

Results

To overexpress GS2 in rice chloroplasts, we used the full length rice GS2 gene which contains the chloroplast targeting sequence. Two transformants (G39-2 and G39-4) showing high levels of GS2 and another transformants (G241-12 and G241-15) showing yellow leaves were used for the detailed analysis in salt stress experiments. Essentially the same results were obtained within the former and also within the latter group, respectively. Western blotting analysis showed that in a transgenic plant line G39-2, the amount of GS2 was about 1.5 times higher than that in the control plant. In contrast, another transgenic plant line, G241-12, showed almost completely loss of GS2.

The photorespiration capacities of rice plants were calculated by measuring the amount of CO_2 released from the intact leaf in CO_2-free air under continuous irradiation with saturating light conditions (500 μE m^{-2} s^{-1}). There was no difference in the amounts of CO_2 released from the dark-adapted control, G39-2 and G241-12 leaves in CO_2-free air. The photorespiration capacity under irradiation was the highest in G39-2 plants which was about 1.5 times higher than that in the control plants. This suggests that the GS2 enzyme from the transgene was transported into the chloroplast and functioned in the photorespiratory nitrogen assimilation cycle.

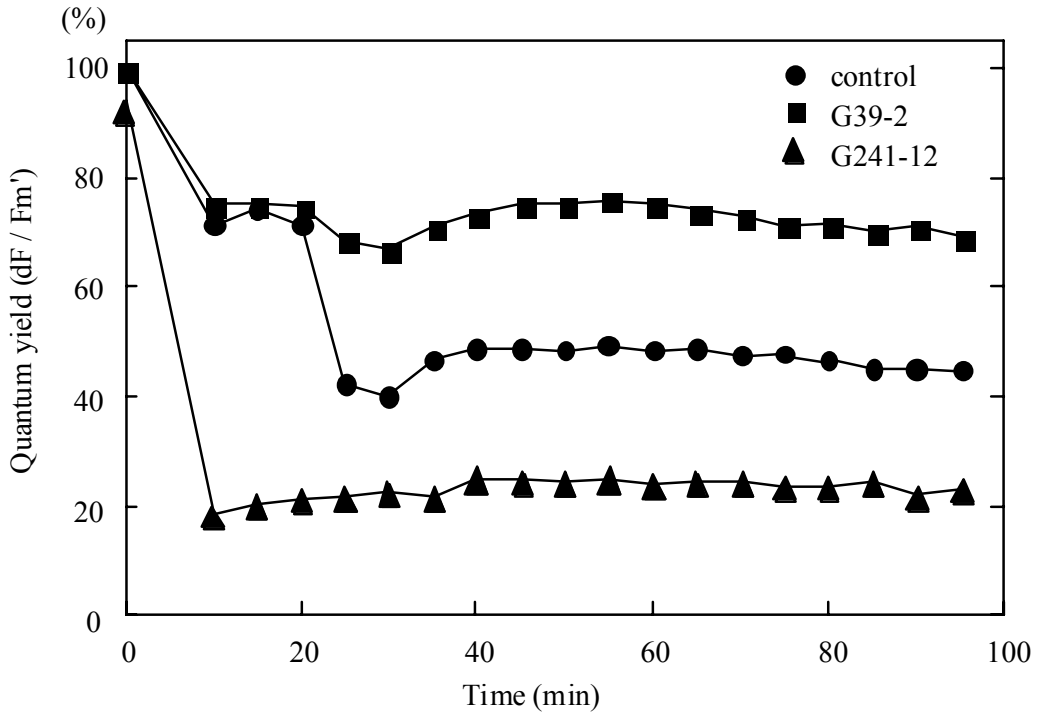

Fig. 2. Effects of salt stress on the quantum yield of PSII.

Next, we examined the effects of overexpression of GS2 on the photosynthetic ET rate in normal and CO_2-free air without salt stress. In both cases, an intact rice leaf was incubated for 30 min in the dark and irradiated at 200 μE m^{-2} s^{-1}. The ET rates are defined as (quantum yield) x (leaf absorbance) x (irradience) x 0.5. In normal air, the quantum yields in PSII of three kinds of rice plants were almost the same. However, the quantum yield of the control plant in CO_2-free air was about half of that in normal air. This residual activity in CO_2-free air could be attributable to the photorespiration and Mehler reaction. The quantum yield of PSII in G241-12 in CO_2-free air was only about 29% of that in normal air, whereas that in G39-2 was about 90% of that in normal air. In both CO_2-free and normal air, the maximum quantum yield in PSII ((Fm-Fo)/Fm) of three kinds of plants were almost the same, indicating that PSII was not damaged (data not shown). These data suggest that if CO_2 was unavailable for Rubisco enzyme due to the salt stress, the ET rates would decrease in the following order, G39-2 > control > G241-12. When the control plants were treated with 150 mM NaCl, the quantum yield in PSII started to decrease at the 7th day of incubation and we could not detect any activity after 2 weeks. However, the PSII quantum yield of G39-2 remained at very high levels and were only lowered by 10% even after 2 weeks of treatment. In contrast, G241-12 rice plants completely lost the activities of ET after 6 days of treatment with 150 mM NaCl. Upon the salt stress, the GS2 contents decreased whereas the GS1 contents remained at a similar level. Thus, overexpression of GS2 conferred the resistance to salt stress, and their resistance was correlated with the photorespiration capacity.

The effects of the overexpression of GS2 on the intracellular NH_4^+ and Na^+ contents in control and transgenic plants were examined. Before NaCl treatment, the intracellular NH_4^+ contents in the control and G39-2 plants were almost the same, but that of G241-12 was 1.6-fold higher (Fig. 3). After 5 days of treatment with 150 mM NaCl, the NH_4^+ contents in control and G39-2 plants were about 2.4 and 1.9-fold higher, respectively, whereas that in G241-12 decreased to about 35%. These results indicate that the overexpression of GS2 reduced the increase of NH_4^+ content at a high salinity. The decrease of NH_4^+ content in

G241-12 upon salt stress was presumably due to the decrease in ET rates. Low ET rates in the G241-12 would produce low amount of NH_3.

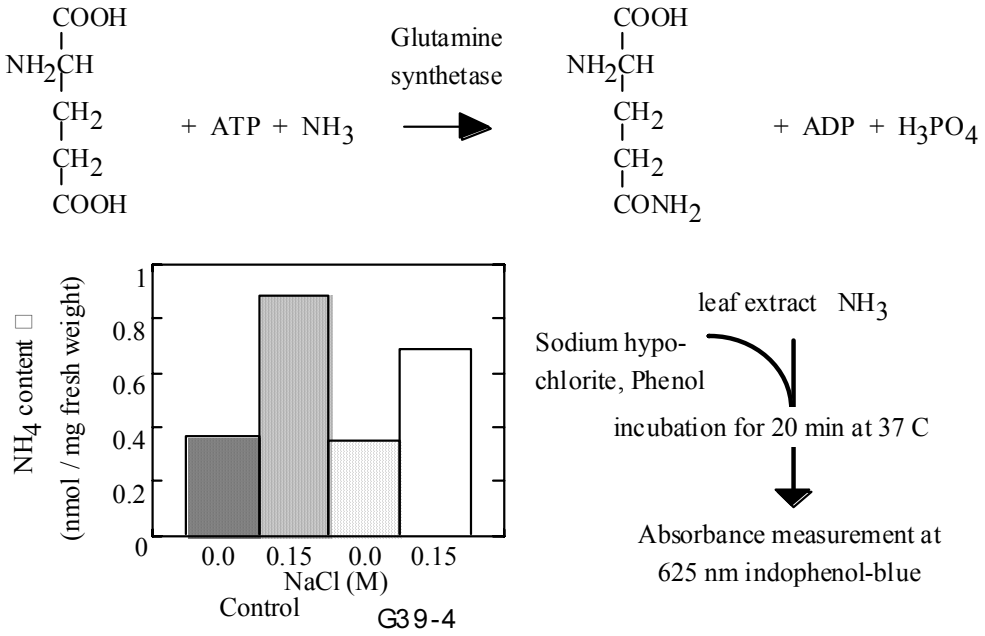

Fig. 3. Effects of salt stress on the ammonium ion accumulation.

The intracellular Na^+ contents in non-stressed plants were similar. After 5 days of treatment with 150 mM NaCl, the Na^+ contents in the control, G39-2, and G241-12 plants were, respectively, about 1.9, 1.0, and 2.8 fold of that in the non-stressed plants. These results indicate that the overexpression of GS2 reduced the increase of intracellular Na^+ content at a high salinity and consequently protects the plants from salt stress. A study using the isonicotinic acid hydrazide (INH), an inhibitor of glycine decarboxylase, also support the above described viewpoint (Hoshida et al. 2000).

Discussion

The results presented here clearly showed that the overproduction of GS2 in rice leaves can increase their photorespiration capacity and improve their salt tolerance. The action mechanisms of GS2 for the protection of rice plants from salt stress may be considered as follows. Upon salt stress, stomatal closure triggered by ABA may limit the CO_2 supply to cells which causes the overreduction of photosynthetic ET chains. Under such conditions, Rubisco operates as an oxygenase and the photorespiration is activated. Ammonium ions thus released may be reassimilated by the increased GS2. The increased photorespiration produces phosphoglycerate and CO_2 which enter Calvin-Benson cycle and consume NADPH and ATP. The consumption of NADPH and ATP may contribute to the decrease in overreduction of the ET chain. These interpretations are similar to those for the increased protection to photoinhibition in transgenic tobacco plants overproducing GS2 (Kozaki and Takeba, 1996).

Ammonium content in the control and G39-2 plants was increased by stress treatment. GS2 might be involved in assimilation of ammonium derived from many sources such as nitrate in the soil, atmospheric N_2, and endogenous amino acids as well as from photorespiration (Ogren, 1984). It is possible that the observed protection against salt stress in transgenic plants overproducing GS2 was due to the increased availability of ammonium derived from the non-photorespiration sources. However, the inhibition experiments by INH showed that

the enhanced tolerance to salt stress was due to the increased reassimilation of ammonium by photorespiration capacity.

Acknowledgements

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