Correlation between Chloroplast Ultrastructure and Chlorophyll Fluorescence Characteristics in the Leaves of Rice (*Oryza sativa* L.) Grown under Salinity

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**Abstract**: The seedlings of *Oryza sativa* L. cv. Nipponbare grown by hydroponic culture for 3 wks were treated with 75, 100, 150 and 200 mM NaCl for 14, 14, 6 and 3 days, respectively, and examined for chloroplast ultrastructure in the region where chlorophyll fluorescence had been recorded. NaCl treatment decreased the ratio of variable to maximum chlorophyll fluorescence yield (Fv/Fm) and caused swelling of thylakoids. The swelling of thylakoids was quantified by the percentage of the length of swollen thylakoids to the total length of thylakoids. This value was increased with increasing NaCl concentration. Although Fv/Fm decreased at all concentrations of NaCl, the minimal fluorescence yield F0 was not increased by the treatment with 75 or 100 mM NaCl. The percentage of the length of swelling was low at 75 and 100 mM NaCl. On the other hand, F0 increased and the swelling of thylakoids was prominent with 150 and 200 mM NaCl treatment. These results suggest that the decrease in Fv/Fm due to the increase in F0 under salt stress correlates with the ultrastructural damage. The decrease in Fv/Fm due to the increase in F0 is expected to be useful as an indicator to evaluate the damage in chloroplasts, especially in thylakoid membranes, under salinity.

**Key words**: Chlorophyll fluorescence, Chloroplast, Rice (*Oryza sativa* L.), Salt stress, Ultrastructure.

An analysis of chlorophyll (Chl) fluorescence emission by using pulse modulated excitation and detection with a pulse amplitude modulation (PAM) instrument has become a powerful tool for quickly and non-intrusively estimating the photosynthetic activity and photoinhibition in leaves (Krause and Weis, 1991; Demmig-Adams and Adams, 1992). In recent years, it has been widely used as an analytical tool for the investigation of photoinhibition under senescence (Wingler et al., 2004) and environmental stresses such as air pollution (Bussotti et al., 2005), water deficit (Araus and Hogan, 1994; Jung, 2004) and salinity (Lutts et al., 1996), because the primary damaging effect of environmental stress might be the impairment of the quinone-binding protein, the D1 protein in the PSII complex (Ohad et al., 1984; Aro et al., 1993). In particular, the maximum photosynthetic quantum yield (Fv/Fm=[Fm−F0]/Fm) is used, because Fv/Fm has been shown to be highly correlated with the quantum yield of net photosynthesis of intact leaves showing various levels of photoinhibition (Bohlhar-Nordenkampf et al., 1989).

Chl fluorescence characteristics under salinity have been well investigated using barley (Belkhodja et al., 1994), rice (Lutts et al., 1996; Dionisio-Sese and Tobita, 2000), wheat (Larcher et al., 1990; Jimenez et al., 1997), celery (Everard et al., 1994), maize (Hasan et al., 2006) and sorghum (Lu and Zhang, 1998; Netondo et al., 2004). Many reports highlighted the effect of salt stress on Fv/Fm. Values of Fv/Fm in the plants grown in a saline condition under relatively low-intensity light showed little change compared with those in the control plants (Belkhodja et al., 1994; Jimenez et al., 1997; Dionisio-Sese and Tobita, 2000). However, a decrease in Fv/Fm was observed with increasing NaCl concentration (Everard et al., 1994; Lutts et al., 1996) and was enhanced by the interaction with other environmental stresses such as high-intensity light and high temperature (Larcher et al., 1990; Jimenez et al., 1997). Chl fluorescence characteristics under salt stress were well studied not only on Fv/Fm but also on other parameters such as efficiency of excitation capture by open reaction centers (Fv'/Fm'), photochemical quenching (qP), non-photochemical quenching (qN) and the actual quantum yield of PSII electron transport (φII) (Li and Ong, 1998; Lu and Zhang, 1998; Dionisio-Sese and Tobita, 2000).

Although the decrease in Fv/Fm is used as an indicator of photoinhibition under salinity, whether the decrease reflects the damage of chloroplasts has not been clarified. Araus and Horgan (1994) suggested in photoinhibited palm trees that the decrease in Fv/Fm involving the increase in F0 accompanies the ultrastructural damage of chloroplasts under drought stress. However, the correlation of the changes in
chloroplast ultrastructure with Chl fluorescence parameters under salinity has not been studied. Such studies are needed to evaluate the use of Chl fluorescence as a probe for chloroplast damage under salinity. Rice is one of the most important crop species and is sensitive to salinity (Yeo et al., 1990). However, Chl fluorescence data available are limited (Dionisio-Sese and Tobita, 2000).

In our previous study, salinity altered chloroplast ultrastructure causing the prominent swelling of thylakoids (Yamane et al., 2004). The ultrastructural changes were induced by ion toxicity or ionic imbalance but not by osmotic effects of salt stress (Yamane et al., 2003). Salt-induced oxidative stress was responsible for the alteration of thylakoid membrane property (Yamane et al., 2004). Since PAM instrument is both non-destructive and non invasive, we can observe the chloroplast ultrastructure in the region where Chl fluorescence parameters were recorded. In addition, we investigated the correlation between chloroplast ultrastructure and Chl fluorescence characteristics under different NaCl concentrations and treatment duration, because the changes in Chl fluorescence characteristics depend on NaCl treatment (Everard et al., 1994; Lutts et al., 1996) and the salt concentration varies with the field. For example, most rice fields in the banks of the Kazibachha River in Bangladesh are moderately (EC_e =4 dS m^{-1}) to highly salinized (16 dS m^{-1} ≥ 150 mM NaCl) (Mondal et al., 2006). To contribute to the screening of salt-tolerant cultivars of rice, we investigated the correlation between the changes in Chl fluorescence parameters and the chloroplast ultrastructure in rice plants under salt stress.

**Materials and Methods**

1. **Plant materials and salinity treatments**

   Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were surface sterilized with a 5% sodium hypochlorite solution for 5 min. After washing several times with distilled water, seeds were imbibed in a beaker containing distilled water in a culture room at 24±2ºC until the appearance of the white tip of the coleoptile.

   After imbibition, the seeds were sown on hydroponic culture according to Mae and Ohira (1981) and grown in a growth chamber under a 14 hr photoperiod (0800-1000) at 250 μmol m^{-2} s^{-1} and 28/20ºC (day/night) for 3 wk.

   The 3-week-old plants were treated with 75, 100, 150, 200 mM NaCl in hydroponic culture for 14, 14, 6 and 3 d, respectively. Nutrient solution without NaCl addition served as the control. After the plants were treated with NaCl, the middle portion of fully expanded uppermost leaves (the 6th leaf blades) was used for following analyses. Eleven plants were used for each treatment and control.

2. **Chl fluorescence measurements**

   Chl fluorescence was measured with a portable Chl fluorometer (PAM-2100, Walz, Effetrich, Germany). The measurements were performed between 0900 and 1400 using eleven plants for each treatment and control. The plants were placed in the dark for 30 min, and then the dark adapted leaves were irradiated by weak modulated measuring beam (2.0 μmol m^{-2} s^{-1}) to determine the minimal fluorescence yield (F_0). Maximum fluorescence yield (F_m') was determined during saturating flash (3000 μmol m^{-2} s^{-1}). The actual fluorescence level (F) was monitored to ensure that it was stable. To obtain the maximal fluorescence yield under illumination (F_m'), the leaf was exposed to a saturating flash during exposure to acting light (210 μmol m^{-2} s^{-1}). To determine the minimal level of fluorescence during illumination (F_0'), we continuously illuminated the leaf with far-red light (750 nm) to rapidly reoxidize the PSI centers. All measurements were conducted at 25ºC.

   The maximal quantum yield of PSII was indicated by F_/F_m', where F_0 = F_m' − F_0. The quantum yield of open PSII was determined by F_/F_m', where F_0 = F_m' − F_0'. The photochemical quenching coefficient (qP) was calculated as (F_m' − F_0')/(F_m' − F_0'). In addition, three chlorophyll fluorescence parameters were estimated to analyze the allocation of the fraction of excitation energy according to Demmig-Adams et al. (1996). They were the fraction of photon energy absorbed by PSI antennae utilized for photosynthetic electron transport, P=qPxF_/F_m'; the fraction of photon energy absorbed by PSI antennae dissipated via thermal energy, D=1−F_/F_m'; and the fraction of excess excitation energy neither dissipated in the PSI antennae nor utilized for photochemistry, Ex=F_/F_m'×(1-qP).

3. **Electron microscopy**

   Electron microscopic studies were made in the region where the Chl fluorescence parameters had been recorded. Small pieces of leaves (2 mm square) were fixed in Karnovski’s fixative (mixture of 4% paraformaldehyde and 5% glutaraldehyde in 50 mM phosphate buffer (pH 7.2)) and post fixed in 2% osmium tetroxide in the same buffer. Samples were dehydrated in a series of graded acetone and propylene oxide and embedded in Spurr’s resin.

   Ultrathin sections (70–90 nm in thickness) were cut with a diamond knife and placed on 200 mesh copper grid. The grids were stained with 2% uranyl acetate for 20 min followed by lead citrate for 5 min. Then the sections were examined on a Hitachi H7500 transmission electron microscope at 100 kV, and photographed with CCD camera (Advanced Microscopy Technique, USA) connected to the electron microscope. Three photographs of chloroplasts per a sample were taken and analyzed for the calculation of percentage in length of swollen thylakoids to total thylakoids. The percentage of swelling was calculated as follows:
the length of swollen thylakoids × 100(%) 

The length of thylakoids was estimated with an Image Analysis program (Image Tool for Windows Version 3.00, UTHSCSA, USA) in three chloroplasts from eleven different plants.

4. Statistic analysis

Data were statistically analyzed using ANOVA followed by Tukey's HSD test (SPSS 14.0; SPSS Chicago, IL, USA). Significant differences were analyzed based on P < 0.05 and P < 0.01. Percentage data were subjected to arc sine transformation prior to statistical analysis.

Results

1. Chlorophyll fluorescence parameters

\( \frac{F_v}{F_m} \) in control plants was around 0.8, whereas the value decreased as rice plants were affected by salt stress. \( \frac{F_v}{F_m} \) in the 75, 100, 150 and 200 mM NaCl treated plants was lower by 14%, 7%, 9% and 22%, respectively compared with that in the control plants (Fig. 1a). Therefore, \( F_v \) in the plants treated with 150 and 200 mM NaCl increased (Fig. 1b). The values were 12% and 24% higher than that of the control plants.
control plants. When the rice plants were affected by salt stress, \( F_m \) decreased under all concentrations of NaCl, while significant decreases were not observed in the plants treated with 100 and 150 mM NaCl (Fig. 1c). The values were lower by 33%, 14%, 11% and 32% in 75, 100, 150 and 200 mM NaCl treatment, respectively compared with that of the control plants.

Figure 2a-c shows the partitioning of absorbed light energy estimated with the model of Demmig-Adams et al. (1996). P decreased and D increased when rice plants were affected by NaCl (Fig. 2a, b). The magnitude of Ex had the tendency to increase when the rice plants were exposed to salt stress, although a significant increase was observed only in the plants treated with 150 mM NaCl (Fig. 2c).

2. Alterations in the chloroplast ultrastructure

Figure 3a-d shows chloroplast ultrastructure in mesophyll cells treated with 0, 100, 150 and 200 mM NaCl, respectively. Chloroplasts in mesophyll cells of
control plants showed no structural distortion and possessed typical well-developed grana and stromal thylakoids (Fig. 3a). However, swollen thylakoids were observed in a few chloroplasts in mesophyll cells treated with 75 and 100 mM NaCl (Fig. 3b arrow). In chloroplasts in mesophyll cells treated with 150 and 200 mM NaCl, swelling was frequently observed (Fig. 3c, d arrows).

Figure 4 shows the percentages of the length of swollen thylakoids. The percentage increased with increasing NaCl concentration, while significant increases were not observed in the plants treated with 75, 100, and 150 mM NaCl (Fig. 4). The values were 1.4, 2.1, 6.8 and 17.2% with 75, 100, 150 and 200 mM NaCl treatment, respectively.

**Discussion**

There are some reports that $F_v/F_m$ is unaffected by salt-stress at relatively low-intensity light (approximately one-fifth of full sunlight) (Morales et al., 1992; Belkhodja et al., 1994; Jimenez et al., 1997; Dionisio-Sese and Tobita, 2000). In the present study, plants were grown in the light at 250 μmol m$^{-2}$ s$^{-1}$, which was approximately one-fifth of full sunlight. Thus, in the plants treated with 75 and 100 mM NaCl relatively long exposure (14 days) was needed until $F_v/F_m$ decreased (Fig. 1a). $F_v/F_m$ may be less affected by salt stress when plants are grown under low-intensity light (Jimenez et
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by lipid peroxidation caused by reactive oxygen species. The swelling of thylakoids under salinity is induced (Fig. 1a). In examining changes in \( F_v/F_m \), it is important to distinguish between the increase in \( F_0 \) and the decrease in \( F_v \) (Bolhar-Nordenkampf et al., 1989). The \( F_0 \) level is theoretically the fluorescence emission when all reaction centers are open and the photochemical quenching is maximal. An increase in \( F_0 \) is characteristic of destruction of PSII reaction centers or inhibition of transfer of excitation energy from the antennae to the reaction centers (Bolhar-Nordenkampf et al., 1989). Especially, the increase in \( F_0 \) observed under environmental stresses such as high irradiance and drought is due to the destruction of reaction centers (Araus and Hogan, 1994; La Porta et al., 2004). On the other hand, a decrease in \( F_v \) caused by a decrease in \( F_m \) may indicate an increase in nonphotochemical quenching (Bolhar-Nordenkampf et al., 1989). Photoinhibition produces both of these changes (Baker and Horton, 1988). However, a decrease in \( F_v/F_m \) due to a decrease in \( F_m \) should be termed photoprotection (Demmig-Adams and Adams, 1992) because it is thought to provide a harmless means of disposal of excess excitation. In the present study, NaCl treatment caused the decrease in \( F_v/F_m \) (Fig. 1a). However, the response of \( F_0 \) and \( F_v \) was different with NaCl concentrations. Although decreases in \( F_m \) were observed at all concentrations of NaCl (Fig. 1c), the value of \( F_0 \) did not increase by the treatment with 75 and 100 mM NaCl (Fig. 1b). The percentage of swollen thylakoid length was low under 75 and 100 mM NaCl (Fig. 4). These results suggest that the decrease in \( F_v/F_m \) observed under 75 and 100 mM NaCl resulted from photoprotection. On the other hand, in the rice plants treated with 150 and 200 mM NaCl, \( F_0 \) increased (Fig. 1b) and the swelling of thylakoids was high (Fig. 4), whereas Ex did not significantly increase except in 150 mM NaCl treatment compared with that in the control plants (Fig. 2c). The thylakoid swelling under salinity is not related to the accumulation of Ex in PSII. In fact, it was suggested that the thylakoid swelling is induced by hydrogen peroxide and hydroxyl radical but not by \(^1\)O\(_2\) (Yamane et al., 2004).

In conclusion, the decrease in \( F_v/F_m \) due to the increase in \( F_0 \) correlates with the swelling of thylakoids, and the decrease in \( F_v/F_m \) without the increase in \( F_0 \) is suggested to be resulted from photoprotection. These results also suggest that the decrease in \( F_v/F_m \) due to the increase in \( F_0 \) can be used as a tool for evaluating the damage of chloroplasts under salinity and the screening of cultivars for salinity tolerance.

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