Photosystem II photochemistry, photoinhibition, and the xanthophyll cycle in heat-stressed rice leaves

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A B S T R A C T

To investigate how high light affects the responses of photosynthesis to heat stress, the effects of high temperature (25–42.5 °C) either in the dark or in the light (1000 μmol m−2 s−1) on photosystem II (PSII) photochemistry and the xanthophyll cycle were investigated in rice plants. At temperatures higher than 35 °C, there was a decrease in the CO2 assimilation rate, and this decrease was greater in the light than in the dark. The maximal efficiency of PSII photochemistry (Fv/Fm) showed no significant change in the dark, but did show a significant decrease in the light. In addition, there was an increase in non-photochemical quenching (NPQ) and this increase was greater in the light than in the dark. Furthermore, the de-epoxidation status of the xanthophyll cycle increased significantly with increasing temperature in the light. Compared to the control leaves, the dithiothreitol-fed leaves showed a greater decrease in Fv/Fm but a very small increase in NPQ and de-epoxidation status of the xanthophyll cycle at temperatures higher than 35 °C. On the other hand, the ascorbate-fed leaves showed less of a decrease in Fv/Fm but a greater increase in NPQ and the de-epoxidation status of the xanthophyll cycle. Ascorbate peroxidase and glutathione reductase activities in leaves and chloroplasts were enhanced and this enhancement was greater in the light than in the dark. Heat stress had no significant effect on the contents of ascorbate and glutathione in leaves and chloroplasts in the dark, but led to an increase in the contents of reduced ascorbate and glutathione in leaves and chloroplasts in the light at the temperatures higher than 35 °C. Our results suggest that the xanthophyll cycle plays an important role in protecting PSII against heat-induced photoinhibition by an increase in the ascorbate pool in the chloroplast.

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Introduction

Heat stress is a major environmental factor that limits plant growth and productivity (Berry and Björkman, 1980; Lobell and Asner, 2003). Among the physiological processes in plants, photosynthesis is one of the most sensitive processes to heat stress, with inhibition occurring at temperatures only slightly higher than those optimal for growth (Allakhverdiev et al., 2008; Yamamoto et al., 2008). Although many studies have investigated the responses of photosynthesis to heat stress, the mechanisms of the inhibition of photosynthesis under heat stress remain unclear (Allakhverdiev et al., 2008; Yamamoto et al., 2008). One proposed mechanism is that heat stress inhibits the ribulose bisphosphate (RuBP) carboxylation rate due to the heat-induced decrease in the activase activity, which regulates the Rubisco activation state (Salvucci and Crafts-Brandner, 2004; Kurek et al., 2007). Another proposed mechanism is that heat stress inhibits photosynthetic electron transport and the decrease in Rubisco activation state under heat stress is a regulated response resulting from other limitations, including electron transport (Cen and Sage, 2005; Makino and Sage, 2007). In addition, photosystem II (PSII) has long been considered the most heat sensitive component of the photosynthetic apparatus (Berry and Björkman, 1980; Havaux, 1996). However, recent studies have suggested that heat stress does not cause serious PSII damage, but inhibits the repair of PSII (Sharkey, 2005; Allakhverdiev et al., 2008).

It should be noted that most studies on the effects of heat stress on photosynthesis have been carried out in the dark (Allakhverdiev et al., 2008; Yamamoto et al., 2008). Under natural conditions, and in particular in the daytime of the summer season where temperatures often rise to 30–40 °C and light intensity can reach 1000–2000 μmol m−2 s−1, light and heat stresses are superimposed (Yamamoto et al., 2008). Indeed, enhanced thermal injury to photosynthesis by high light is observed under such conditions (Al-Khatib et al., 2008; Yamamoto et al., 2008).
and Paulsen, 1989; Ohira et al., 2005). However, it is unknown how heat stress under high light conditions affects PSII photochemistry and dissipation of excess light.

Rice is one of the world’s most important cereals and is grown mainly in tropical and subtropical zones. Global warming may increase the instability of rice yields even in temperate regions (Horie et al., 1996). It has been reported that high temperature may cause deleterious effects on the rice yield (Peng et al., 2004). Thus, studies concerning physiological effects of high temperature on photosynthesis should be of significance and are indispensable.

In this study, we investigated the effects of heat stress on CO₂ assimilation, Rubisco activation state, PSII photochemistry and the xanthophyll cycle in rice plants in the dark and under high light conditions. Our results show that, although heat-induced photoinhibition was aggravated by high light, the xanthophyll cycle was enhanced through an increase in the ascorbate pool in the chloroplast in order to protect the photosynthetic apparatus from further damage.

**Materials and methods**

**Plant material and growth conditions**

Rice (Oryza sativa, L. cv. 9311) plants were grown in a greenhouse at 25 ± 1 °C with 70–80% relative humidity under natural sunlight conditions. The maximal irradiance at noon was about 1000 μmol m⁻² s⁻¹. Plants were grown in watered soil with plastic seedling cases (25 cm × 20 cm × 10 cm). The soil was sandy loam that contained organic matter at 2.6% and available N–P–K at 112, 88.9 and 36.2 mg kg⁻¹, respectively. Each plastic seedling case contained approximately 40 rice plants.

**Heat stress treatments**

The rice seedlings were subjected after growth for one month to various experiments. To study the effects of heat stress on photosynthetic physiology and biochemical parameters, whole plants were exposed to various temperatures (25–42.5 °C) for 1 h in the dark. In addition, to examine the role of high light in the responses of photosynthesis to heat stress, heat treatments on the whole plants were also imposed in the light (1000 μmol m⁻² s⁻¹). For the heat treatment in detached leaves, the leaf disc (ca. 2 cm²) was directly placed into the smooth bottom of a small hole (5.5 cm in height × 3 cm in diameter) in a block of brass, of which the temperature was regulated by circulation of water from a thermostated water bath. At the same time, the up-face of the leaf disc was pressed directly by a block of glass so that the water evaporation of the leaf could be prevented and the heat equilibrium between the leaf disc and the brass block could be reached immediately. The leaf discs were exposed to different elevated temperatures for 20 min under high light (1000 μmol m⁻² s⁻¹). All measurements of physiological and biochemical parameters were carried out on the youngest fully expanded leaves.

**Analysis of gas exchange**

Measurements of net photosynthetic gas exchange were performed on a fully expanded attached leaf of rice seedlings using an open system (Ciras-1, PP systems, UK). After exposure to different elevated temperatures for 1 h, the whole plants were returned to 25 °C and gas exchange was then analyzed. The light-saturating photosynthetic rate was made at a CO₂ concentration of 360 μL L⁻¹ and at a temperature of 25 °C with a relative humidity 80% and saturating light (1200 μmol m⁻² s⁻¹).

**Determination of light-dependent activation of Rubisco**

To determine light-dependent activation of Rubisco, whole plants were irradiated with saturating light 1200 μmol m⁻² s⁻¹ for 10 min at 25 °C to promote full activation of Rubisco following 1 h high temperature treatment. After illuminaion, leaf tissues were harvested immediately for the determination of the initial and total Rubisco activities. Determination of light-dependent activation of Rubisco was followed essentially according to Feller et al. (1998). The activation state of Rubisco (Perchorowicz et al., 1981) was calculated as the relative ratio of initial to total Rubisco activities.

**Measurements of chlorophyll fluorescence**

Chlorophyll fluorescence was measured at room temperature with a portable fluorometer (PAM-2000, Walz, Germany) after the leaves had been dark-adapted for 10 min. The experimental protocol of Genty et al. (1989) was followed.

The minimal fluorescence level (Fₒ) with all PSII reaction centers open was measured by the measuring modulated light, which was sufficiently low (<0.1 μmol m⁻² s⁻¹) not to induce any significant variable fluorescence. The maximal fluorescence level (Fₘ) with all PSII reaction centers closed were determined by a 0.8 s saturating pulse at 8000 μmol m⁻² s⁻¹ in dark-adapted leaves. Then, the leaf was continuously illuminated with a white actinic light (1000 μmol m⁻² s⁻¹). After about 5 min, the steady-state value of fluorescence (Fₛ) was thereafter recorded and a second saturating pulse at 8000 μmol m⁻² s⁻¹ was imposed to determine maximal fluorescence level in the light-adapted state (Fₘ′). The actinic light was removed and the minimal fluorescence level in the light-adapted state (Fₒ′) was determined by illuminating the leaf with a 3 s pulse of far-red light. All measurements of Fₘ, Fₒ and Fₛ were performed with the measuring beam set to a frequency of 600 Hz, whereas all measurements of Fₘ′ and Fₒ′ were performed with the measuring beam automatically switching to 20 kHz during the saturating flash.

Using both light and dark fluorescence parameters, we calculated: (1) the maximal efficiency of PSII photochemistry in the dark-adapted state, Fₘ/Fₘ′ = (Fₘ′ – Fₒ)/Fₘ, (2) the photochemical quenching coefficient, qP = (Fₘ′ – Fₒ)/Fₘ, (3) non-photochemical quenching, NPQ = (Fₘ/Fₘ′) – 1 (van Kooten and Snel, 1990), (4) the actual PSII efficiency in the light-adapted state, Φₚₛₛ = (Fₘ′ – Fₒ)/Fₘ′ (Genty et al., 1989). Fluorescence nomenclature was according to van Kooten and Snel (1990).

**Assays of the activities of the enzymes involved in ascorbate-glutathione cycle**

Antioxidant enzyme assays were performed essentially according to Jiang and Zhang (2001). Soluble proteins were extracted in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP), with addition of 1 mM ascorbate in the case of ascorbate peroxidase assay. The homogenate was centrifuged at 15,000 × g for 30 min at 4 °C and the supernatant was used for the following enzyme assays.

Ascorbate peroxidase (APX, EC1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM⁻¹ cm⁻¹) and correction was done for the low, non-enzymatic oxidation of ascorbate by H₂O₂ (Nakanod and Asada, 1981). Glutathione reductase (GR, EC1.6.4.2) activity was assayed following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM⁻¹ cm⁻¹) and corrections were made for the background absorbance at 340 nM, without NADPH (Schaadle and Bassham, 1977). Dehydroascorbate reductase (DHAR, EC1.8.5.1) activity was measured by monitoring increases in absorbance at 265 nm (extinction coefficient 14 mM⁻¹ cm⁻¹) due to the for-
mation of ascorbate and the rate of reaction was corrected for the non-enzymatic reduction of dehydroascorbate by reduced glutathione (Hossain and Asada, 1984). Monodehydroascorbate reductase (MDHAR, EC1.6.5.4) activity was measured by monitoring decreases in absorbance at 340 nm (extinction coefficient 2.8 mM$^{-1}$ cm$^{-1}$) due to the oxidation of NADPH (Arrigoni et al., 1981).

Assays of ascorbate and glutathione

ASC (reduced ascorbate) content was measured according to Arakawa et al. (1981). Total glutathione was determined by an enzymatic cycling assay method according to Griffiths (1980). GSSG (oxidized glutathione) was determined after removal of GSH (reduced glutathione) by 2-vinylpyridine derivatization. GSH was determined by subtraction of GSSG from the total glutathione content.

Isolation of chloroplasts

Intact chloroplasts were isolated by centrifugation on a Percoll density gradient according to the protocol of Mullet and Chua (1983). The percentage of intact chloroplasts was determined by measuring ferricyanide photoreduction before and after osmotic shock. In this study, the percentage of intact chloroplasts was about 85%.

Ascorbate and dithiothreitol feeding

Leaves were excised at the petiole with a razor blade under water to embolism. The detached leaves were placed into 1 ml of 10 mM ascorbic acid, or 5 mM dithiothreitol, or water (control) and incubated in the dark at 25 °C for 2 h.

Pigment and protein analyses

Leaf samples were taken and immediately frozen in liquid nitrogen. Leaf samples were extracted in ice-cold 100% acetone and the pigments extracts were filtered through a 0.45 μm membrane filter. Pigments were separated and quantified by HPLC essentially as described by Thayer and Björkman (1990). Protein content was determined according to the method of Bradford (1976).

Results

CO$_2$ assimilation and Rubisco activase-mediated activation of Rubisco

Fig. 1A shows the effects of high temperature on CO$_2$ assimilation in rice seedlings. When temperatures were higher than 35 °C, the CO$_2$ assimilation rate decreased significantly both in the dark and in the light. However, the decrease in the CO$_2$ assimilation rate was greater in the light than in the dark. These results indicate that the sensitivity of CO$_2$ assimilation to high temperature was significantly increased when high temperature treatment was imposed in the light.

Since it has been reported that Rubisco activase is very sensitive to high temperature and plays an important role in limiting photosynthesis at high temperature (Feller et al., 1998; Salvucci and Crafts-Brandner, 2004), we examined the effects of high temperature on the activity of Rubisco activase in the dark and in the light. In this study, we observed that the activity of fully carbamylated Rubisco, i.e. total Rubisco activity, was not affected in the temperature range between 25 and 42.5 °C either in the dark or in the light (data not shown). On the other hand, the initial Rubisco activity clearly decreased with increasing temperature in the dark and in the light. However, a greater decrease in CO$_2$ assimilation induced by high temperature in the light than in the dark was associated with Rubisco activase activation. 

PSII photochemistry

To investigate whether the greater decrease in CO$_2$ assimilation induced by high temperature in the light than in the dark was associated with PSII photochemistry, we examined the effects of high temperature on $F_v/F_m$, $\Phi_{PSII}$, $q_p$, and NPQ in the dark and in the light. Fig. 2 shows that there was no significant change in $F_v/F_m$ when high temperature treatment was imposed in the dark. However, $F_v/F_m$ decreased significantly at temperatures higher than 35 °C when high temperature treatments were imposed in the light. Heat stress caused a decrease in $\Phi_{PSII}$ at temperatures higher than 35 °C and the decrease was much greater in the light than in the dark. There was a decrease in $q_p$ at temperatures higher than 35 °C, but no significant difference in $q_p$ was observed between the high temperature treatments in the dark and in the light. NPQ increased significantly when the temperature was higher than 35 °C and the increase was greater in the light than in the dark.
Fig. 2. Effects of heat stress on the maximal efficiency of PSII photochemistry ($F_v/F_m$, A), the actual PSII efficiency ($\Phi_{PSII}$, B), the photochemical quenching coefficient ($q_p$, C), and non-photochemical quenching (NPQ, D) in rice plants. Seedlings grown at 25 °C in the greenhouse were exposed to different temperatures 25, 30, 35, 37.5, 40 or 42.5 °C in the chambers for 1 h in the dark and in the light (1000 μmol m$^{-2}$ s$^{-1}$), respectively. The values are mean ± SE of three independent experiments.

The xanthophyll cycle

The greater increase in NPQ induced by high temperature in the light may be associated with a greater increase in the de-epoxidation of the pigment interconversion within the xanthophyll cycle. To assess this possibility, we examined the changes in the contents of violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z) during high temperature in the dark and in the light (Fig. 3). During heat stress, there was no significant change in the content of V in the dark, but we observed a decrease in the light at temperatures higher than 35 °C. No significant changes were observed in the content of A when heat stress was performed either in the dark or in the light. The content of Z was very low in the dark and increased significantly in the light at temperatures higher than 35 °C. The extent of the de-epoxidation of the pigment interconversion within the

Fig. 3. Effects of heat stress on the contents of violaxanthin (A), antheraxanthin (B), zeaxanthin (C), and the $(A+Z)/(V+V+2A+2Z)$ ratio (D) in rice plants. Seedlings grown at 25 °C in the greenhouse were exposed to different temperatures 25, 30, 35, 37.5, 40 or 42.5 °C in the chambers for 1 h in the dark and in the light (1000 μmol m$^{-2}$ s$^{-1}$), respectively. The values are mean ± SE of three independent experiments.
Dithiothreitol is a widely used inhibitor of violaxanthin de-epoxidase and ascorbate is a co-substrate for violaxanthin de-epoxidase (Xu et al., 2000; Jahns et al., 2009). The effects of dithiothreitol and ascorbate on the xanthophyll cycle and NPQ under heat stress indicate that dithiothreitol and ascorbate exist mainly in their reduced status after the detached leaves were fed with them.

### Ascorbate content

To examine whether the enhanced xanthophyll cycle metabolism under heat stress is associated with an increase in the ascorbate content, we also investigated the changes in the ASC content during high temperature treatment in the dark and in the light (Fig. 5). When high temperature treatment was performed in the dark, there was no significant change in the ASC content, either in leaves or in isolated chloroplasts. However, when high temperature treatment was performed in the light, the ASC content in leaves and in isolated chloroplasts increased with increasing temperature at temperatures higher than 35 °C. The changes in the GSH content were similar to these in the ASC content (Fig. 5).

Since the ascorbate pool in the chloroplast is regulated by ascorbate-glutathione cycle and four enzymes, i.e. APX, GR, DHAR, and MDHAR are involved in this cycle (Noctor and Foyer, 1998; Asada, 1999, 2006), we examined the changes in the activities of these four enzymes during heat stress in the dark and in the light. Fig. 6 shows that there was an increase in the activities of APX and GR in leaves and in isolated chloroplasts with increasing temperature when temperature was higher than 35 °C. The increase was greater in the light than in the dark. However, high temperature had no effects on the activities of DHAR and MDHAR, either in the dark or in the light.

### Discussion

It is well known that heat stress often inhibits photosynthesis in higher plants. Under natural conditions, plants are often subjected to heat stress and high light simultaneously during the daytime in the summer season. However, most studies on the response of photosynthesis have been performed in the dark (Allakhverdiev et al., 2008; Yamamoto et al., 2008). Although it has been reported that inhibition of photosynthesis induced by heat stress was enhanced when heat stress was imposed by high light (Al-Khatib and Paulsen, 1989), it is unclear how photosynthesis, and in particular PSII photochemistry and the dissipation of excess light, respond to heat stress and high light conditions simultaneously during the daytime.

In this study, our results show that there was no decrease in Fv/Fm when heat stress was imposed in the dark, but a significant decrease in Fv/Fm was observed when heat stress was performed...
Fig. 5. Effects of heat stress on the contents of ASC and GSH in leaves (A and B) and in chloroplasts (C and D), respectively, in rice plants. Seedlings grown at 25 °C in the greenhouse were exposed to different temperatures 25, 30, 35, 37.5, 40 or 42.5 °C in the chambers for 1 h in the dark and in the light (1000 μmol m⁻² s⁻¹), respectively. The values are mean ± SE of three independent experiments.

under high light conditions (Fig. 2). Obviously, the greater decrease in CO₂ assimilation rate induced by heat stress under high light was associated with a greater decrease in Fv/Fm, since there was no significant difference in the Rubisco activation state between heat treatments imposed in the dark and in the light (Fig. 1). These results suggest that high light has profound effects on the responses of CO₂ assimilation rate and PSII to heat stress.

Our results show that heat stress alone had no effect on Fv/Fm but resulted in a decrease in CO₂ assimilation rate (Figs. 1 and 2). A decrease in the CO₂ assimilation rate accompanied by no decrease in Fv/Fm induced by heat stress can potentially expose the plants to excess excitation energy under light conditions, which, if not safely dissipated, may result in photodamage to PSII because of an overreduction of reaction centers (Demmig-Adams and Adams, 1992, 2006). Excess excitation energy can be harmlessly dissipated in the antennae complexes of PSII as heat through a process that involves the xanthophyll cycle and a low thylakoid pH. The xanthophyll cycle pigments zeaxanthin (Z) and antheraxanthin (A) are formed from violaxanthin (V) under conditions of excess excitation energy and are both thought to be involved in the photoprotective dissipation process (Gilmore, 1997; Demmig-Adams and Adams, 2006). Our results show that heat stress induced a greater increase in both NPQ and de-epoxidation status of the xanthophyll cycle in the light than in the dark (Fig. 3). The inhibition of violaxanthin de-epoxidase resulted in an increase in the sensitivity of PSII to photoinhibition under stress (Fig. 4). Thus, the results in this study suggest that the xanthophyll cycle plays an important role in dissipating excess light in order to avoid possible photodamage to PSII when heat stress was imposed under high light conditions.

Our results show that, compared to the control leaves, the artificially increased ASC led to a decrease in the sensitivity of PSII to heat-induced photoinhibition and a greater increase in NPQ and the (Z+A)/(V+Z+A) ratio under heat stress (Fig. 4). Moreover, our results show that heat stress induced a significant increase in the content of ASC in the chloroplast in the light (Fig. 5). It has been reported that ascorbate is a co-substrate for violaxanthin de-epoxidase, and artificially increased ascorbate content results in an increase in Z formation (Leipner et al., 2000; Jahns et al., 2009). Therefore, our results suggest that the enhanced xanthophyll cycle under heat stress is associated with an increase in ASC content in the chloroplast.

The results in this study suggest that the increase in the content of ASC in the chloroplast may be due to the increase in the activities of APX and GR.

It has been shown that enhanced chloroplastic GR activity in transgenic plants results in increased protection against oxidative stress (Broadbent et al., 1995; Foyer et al., 1995; Pilon-Smit et al., 2000). In addition, transgenic plants over-expressing superoxide dismutase (SOD) and APX were found to be more tolerant than wild-type plants to a combination of temperature and high light (Allen, 1995; Yabuta et al., 2002). Furthermore, ascorbate and glutathione are the two major soluble antioxidants in the chloroplast and have another photoprotective function (Noctor and Foyer, 1998). Evidently, the enhanced activities of GR and APX, concomitant with the enhanced content of ASC and GSH observed in this study could help to quench reactive oxygen species, thus protect...
Fig. 6. Effects of heat stress on the activities of APX, GR, DHAR, and MDHAR in leaves (A–D) and in chloroplasts (E, F, G, and H), respectively, in rice plants. Seedlings grown at 25°C in the greenhouse were exposed to different temperatures 25, 30, 35, 37.5, 40 or 42.5°C in the chambers for 1 h in the dark and in the light (1000 μmol m⁻² s⁻¹), respectively. The values are mean ± SE of three independent experiments.

Previous studies have also reported that heat stress either in the dark or in the light induced enhanced APX and GR activities (Gong et al., 2001; Dash and Mohanty, 2002; Kreslavski et al., 2008). It is not clear how heat stress induces enhanced activities of GR and APX, as observed in this study. Presumably, enhanced activities of these enzymes could be due to the increased levels of the steady-state transcripts and proteins of these enzymes induced by heat stress, since it has been reported that the steady-state transcript and protein levels of many ROS-scavenging enzymes were found to be elevated by heat stress (Rainwater et al., 1996; Sato et al., 2001; Rizhsky et al., 2002; Vacca et al., 2004).

It has been shown that heat stress causes the generation of reactive oxygen species (ROS) and injures cell membranes and proteins (Larkindale and Knight, 2002). High light has the potential to enhance the production of ROS in cells and cause oxidative damage to chloroplasts (Niyogi, 1999; Li et al., 2009). Thus, high light could enhance ROS-mediated damage during heat stress. In this study, we observed that heat stress induced an accumulation of O₂⁻ and H₂O₂ either in the dark or in the light and accumulation of O₂⁻ and H₂O₂ was greater in chloroplasts in the light than in the dark under heat stress (data not shown). Although the enhanced xanthophyll cycle played an important role in dissipating excess excitation energy under heat stress and high light conditions, it seems that such an enhancement of the xanthophyll cycle is not enough to dissipate all excess excitation energy, as we still observed an accumulation of O₂⁻ and H₂O₂.

In conclusion, our results show that high light had a profound effect on CO₂ assimilation and PSII photochemistry under heat stress and that heat stress led to increased sensitivity of PSII to high light. Our results suggest that the xanthophyll cycle plays an important role in protecting PSII against heat-induced photoinhibition by an increase in the ascorbate pool in the chloroplast.
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