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Photosynthetic Characteristics of Rice Leaves Aged under Different Irradiances from Full Expansion through Senescence[1]

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ABSTRACT

Effects of irradiance on photosynthetic characteristics were examined in senescent leaves of rice (*Oryza sativa* L.). Two irradiance treatments (100 and 20% natural sunlight) were imposed after the full expansion of the 13th leaf through senescence. The photosynthetic rate was measured as a function of intercellular CO_2 pressure with a gas-exchange system. The amounts of cytochrome *f*, coupling factor 1, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and chlorophyll were determined. The coupling factor 1 and cytochrome *f* contents decreased rapidly during senescence, and their rates of decrease were much faster from the 20% sunlight treatment than from the full sunlight treatment. These changes were well correlated with those in the photosynthetic rate at CO_2 pressure = 600 microbars, but not with those under the ambient air condition (350 microbars CO_2) and 200 microbars CO_2. This suggested that the amounts of coupling factor 1 and cytochrome *f* from the full sunlight treatment cannot be limiting factors for the photosynthetic rate at ambient air conditions. The Rubisco content also decreased during senescence, but its decrease from the 20% sunlight treatment was appreciably retarded. However, this difference was not reflected in the photosynthetic rates at the ambient and 200 microbars CO_2. This implied that *in vivo* Rubisco activity may be regulated in the senescent leaves from the 20% sunlight treatment. The chlorophyll content decreased most slowly. In the 20% sunlight treatment, it remained apparently constant with a decline in chlorophyll *a*/*b* ratio. These photosynthetic characteristics of the senescent rice leaves under low irradiance were discussed in relation to acclimation of shade plants.

Leaf photosynthesis decreases during senescence. Various attempts have been made to elucidate the mechanisms of decrease in photosynthesis during senescence. Several quantitative analyses indicated that decrease in photosynthesis under ambient air conditions can be predicted from changes in the amounts and kinetics of Rubisco (5, 23). Electron transport activities and the reaction-center complexes also decreased during leaf senescence, and their positive correlations with photosynthesis have been frequently reported (3, 12–14). Thus, it can be expected that a decline in photosynthesis during senescence is mainly caused by a decrease in a functional unit of the photosynthetic system.

When plants are transferred to a different irradiance, they show acclimation of the photosynthetic system. The characteristics of plants acclimated to low irradiance have been reviewed in detail (1, 2, 7). These plants generally have relatively more Chl *b*, low capacities of electron transport per unit of Chl, and a reduction in soluble protein relative to Chl. However, the photosynthetic characteristics of leaves aged under low irradiance after full expansion are not known. In addition, it is unclear whether the senescent leaves show the regulatory light acclimation.

In this study, we examined the effects of irradiance on the *in vivo* and *in vitro* photosynthetic characteristics of senescent leaves of rice. Two irradiance treatments (100 and 20% sunlight) were imposed after the full expansion of the 13th leaf through senescence. To deduce the *in vivo* balance between Rubisco and electron transport limitation, we first examined the rate of CO_2 assimilation as a function of C_i[2] according to the photosynthetic model of Farquhar and Caemmerer (9). Second, we determined the amounts of Rubisco, CF_1, and Cyt *f* proteins in relation to the gas-exchange data. The limitation of CF_1(ATP-ase) and Cyt *f* contents for electron transport capacities has been repeatly reported (6, 12, 17, 35). In addition, we analyzed their relationships to leaf-nitrogen content, and characterized the photosynthetic system of the leaves aged under low irradiance in relation to acclimation of shade plants.

MATERIALS AND METHODS

Plant Culture

Rice (*Oryza sativa* L. cv Sasanishiki) plants were grown hydroponically to the ripening stage in a greenhouse under natural light conditions. The basal nutrient solution used was described in Mae and Ohira (19). The solution was renewed once a week and its pH was adjusted to 5.0 to 5.5. The strength of the nutrient solution was varied depending on the age of the plants (19). The 13th leaves on the main stems were used as samples throughout the experiments. The emer-

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[2] Abbreviations: C_i, intercellular CO_2 partial pressure; CF_1, coupling factor 1; G3P-DH, glyceraldehyde 3-phosphate dehydrogenase; RuBP, ribulose 1,5-bis-phosphate; LHC, light-harvesting Chl protein complex.

gence of these leaves was on 90th d after germination. From 5 d after the full expansion of the 13th leaves, the plants were grown under two different light conditions: 100 and 20% natural sunlight. To obtain the 20% sunlight treatment, the plants were covered with shade cloth. The average irradiances in both treatments were 2000 and 400 μmol quanta PAR m^{-2} s^{-1} at noon on a sunny day, respectively. All collections and photosynthetic measurements were made between 9:00 and 15:00 h.

Photosynthetic Measurements

The rates of CO_2 exchange and transpiration of the leaf attached to the plant were determined with an open gas-exchange system using a temperature-controlled chamber equipped with two fans. This system was detailed in Makino *et al.* (24). Differences in the partial pressures of CO_2 and H_2O entering and leaving the chamber were measured with an infrared gas analyzer (Horiba ASSA-1110, Horiba, Kyoto, Japan) and a dew point hygrometer (EG&G model 911, EG&G, Waltham, MA), respectively. The photon fluence rate at the position of the leaf in the chamber was measured with a Li-Cor quantum sensor (LI-190SA and LI-1000, Li-Cor, Lincoln, NE), and was adjusted to 1800 μmol quanta m^{-2} s^{-1}. Leaf temperature was controlled at 25°C. Gas-exchange parameters were calculated according to the equations in von Caemmerer and Farquhar (37).

Determinations of Chl, Rubisco, and Total Leaf Nitrogen

These were determined as described elsewhere (25). The leaf (about 0.3 g) was homogenized in 50 mM Li-phosphate buffer (pH 7.0) containing 10 mM DTT, 5 mM iodoacetic acid, and 12.5% (v/v) glycerol at a ratio of leaf to buffer of 1:10 (g:mL) in a chilled mortar and pestle with acid-washed quartz sand. The Chl and total leaf nitrogen contents were measured from part of this homogenate. The remaining homogenate for the determination of Rubisco was centrifuged at 39,000*g* for 10 min at 0 to 4°C. The amount of Rubisco protein in the supernatant was determined by SDS-PAGE and densitometry (Shimadzu chromatoscanner CS-930, Shimadzu, Kyoto, Japan). Calibration curve was made with the Rubisco purified from rice leaves (20).

Assay of NADP-G3P-DH

NADP-G3P-DH activity was measured at 25°C spectrophotometrically (340 nm) according to the method described by Makino *et al.* (21). Assay was started by the addition of enzyme solution.

Determination of CF_1 and Cyt *f* by Western Blotting

Antisera against CF_1 and Cyt *f* were raised separately in rabbits with purified spinach CF_1 and Cyt *f*. CF_1 was purified from spinach leaves according to the methods of Lien and Racker (18) and Nelson *et al.* (27) and then prepared by SDS-PAGE. The spinach Cyt *f* was purchased from Sigma Chemical Co. and additionally purified by SDS-PAGE. The gel sections containing CF_1 (α and β subunits) or Cyt *f* were excised and then dispersed in PBS with the aid of a syringe. These proteins (about 300 μg) were emulsified with Freund's complete adjuvant and injected subcutaneously four times at 2-week intervals into rabbits. The rabbits were bled by cardiac puncture at 10 d after the last injection, and antisera were prepared. The monospecificity of the respective antisera to rice CF_1 and Cyt *f* was verified by Western blotting after SDS-PAGE of the thylakoid fraction from rice leaves.

The thylakoid fraction used for the determination of CF_1 and Cyt *f* was prepared mechanically. Fresh leaves were immediately cut into small pieces with a razor blade, vacuum-infiltrated for 30 min at 0°C in 50 mM Hepes-NaOH buffer (pH 7.6) containing 0.33 M sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, and 5 mM Na-ascorbate, then homogenated in a VirTis-45 Homogenizer (VirTis Co. Gordiner, NY) at maximum speed for 2 min. After filtration through 0.5-mm stainless net and 20-μm nylon mesh, the crude thylakoids were collected at 2500*g* for 1 min and then suspended in 50 mM Hepes-NaOH buffer (pH 7.8). A 100-μL portion of this suspension was treated with lithium dodecylsulfate and iodoacetic acid solution, added to final concentrations of 2% (w/v) and 5 mM, respectively, and then centrifuged to remove insoluble materials. After adding 2-mercaptoethanol (2% [v/v]), the supernatant was heated at 100°C for 45 s. This preparation was used for the determination of CF_1 and Cyt *f* by SDS-PAGE, followed by immunoblotting. Blotting from SDS-PAGE to nitrocellulose filter and immunodetection were carried out according to the instruction manual of Bio-Rad Immuno Blot Assay Kit. Horseradish peroxidase-conjugated goat-anti-rabbit secondary antibodies were used for immunodetection. The intensity of developed color on the nitrocellulose was measured with a reflective densitometor (Shimadzu Chromatoscanner CS-930) by scanning it at 560 nm. A linear correlation between color density and CF_1 and Cyt *f* contents was found at thylakoid amounts corresponding to 0.8 μg and 2.0 μg Chl, respectively. Recoveries of CF_1 and Cyt *f* contents were calculated from the Chl content in the thylakoid fraction.

RESULTS

In this report, we define the beginning of leaf senescence as the time when the photosynthetic rate begins to decrease. Because preliminary experiments showed that the photosynthetic rate begins to decrease about 5 d after full expansion, the plants were transferred to two different irradiances (100 and 20% sunlight) from 5 d after full expansion of the 13th leaves through senescence.

The rate of CO_2 assimilation as a function of C_i was examined. The purpose of this analysis was to deduce the effects of irradiance on the *in vivo* balance between capacities of Rubisco and other photosynthetic limiting factors in the leaves during senescence. According to the photosynthetic model developed by Farquhar and von Caemmerer (9), the photosynthetic rate at low CO_2 pressures ($C_i \leqq 200$ μbar) is limited by Rubisco capacity, and the rate at high CO_2 pressures ($C_i \approx 600$ μbar) is limited by other factors such as the electron transport capacity (8, 37). In some cases, the photosynthetic rate above 600 μbar CO_2 is also limited by the capacity of orthophosphate regeneration by starch and sucrose synthesis (31, 34). The relative capacities of Rubisco and electron transport, therefore, can be deduced from the ratio

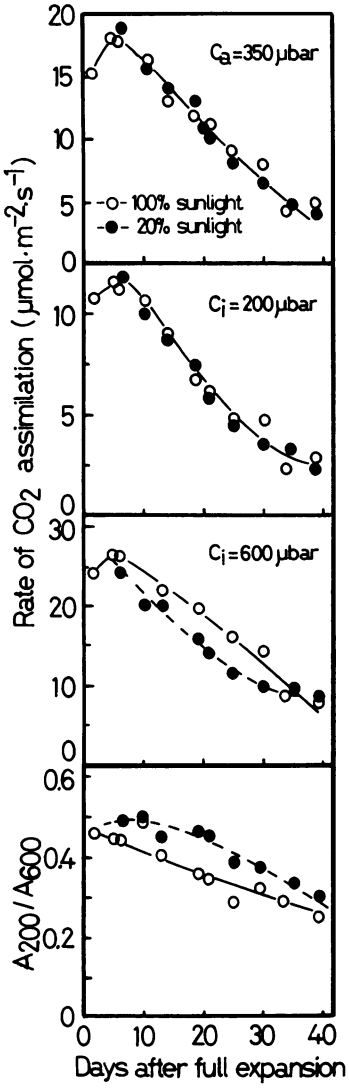

Figure 1. Changes in the photosynthetic rates at an ambient CO_2 pressure (C_a) of 350 μbar, and at intercellular CO_2 pressures (C_i) of 200 μbar and 600 μbar, and in the ratio of the rates at C_i = 200 to 600 μbar (A_{200}/A_{600}) in the 13th leaves aged under full sunlight (○) and 20% sunlight (●) conditions. Measurements were made at a leaf temperature of 25°C and an irradiance of 1800 μmol quanta m^{-2} s^{-1}.

of the photosynthetic rates at C_i = 200 and C_i = 600 μbar. Figure 1 shows the changes in the photosynthetic rates at an external CO_2 pressure of 350 μbar (ambient CO_2 pressure) and intercellular CO_2 pressures of 200 and 600 μbar, and in their ratio during senescence. The C_i obtained at ambient air was in the range of 250 to 300 μbar. There was no difference in the rates at an ambient air and C_i = 200 μbar between the light treatments. The rate at C_i = 600 μbar, however, decreased faster in the 20% sunlight treatment than in the full sunlight treatment. This means that although there is no difference in the change in the Rubisco capacity between the treatments, the RuBP regeneration capacity from the shade treatment decreased at a faster rate than the full sunlight treatment during senescence. The ratio of the photosynthetic rates at C_i = 200 and C_i = 600 μbar decreased with leaf age in both treatments.

Despite no difference in the photosynthetic rates at 200 μbar between the treatments, the Rubisco content from the full sunlight treatment decreased sooner, at the same rate than from the shade treatment during senescence (Fig. 2). The changes in the activity of NADP-G3P-DH was almost coordinated with that in Rubisco protein. In contrast, CF_1 and Cyt *f* contents decreased more rapidly from the shade treatment. These changes in CF_1 and Cyt *f* were very similar to those in the photosynthetic rate at 600 μbar with leaf age,

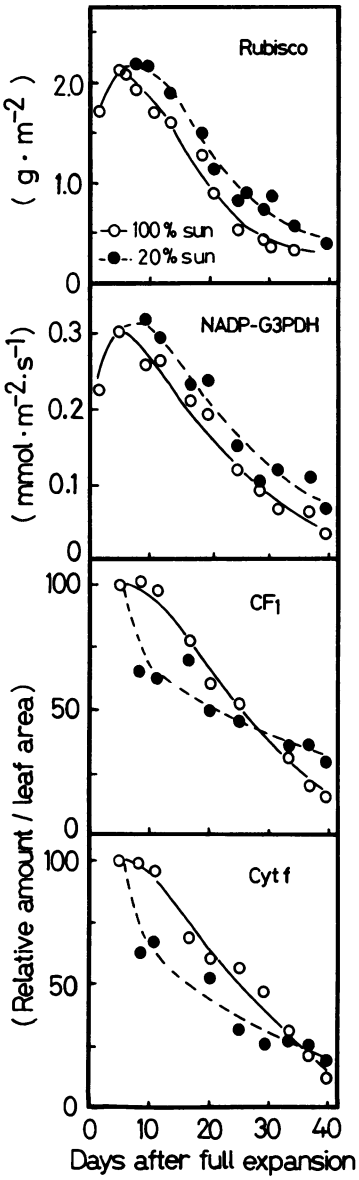

Figure 2. Changes in Rubisco content, NADP-G3P-DH activity, and CF_1 and Cyt *f* contents in the 13th leaves aged under full sunlight (○) and 20% sunlight (●) conditions. Rubisco content was measured by SDS-PAGE. The CF_1 and Cyt *f* contents were determined by Western blotting, and represented as relative content to those in the leaf on the 5th d after full expansion.

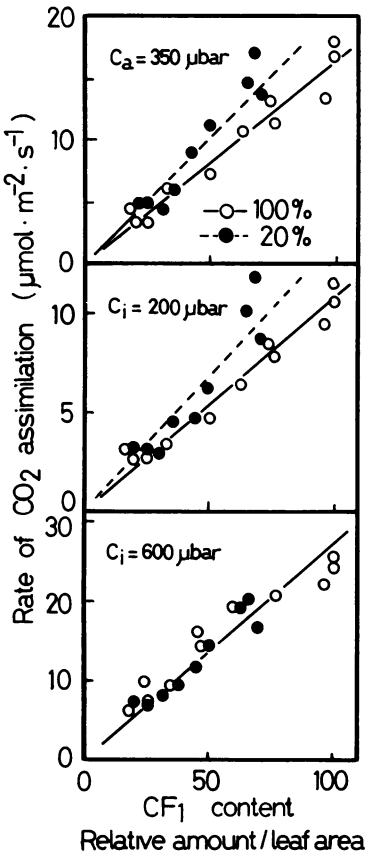

Figure 3. Photosynthetic rates at C_a = 350 μbar, C_i = 200 μbar, and C_i = 600 μbar *versus* CF_1 contents. Symbols are the same as in Figure 1. Data are taken from Figures 1 and 2.

except in the late senescent leaves. The slightly greater contents of CF_1 and Cyt *f* in the late senescent leaves from the shade treatment may have been caused by the delay of the overall senescence with enervation of sink organs. Low irradiance strongly reduced grain development.

The relationships between photosynthetic rate and CF_1 contents were analyzed (Fig. 3). The CF_1 content from the respective treatments did not fall on the same line when plotted against the photosynthetic rates at the ambient air and 200 μbar CO_2, but the relationship to the rate at 600 μbar CO_2 yielded a single line irrespective of irradiance treatment. Similar trends were observed for the relationship to Cyt *f* content (data not shown). This means that the amounts of CF_1 and Cyt *f* in the natural sunlight conditions cannot be limiting factors for the photosynthetic rate at ambient CO_2 pressure, even if they are limiting RuBP regeneration capacity. Thus, the rapid decrease in the photosynthetic rate at 600 μbar CO_2 from the shade treatment may have been caused by a rapid decrease in CF_1 and Cyt *f* contents.

Changes in Chl content and Chl *a/b* ratio are shown in Figure 4. The Chl content from shade treatment remained apparently constant until late senescence, then decreased slightly. The loss of Chl from the full sunlight treatment was also relatively slow. These changes in Chl content were not apparently correlated with those of other photosynthetic components and photosynthesis (see Figs. 1 and 2). The Chl *a/b* ratio from the full sunlight treatment remained relatively constant, but that from the shade treatment decreased with leaf age.

Nitrogen costs for Rubisco, CF_1, and Chl during leaf senescence were analyzed (Fig. 5). No difference in the proportion of nitrogen in Rubisco was found between the treatments, although the absolute amount of Rubisco from the shade treatment was greater (see Fig. 2). The CF_1 content from both treatments was proportional to leaf-nitrogen content, but the nitrogen cost was smaller from the shade treatment. In contrast, the nitrogen cost for Chl was greater from the shade treatment and the relationship from both treatments was curvilinear.

DISCUSSION

Rice leaves aged under the low irradiance showed the following three characteristics for the inactivation mechanisms of photosynthesis.

Rapid Decrease in CF_1 and Cyt *f*

The rapid decrease in CF_1 and Cyt *f* contents from the shade treatment during senescence is similar to one of the phenomena shown by the plants acclimated to low irradiance. It is well known that shade plants have lower electron transport capacities relative to Chl or total thylakoid nitrogen (1, 2, 7). This is mainly caused by a relative reduction in the

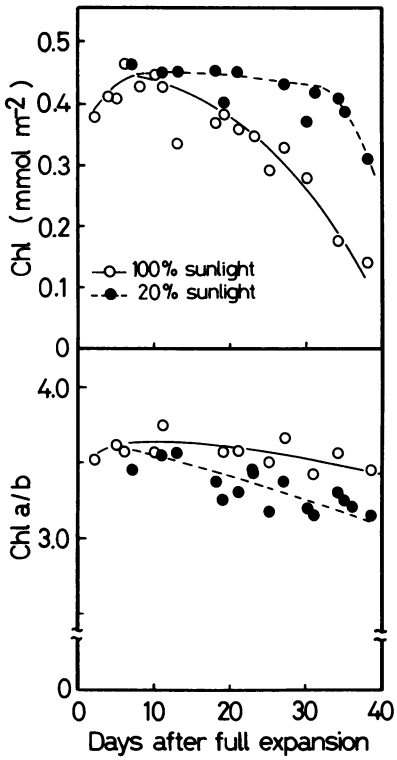

Figure 4. Changes in the Chl content and Chl *a/b* ratio in the 13th leaf aged under full sunlight (○) and 20% sunlight (●) conditions.

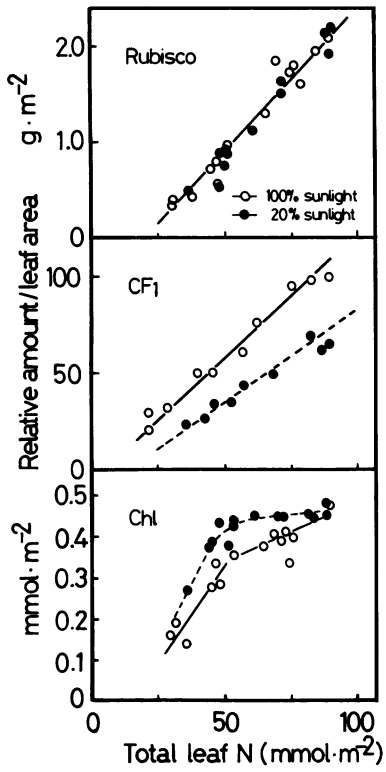

Figure 5. Rubisco, CF_1, and Chl contents *versus* leaf-nitrogen content. Symbols are the same as in Figure 1.

amounts of electron transport components such as CF_1 (ATPase) and Cyt *f* (6, 17, 35, 36). Reduction of CF_1 and Cyt *f* contents causes low rates of the photosynthetic O_2 evolution under saturating CO_2 conditions in shade plants (6, 35). We also found that a rapid decrease in CF_1 and Cyt *f* contents from the shade treatment results in a similar reduction in the photosynthetic rate at C_i = 600 μbar (Figs. 1 and 2).

Retardative Decrease in Rubisco

There was more Rubisco in shaded than in sunlight-illuminated leaves as a result of a retardative decrease (Fig. 2). The ratio of Rubisco to CF_1 or Cyt *f* contents from the shade treatment also increased, but the ratio to leaf nitrogen did not differ (Fig. 5) and the ratio to Chl declined (Fig. 4). Shade plants generally have less Rubisco content for a given Chl or total leaf-nitrogen content (10, 28, 33, 35). Concerning the ratio to electron transport, Terashima and Evans (35) reported that spinach plants adapted to low irradiance have reduced Rubisco content but that the decrease is less than that in electron transport activity. A similar observation was reported by Osmond (28) in *Solanum*. In addition, several gas-exchange studies show a decrease in the CO_2-limited photosynthesis and a greater decrease in the CO_2-saturated photosynthesis in shade plants (6, 10, 15, 28, 29, 33). Thus, the effects of low irradiance on change in Rubisco content in senescing leaves differed from the characteristics of shade plants, with the exception of the ratio to electron transport.

Despite the greater Rubisco content from the shade treatment, there was no apparent difference between the treatments in the photosynthetic rates at ambient and 200 μbar CO_2 (Fig. 1). The reason for this is unknown, but one possible reason is that the activation capacity of Rubisco may decline in the leaves from the shade treatment. Seemann *et al.* (33) reported that RuBP pool size per unit of Rubisco active site at the steady rate of photosynthesis in ambient air was saturating irrespective of growth irradiance. Thus, RuBP limitation did not occur at maximum photosynthesis, even in the shade plants. Because Rubisco activation is regulated by Rubisco activase with ATP supply that depends on electron transport activity (30, 32), it is possible that low activation of Rubisco may have been caused by a decline in electron transport from the shade treatment.

Although NADP-G3P-DH is not related to RuBP carboxylation but to RuBP regeneration, its change was highly correlated with that of Rubisco (Fig. 2). Similar results were reported by Camp *et al.* (3) in senescent wheat leaves. Also, Makino *et al.* (21) found a strong correlation between Rubisco and ribulose 5-phosphate kinase during leaf senescence. However, a correlation between stromal enzymes like this is only recognized during leaf senescence and not necessarily found within young expanded leaves. For example, Makino and Osmond (25) observed that, despite a correlation between ribulose 5-phosphate kinase and electron transport, this kinase was not correlated with Rubisco in young expanded leaves when the plants were grown with different nitrogen levels. Thus, these results may suggest that there is no selective degradation mechanism for each enzyme in chloroplastic stroma (26).

No Apparent Decline in Chl

Although loss of Chl is a commonly observed symptom of senescence, Chl frequently disappears most slowly during senescence among the photosynthetic components (11, 12, 14, 21). As shown in Figure 4, low irradiance strongly retarded the decline in the Chl content and it remained almost constant until late senescence. In addition, the Chl *a*/*b* ratio from the shade treatment decreased during this period. A decline in Chl *a*/*b* ratio during senescence is mainly caused by a more rapid decrease in the reaction centers and core Chl than in the LHC of PSII (13, 14). No apparent change in the Chl content may be ascribed to changes in the relative amounts of several Chl proteins, especially a slight increase in LHCII. Shade plants have relatively more Chl *b* and LHC per PSII reaction center (16). This increase in LHC is associated with a decline in PSII reaction centers and core Chl, and with relatively little change in PSI reaction center content (6, 17, 35). Thus, it is possible that changes in the respective Chl components similar to those of shade plants occurred during leaf senescence under shade conditions, resulting in no apparent change in total Chl.

CONCLUSION

The photosynthetic characteristics of rice leaves aged under low irradiance were approximately similar to those of shade plants. This means that leaf senescence under light stress has some of the regulatory characteristics of acclimation, but with notable differences. One of the most striking points is the

greater content of Rubisco from the shade treatment. This was not reflected in the rate of photosynthesis. A plausible explanation is that *in vivo* Rubisco activity is coordinated with a reduction in electron transport components such as CF_1 and Cyt *f*. Previously, Makino *et al.* (22) reported that Rubisco acts as the most easily remobilizable nitrogen source in leaves. Low irradiance strongly reduced sink capacities, which in turn may result in a slower decrease in Rubisco. In addition, Crafts-Brandner *et al.* (4) found that in some soybean cultivars, fruit removal induces the formation of an insoluble form of Rubisco. Although such an induction of insoluble Rubisco was not found for rice leaves from the shade treatment (data not shown), the changes in Rubisco content in a single leaf aged under low irradiance may be closely related to sink capacities.

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