Gene Expression and Accumulation of Rubisco in Bundle Sheath and Mesophyll Cells during Leaf Development and Senescence in Rice, a C₃ Plant

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Abstract: Gene expression of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (rbcL) and small subunit (rbcS) in bundle sheath and mesophyll cells of rice, a C₃ plant, was examined during leaf development and senescence by in situ hybridization. Localization of Rubisco protein in both cells was also examined by immuno-electron microscopy. Gene expression and accumulation of Rubisco were related with the chlorophyll fluorescence parameters. The chlorophyll fluorescence parameters, such as Fᵥ/Fₚ and Φₚₛₛ, gradually increased during leaf development with the increase in the accumulation of Rubisco. However, the chlorophyll fluorescence parameters decreased earlier than the Rubisco content during leaf senescence. The expression of rbcS decreased earlier in bundle sheath cells than in mesophyll cells during leaf development, whereas the expression of rbcL in both cells was retained during leaf development and decreased during leaf senescence. On the other hand, Rubisco content of bundle sheath and mesophyll cells increased during leaf development and decreased during leaf senescence. Rubisco was retained even after the disappearance of the expression of rbcS and rbcL detectable by in situ hybridization. The present results suggest that the expression pattern of rbcS in bundle sheath cells was somewhat different from that in mesophyll cells, but this difference was not reflected in Rubisco content.

Key words: Bundle sheath, C₃ photosynthesis, Chlorophyll fluorescence, Immuno-localization, In situ hybridization, Leaf development, Leaf senescence, Mesophyll.

In C₄ plant leaves, structural and functional differentiation between bundle sheath and mesophyll cells has been the object of numerous investigations on the regulation of gene expression (Dengler and Nelson, 1999; Sheen, 1999). Chlorenchyma of leaves in most C₄ plants is also differentiated into bundle sheath and mesophyll cells (Esau, 1953). However, the function of the bundle sheath cells of C₃ plants is almost unknown (Yoshimura et al., 2004), although the bundle sheath cells of C₃ plants is intriguing for the investigation on introducing C₄ genes into C₃ crop species and the evolutionary studies of C₄ photosynthesis (Miyake, 1999).

In the emerging leaves of rice, a C₃ plant, bundle sheath chloroplasts accumulate large amounts of starch in contrast to mesophyll chloroplasts (Miyake and Maeda, 1976). This fact suggests that the function of bundle sheath cells is different from that of mesophyll cells in rice. It may also be expected that bundle sheath cells of rice possess functions different from mesophyll cells other than starch accumulation.

We previously examined the expression of photosynthetic genes, rbcS and cab, which encode the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and light harvesting chlorophyll a/b binding protein, respectively, in the emerging leaves and lamina joint of rice (Tsutsumi et al., 2006, 2007). The photosynthetic genes were expressed in both bundle sheath and mesophyll cells in the immature basal region of the emerging leaves whereas the genes were expressed only in mesophyll cells in more mature middle and tip regions. The expression of photosynthetic genes was suppressed in the lamina joint. This fact suggests that the photosynthetic gene expression in bundle sheath and mesophyll cells is controlled differently during the leaf development. However, in situ hybridization is not suitable for quantitative analysis. To analyze the difference between bundle sheath and mesophyll cells quantitatively, we used immuno-electron microscopy, which has been used for quantitative analysis by estimating the labeling density in electron-micrographs (Ueno, 1996; Yamane et al., 2003; Yoshimura et al., 2004; Wakayama et al., 2006). In addition, it is not known whether the expression of photosynthetic genes reflects photosynthetic activity during leaf development, in which the expression of photosynthetic genes becomes mesophyll specific.
Since stomatal conductance is known to fluctuate during leaf development and senescence (Makino et al., 1984), gas exchange is invalid as a parameter of photosynthetic activity in chlorenchyma. Therefore, chlorophyll fluorescence parameters were examined as an indicator of photosynthetic activity in the present study.

The Rubisco content of rice leaves rapidly increases during leaf emerging to expansion stage and gradually decreases during senescence after the leaf expansion (Mae et al., 1983; Makino et al., 1984; Fukayama et al., 1996). However, the distribution of Rubisco protein in bundle sheath and mesophyll cells has not been examined. Yamane et al. (2003) investigated the distribution of Rubisco protein in drought-stressed rice leaves and showed that Rubisco content of bundle sheath chloroplasts decreased more greatly than that of mesophyll chloroplasts. However, Yamane et al. (2003) did not investigate the distribution of Rubisco during the leaf development.

In this study, we examined the Rubisco gene expression and Rubisco protein accumulation in bundle sheath and mesophyll cells in emerging and senescing leaves. We also examined the fluorescence characteristics to estimate the photochemical activities of the tissues.

Materials and Methods

1. Plant materials

Seeds of rice (Oryza sativa L. cv. Nipponbare) were surface sterilized with sodium hypochlorite solution (0.5 g L\(^{-1}\) available chlorine) for 10 min. After washing several times with distilled water, seeds were imbibed in distilled water in a growth chamber at 28°C for 3 days. Then, seeds were sown on plastic nets placed on the surface of 300 mL Kimura’s B solution in tall beakers. The pH of the solution was adjusted to 5.5 at the day of imbibition. Seeds were imbibed in distilled water in a growth chamber at 28°C for 3 days. Rice plants were grown for 10 days thereafter to obtain the expanded third leaves as the sample for western blotting.

2. Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a PAM-2100 chlorophyll fluorometer (Walz, Germany) on tip, middle and basal regions of intact plants (Yamane et al., 2008). Intact leaves of rice plants, which were kept in the dark for 30 min, were exposed to a weak modulated irradiance (2.0 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)), and minimal fluorescence (\(F_0\)) was measured. Thereafter, maximal fluorescence (\(F_{\text{m}}\)) was measured by exposing the leaves to a saturating light pulse (3000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) for 400 ms. After the measurement of \(F_{\text{m}}\), actinic light (210 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) was immediately applied and the F value was recorded every 20 s. At each time of F measurement, \(F_{\text{m}}'\) values were measured by applying saturating pulse. When the F value reached a plateau, actinic light was turned off and far-red light was turned on for the measurement of the \(F_0'\) value. The second leaf sheaths, which covered the emerging third leaves, were carefully removed not to damage the third leaves using a razor blade and tweezers before measurement.

The parameters of photosynthetic performance were calculated by the following formula according to the manufacturer’s instructions.

The maximal quantum yield (\(\Phi_{\text{PSII}}\)) = \((F_{\text{m}}' - F_0')/F_{\text{m}}'\).

The effective quantum yield (\(\Phi_{\text{max}}\)) = \((F_{\text{m}} - F)/F_{\text{m}}'\).

where \(F\) and \(F_{\text{m}}'\) were the values obtained in the time when F value reached a plateau.

3. Preparation of probes for In situ hybridization

RNA probe for \(rbcS\) was produced as described in Tsutsumi et al. (2006). As the template for \(rbcL\) RNA probe, the rice cDNA clone (accession number AK105600) inserted in pME18SFL-3 was used (provided by National Institute of Agrobiological Sciences). Eco RI fraction (450 bp within the 5' region of the coding region) of AK105600 was inserted in pBluescript II KS (−) (Stratagene) and cloned. The cloned vector was digested with Hind III to produce T7 RNA polymerase template for sense probe and with Bam HI to produce T3 RNA polymerase template for anti-sense probe. The probes were produced with DIG-11-UTP Labeling Mix (Roche).

The probes used in the present report are made from 5' coding region of \(rbcS\) and \(rbcL\) and expected to recognize the expression of all members of respective gene families.

4. In situ hybridization

Segments 5 mm in length were cut with a razor blade from the tip, middle and basal regions of the emerging and senescing third leaves. The in situ hybridization was performed according to Tsutsumi et al. (2006).

5. Western blotting

The expanded rice leaves were ground in the grinding buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged and the supernatant was collected. The protein content was determined by the method of Bradford (1976). Two micrograms of the protein
was separated on the SDS polyacrylamide gel and transferred to polyvinylidenedifluoride membrane (Millipore). Rubisco protein was immuno-labeled with 1:2000 diluted antiserum raised against Rubisco whole molecule of maize in rabbit with blocking buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.3 g L⁻¹ BSA). The immuno-labeling with rabbit antibody was detected with anti-rabbit alkaline phosphatase-conjugated goat antibody and stained with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate.

6. Immuno-electron microscopy
Segments 1 mm in length were cut with a razor blade from the tip, middle, and basal regions of the emerging and senescing third leaf blades. The samples were fixed in 0.4 g L⁻¹ paraformaldehyde and 5 mL L⁻¹ glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2) for 3 hr, washed with the 50 mM sodium phosphate buffer, dehydrated with grading ethanol series and embedded in LR White resin. Ultrathin sections (ca. 80 nm in thickness) were immuno-labeled with 1:200 diluted antiserum raised against Rubisco with TBS (Tris-HCl buffered saline, 50 mM Tris-HCl and 150 mM NaCl, pH 7.3) or, as a control, non-immune rabbit serum for 2 hr at room temperature. The rabbit antibody was localized with goat anti-rabbit IgG antibody-colloidal gold complex (15 nm in diameter) (Ultra Biosols, U.K.). The sections were stained with 0.2 g L⁻¹ uranyl acetate and observed with a transmission electron microscope (Hitachi H7500).

7. Quantitative analysis
Electron-micrographs of chloroplasts were analyzed quantitatively using the ImageJ program (Rasband, 1997-2007). The cross-sectional areas of chloroplasts were measured and the gold particles observed on the chloroplast cross-sections were counted. When the cross-sectional areas of chloroplasts were measured, the areas occupied by starch grains were excluded. The labeling density was calculated as the number of gold particles per unit area (μm²).

For these quantitative analyses, more than 30 chloroplasts from five or more cells were used for each cell type.

8. Statistical analysis
Data obtained in the present examination were statistically analyzed using one-way ANOVA followed by Tukey’s HSD test (SPSS for Windows ver. 14.0J, SPSS Inc., Japan). Significant difference was analyzed based on P-value < 0.05.

Results

1. Chlorophyll fluorescence
To examine the photosynthetic performance of different regions of the leaves, we measured chlorophyll fluorescence. In emerging leaves, Fv/Fm increased from basal to tip region (Fig. 1A). This result indicates that Fv/Fm increased during leaf development. Fv/Fm of tip and middle regions decreased when the leaves were in the senescence stage, whereas that of the basal region was retained at high level. ΦPSII also increased during leaf development and decreased during leaf senescence as Fv/Fm did (Fig. 1B). These results indicate that PSII activities increased during leaf development and declined during leaf senescence.

2. Expression of photosynthetic genes during leaf development and senescence
To examine the distribution of photosynthetic gene expression during leaf emergence and senescence, we conducted in situ hybridization on the leaf tissues as examined for fluorescence analysis (Figs. 2, 3). Figures 2 and 3 show that signals of rbcS were located in cytosol and those of rbcL in plastids. This is because rbcS is encoded by nuclear genome (Matsuoka et al., 1988) and rbcL by plastid genome (Nishizawa and Hirai, 1987). In the basal region of the emerging leaves, photosynthetic genes, rbcS and rbcL, were expressed in both bundle sheath and mesophyll cells (Fig. 2A, D). In the middle and tip regions of the emerging leaves, rbcS was expressed in mesophyll cells but not in bundle
sheath cells (Fig. 2E, F), whereas rbcL was expressed in both bundle sheath and mesophyll cells (Fig. 2B, C). The results of the expression of rbcS are consistent with our previous report (Tsutsumi et al., 2006). These results indicate that the expression of rbcS in bundle sheath cells decreased earlier than that in mesophyll cells during leaf development while the expression of rbcL continued in both bundle sheath and mesophyll cells.

In the basal region of senescing leaves, rbcL was expressed in both bundle sheath and mesophyll cells (Fig. 3A), whereas rbcS was expressed in mesophyll cells but weakly in bundle sheath cells (Fig. 3D). In the middle region, the expression of rbcL and rbcS decreased (Fig. 3B, E). The expression of rbcS was hardly observed in bundle sheath cells (Fig. 3E). In the tip region of the senescing leaves, rbcS and rbcL
were not expressed in either cells (Fig. 3C, F). These results indicate that the expression of \textit{rbcL} decreased at a similar rate in both bundle sheath and mesophyll cells during leaf senescence while the expression of \textit{rbcS} in bundle sheath cells decreased faster during leaf senescence. No signal was observed in bundle sheath and mesophyll cells when sense probes were used as in our previous report (Tsutsumi et al., 2006) (data not shown).

### 3. Localization of Rubisco proteins during leaf development and senescence

The results of \textit{in situ} hybridization indicated that the expression of \textit{rbcS} in bundle sheath cells decreased earlier than in mesophyll cells during leaf development, and the expression of both \textit{rbcS} and \textit{rbcL} decreased during leaf senescence. To examine whether the gene expression is reflected in the Rubisco content of bundle sheath and mesophyll cells, we conducted immuno-electronmicroscopy. The specificity of the anti-Rubisco antibody was confirmed by western blotting. Two clear bands, approx. 55 kDa and 12 kDa, were detected (Fig. 4). These are corresponding to large and small subunits of Rubisco, respectively (Gatenby and Ellis, 1990).

The signal of Rubisco in bundle sheath and mesophyll chloroplasts in emerging leaves increased from the basal to tip region (Fig. 5A-F). In contrast, in the chloroplasts of both cells within senescing leaves, the signal decreased from the leaf basal to tip region (Fig. 5G-L). These results indicate that the Rubisco content increased during leaf development and decreased during leaf senescence. However, the difference in Rubisco content between bundle sheath and mesophyll cells is not clearly distinguishable from these morphological observations.

To analyze these morphological data quantitatively, we estimated the number of the gold particles of

![Fig. 5. Immuno labeling of Rubisco protein in bundle sheath (A-C, G-I) and mesophyll chloroplasts (D-F, J-L) in the basal (A, D), middle (B, E), and tip region (C, F) of emerging leaves and in the basal (G, J), middle (H, K) and tip region (I, L) of senescing leaves. Bars: 0.5 \( \mu \text{m} \). S: starch granule.](image)
Rubisco per unit area of chloroplasts as an indicator of Rubisco content of bundle sheath and mesophyll chloroplasts (Fig. 6). Mesophyll-1 is the mesophyll cells adjacent to bundle sheath cells and mesophyll-2 cells are located farther from the vascular bundles than mesophyll-1. Figure 6 shows that Rubisco content in chloroplasts was increased during leaf development and decreased during leaf senescence. However, the Rubisco content of bundle sheath chloroplasts did not significantly differ from that of mesophyll chloroplasts. This result is inconsistent with the results of rbcS expression. Significant difference was not observed between mesophyll-1 and mesophyll-2 cells, either.

The results of the present study are summarized in Table 1.

### Discussion

Our previous report showed that rbcS and cab were expressed in both bundle sheath and mesophyll cells in the basal region of the emerging rice leaves while both genes were expressed only in mesophyll cells in tip and middle regions (Tsutsumi et al., 2006). This fact suggests that the expression of photosynthesis-related genes in emerging rice leaves is less tissue specific at very early stage but gradually becomes mesophyll-specific during leaf development. However, whether the difference between bundle sheath and mesophyll cells is reflected to the photosynthetic enzyme level remained unsolved.

Chlorophyll fluorescence parameters, $F_{v}/F_{m}$ and $\Phi_{PSII}$, increased during leaf development and decreased during leaf senescence (Fig. 1). This suggests that photosynthetic activity increased during leaf development and decreased during leaf senescence. Rubisco content also increased during leaf development and decreased during leaf senescence. In emerging leaves, the chlorophyll fluorescence parameters and Rubisco content gradually increased from the basal region to tip region (Figs. 1, 6A), where the expression of rbcS and rbcL was prominent (Fig. 2). On the other hand in the senescing leaves, the Rubisco content and gene expression gradually decreased from leaf base to tip region, whereas the chlorophyll fluorescence parameters drastically decreased from leaf base to middle region (Figs. 1, 3 and 6B). These results suggest that the photochemical activity decreases earlier than the Rubisco content during leaf senescence.

The earliest event of structural degradation in senescing leaves occurs in chloroplasts, especially in grana (Lim et al., 2007), because the light-harvesting chlorophyll molecules localized in thylakoid membranes degrade rapidly to escape from photooxidative damages (Matile et al., 1996).
Therefore, the present results of chlorophyll fluorescence measurement are reasonable and suggest that photochemical activity in chloroplasts is declined faster than Rubisco degradation during leaf senescence.

The Rubisco content of rice leaves has already been examined from emerging to senescence by several researchers (Mae et al., 1983; Makino et al., 1984; Fukayama et al., 1996). These researchers reported that the Rubisco content of rice leaves rapidly increases from leaf emerging to expansion stage and gradually decreases during senescence after the leaf expansion. However, the distribution of Rubisco protein in bundle sheath and mesophyll cells has not been examined. Bundle sheath cells of \(C_4\) plant are suggested to participate in a small amount of whole-leaf photosynthesis, since relative volume of chloroplasts to the cells is lower than that of mesophyll cells (Kinsman and Pyke, 1998; Yoshimura et al., 2004). This suggestion implies that the expression of photosynthesis-related genes and enzymes in bundle sheath cells would become lower than those in mesophyll cells at a certain stage of leaf development. Indeed, the expression of \(rbcS\) in bundle sheath cells decreased earlier than that in mesophyll cells, whereas the expression of \(rbcL\) did not. In addition, Rubisco content was increased similarly in both bundle sheath and mesophyll cells during leaf development (Fig. 6A). It has been shown that \(rbcL\) translation was reduced in the transgenic tobacco plants in which \(rbcS\) mRNA levels were reduced by antisense inhibition (Rodermel et al., 1996). Therefore, the increase in Rubisco content of emerging leaves suggests the expression of both \(rbcS\) and \(rbcL\) even in bundle sheath cells. It is suggested that the expression of \(rbcS\) might be sustained at a rate below the detectable limit. More sensitive and quantitative analysis is needed to verify this possibility. Another possibility is that \(rbcS\) protein production decreased earlier in bundle sheath cells than in mesophyll cells. In this case, \(rbcS\) content should be lower than \(rbcL\) content in bundle sheath chloroplasts at maturing. This possibility may be examined by using antibodies specific to respective subunits.

The Rubisco content of bundle sheath chloroplasts decreased at a rate similar to that of mesophyll chloroplasts during leaf senescence (Figs. 5, 6). Yamane et al. (2003) also showed that Rubisco contents of bundle sheath and mesophyll chloroplasts were almost the same in the fully expanded leaves of rice. This indicates that the change in Rubisco content during leaf development and senescence is similar in both bundle sheath and mesophyll cells. Together with the fact that the morphological characteristics of bundle sheath and mesophyll chloroplasts are similar (Yamane et al., 2003; Yoshimura et al., 2004), it is suggested that bundle sheath and mesophyll chloroplasts might possess similar function in photosynthesis although the contribution of bundle sheath cells might be less than that of mesophyll cells. There was not significant difference in Rubisco content between mesophyll-1 and mesophyll-2 cells. Langdale and Nelson (1991) suggested a factor passing from vein to bundle sheath and mesophyll cells and regulating the gene expression in the cells around the vein in maize, a \(C_4\) plant. However, in our study on rice, a \(C_4\) plant, the gradient of Rubisco content in relation to vein was not observed.

We found that Rubisco content increased similarly in bundle sheath and mesophyll chloroplasts during leaf development and decreased similarly during leaf senescence. The expression pattern of \(rbcS\) was somewhat different between bundle sheath and mesophyll cells, but this difference was not reflected in Rubisco content. It is believed that \(C_4\) plants were evolved from \(C_3\) plants (Sage, 2004). Rubisco is confined to bundle sheath chloroplasts in \(C_4\) plants. The present observation suggests that the bundle sheath chloroplasts possessed Rubisco at the beginning of \(C_4\) plant evolution. The next step of \(C_4\) plant evolution seems to be concentration of Rubisco in bundle sheath chloroplasts and suppression of Rubisco expression in mesophyll cells.

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