Expression of Photosynthesis-Related Genes during the Leaf Development of a C₃ Plant Rice as Visualized by In Situ Hybridization

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Abstract: The expression of photosynthesis-related genes, rbcS (small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase) and cab (light-harvesting chlorophyll a/b-binding protein), in emerging rice leaves was examined. We performed in situ hybridization to visualize the spatial expression pattern of the photosynthesis-related genes. In the basal region of the leaf blade, which is the youngest region in a leaf blade of monocotyledonous plants, the expression of the genes was observed in both bundle sheath and mesophyll cells, while in the older middle and the oldest tip regions, the expression was only observed in mesophyll cells and not in bundle sheath cells. These results indicate that the expression of these photosynthesis-related genes is developmentally regulated and becomes mesophyll-specific in mature leaves. The expression of the photosynthesis-related genes in the lamina joint was also examined. These genes were not expressed in the lamina joint of immature leaves nor in the mature leaves. Therefore, the lamina joint was considered to be a photosynthetically inactive region.

Key words: Bundle sheath, Chlorophyll a/b-binding protein, in situ hybridization, Lamina joint, Leaf development, Mesophyll, Rice, Rubisco small subunit.

Structural and functional differentiation in C₄ plant leaves has been the object of numerous investigations on the regulation of the expression of photosynthesis-related genes in bundle sheath and mesophyll cells (Evert et al., 1996). In addition, the expression of photosynthetic gene in developing C₄ plant maize leaves has been reported in detail (Nelson and Langdale, 1989).

Chlorenchyma of leaves in most C₃ plants is also differentiated into bundle sheath and mesophyll cells (Esau, 1953). Bundle sheath cells of C₃ plants are smaller and contain only a fewer chloroplasts and mitochondria than those of C₄ plants, although bundle sheath cells of C₃ plants surround vascular bundles (Ueno et al. 2003; Yoshimura et al. 2004). However, granal development in bundle sheath chloroplasts are similar to those in C₄ plants as those of mesophyll. Rubisco protein is accumulated in both bundle sheath and mesophyll chloroplasts in mature leaves of a C₃ plant rice (Yamane et al., 2003). However, detailed analysis of the expression of photosynthesis-related genes in different cell types has not been conducted in C₃ plant leaves.

Bundle sheath chloroplasts accumulate large amounts of starch in contrast with mesophyll chloroplasts during the leaf development in rice seedlings (Miyake and Maeda, 1976). This fact indicates that bundle sheath cells of rice are specialized in carbohydrate storage (Miyake and Maeda, 1978). Therefore, it is suggested that the bundle sheath cells possess functions different from those of mesophyll cells in rice seedlings, at least with respect to sugar transport or starch synthesis (Miyake, 1999). It may also be expected that bundle sheath cells of rice possess functions different from mesophyll cells in addition to starch synthesis.

The lamina joint, which is the lowermost region of a leaf blade located just above the junction between a sheath and a blade and is known as gravitropic region, possesses a specialized anatomy different from normal leaf blade or sheath in rice plants (Maeda, 1961). In the lamina joint, sclerenchyma is denigrated to become collenchyma and vascular bundles are distant from epidermis. Counterparts of bundle sheath cells in the lamina joint predominantly accumulate a large amount of starch compared with other tissues (Nakano and Maeda, 1978). Therefore, there may also be a functional differentiation between bundle sheath and mesophyll cells in the lamina joint.

The function of the bundle sheath cells in leaves
of C\textsubscript{4} plants is almost unknown (Yoshimura et al., 2004) and is intriguing for investigation in regard to evolution of C\textsubscript{4} photosynthesis and to introducing C\textsubscript{4}
genes in C\textsubscript{3} plants (Miyake, 1999). We investigated the functional difference between bundle sheath and mesophyll cells in C\textsubscript{3} plants, by examining the expression of photosynthesis-related genes, \textit{rbcS} and \textit{cab}, in different tissues, especially in bundle sheath and mesophyll cells, of emerging leaf blades and the lamina joint in rice seedlings by \textit{in situ} hybridization. \textit{rbcS} encodes small subunit of Rubisco. Rubisco is one of the major proteins in plants and acts as a key enzyme in the photosynthesis fixing CO\textsubscript{2} in the Calvin cycle (Sharkey, 1985; Nishizawa and Hirai, 1987). \textit{cab} encodes type-I light-harvesting chlorophyll a/b binding protein (LHCP) that is embedded in the thylakoid membrane where it associates with chlorophyll a and b and carotenoids to form a pigment-protein complex (Chang and Walling, 1992). This complex harvests light energy and transfers to the photosystem reaction centers. This complex also mediates grana stacking (Tobin and Silverthorne, 1985; Thornber, 1986). Both genes are encoded in nuclear genome; the proteins are synthesized as precursors in the cytoplasm and these pre-proteins are modified upon transport into the chloroplast (Schmidt et al., 1981; Thornber, 1986). Therefore, examination of these two genes seems to be suitable for studying functional differentiation in photosynthesis during cellular development in leaf tissues.

In rice plants, there are two-layered bundle sheaths, the inner sheath called the mestome sheath and outer sheath called the parenchymatous bundle sheath. The cells of the mestome sheath are smaller than those of the outer sheath in cross sectional diameter and contain few plastids, if present, which are small and little differentiated. In addition, the walls are thickened and contain suberized lamellae (Dengler et al., 1985; Fahn, 1990). In this paper, the term "bundle sheath" represents the parenchymatous bundle sheath.

\section*{Materials and Methods}

\subsection*{1. Plant materials}

Seeds of rice (\textit{Oryza sativa} L. cv. Nipponbare) were surface sterilized with 5\% sodium hypochlorite solution for 10min. After washing several times with distilled water, seeds were imbibed in distilled water in a growth chamber at 28\textdegree C for 3 days. After imbibition, seeds were sown on plastic nets placed on the surface of 250ml distilled water (water culture) in tall beakers. Seedlings were grown in a culture room under a 16h photoperiod (ca. 75 \textmu mole m\textsuperscript{-2} s\textsuperscript{-1} of photosynthetically active radiation (PAR) at 28\textdegree C for 5 days until the third leaf emerging stage.

For starch staining experiments, rice plants were grown under various conditions, that is, water culture, water culture under dark condition and soil culture. The soil culture was conducted in a glasshouse until the fifth leaves emerged.

\subsection*{2. Starch staining}

Segments of 5mm in length were cut with a razor blade from the tip, middle, and basal regions of the emerging third leaf blades of about 4cm long. Segments including the lamina joint of the mature second leaves and of the emerging third leaves were also used. At the sampling time, the tip region of the third leaves had emerged from the sheath of the second leaves but the basal region was covered by the sheath. The middle region was the transition zone emerging from the sheath. The samples were fixed in FAA (95\% ethanol: acetate: 57\% formaldehyde solution: sterilized water = 10: 1: 2: 7) and embedded in paraffin after ethanol and t-butanol dehydration. Tissue sections (10 \textmu m thick) were mounted on glass slides. Then, starch grains were stained with I-KI solution containing 10 g L\textsuperscript{-1} iodine and 20 g L\textsuperscript{-1} potassium iodide for 20min. These sections were observed under a microscope (Nikon OPTIPHOT-2).

\subsection*{3. Preparation of probes}

A 439-bp fragment containing open reading frame of \textit{rbcS} (Matsuoka et al., 1988, Accession number D00644) and a 527-bp fragment containing open reading frame of \textit{cab} (Matsuoka, 1990, Accession number D00641) were amplified using the polymerase chain reaction (PCR). cDNA from rice leaves was used as the template for PCR. The gene-specific primers for \textit{rbcS} and \textit{cab} were as follows: 5\textquoteright-AATCCAGGGCTCAAGTCCAC-3\textquoteright in sense and 5\textquoteright-GAAGCTAATCAACTGCACTGTG-3\textquoteright in anti-sense orientation for the open reading frame of \textit{rbcS} and 5\textquoteright-CACAAGACGGAGCTGGA-3\textquoteright in sense and 5\textquoteright-ACCTCAGTTGCCGGGGACGAA-3\textquoteright in anti-sense orientation for the open reading frame of \textit{cab}. The products were cloned in TOPO vector (Invitrogen) at EcoR I site. The cloned vector was digested with Xba I to produce SP6 RNA polymerase template for sense probe and with \textit{Hind III} to produce T7 RNA polymerase template for anti-sense probe. The probes were produced with DIG-11-UTP Labeling Mix (Roche).

\subsection*{4. \textit{in situ} hybridization}

Samples embedded as above were sectioned and mounted on glass slides treated with silane. Then, the sections were hybridized with RNA probes produced as above and the hybridized probes were detected immunologically with anti-DIG alkaline phosphatase (Roche) and stained with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyI-phosphate. Microscopic observation was conducted with a Nikon OPTIPHOT-2 microscope.
5. **Electron microscopy**  
The lamina joint chloroplasts were observed by electron microscopy according to Yamane et al. (2003), except that 5% glutaraldehyde in 0.05M phosphate buffer (pH 7.2) was employed as the fixative.

6. **Chlorophyll content**  
Chlorophyll content was determined as an approximate indicator of chloroplast development. Segments of 1 cm in length were cut from the tip, middle, and basal regions of the emerging third leaf blades. The chlorophyll content of the segments was assayed according to Knudson et al. (1977), except that we determined the chlorophyll content per fresh weight of leaves. Data obtained were statistically analyzed by Tukey’s HSD test.

**Results**

1. **Starch accumulation in bundle sheath cells in young leaf blades and the mature lamina joint**  
Emerging leaves of rice accumulate starch grains in bundle sheath cells at early developmental stages (Miyake and Maeda, 1976). We examined whether different cells of young leaves grown under different environments accumulate starch grains. Since the leaf tissues showed similar starch accumulation pattern in all growth conditions, the results of water culture were shown as representation. In emerging young leaf blades, starch grains predominantly accumulated in bundle sheath cells (Fig. 1). This indicates that the function of bundle sheath cells is different from that of mesophyll cells at least with respect to starch synthesis or sugar transport in rice plants. Starch grains were scarcely observed in mestome sheath cells. The starch accumulation in bundle sheath cells was also observed in rice seedlings grown in the dark (data not shown). This means that the starch accumulated in bundle sheath is derived from other organs such as endosperm (Miyake and Maeda, 1976).

The distribution of starch grains were also investigated in the lamina joint of mature leaves. To detect the lamina joint, we observed the ligule in serial sections (Fig. 4C, D). The ligule is a thin, tongue-like white organ with a triangular shape and originated from a degenerated leaf sheath (Matsuoka et al., 1995). Ligule is localized at the adaxial side between a sheath and a blade and separates the leaf into these two regions. Therefore, the lamina joint is detected just above the insertion point of the ligule. The lamina joint is known as a gravitropic region. We confirmed the report of Nakano and Maeda (1978) that starch grains were accumulated in bundle sheath cells in the lamina joint of mature leaves (Fig. 2A). This also suggests that bundle sheath cells of this region have functions different from those of mesophyll cells. Plastids in bundle sheath cells of the lamina joint developed a few thylakoids and contained large starch grains (Fig. 2B), showing features of amylochloroplasts in bundle sheath cells of leaf blades (Miyake and Maeda, 1976). Similar plastids were not observed but small chloroplasts existed in mesophyll cells. On the other hand, small starch grains were evenly distributed in the mesophyll and bundle sheath cells of the lamina joint of emerging young leaves (Fig. 2C). This portion corresponds to the portion below the basal region of the leaf blade shown in Fig. 1C. Around this stage, the differentiation between bundle sheath and mesophyll cells is obscure.

![Fig. 1. The distribution of starch grains in the emerging leaf blade of rice. Starch grains are stained with I-KI. A. Tip region. B. Middle region. C. Basal region. Bars: 20µm.](image-url)
2. The expression of photosynthesis-related genes in young leaf blades and the lamina joint of mature leaves

Our starch-staining analysis confirmed the previous report that emerging leaves of rice accumulated starch in bundle sheath cells and suggested that these cells have a function different from that of mesophyll cells. To examine whether this functional difference between bundle sheath and mesophyll cells is reflected in the expression of photosynthesis-related genes, we examined the expression of rbcS and cab by in situ hybridization. No signal was observed with sense probes (Fig. 3A, B; 4A, B).

The expression of rbcS as visualized by in situ hybridization was localized in mesophyll cells but the signal was scarce if any in bundle sheath cells of the leaf tip, the oldest region of a leaf blade (Fig. 3C). The expression in mesophyll cells was also observed in the middle region (Fig. 3E). In the basal region of leaf blade, however, rbcS expression was observed not only in mesophyll cells but also in bundle sheath cells (Fig. 3G). No signal of gene expression by in situ hybridization was observed in other tissues, such as epidermis including guard cells, sclerenchyma, vascular tissues and mestome sheath cells. These results indicated that the expression of rbcS first appears in both bundle sheath and mesophyll cells and becomes restricted to the mesophyll cells in later stages.

The expression pattern of cab was similar to that of rbcS. The expression of cab was localized in mesophyll cells in the tip and the middle regions of leaf blades (Fig 3D, F). However, the expression of cab was observed in both bundle sheath and mesophyll cells in the basal region (Fig. 3H). Weak signals of gene expression were observed in vascular cells.

Starch accumulation in bundle sheath cells (Fig. 1C) precedes the decrease in expression of photosynthetic genes in bundle sheath cells (Fig. 3G, H).

The expression of photosynthesis-related genes in the lamina joint of mature leaves was also examined (Fig. 4). No expression of rbcS in the lamina joint was detected (Fig. 4C, E). The expression of cab was not observed, either, although weak signals were detected in vascular cells especially in companion cells (Fig. 4D, F). These results suggest that the mature lamina joint is not competent for photosynthesis. In addition, the expression of rbcS and cab was weak even in the lamina joint of emerging young leaves (Fig. 5), which is below the basal region shown in Fig. 3G and H. Within the lamina joint of emerging young leaves, weak expression of rbcS (Fig. 5C) and cab (data not shown) was observed in the abaxial region of the ground tissue.

3. Chlorophyll content in young leaf blades

Chlorophyll content in the tip, middle and basal regions of emerging third leaves were determined
Fig. 3. The expression of *rbcS* (A, C, E, G) and *cab* (B, D, F, H) in the emerging third leaf detected by *in situ* hybridization. Sections were hybridized with sense (A, B) or anti-sense probes (C-H). A, B. Middle region. C, D. Tip region. E, F. Middle region. G, H. Basal region. Bars: 20 µm.
as an indicator of chloroplast development and the results are shown in Fig. 6. The chlorophyll content was increased from the base to the tip. Even the basal region contained chlorophyll. Lamina joint used in this examination was too small to determine chlorophyll content.

Discussion

In developing leaf blades of a C₄ plant maize, Rubisco large and small subunit genes are expressed in a ring of cells tightly surrounding the region of provascular cell division (Nelson and Langdale, 1992). This position-specific expression occurs at a time in the leaf primordium when neither bundle sheath nor mesophyll is morphologically distinct. This fact suggests that positional control of photosynthetic development in maize bundle sheath and mesophyll cells must occur very early in the leaf development, concurrent with or just after the initiation of veins (Nelson and Langdale, 1989). However, there has been no detailed study on the expression of photosynthesis-related genes during the leaf development of C₃ plants. Therefore, we examined the expression pattern of photosynthesis-related genes, \( rbcS \) and \( cab \), in different regions of the leaf blade in rice with \textit{in situ} hybridization.

Fig. 4. The expression of \( rbcS \) (A, C, E) and \( cab \) (B, D, F) in lamina joint detected by \textit{in situ} hybridization. Sections were hybridized with sense (A, B) or anti-sense probes (C-F). A, B, Vascular bundle and the surroundings in lamina joint of second leaf. C, D, An emerging third leaf and the lamina joint of mature second leaf are observed. E, F, Vascular bundle and the surroundings in the lamina joint of second leaf. Bars: 50 \( \mu \text{m} \).
We confirmed that starch grains predominantly accumulate in bundle sheath cells in young emerging leaves of rice (Fig. 1), which coincides with the previous reports (Miyake and Maeda, 1976; Miyake and Maeda, 1978). These facts indicate that bundle sheath cells possess functions different from mesophyll cells in immature leaves of seedlings. Therefore we examined whether the expression of photosynthesis-related genes is different between these cells during leaf development.

The expression of \textit{rbcS} was observed in mesophyll cells at the older tip and the middle regions of leaf blades in rice seedlings (Fig. 3C, E). This fact is consistent with a previous report (Kyozuka et al., 1993). However, in the younger basal region, \textit{rbcS} was expressed in both bundle sheath and mesophyll cells (Fig. 3G). The expression pattern of \textit{cab} was similar to that of \textit{rbcS}. The expression of \textit{cab} was observed in mesophyll cells in the tip and the middle regions (Fig. 3D, F), whereas it was observed in both bundle sheath and mesophyll cells in the young basal region (Fig. 3H). These results indicate that the expression pattern of two genes is less tissue-specific at the early developmental stage and gradually becomes mesophyll-specific. However, Rubisco protein as examined by immunoelectron microscopy is reported to be located also in bundle sheath chloroplasts of mature rice leaves (Yamane et al., 2003). These facts suggest that transcription of \textit{rbcS} occurs in bundle sheath cells at immature stages and ceases at mature stages whereas Rubisco protein remains even at mature stages.

Yamane et al. (2003) and Yoshimura et al. (2004) observed well-developed grana in bundle sheath chloroplasts of mature rice leaves, indicating that LHCP II, which is encoded by \textit{cab} and plays a role for stacking of grana (Tobin and Silverthorne, 1985; Thornber, 1986), exists in bundle sheath chloroplasts of mature rice leaves. Therefore, the transcription of \textit{cab} may occur in bundle sheath cells at immature stages and cease at mature stages whereas LHCP II remains, as observed for \textit{rbcS}. Bundle sheath cells of several C₃ plant species contain small amount of photosynthetic organelles (Kinsman and Pyke, 1998; Koroleva et al., 2000; Ueno et al., 2003; Yoshimura et al., 2004). From these facts, it is suggested that bundle sheath cells of rice leaves possess lower photosynthetic activity than mesophyll cells if they possess photosynthetic competency.

Bundle sheath and mesophyll may differentiate from ground meristem in C₃ Poaceae plants while vascular bundles differentiate from procambium (Dengler et al., 1985). The present results suggest that the cells from ground meristem express the photosynthesis-related genes in the early stages and that the tissue adjacent to the vascular bundle ceases to express these genes in later stages. The photosynthesis-related gene expression in rice leaves seems to be affected by the origin of cells in the early stages of leaf development and by positional information in later stages.

No expression of \textit{rbcS} and \textit{cab} were detected in bundle sheath or mesophyll cells of the lamina joint (Fig. 4). These facts suggest that the lamina joint is not a photosynthetically-competent region. Furusawa et al. (1996) suggests that lamina joint is involved in the response to gravity as the unit of "pulvinus-leaf
sheath-lamina joint”. The starch grains accumulated in bundle sheath cells in the lamina joint may function as specialized statolith in the gravity perception mechanism (Nakano and Maeda, 1978). In addition, Nakano and Maeda (1978) observed these starch grains accumulated in the seedlings grown in darkness. This means that these starch grains are not derived from photosynthesis in this region but derived from other organs, e.g. endosperm and/or other highly photosynthetically competent region in a leaf as in the bundle sheath cells of a leaf blade. The expression of these genes was suppressed even in the lamina joint of emerging young leaves, which is a marked contrast with the leaf blade (Fig. 5). These results suggest that the lamina joint is destined for the gravity perception from the early stages of leaf development. The weak expression of cab was observed in vascular cells (Fig. 4F). Further studies are needed to explain this result.

| Starch Accumulation | rbcS and cab Expression | Chlorophyll Content (mg · g⁻¹ FW) |
|---------------------|--------------------------|----------------------------------|
| Mesophyll           | Bundle Sheath            | Mesophyll | Bundle Sheath |
| tip                 | -                        | +         | -             | 1.97±0.44ᵃ |
| middle              | -                        | ++        | +             | 1.17±0.23ᵇ |
| base                | +                        | ++        | +             | 0.45±0.03ᵇ |
| Lamina Joint (2nd leaf) | -                      | ++        | -             | nd          |
| Lamina Joint (3rd leaf) | +                      | +         | -             | nd          |

Fig. 6. Diagram representing a shoot of a rice seedling examined and table indicating starch accumulation, the expression of photosynthesis-related genes and chlorophyll content. Solid region indicates leaf blade and open region indicates leaf sheath on the diagram. Data of chlorophyll content are means ± SE. The same letters represent no significant difference at P<0.05 by Tukey’s HSD test.
Weak expression of \( rbcS \) and \( cab \) was observed in the abaxial region of the ground tissue in the immature lamina joint (Fig. 5C). It was reported that \( sps1 \) (a gene encoding sucrose phosphate synthase) expression shows transversal gradient in sheathed leaves, being higher in the outermost and lower in the innermost (Chávez-Bárcenas et al., 2000). These authors suggested that light, in part, has a major influence on the pattern of \( sps1 \) expression during leaf development. Therefore, the weak expression of \( rbcS \) and \( cab \) in the abaxial region is also possibly to be attributed to the light intensity.

Our data showed that mesophyll-specific expression of photosynthesis-related genes is preceded by morphological differentiation between bundle sheath and mesophyll tissue, whereas the bundle sheath-specific expression of the gene coding Rubisco small subunit is observed in \( C_4 \) maize leaves before morphological differentiation between bundle sheath and mesophyll tissue becomes apparent (Nelson and Langdale, 1992). It is suggested that there is a factor passing from vein to bundle sheath and restricting Rubisco gene expression to bundle sheath cells in \( C_4 \) maize leaves (Nelson and Langdale, 1992). Our data suggest that such a factor, if any, may begin functioning at later stages of leaf development in rice. This factor may function in restricting the expression of \( rbcS \) and \( cab \) to mesophyll cells.

In conclusion, our data revealed the pattern of photosynthesis-related gene expression and of starch accumulation, and showed that the expression of photosynthesis-related genes in rice leaves is less tissue specific at very early stage but gradually becomes mesophyll-specific during leaf development after the starch accumulation in bundle sheath cells begins.

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