Structural and Functional Differentiation of Bundle Sheath and Mesophyll Cells in the Lamina Joint of Rice Compared with that in the Corresponding Region of the Liguleless Genotype

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Abstract: The structural and functional characterization of the blade-sheath boundary region of a rice cultivar T65 and its near-isogenic line T65lg were examined by light and electron microscopy and in situ hybridization. Starch accumulation in bundle sheath cells was compared between the lamina joint of T65 and the corresponding region of T65lg and also between the lamina joint and the leaf blade. In the lamina joint of T65, starch grains were predominantly accumulated in bundle sheath cells, and the starch-containing chloroplasts within these cells were spherical in shape. On the other hand, in the blade-sheath transition region of T65lg, little starch accumulation was observed and the chloroplasts were oval in both mesophyll and bundle sheath cells. Furthermore, photosynthesis-related genes, *rbcS* and *cab*, were expressed in mesophyll cells within the blade-sheath transition region of T65lg as in the leaf blade and sheath, while no expression of these genes was found within the lamina joint of T65. These facts indicate that T65lg can not develop the lamina joint from either structural or functional aspect. The present results suggest that the control mechanism of starch accumulation in bundle sheath chloroplasts in the lamina joint differs from that in leaf blade in rice.

Key words: Blade-sheath boundary region, Bundle sheath, C₃ plant, Lamina joint, Liguleless, Photosynthesis-related gene expression, Plastid structure, Starch accumulation.
carboxylase/oxygenase (rbcS) and the light harvesting chlorophyll a/b binding protein (cab), was suppressed in the lamina joint despite the occurrence of granal chloroplasts, while these genes were expressed in mesophyll cells of mature leaf blades (Tsutsumi et al., 2006). These facts also suggest that the lamina joint is functionally different from the leaf blade.

It is known that there are spontaneous liguleless lines in rice. These liguleless lines are not competent in bending leaf blades, while auxin-treated normal rice leaf blades bend at the lamina joint (Maeda, 1962). This fact indicates that liguleless rice lacks the function of the lamina joint. There are also several liguleless lines in maize. These liguleless maize lines have been studied to investigate the mechanisms regulating leaf development (Sylvester et al., 1996). However, structural and functional differentiation in the inner structure, especially with respect to bundle sheath and mesophyll cells, have not been investigated in detail. Although the lamina joint of rice has specialized structure and function as mentioned above, the blade-sheath transition region of liguleless rice has not been studied in detail.

In this paper, we report the structural features and the expression of photosynthetic genes in the region corresponding to the lamina joint of a liguleless line of rice. We discuss the difference in the function of bundle sheath cells between the lamina joint and the leaf blade.

Materials and Methods

1. Plant materials

A rice cultivar (Oryza sativa L. cv. Taichung 65) (T65) and its near-isogenic liguleless line T65lg were used. The liguleless gene of T65lg had been introduced from H-79, a linkage tester of Hokkaido University (Oka, 1974), into T65 as a recurrent parent by backcrossing eight times. Seeds of T65 and T65lg were surface sterilized with 5% sodium hypochlorite solution for 10 min. After washing several times with distilled water, seeds were imbibed in distilled water in a growth chamber at 28ºC for 3 days. After imbibition, seeds were sown in plastic nets placed on the surface of 250 mL distilled water in tall beakers. Seedlings were grown in a culture room under continuous light (ca. 75 µmole m⁻² s⁻¹ of photosynthetically active radiation (PAR)) at 28ºC for 6 days until the second leaves expanded.

2. Scanning electron microscopy (SEM)

Segments of 2 mm in length were cut with a razor blade from mature second leaves including the lamina joint in T65 or its corresponding region in T65lg. The samples were fixed in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) and then dehydrated with grading ethanol series. For critical point drying, isopentyl acetate was used as intermediate liquid for introducing CO₂. Critical point drying was carried out in a Hitachi HCP-2 apparatus using CO₂ as the transition. These specimens were coated with platinum with ion sputtering apparatus, Eiko IB-3, and observed under a scanning electron microscope (Hitachi S-4200).

3. Starch staining

Segments of 5 mm in length were cut with a razor blade from the lamina joint and its corresponding region of the mature second leaves including the emerging third leaves. The samples were fixed in FAA (70% ethanol: acetate: 37% formaldehyde solution = 18 : 1 : 1) and embedded in paraffin after ethanol and t-butanol dehydration. Tissue sections (10 µm thick) were mounted on glass slides. Then, starch grains were stained with I-KI solution containing 2.5 g L⁻¹ iodine and 5 g L⁻¹ potassium iodide for 20 min. These sections were observed under a microscope (Olympus BX51).

4. In situ hybridization

The samples embedded as above were sectioned and mounted on glass slides treated with silane. Then,
the sections were hybridized with RNA probes for rbcS and cab prepared as Tsutsumi et al. (2006) 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-indolyl-phosphate. Microscopic observation was conducted as above.

5. Transmission electron microscopy (TEM)

The method of electron microscopic observation of lamina joint chloroplasts was according to Tsutsumi et al. (2006).

6. Stability of bundle sheath starch grains

To examine the stability of starch grains in the lamina joint and the emerging leaf blades, we separated the shoots of rice plants grown as above from the seeds and roots and kept them in the dark for 1 to 3 d with the basal 1 cm portion immersed in distilled water. Observation of starch grains was conducted as above.

Results

1. Surface structure of the lamina joint of T65 and the corresponding region of T65lg

The blade of T65 was inclined at the lamina joint by sensing gravity force (Fig. 1A). The inclination of blade was increased by treatment with 5 mg L\(^{-1}\) IAA (Fig. 1C; Maeda, 1962). In contrast, the inclination of blade was not induced in T65lg either by gravity force or IAA (Fig. 1B, D).

The leaf of T65 consisted of the blade (Fig. 2B), sheath (Fig. 2E) and the lamina joint (Fig. 2C, D) with the ligule (Fig. 2A). The adaxial surface of leaf blade of T65 showed crenulated cell shape and had a great number of verrucas, occasional hairs and several rows of stomata (Fig. 2B). On the other hand, the cells on the adaxial surface of the leaf sheath were noncrenulated rectangular in shape and possessed no specialized structures on the surface (Fig. 2E), except that stomata were occasionally observed at a much lower frequency than the leaf blade. The adaxial surface of the lamina joint showed cell bulges but no other specialized structures (Fig. 2C, D). The abaxial surface of the lamina joint of T65 was smooth in contrast with that of leaf blade and sheath in which many verrucas were observed (data not shown).

The leaf blade-sheath boundary of T65lg is indistinguishable when observed macroscopically (Fig. 1). To clarify the blade-sheath boundary, we observed on the surface of T65lg (Fig. 3). The adaxial surfaces of both leaf blade and sheath of T65lg were similar to those of T65 (Figs. 2B, E and 3B, E): The adaxial surface of the leaf blade showed a crenulated cell shape and possessed hairs, stomata and many verrucas, whereas adaxial surface of leaf sheath showed a noncrenulated rectangular cell shape and possessed no specialized structures on the surface. These results indicate that we can distinguish leaf blade from leaf sheath in T65lg based on the adaxial surface structures, especially the verrucas.

In addition, it was evident that T65lg leaf lacked the characteristic lamina joint region (Fig. 3A compared with Fig. 2A). The blade-sheath transition region of T65lg exhibited transitional feature from blade to sheath (Fig. 3D). Wax and verrucas were reduced basipetally from the leaf blade. The blade-sheath boundary of T65lg was not straight but irregular through the leaf width (Fig. 3A, C), in contrast to the more or less straight boundary between leaf blade and the lamina joint of T65 (data not shown).

There is no obvious difference between the abaxial surface of leaf blade and sheath in either T65 or T65lg. The abaxial surface of leaf blade and sheath was similar to the adaxial surface of leaf blade (data not shown).

2. Anatomy and cell specific starch accumulation in the lamina joint of T65 and the corresponding region of T65lg

The anatomy of the lamina joint of T65 and the corresponding region of T65lg was different. In the lamina joint of T65, several small vascular tissues were observed on the adaxial side near the midvein region of the leaves (Fig. 4C, adIV), in addition to the vascular bundles in the abaxial side which were distant from epidermis (Fig. 4C; Maeda, 1961). On the other hand, vascular tissues in the adaxial side of the corresponding region of T65lg did not develop as in the lamina joint of T65 and the vascular bundles in the abaxial side were not apart from epidermis (Fig. 4G). The lamina joint of T65 was thicker in the transverse section than the corresponding region of T65lg (Fig. 4C, G). In a transverse section of the blade-sheath transition region of T65lg, both the blade-like region with verrucas and the sheath-like region without verrucas were observed (Fig. 4G).

Starch accumulation was observed in bundle sheath cells including bundle sheath extension cells in the lamina joint of T65 (Fig. 4C), the result being consistent with the previous reports (Nakano and Maeda, 1978; Tsutsumi et al., 2006). On the other hand, bundle sheath cells of the blade-sheath transition region of T65lg accumulated few starch grains (Fig. 4G).

Bundle sheath and mesophyll cells of the mature leaf blade of both T65lg and T65 accumulated few starch grains (Fig. 4B, F). The bundle sheath and mesophyll cells of mature leaf sheath of both T65lg and T65 also accumulated few starch grains (Fig. 4D, H). In addition, common adaxial surface characteristics of T65 and T65lg, namely verrucas on the blade and a lack of verrucas on the sheath observed by SEM, were also recognized in longitudinal sections under a light
Fig. 2. Adaxial structure of ligule region and its vicinity in T65 observed with SEM. The top of all figures indicates distal side. A, The vicinity of lamina joint. To unveil the lamina joint, leaf marginal region was broken; B, Leaf blade; C, Leaf blade-lamina joint boundary; D, Lamina joint. E, Leaf sheath. Bars: A, 200 µm; B-E, 20 µm.
Fig. 3. Adaxial surface of T65lg observed with SEM. The top of all figures indicates distal side. A, The blade-sheath transition region; B, Leaf blade; C, The blade–sheath transition boxed in A; D, The blade-sheath transition region boxed in C; E, Leaf sheath; Bars: A, 200 µm; B-E, 20 µm.
Fig. 4. The distribution of starch grains in lamina joint of T65 (A-D) and in blade-sheath transition region of T65lg (E-H). Starch grains are stained with I-KI. A, E, Longitudinal sections of leaf blade-sheath transition region; B, F, Transverse sections of leaf blades; C, Lamina joint; D, H, Leaf sheaths; G, The leaf blade-sheath transition region. An arrow shows adaxial epidermal cells with verrucas. An arrowhead shows adaxial epidermal cells without verrucas. Bars: 100 $\mu$m (A, E), 50 $\mu$m (B-D, F-H).

Fig. 5. Lamina joint of 2nd leaf and leaf blade of emerging 3rd leaf of T65 incubated for 1d (A) or 3d (B) under dark condition. Bars: 50 $\mu$m.
microscope (Fig. 4A, E).

Starch grains in bundle sheath and bundle sheath extension cells in the lamina joint of T65 were more stable than those in emerging third leaf blades when the plants were kept in the dark. The starch grains that had been accumulated in bundle sheath cells of emerging third leaves completely disappeared within 3d of darkness while those of the lamina joint remained (Fig. 5). This indicates that the starch grains in bundle sheath cells of lamina joint are more...
than reserve material and suggests that a different mechanism is involved in the starch metabolism in bundle sheath cells between the emerging leaf blade and the mature lamina joint.

3. Expression of photosynthesis-related genes in the mature lamina joint of T65 and the corresponding region of T65lg

We previously reported that the expression of photosynthesis-related genes was not found in mature lamina joint, despite the existence of mature chloroplasts (Tsutsumi et al., 2006). To examine the expression of photosynthesis-related genes in blade-sheath transition region of T65lg, we examined the expression of rbcS and cab by in situ hybridization.

The expression of rbcS was observed in mesophyll cells in mature leaf blades of both T65 and T65lg but not in bundle sheath cells (Fig. 6A, D), which coincided with previous reports (Kyozuka et al., 1993; Matsuoka et al., 1994). The rbcS expression in mesophyll cells was also observed in the leaf sheath (Fig. 6C, F). On the other hand, the expression of rbcS was not found in the lamina joint of T65 (Fig. 6B), which coincided with our previous results (Tsutsumi et al., 2006). In the blade-sheath transition region of T65lg, the expression of rbcS was observed only in mesophyll cells (Fig 6E), the result being comparable with that in the blade and sheath (Fig. 6D, F). The region in which the expression of photosynthetic genes was suppressed as in the lamina joint of T65 was not found. This indicates that T65lg could not develop the lamina joint region from the aspect of photosynthetic gene expression.

The expression pattern of cab was almost the same as that of rbcS (data not shown).
4. **Ultrastructure of bundle sheath plastids within the lamina joint of T65 and the corresponding region of T65lg**

To examine the structure of photosynthetic and starch accumulating apparatus within the blade-sheath transition regions of T65 and T65lg, we conducted TEM observation (Fig. 7). In the lamina joint of T65, chloroplasts of bundle sheath cells were spherical in shape and possessed large starch grains and several grana (Fig. 7A), showing features of amylochloroplasts in bundle sheath cells of developing leaf blades (Miyake and Maeda, 1976), whereas mesophyll chloroplasts possessed small or no starch grains (Fig. 7B).

In the leaf blade-sheath transition region of T65lg, starch grains in bundle sheath plastids were smaller than those in the lamina joint of T65 (Fig. 7C). This observation corresponds to the results obtained by light microscopy (Fig. 4G). The ultrastructural features of bundle sheath chloroplasts of T65lg are similar to those of the mesophyll chloroplasts (Fig. 7D). The ultrastructure of bundle sheath and mesophyll chloroplasts of T65lg was similar to that of the basal region of the leaf blade just above the lamina joint of T65 (Fig. 7E, F).

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

### Discussion

1. **Ligule of grass species and liguleless mutant**

Grass species develop the ligule between the leaf blade and the sheath, although several species such as some species in *Echinochloa* normally lack the ligule (Chaffey, 2000). Many genes are involved in the ligule development in the grass species. In rice, there are three genes known to affect the development of the ligule: *auricleless* (*aul*), *liguleless* (*lg*) and *collarless* (*col*) (Kurata et al., 2005). The *aul* mutant does not possess auricles but rudimental ligules. The *col* mutant lacks collars (Sanchez and Khush, 1998). The *lg* rice lacks the ligule, auricle and lamina joint (Maekawa, 1988). T65lg used in the present investigation carried *lg* gene in the genetic background of T65.

In grass species, maize has been the most frequently used for studying the leaf development mechanisms. The morphological events during the development of lamina joint region have been clarified by using a liguleless mutant (Becraft et al., 1990; Moreno et al., 1997). In addition, Sylvester et al. (1990) suggested that the ligule itself is not required to differentiate blade from sheath. On the other hand, the lamina joint region of rice has not been investigated in detail.

2. **Structural features of the blade-sheath transition region of liguleless rice**

We observed the adaxial surface of the blade-sheath boundary region of both liguleless and normal rice by SEM. The blade-sheath boundary region of liguleless maize has been observed by SEM in detail during leaf development (Becraft et al., 1990; Sylvester et al., 1990; Harper and Freeling, 1996; Walsh et al., 1998). Our findings obtained by SEM from T65lg are similar to those of liguleless maize in that gradual transition from blade to sheath occurs in longitudinal axis, there is a non-straight blade-sheath boundary and the ligule region consisting of ligule, auricle and lamina joint is absent. These features were confirmed in both transverse and longitudinal sections by light microscopy (Fig. 4). These facts indicate that T65lg can not develop lamina joint and that the leaf blade gradually transforms to sheath along the longitudinal axis.

3. **Starch accumulation and the expression of photosynthesis-related genes**

Large starch grains were accumulated in bundle sheath cells of the lamina joint (Fig. 4C). They were shown to move along the gravity vector and are speculated to function in gravity-sensing (Nakano and Maeda, 1978). In contrast, little starch accumulation was observed in bundle sheath cells of blade-sheath transition region of T65lg (Fig. 4E, G). These small starch grains did not move along the gravity vector (data not shown). These results indicate that bundle sheath specific starch accumulation in mature leaves is correlated with the differentiation of lamina joint.

The bundle sheath cells of the emerging leaves accumulate large starch grains (Miyake and Maeda, 1976; Tsutsumi et al., 2006). The starch grains accumulated in bundle sheath cells of the lamina joint are more stable than those of emerging leaf blades under a dark condition (Fig. 5). These facts suggest that starch grains accumulated in bundle sheath...
cells in the lamina joint are not for temporal storage as in emerging leaf blades but are the functional component for gravity-sensing.

The expression of photosynthesis-related genes, \textit{rbcS} and \textit{cab}, was observed in mesophyll cells in the blade-sheath transition region of T65lg. This feature is similar to that in leaf blades and sheaths of both T65 and T65lg and indicates that the leaf blade is gradually replaced by the sheath along the longitudinal axis in T65lg. On the other hand, no expression of photosynthetic genes was found in either mesophyll or bundle sheath cells of the lamina joint of T65, which corresponds to our previous report (Tsutsumi et al., 2006). The results of starch accumulation and photosynthetic gene expression indicate that T65lg lacks the lamina joint from both structural and functional aspects.

Plastids observed in bundle sheath cells of lamina joint of T65 were spherical in shape, accumulated large starch grains and possessed grana and thylakoids (Fig. 7A). Mesophyll chloroplasts in lamina joint of T65 also possessed developed grana and thylakoids (Fig. 7B). However, photosynthetic genes were not expressed either in bundle sheath or mesophyll cells of lamina joint (Fig. 6B). These results suggest that the lamina joint possessed very low photosynthetic activity if any.

4. Bundle sheath cells within the lamina joint are functionally different from those in emerging leaf blades

The bundle sheath cells of both lamina joint and emerging leaf blades accumulate large starch grains. The starch grains in bundle sheath cells of leaf blades were accumulated during emerging stage and disappeared in expanded stage, whereas the bundle sheath cells of the lamina joint accumulated starch grains in mature leaves.

The starch grains accumulated in bundle sheath cells of emerging leaves disappeared under the dark condition, while those of mature lamina joint persisted. The expression of photosynthesis-related genes in the lamina joint of T65 was suppressed, whereas that in the blade-sheath boundary region of T65lg was not. The present results suggest that the properties of lamina joint, such as bundle sheath-specific starch accumulation and suppression of photosynthetic gene expression, are regulated in the down-stream of \textit{Lg} gene. The present findings also suggest that starch accumulation in the lamina joint is controlled by mechanisms different from those in the bundle sheath cells of emerging leaf blades.

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References

Becraft, P.W., Bongard-Pierce, D.K., Sylvester, A.W., Poething, R.S. and Freeling, M. 1990. The \textit{liguleless-1} gene acts tissue specificity in maize leaf development. Dev. Biol. 144 : 220-232

Chaffey, N. 2000. Physiological anatomy and function of the membranous grass ligule. New Phytol. 146 : 5-21.

Dengler, N. and Nelson, T. 1999. Leaf structure and development in \textit{C}_{4} plant. In R.F. Sage and R.K. Monson eds., \textit{C}_{4} Plant Biology. Academic Press, New York. 133-172.

Esau, K. 1953. Plant Anatomy. John Willy & Sons Inc. New York. 411-469.

Furusawa, K., Takahashi, K. and Hoshikawa, K. 1996. Analysis of gravity-induced growth response of shoot in rice (\textit{Oryza sativa L.}): Response of leaf pulvinus, lamina joint and torsion of leaf sheath. Jpn. J. Crop Sci. 65 : 14-40.

Harper, L. and Freeling, M. 1996. Interaction of \textit{liguleless1} and \textit{liguleless2} function during ligule induction in maize. Genetics 144 : 1871-1882.

Kurata, N., Miyoshi, K., Nonomura, K., Yamazaki, Y. and Ito, Y. 2005. Rice mutants and genes related to organ development, morphogenesis and physiological traits. Plant Cell Physiol. 46 : 48-62.

Kyozuka, J., McElroy, D., Hayakawa, T., Xie, Y., Wu, R. and Shimamoto, K. 1993. Light-regulated and cell-specific expression of tomato \textit{rbcS-gusA} and rice \textit{rbcS-gusA} fusion genes in transgenic rice. Plant Physiol. 102 : 991-1000.

Maeda, E. 1961. Studies on the mechanism of leaf formation in crop plants II. Anatomy of the lamina joint in rice plant. Proc. Crop Sci. Soc. Jpn. 29 : 234-239.

Maeda, E. 1962. Studies on the mechanism of leaf formation in crop plants. III. Effects of gibberellin on the extension of lamina joints in intact rice seedlings. Proc. Crop Sci. Soc. Jpn. 31 : 49-54.

Maekawa, M. 1988. A new allele at the \textit{lg} locus conferring short ligule. Rice Genet. Newsl. 5 : 87-89.

Matsuoka, M., Kyozuka, J., Shimamoto, K. and Kano-Murakami, Y. 1994. The promoters of two carboxylases in a \textit{C}_{4} plant (maize) direct cell-specific, light-regulated expression in a \textit{C}_{3} plant (rice). Plant J. 6 : 311-319.

Miyake, H. and Maeda, E. 1976. Development of bundle sheath chloroplasts in rice seedlings. Can. J. Bot. 54 : 556-565.

Miyake, H. 1999. C, photosynthesis and crop plants. Jpn. J. Crop Sci. 68 : 1-9.

Moreno, M.A., Harper, L.C., Krueger, R.W., Dellaporta, S.L. and Freeling, M. 1997. \textit{Liguleless1} encodes a nuclear-localized protein required for induction of ligules and auricles during maize leaf organogenesis. Genes Dev. 11 : 616-628.

Nakano, H. and Maeda, E. 1978. On starch statolith in the lamina joint of rice plants. Jpn. J. Crop Sci. 47 : 262-266.

Oka, H. 1974. Analysis of genes controlling F, sterility in rice by the use of isogenic lines. Genetics 77 : 521-534.

Paiva, E.A.S. and Machado, S.R. 2003. Collenchyma in \textit{Panicum maximum} (Poaceae): localisation and possible role. Aust. J. Bot. 51 : 69-73.

Sanchez, A.C. and Khush, G.S. 1998. A gene for collarless phenotype in rice. Rice Genet. Newsl. 15 : 999.
Sheen, J. 1999. C_{4} gene expression. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 : 187-217.

Sylvester, A.W., Cande, W.Z. and Freeling, M. 1990. Division and differentiation during normal and liguleless-1 maize leaf development. Development 110 : 985-1000.

Sylvester, A.W., Smith, L. and Freeling, M. 1996. Acquisition of identity in the developing leaf. Annu. Rev. Cell Dev. Biol. 12 : 257-304.

Tsutsumi, K., Taniguchi, Y., Kawasaki, M., Taniguchi, M. and Miyake, H. 2006. Expression of photosynthesis-related genes during the leaf development of a C_{3} plant rice as visualized by in situ hybridization. Plant Prod. Sci. 9 : 232-241.

Walsh, J., Waters, C.A. and Freeling, M. 1998. The maize gene liguleless2 encodes a basic leucine zipper protein involved in the establishment of the leaf blade-sheath boundary. Genes Dev. 12 : 208-218.

Yoshimura, Y., Kubota, F. and Ueno, O. 2004. Structural and biochemical bases of photorespiration in C_{4} plants : quantification of organelles and glycine decarboxylase. Planta 220 : 307-317.

* In Japanese with English summary.
** In Japanese.