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ULTRASTRUCTURE AND CARBON ISOTOPE RATIOS OF LEAVES IN C_4 SPECIES OF *RHYNCHOSPORA* (CYPERACEAE) THAT DIFFER IN THE LOCATION OF KRANZ CELLS

Osamu Ueno[1,*]

*Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan

The genus *Rhynchospora* in the Cyperaceae includes both C_3 and C_4 species. The C_4 species shows two distinct Kranz anatomies: the rhynchosporoid and the fimbristyloid–chlorocyperoid intermediate. This study reports the use of herbarium specimens to obtain the leaf ultrastructure in eight *Rhynchospora* C_4 species in relation to their C_4 biochemical subtypes, together with carbon isotope ratios. Despite the use of dried materials, electron microscopic observation revealed the structures of chloroplasts and cell walls. In C_4 species with the fimbristyloid–chlorocyperoid intermediate anatomy, Kranz cell chloroplasts had reduced grana and convoluted thylakoids, and suberized lamellae occurred in the mestome sheath cell walls. In C_4 species with the rhynchosporoid anatomy, the Kranz cell chloroplasts also had reduced grana and convoluted thylakoids but with less convolution; suberized lamellae occurred in the Kranz cell walls. These data show that the *Rhynchospora* C_4 species have ultrastructural features present in the NADP–malic enzyme–type C_4 sedges. The Kranz species had $\delta^{13}C$ values typical of C_4 plants, as expected, and the values did not differ significantly between the two Kranz anatomies. This study demonstrates that dried herbarium specimens can be used for ultrastructural observation of C_4 leaves and provide valuable information for understanding of the photosynthetic diversity.

Keywords: carbon isotope ratio, C_4 species, C_4 biochemical subtype, Cyperaceae, leaf ultrastructure, *Rhynchospora*.

Introduction

Plants are divided into three photosynthetic types according to differences in their photosynthetic carbon metabolism: C_3, C_4, and CAM (crassulacean acid metabolism) plants. Recent phylogenetic studies have revealed that C_4 plants evolved independently from C_3 progenitors among at least 62 lineages of higher plants (Sage et al. 2011). The C_4 pathway functions as a CO_2-concentrating mechanism for the C_3 cycle, thereby reducing photorespiration. Therefore, C_4 plants are thought to have evolved primarily in response to a decrease in atmospheric CO_2 levels after the end of the Cretaceous (Ehleringer and Monson 1993; Sage 2004). In general, C_4 plants thrive in warm, water-deficient environments with high intensities of light, where C_4 plants can exhibit higher photosynthetic efficiency than C_3 plants (Ehleringer and Monson 1993). C_4 plants are further divided into three C_4 subtypes, depending on which C_4 acid-decarboxylating enzyme operates in the decarboxylation process of C_4 acids: the NADP–malic enzyme (NADP-ME), NAD–malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PCK) subtypes (Gutierrez et al. 1974; Hatch et al. 1975). Although the ecophysiological and adaptive significance of each C_4 subtype remains to be elucidated (Ghannoum et al. 2011), the C_4 subtypes can provide insights into the evolutionary history among taxa of C_4 families (Christin et al. 2009; Roalson 2011).

In the Cyperaceae, there are at least five separate origins of C_4 species in four lineages: the tribes Rhynchosporeae, Abildgaardieae, Eleocharidae, and Cypereae (Roalson 2011 and literature cited therein). The C_4 species of the Cyperaceae are distributed in warm and open habitats (Lerman and Raynal 1972; Teeri et al. 1980; Takeda et al. 1985) but tend to prefer somewhat wetter habitats than C_4 species of other families (Ueno and Koyama 1987; Ueno and Takeda 1992). Although the C_4 subtypes of the Cyperaceae still have not been investigated fully, previous studies have shown that most C_4 species are of the NADP-ME-type (Ueno et al. 1986; Bruhl et al. 1987), with the exception that C_4 species of *Eleocharis* are of the NAD-ME-type (Bruhl et al. 1987; Ueno et al. 1988*a*; Ueno and Samejima 1989).

Within the tribe Rhynchosporeae, *Rhynchospora* is known to include both C_3 and C_4 species (Takeda et al. 1980). This genus contains 250–300 species whose principal center of distribution is tropical America (Koyama 1972; Thomas et al. 2009). Within *Rhynchospora*, C_4 species occur in two sections, Pluciflorae and Pauciflorae, of the Capitatae group, which belongs to the subgenus Haplostyleae (Ueno and Koyama 1987), but phylogenetic relationships among members of this genus (Thomas et al. 2009) and between the C_3 and C_4 groups (Besnard et al. 2009) have not yet been clarified. The C_4 species in *Rhynchospora* show two types of Kranz anatomy in their leaves: the rhynchosporoid and fimbristyloid–chlorocyperoid intermediate (Ueno and Koyama 1987). These anatomies differ in the location of the Kranz cells (so-called bundle sheath cells in C_4 plants; see Brown 1975 for the definitions of Kranz cells and Kranz sheath). In the rhynchosporoid anatomy (fig. 1*A*),

[1] E-mail: uenoos@agr.kyushu-u.ac.jp.

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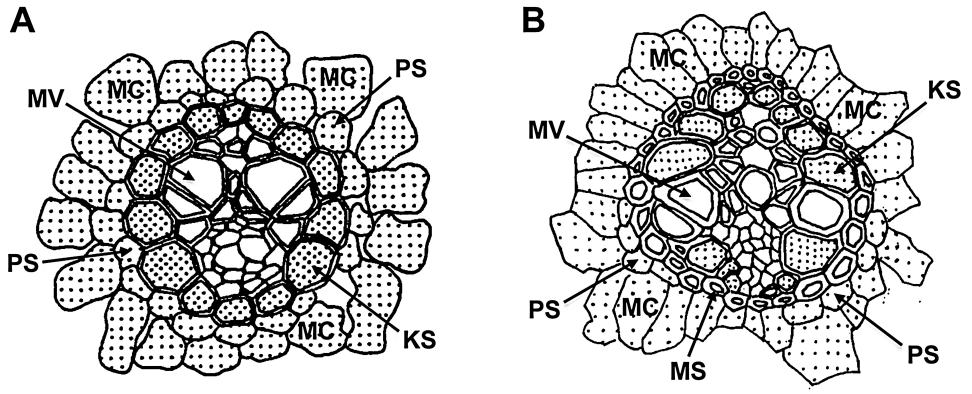

Fig. 1 Two types of Kranz anatomy in the genus *Rhynchospora*. *A*, Rhynchosporoid anatomy (*Rhynchospora rubra*). *B*, Fimbristyloid–chlorocyperoid intermediate anatomy (*Rhynchospora subpulmosa*). KS = Kranz sheath; MC = mesophyll cell; MS = mestome sheath; MV = metaxylem vessel; PS = parenchyma sheath.

a complete Kranz sheath surrounds the vascular bundle and is in direct contact with metaxylem vessel elements. Furthermore, irrespective of large and small vascular bundles, there is an incomplete parenchyma sheath outside the Kranz sheath (Takeda et al. 1980). In the fimbristyloid–chlorocyperoid intermediate anatomy (fig. 1*B*), there are three sheaths (an outermost parenchyma sheath, a middle mestome sheath, and an innermost Kranz sheath), and the Kranz sheath is interrupted by metaxylem vessel elements. In addition, the parenchyma sheath in the fimbristyloid–chlorocyperoid intermediate anatomy is incomplete in both large and small vascular bundles because of the partial absence of cells (Ueno and Koyama 1987).

In C_4 *Rhynchospora* species, several C_4 species with rhynchosporoid anatomy (*Rhynchospora rubra*, *Rhynchospora subtenuifolia*, and *Rhynchospora wightiana*) were biochemically confirmed to be of the C_4 NADP-ME subtype (Ueno et al. 1986; Bruhl et al. 1987). In addition, ultrastructural studies of these species (Ueno et al. 1988*b*; Bruhl and Perry 1995) showed that the Kranz cells contain chloroplasts with reduced grana but no significant increases in the size and number of mitochondria compared with those of the mesophyll cells, which are traits of NADP-ME-type C_4 species (Hatch et al. 1974; Gutierrez et al. 1975; Yoshimura et al. 2004). Suberized lamellae, which presumably play a role as a diffusional barrier to CO_2 leakage (Hattersley and Browning 1981; Dengler and Nelson 1999), occur in the Kranz cell walls (Ueno et al. 1988*b*; Bruhl and Perry 1995). However, C_4 *Rhynchospora* species with the fimbristyloid–chlorocyperoid intermediate anatomy have not yet been characterized biochemically or ultrastructurally in regard to their C_4 subtype. This may be because of the difficulty of collecting plants. In contrast with widespread distribution of C_4 species with rhynchosporoid anatomy in tropics and subtropics of the world, the distribution of C_4 species with the fimbristyloid–chlorocyperoid intermediate anatomy is restricted in South and Central America (Ueno and Koyama 1987). Although fresh plants are required for the biochemical characterization of the C_4 subtype, electron microscopic observation of leaves from dry herbarium specimens may provide valuable structural insight into the C_4 subtype. Ultrastructural observation of herbarium specimens has been successful for the leaves of C_4 grasses (Hattersley and Perry 1984) and C_4 sedges (Bruhl and Perry 1995).

One of the objectives in this study is to clarify whether leaf materials obtained from herbarium specimens can be used for ultrastructural observation of photosynthetic cells in C_4 plants using *Rhynchospora* C_4 species. Another objective is to characterize the leaf ultrastructure of *Rhynchospora* C_4 species of the two Kranz anatomical types relative to the C_4 subtype by using herbarium materials. In addition, this study provides carbon isotope ratios for species of the Capitatae group in *Rhynchospora*.

Material and Methods

Plants

The herbarium specimens stored in the New York Botanical Garden were the source of leaves used in this study (table 1). All samples except for *Rhynchospora armerioides* and *Rhynchospora globosa* were obtained from the same specimens that had been used for a previous leaf anatomical study (Ueno and Koyama 1987). Carbon isotope ratios were assessed in 17 Kranz and 5 non-Kranz species of the Capitatae group (table 1). From these stored samples, eight samples of Kranz species were selected for electron microscopic observation, because they had remained relatively green and were expected to maintain an intact cellular structure (table 1).

In addition, fresh plants of *Rhynchospora rubra* were examined to investigate the effect of drying on leaf ultrastructure. The plants were collected from the field (Khoradai, Kurume, Fukuoka, Japan) and transplanted into pots with field soil. They were grown in a naturally illuminated greenhouse maintained at 25°–30°C/15°–20°C (day/night temperatures). Fully expanded leaf blades were collected and dried to use as controls for fresh leaves.

TEM

For dried specimens, leaf samples were taken from midway between the apex and the base of fully expanded leaf blades

Table 1

Carbon Isotope Ratios and Ultrastructural Features of Leaves of Capitatae Group in *Rhynchospora*

Anatomy and species	Locality	Specimen	$\delta^{13}C$ (‰)	Grana in Kranz cell chloroplasts	Suberized lamella
Fimbristyloid–chlorocyperoid intermediate:					
R. armerioides Presl.	Brazil	Irwin et al. 15166	−10.4	Reduced	KC
R. barbata (Vahl) Kunth	Dominica	Basilio & Lavastre 1963	−10.6	Reduced	KC
R. hirta (Nees) Böck.	Brazil	Irwin et al. 21544	−11.2	Reduced	KC
R. mexicana (Liebm.) Steudel	Honduras	Willams & Molina 10756	−10.1	...	...
R. subimberbis Griseb.	Cuba	Britton et al. 7072	−10.1	...	...
R. subplumosa C.B. Clarke	Suriname	Maas 11039	−11.4	Reduced	KC
R. trichochaeta C.B. Clarke	Brazil	Plowman et al. 9236	−11.1	Reduced	KC
Rhynchosporoid:					
R. albo-tuberculata Kükenth.	Brazil	Duarte 118654	−10.4	...	...
R. capitatae Römer & Schlt.	Venezuela	Davidse et al. 17051	−12.2	...	...
R. confusa F. Ballard	Brazil	Irwin et al. 11995	−10.6	Reduced	MSC
R. culvula Griseb.	Trinidad	Richardson 527	−11.5	...	...
R. dentinux C.B. Clarke	Venezuela	Davidse et al. 16769	−13.0	...	...
R. diamontiana (C.B. Clarke) Kükenth.	Brazil	Pereira 98013	−9.7	...	...
R. elatior Kunth	Brazil	Goodland 247	−10.6	...	...
R. globosa (H.K.B.) Roem. et Schult	Paraguay	Woolston 1181	−10.7	Reduced	MSC
R. terminalis (Nees) Steudel	Brazil	Hatschbach 20160	−11.2	Reduced	MSC
R. wightiana Nees	Philippines	Ramos 21743	−9.5	...	...
Non-Kranz:					
R. albiceps Kunth	Brazil	Eiten & Eiten 2723	−26.7	...	...
R. consanguinea (Kunth) Böck.	Brazil	Shepherd et al. 3603	−27.5	...	...
R. grisebachii Böck.	Cuba	Ekman 17821	−26.7	...	...
R. longibracteata Böck.	Trinidad	Davidse 2560	−23.3	...	...
R. warmingii Böck.	Brazil	Mori & Boom 14345	−27.3	...	...

Note. KC = Kranz cell; MSC = mestome sheath cell.

and immersed in distilled water for 1 d before fixation. Samples were fixed in 3% glutaraldehyde in 50 mM sodium phosphate (pH 6.8) for 2 h. Similar samples were obtained from fresh leaves of *R. rubra* and fixed for 2 h. Subsequently, all samples from dried and fresh leaves were washed in phosphate buffer three times for 30 min each and postfixed in 2% OsO_4 in phosphate buffer for 2 h at room temperature. They were dehydrated through an ethanol series (10%, 30%, 50%, 70%, 95%, and 100%; three times for 10 min each), infiltrated in Spurr's resin for 1.5 d at room temperature, and embedded in fresh Spurr's resin at 70°C (Spurr 1969). Ultrathin sections were double-stained with uranyl acetate and lead citrate and viewed by a transmission electron microscope (model H-7000, Hitachi, Tokyo). Electron micrographs were obtained as film images.

Carbon Isotope Ratio

Carbon isotope ratios were measured at SI Science, Kitakatsushika, Saitama, Japan. For determination of $\delta^{13}C$ values, 2 mg of each leaf sample was used for measurement of ^{12}C and ^{13}C contents. The carbon isotope values of leaf tissue were determined by using the elemental analyzer–isotope ratio mass spectrometer (EA-IRMS) system (Thermo Fisher Scientific, Waltham, MA), as described elsewhere (Sato and Suzuki 2010). The isotope ratio was expressed in δ notation in parts per million (‰) with respect to the Pee Dee belemnite standard.

Results

Comparison between Fresh and Dry Materials

Examination with TEM of fresh leaves of *Rhynchospora rubra* confirmed previous results (Ueno et al. 1988*b*; Bruhl and Perry 1995). This species has rhynchosporoid anatomy (fig. 1*A*). The chloroplasts of the Kranz cells had a parallel-arranged thylakoid system with few reduced grana (fig. 2*A*). The Kranz cell chloroplasts tended to be centrifugally located, as reported in previous studies (Ueno et al. 1988*b*; Bruhl and Perry 1995). Occasionally, they did not show a constant location within cells (fig. 2*A*). No significant increase in the size or frequency of mitochondria was observed in the Kranz cells relative to the mesophyll cells (fig. 2*A*). The cell walls of the Kranz cells were thicker than those of other cells and had suberized lamellae (fig. 2*A*). The transverse sectional view revealed that the suberized lamellae occurred in the cell walls of the Kranz cells but often were intermittent or indistinct in the middle regions of radial walls (fig. 2*A*). In sections from fresh leaves, the mesophyll cells contained granal chloroplasts, and the cell walls were thinner than those of the Kranz cells (fig. 2*A*). The parenchyma sheath cells contained chloroplasts similar to those of the mesophyll cells and had thinner cell walls than the Kranz cells (data not shown; see fig. 4 in Ueno et al. 1988*b*).

In sections prepared from dried leaves of *R. rubra*, the chloroplasts were present in the Kranz cells, although the protoplasm was shrunken (fig. 2*B*). The centrifugal location of

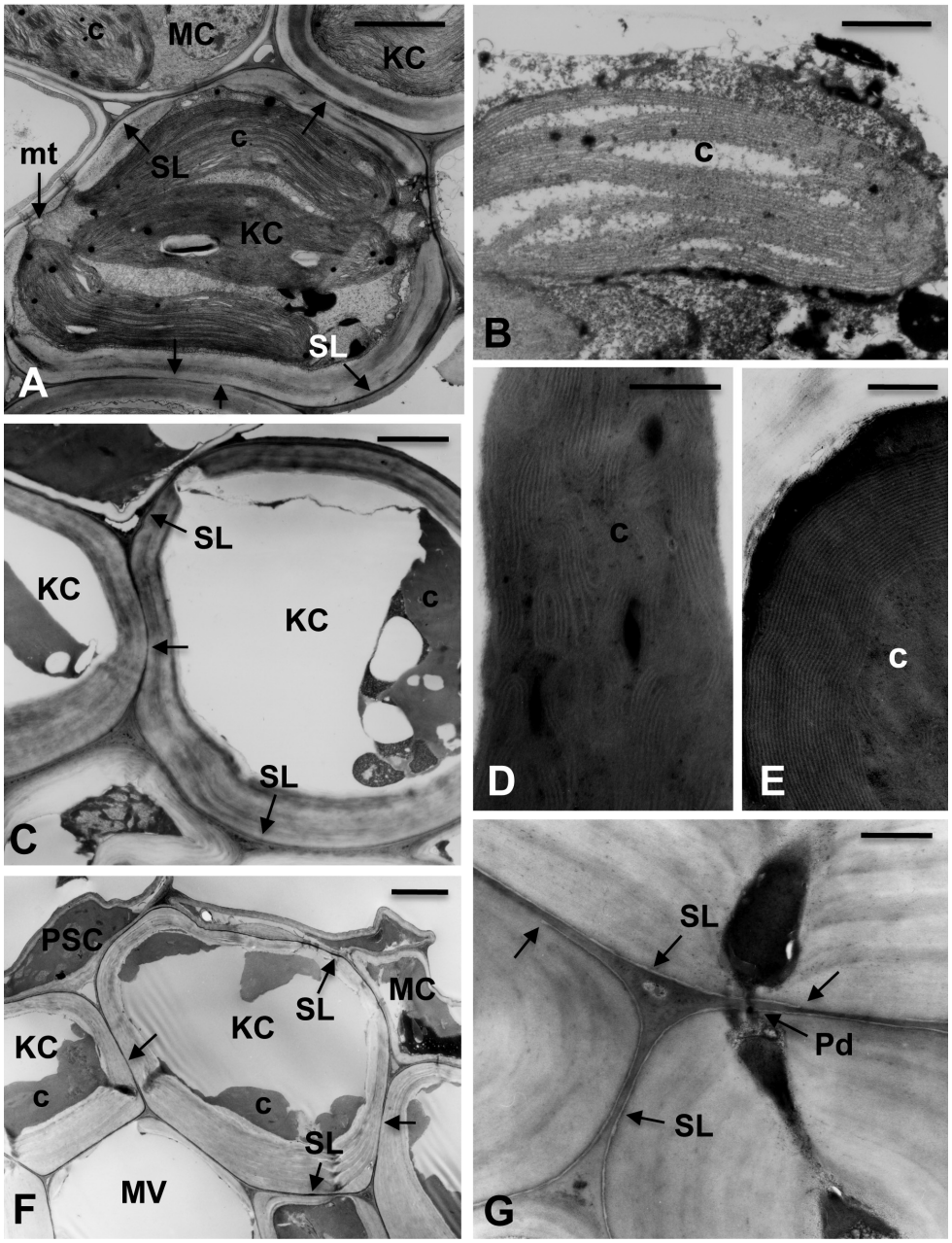

Fig. 2 Transverse sections of leaves of Kranz species in *Rhynchospora*, which have rhynchosporoid anatomy. The sample for the image shown in *A* was prepared from fresh leaves, whereas that in *B* was from dried leaves. The samples for the images in *C–G* were from herbarium materials. *A*, Kranz cells and parts of mesophyll cells of *Rhynchospora rubra*. The unlabeled arrows indicate partial absence of suberized lamellae in the middle parts of radial walls of the Kranz cells. Scale bar = 2 μm. *B*, A chloroplast of a Kranz cell in *R. rubra*. Compare the thylakoid structure with that shown in *A*. Scale bar = 1 μm. *C*, Kranz cells of *Rhynchospora globosa*. The unlabeled arrow indicates partial absence of suberized lamellae in the middle parts of radial walls. Scale bar = 2 μm. *D*, A chloroplast with convoluted thylakoids of a Kranz cell in *R. globosa*. Scale bar = 0.5 μm. *E*, Condensed arrangement of thylakoids in a Kranz cell chloroplast of *Rhynchospora terminalis*. Scale bar = 0.5 μm. *F*, Kranz cells, a mesophyll cell, a parenchyma sheath cell, and vascular tissue of *Rhynchospora confusa*. The cell walls of Kranz cells are thicker in the inner tangential walls than in the outer tangential walls. The unlabeled arrows indicate partial absence of suberized lamellae in the radial walls of the Kranz cells. Scale bar = 2 μm. *G*, An outer Kranz cell (*top*) and two inner thick-walled cells (*bottom*). The plasmodesmata penetrate two adjacent suberized lamellae. The unlabeled arrows indicate the partial absence of suberized lamellae. Scale bar = 0.5 μm. c = chloroplast; G = grana; KC = Kranz cell; MC = mesophyll cell; mt = mitochondrion; MV = metaxylem vessel; Pd = plasmodesmata; PSC = parenchyma sheath cell; SL = suberized lamella.

Kranz cell chloroplasts noted in fresh leaves could not be discerned from dried leaves. The inner structure of the chloroplasts in dried leaves, including the thylakoid system and the grana, was essentially similar to that in fresh leaves (fig. 2*A*, 2*B*). Mitochondria were not visible, probably owing to collapse during drying. Suberized lamellae were well preserved in the cell walls of the Kranz cells, with the same appearance as in fresh leaves (data not shown). In sections from dry leaves, the inner structure of chloroplasts in mesophyll cells was not preserved sufficiently for analysis, unlike the situation for fresh samples. This comparative study between fresh and dry materials in *R. rubra* leaves indicates that dry leaves can be used to study some structures of the Kranz cells and the suberized lamellae.

Fimbristyloid–Chlorocyperoid Intermediate Anatomy

The dried leaves of five Kranz species with fimbristyloid–chlorocyperoid intermediate anatomy were examined for electron microscopic observation (table 1). In all species, chloroplasts were observed within the Kranz cells, but the mitochondria were not recognizable (fig. 3*A*–3*C*). The location of chloroplasts within the Kranz cells was unclear owing to shrinkage of the protoplasm. The thylakoids occurred in electron-dense stroma, but the peripheral reticulum-like structure could only rarely be noted in the margin of chloroplasts of the Kranz cells (fig. 3*C*). The chloroplasts had a convoluted thylakoid system (fig. 3*A*–3*C*), in which only reduced grana were seen (fig. 3*D*). In some cases, the thylakoids formed concentric circles (fig. 3*C*). Chloroplasts were also observed in mesophyll and parenchyma sheath cells, but the inner structure was generally indistinct, except that well-developed grana were found in several species (fig. 3*E*).

In the fimbristyloid–chlorocyperoid intermediate anatomy, the mestome sheath externally surrounds the Kranz sheath (fig. 1*B*). The cell walls of mestome sheath cells were thicker than those of other cells (fig. 3*G*). In the mestome sheath cells of all of the species examined, suberized lamellae were always located in the inner and outer tangential walls and radial walls (fig. 3*B*, 3*F*, 3*G*). However, in the midregions of the radial walls, the suberized lamellae were sometimes intermittent or became indistinct (data not shown). The plasmodesmata penetrated the suberized lamellae in the mestome sheath cells (fig. 3*F*, 3*G*).

Rhynchosporoid Anatomy

The dried leaves of three Kranz species with rhynchosporoid anatomy were examined for electron microscopic observation (table 1). As for the samples with fimbristyloid–chlorocyperoid intermediate anatomy, the chloroplasts of the rhynchosporoid species were visible, but their precise location in the Kranz cells was unclear (fig. 2*C*, 2*F*). The inner structure of the chloroplasts was generally indistinct. However, the structure of thylakoids in some chloroplasts of the Kranz cells was discernible; these chloroplasts had convoluted thylakoids (fig. 2*D*), but the degree of convolution was generally less than that in the Kranz cell chloroplasts of the fimbristyloid–chlorocyperoid intermediates. In the chloroplasts of the Kranz cells in *Rhynchospora terminalis*, multiple layers of condensed, parallel-arranged thylakoids could be appreciated infrequently (fig. 2*E*). Well-developed grana in the chloroplasts of the Kranz cells were not visible (fig. 2*D*, 2*E*). The inner structure of the chloroplasts in the mesophyll and parenchyma sheath cells was indistinct. No mitochondria were seen.

In the rhynchosporoid species, the cell walls of the Kranz cells were thicker than those of the mesophyll cells and parenchyma sheath cells (fig. 2*C*, 2*F*). The inner tangential walls were thicker than the outer tangential walls. Suberized lamellae occurred in the cell walls of the Kranz cells (fig. 2*C*, 2*F*), as seen in the Kranz cells of *R. rubra* (fig. 2*A*). These suberized lamellae were disrupted or became indistinct, however, in the midregions of the radial walls (fig. 2*C*, 2*F*). In *Rhynchospora confusa* and *Rhynchospora globosa*, small thick-walled cells were often present inside the Kranz sheath, especially the phloem side, and formed an incomplete inner sheath (data not shown; see figs. 1*B*, 2*A* in Ueno and Koyama 1987). In these cells also, suberized lamellae generally were located in the cell walls (fig. 2*G*). The plasmodesmata penetrated the suberized lamellae in the Kranz cells and thick-walled cells (fig. 2*G*).

Carbon Isotope Ratios

The 17 Kranz species that we evaluated showed $\delta^{13}C$ values of $-9.5‰$ to $-13.0‰$, whereas the five non-Kranz species showed $\delta^{13}C$ values of $-23.3‰$ to $-27.5‰$ (table 1). In the Kranz species with different anatomical types, the mean $\delta^{13}C$ values did not significantly differ from each other: $-10.9‰ \pm 1.1‰$ ($n = 10$) for the Kranz species with rhynchosporoid anatomy and $-10.7‰ \pm 0.5‰$ ($n = 7$) for those with fimbristyloid–chlorocyperoid intermediate anatomy.

Discussion

This study indicated that the Kranz cell chloroplasts in C_4 *Rhynchospora* species with fimbristyloid–chlorocyperoid intermediate anatomy had a convoluted thylakoid system with reduced grana. These structural features also occurred in the Kranz cell chloroplasts of NADP-ME-type C_4 sedges with either fimbristyloid or chlorocyperoid anatomy (Carolin et al. 1977; Estelita-Teixeira and Handro 1987; Ueno et al. 1988*b*; Li and Jones 1994; Bruhl and Perry 1995). In contrast to the Kranz cell chloroplasts of the NAD-ME and PCK types, those of the NADP-ME-type are agranal or have reduced grana only (Gutierrez et al. 1974; Hatch et al. 1975; Yoshimura et al. 2004). In plants of the NADP-ME-type, therefore, malate transport from mesophyll to Kranz cells provides the reductive power for CO_2 assimilation (Edwards and Walker 1983). The possibility could not be excluded that the structure of the thylakoids might have been modified during the process of drying leaf materials. However, the circular arrangement of the thylakoids is unlikely to be an artifact. This anomalous feature was also observed in earlier studies with fresh leaves (Carolin et al. 1977; Ueno et al. 1988*b*). In the Kranz cell chloroplasts of the fimbristyloid and chlorocyperoid anatomy, well-developed peripheral reticulum was often observed (Carolin et al. 1977; Estelita-Teixeira and Handro 1987; Ueno et al. 1988*b*). In our study, a peripheral reticulum-like structure was noted only in the Kranz cell chloroplasts of *Rhynchospora subplumosa*. A limitation of using herbarium specimens is the inability to observe unstable structures, such as the peripheral

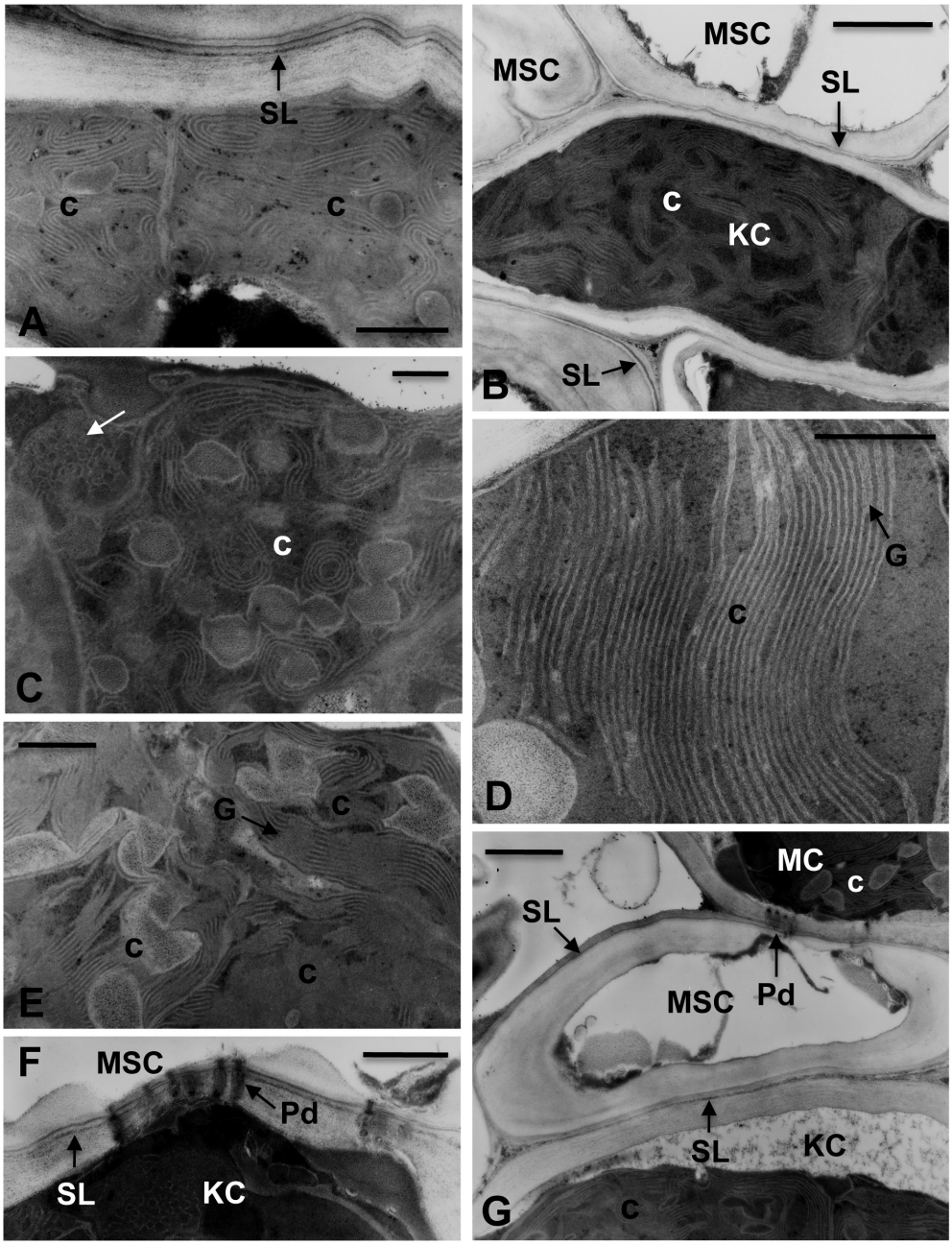

Fig. 3 Transverse sections of leaves of Kranz species in *Rhynchospora*, which have fimbristyloid–chlorocyperoid intermediate anatomy. All samples were prepared from herbarium materials. *A*, Chloroplasts with convoluted thylakoids of a Kranz cell in *Rhynchospora trichochaeta*. A suberized lamella is visible in the inner tangential wall of a mestome sheath cell (upper margin). Scale bar = 0.5 μm. *B*, Kranz cells and parts of mestome sheath cells of *Rhynchospora armerioides*. The Kranz cell chloroplast shows a convoluted thylakoid system. Scale bar = 1 μm. *C*, Chloroplasts with convoluted thylakoids of a Kranz cell in *Rhynchospora subplumosa*. The white arrow indicates a peripheral reticulum-like structure. Scale bar = 0.5 μm. *D*, Chloroplast with reduced grana of a Kranz cell in *R. subplumosa*. Scale bar = 0.5 μm. *E*, Chloroplasts with well-developed grana in a mesophyll cell of *R. subplumosa*. Scale bar = 0.5 μm. *F*, Plasmodesmata between a mestome sheath cell and a Kranz cell in *R. subplumosa*. Scale bar = 0.5 μm. *G*, Mestome sheath cell and parts of a mesophyll cell and a Kranz cell of *R. subplumosa*. The suberized lamellae occur in the mestome sheath cell. Scale bar = 1 μm. c = chloroplast; G = grana; KC = Kranz cell; MC = mesophyll cell; MSC = mestome sheath cell; mt = mitochondrion; MV = metaxylem vessel; Pd = plasmodesmata; PSC = parenchyma sheath cell; SL = suberized lamella.

reticulum and mitochondria, which could not be discerned in this study.

With regard to C_4 species with the rhynchosporoid anatomy, *Rhynchospora rubra* has been reported to have Kranz cell chloroplasts with a parallel thylakoid system and reduced grana (Ueno et al. 1988*b*; Bruhl and Perry 1995). Therefore, other C_4 species with the rhynchosporoid anatomy likely have a similar thylakoid system. However, this study revealed that these Kranz cell chloroplasts also had convoluted thylakoids with reduced grana, as do those of the fimbristyloid–chlorocyperoid intermediates. The degree of convolution in the Kranz cell chloroplasts varied among species of C_4 sedges with either fimbristyloid or chlorocyperoid anatomy (Carolin et al. 1977; Ueno et al. 1988*b*). In addition, Kranz cell chloroplasts of *Remirea maritima* (tribe Cypereae) with chlorocyperoid anatomy had a parallel-arranged thylakoid system (Estelita 1993). It seems that C_4 species of *Rhynchospora* show similar gradiation in the degree of convolution of thylakoids from a parallel arrangement to a remarkably convoluted pattern.

The functional significance of convoluted thylakoids in the Kranz cell chloroplasts of C_4 sedges is still unknown. Carolin et al. (1977) suggested that the convoluted thylakoids might increase the surface area for stroma. This particular thylakoid system has been observed in many NADP-ME-type C_4 sedges but not in the NAD-ME-type C_4 sedges (*Eleocharis*; Ueno and Samejima 1989; Bruhl and Perry 1995; Ueno 1996). However, it is unlikely that the thylakoid system is directly associated with the NADP-ME-type photosynthetic metabolism, because some NADP-ME-type C_4 sedges do not possess it, as described above. It remains unknown whether some environmental factors, such as light intensity, affect the thylakoid structure in Kranz cell chloroplasts of C_4 sedges.

In leaves of C_4 grasses and C_4 sedges, suberized lamellae are often present in the cell walls of Kranz cells and mestome sheath cells (Hattersley and Browning 1981; Dengler and Nelson 1999). Our study confirmed that herbarium materials are useful for assessing the presence of suberized lamellae. Suberized lamellae occurred in the mestome sheath of the fimbristyloid–chlorocyperoid intermediate anatomy but in the Kranz cells of the rhynchosporoid anatomy. These distribution patterns correspond well with those from earlier studies of other C_4 sedges (Carolin et al. 1977; Ueno et al. 1988*b*; Ueno and Samejima 1989; Bruhl and Perry 1995). In C_4 photosynthesis, it is important to create a high-CO_2 environment within Kranz cells because of the suppression of photorespiration (Hatch 1987). It is thought that the suberized lamellae are present to lower the leakage of CO_2 from Kranz cells (Hattersley and Browning 1981). In the two Kranz anatomical types of *Rhynchospora* as well, the suberized lamella always surrounded the sites of decarboxylation of C_4 acids and carboxylation of released CO_2 by the C_3 cycle, irrespective of the difference in cellular location of the lamella.

Takeda et al. (1980) suggested that the Kranz cells of C_4 species with rhynchosporoid anatomy are homologous to mestome sheath cells. Subsequent ontogenetic studies supported this suggestion: Kranz cells are derived from the procambium but not from the ground meristem (Soros and Dengler 2001; Martins and Scatena 2011). This study confirmed again that the structure of cell walls and distribution pattern of suberized lamellae in the Kranz cells are similar to those in the mestome sheath cells. In *Rhynchospora globosa* and *Rhynchospora confusa*, thick-walled cells frequently occurred inside the Kranz sheath and generally had suberized lamellae, as had the Kranz cells. These structural features have been reported to occur in the leaves of the NADP-ME-type grass *Saccharum officinarum* (Robinson-Beers and Evert 1991).

The carbon isotopic composition differs between C_3 and C_4 plants. The $\delta^{13}C$ values of C_4 plants range from $-7‰$ to $-15‰$ and those of C_3 plants range from $-20‰$ to $-35‰$ (Ehleringer and Osmond 1991). This is principally associated with the photosynthetic carboxylation enzymes: the primary carboxylase of C_3 photosynthesis, ribulose 1,5-bisphosphate carboxylase-oxygenase, discriminates strongly against ^{13}C, whereas the primary carboxylase of C_4 photosynthesis, phosphoenolpyruvate carboxylase, discriminates much less strongly against ^{13}C (Ehleringer and Osmond 1991). Thus, the $\delta^{13}C$ value is used to determine whether a plant used the C_4 photosynthetic pathway. The Kranz species of *Rhynchospora* examined showed $\delta^{13}C$ values typical of C_4 plants. In these species, the mean $\delta^{13}C$ values did not significantly differ between the two Kranz anatomical types. At present, the factors influencing CO_2 leakage from Kranz cells are a topic of debate (von Caemmerer and Furbank 2003). However, if CO_2 leakiness is a key factor that influences carbon isotope discrimination, the amount of leakage from Kranz cells may not differ greatly between the two Kranz anatomical types. On the other hand, the non-Kranz species of the Capitatae showed $\delta^{13}C$ values typical of C_3 plants. All non-Kranz species of the Capitatae except for *Rhynchospora longibracteata* were reported to have a remarkable radial arrangement of mesophyll cells surrounding the vascular bundles (Ueno and Koyama 1987). The $\delta^{13}C$ values of these species suggest that they basically perform C_3 photosynthesis.

In taxonomic and morphological studies of plants, dried specimens often are used as materials for scanning electron microscopy. In contrast, fresh material from living plants is used to observe the leaf inner structure by transmission electron microscopy. However, collecting and cultivating the necessary living plants would be difficult if an investigator wanted to examine interspecies variations on a large scale. Leaf ultrathin sections prepared from herbarium specimens of C_4 plants have provided useful information on the distribution of suberized lamellae and granal stacks of chloroplasts in the bundle sheath cells (Hattersley and Perry 1984; Bruhl and Perry 1995). Our study of herbarium specimens of *Rhynchospora* supplied considerable valuable data on the structure of Kranz cell chloroplasts and distribution of suberized lamellae. Future studies using fresh leaves will enable more detailed assessment of structural features, together with biochemical determination of the C_4 subtype. Nevertheless, dry herbarium specimens can provide valuable data for understanding of the diversity and evolution of biochemical and physiological traits, such as photosynthetic type.

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