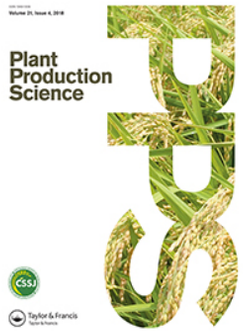

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SHORT REPORT

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Presence of chloroplasts in mestome sheath cells of the C_3 Pooid grass *Elymus tsukushiensis*

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ABSTRACT

The successful introduction of the C_4 pathway into C_3 crops would increase photosynthetic rates and crop productivity. However, our poor understanding of how Kranz leaf anatomy develops poses a great obstacle. In particular, the origin, development, and genetics of bundle sheath (BS) cells in C_4 plants are key points to elucidate. Here we report that *Elymus tsukushiensis*, a common C_3 grass of the subfamily Pooideae, contains chloroplasts in the mestome sheath (MS) cells of the leaf, unlike most MS cells of C_3 grasses. The chloroplasts are smaller than those of mesophyll cells. Immunogold localization showed that the chloroplasts and mitochondria of MS cells, respectively, accumulate ribulose 1,5-bisphosphate carboxylase/oxygenase and a photorespiratory enzyme, glycine decarboxylase, as in mesophyll cells. Thus, we suggest that the MS cells have weak photosynthetic and photorespiratory functions. This finding provides an insight into the development and evolution of C_4-type BS cells in leaves of C_3 grasses.

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Chloroplast; C_3 grass; *Elymus*; mestome sheath cell; leaf anatomy; Poaceae; Pooideae

Introduction

Photosynthesis is one of the major factors determining plant productivity. C_4 plants, which use the C_4 pathway to concentrate CO_2, have greater photosynthetic rates and productivity than C_3 plants in environments where photorespiration is high (Ehleringer et al., 1991). In the leaves of most C_4 plants, two concentric layers of chlorenchyma – an outer mesophyll layer and an inner bundle sheath (BS) layer – surround the vascular bundle; this structure is referred to as Kranz leaf anatomy (Brown, 1975; Lundgren et al., 2014). The greater photosynthetic rate in C_4 plants is attained by the cooperation of the mesophyll and BS cells (Leegood, 2013). Researchers have attempted to introduce the C_4 pathway into C_3 crops such as rice to improve their photosynthetic efficiency. Thus far, several genes for C_4 photosynthetic enzymes have been introduced (Burnell, 2011, and references therein), but photosynthesis has not yet been significantly improved (Leegood, 2013; von Caemmerer et al., 2012). These results suggest that a biochemical approach alone may not be enough, and that the cellular differentiation of leaves should be also considered (von Caemmerer et al., 2012). However, how Kranz leaf anatomy develops is largely unknown. Progress in C_4 engineering will require a better understanding of the developmental and evolutionary aspects of Kranz leaf anatomy (Langdale, 2011; Nelson, 2011; Wang et al., 2016).

Evidence suggests that C_4 plants evolved from C_3 plants, primarily in response to a reduction in atmospheric CO_2 levels that began during the Cretaceous and continued until the Miocene (Ehleringer et al., 1991). Comparative studies of C_3, C_3–C_4 intermediate, and C_4 species in *Flaveria* and several other genera have led researchers to propose models of evolution from a C_3 ancestor to the C_4 state (Sage et al., 2014; Schlüter & Weber, 2016). These models suggest that the structural and biochemical traits in C_4 plants were gradually modified from those in C_3 plants through various intermediate stages.

In the leaves of C_3 grasses, two concentric BSs generally surround the vascular bundle (Brown, 1975). The outer BS, called the parenchyma sheath (PS), consists of large vacuolated parenchyma cells with a few small chloroplasts, whereas the inner BS, called the mestome sheath (MS), consists of small, thick-walled cells without chloroplasts (Brown, 1975; Dengler et al., 1985; Hatakeyama & Ueno, 2016; Leegood, 2008). It is thought that in most C_4 grasses the chlorophyllous BS cells (Kranz cells) originated from either PS or MS cells (Brown, 1975; Dengler et al., 1985). It is easy to speculate that the BS cells in typical NAD-malic enzyme (NAD-ME)-type and phospho*enol*pyruvate carboxykinase (PCK)-type C_4 grasses originated

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from PS cells of C_3 grasses, because these C_4 grasses have outer chlorophyllous BS and inner non-chlorophyllous MS layers. On the other hand, it is thought that the BS cells in typical NADP-ME-type C_4 grasses such as maize and sorghum originated from MS cells of C_3 grasses probably with reduction of PS cells (Brown, 1975; Dengler et al., 1985). However, C_3 grasses containing chloroplasts in the MS cells are very rare and have been discovered only in clades that include C_3, C_3–C_4 intermediate, and C_4 types (Hattersley et al., 1986; Lundgren et al., 2016; Ueno & Sentoku, 2006). It is important to know the origin of BS cells in C_4 grasses in order to understand how Kranz leaf anatomy develops.

Here, we report that *Elymus tsukushiensis*, a C_3 grass common in Japan, has chloroplasts in MS cells. We found this in a comprehensive study of the leaf anatomy of grasses occurring in Japan. This grass belongs to the subfamily Pooideae, in which no C_4 or C_3–C_4 intermediate species have been found (Grass Phylogeny Working Group II, 2012). We discuss the significance of this finding.

Materials and methods

Plant materials

We collected leaves of *E. tsukushiensis* Honda var. *transiens* (Hack.) Osada (*kamojigusa* in Japanese) from plants growing naturally on roadsides on the campus of the National Institute of Agrobiological Sciences, Tsukuba city, Ibaraki, Japan. We also examined two C_3 species, *Phragmites communis* Trinius (Arundinoideae) and *Leersia japonica* Makino (Ehrhartoideae), which we had not examined in our previous study of grass leaf anatomy (Hatakeyama & Ueno, 2016). We collected tillers of these from plants growing at pond edges in Tsukuba city and transplanted them into 5-L pots filled with a 2:1 (v/v) mix of commercial vegetable soil (Iseki, Tokyo, Japan) and sandy loam. These were grown under water-saturated soil conditions in a naturally illuminated greenhouse at 25–30°C during the day and at 15–18°C during the night. Three plants of each species were examined.

Anatomical and ultrastructural observations

Samples taken from the middle of one leaf from each plant were fixed in glutaraldehyde and osmium tetroxide, dehydrated through an acetone series, and embedded in Spurr's resin as described by Ueno (2011). Transverse ultrathin sections were stained with phosphotungstic acid and then with lead citrate and viewed under a transmission electron microscope (H-7000, Hitachi Co. Ltd., Tokyo, Japan) at 75 kV. Semithin sections (~1 μm) on glass slides were stained with toluidine blue O.

We investigated the size and number per cell of chloroplasts and mitochondria in mesophyll, PS, and MS cells surrounding small vascular bundles in *E. tsukushiensis*. In electron micrographs, we measured the length of the long axis of the chloroplasts at 2000× magnification (n = 26–40) and the diameter of the mitochondria at 25,000× (n = 9–50). We counted the chloroplasts and mitochondria in each of 14–20 cells.

Protein A-immunogold electron microscopy

We examined the accumulation of the large subunit (LS) of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplasts and of the P-protein of glycine decarboxylase (GDC) in mitochondria of the mesophyll, PS, and MS cells of *E. tsukushiensis* by immunogold labeling and electron microscopy. Leaf samples were fixed in glutaraldehyde, dehydrated through an ethanol series, and embedded in Lowicryl K4M resin as described by Ueno (2011). Ultrathin sections were immunolabeled with antiserum for each enzyme and with 15 nm of protein A-colloidal gold particles. Antiserum against Rubisco LS from pea leaves was a gift of the late Dr. S. Muto (Nagoya University, Nagoya, Japan). Antiserum against the P-protein of GDC isolated from pea leaf mitochondria was provided by Dr. D. J. Oliver (University of Idaho, Moscow, ID, U.S.A.).

The density of enzyme labeling was determined by counting the gold particles on electron micrographs at 25,000× magnification and calculating the number per unit area (μm^{-2}). The mean labeling density for Rubisco LS was calculated from 8 or 9 measurements of the chloroplasts in 6–8 cells in several immunolabeled sections. Areas occupied by starch grains were excluded from the estimation of the sectional area of the chloroplasts. The mean labeling density for GDC P-protein was calculated from 20–40 measurements of the mitochondria in 8–14 cells in several immunolabeled sections. The measurements were for mesophyll, PS, and MS cells surrounding small vascular bundles.

Statistical analysis

The statistical significance of differences in organelle size and labeling densities of enzymes among mesophyll, PS, and MS cells was assessed by Tukey's HSD test at $p < 0.05$.

Results

In the three C_3 grasses examined, an outer PS and an inner MS generally surrounded the vascular bundle

(Figure 1). However, the MS cells of *Leersia japonica* were partially lacking on the xylem side of small vascular bundles (Figure 1(c)). The PS cells and the much smaller MS cells appeared generally round in transverse sections. The PS cells had smaller and fewer chloroplasts than mesophyll cells had (Figures 1–3). Although some ecotypes of *Phragmites communis* have recently been reported to have C_3–C_4 intermediate or C_4-like traits (Gong et al., 2011; Zhu et al., 2012), the PS cells of our plants had only a few small chloroplasts, as is typical of C_3 grasses (Figures 1(b) and 3 (a)). Under the light microscope, it was not easy to determine the presence or absence of small chloroplasts in MS cells: they were barely visible in *E. tsukushiensis* (Figure 1(a)) and not visible in *P. communis* (Figure 1(b)) and *L. japonica* (Figure 1(c)). Under the electron microscope, it was confirmed that the MS cells of *E. tsukushiensis* had small chloroplasts (Figure 2) but those of *P. communis* (Figure 3(a)) and *L. japonica* (Figure 3(b)) had none. The chloroplasts of MS cells in *E. tsukushiensis* had granal thylakoids, but were smaller than those of mesophyll and PS cells (Figure 2(b) and Table 1). The MS cells of *E. tsukushiensis* had thick walls with suberized lamellae (Figure 2 (b)). These cells had mitochondria that were smaller than those in mesophyll cells but larger than those in PS cells (Figure 2(b) and Table 1). The chloroplasts and mitochondria were scattered in MS cells without particular positioning (Figure 2).

In *E. tsukushiensis*, Rubisco LS was labeled in all chloroplasts of mesophyll, PS, and MS cells at similar density (Figure 4(a–c) and Table 2); GDC P-protein was also labeled in all mitochondria, at highest density in mesophyll mitochondria and lowest in MS mitochondria (Figure 4(d–f) and Table 2).

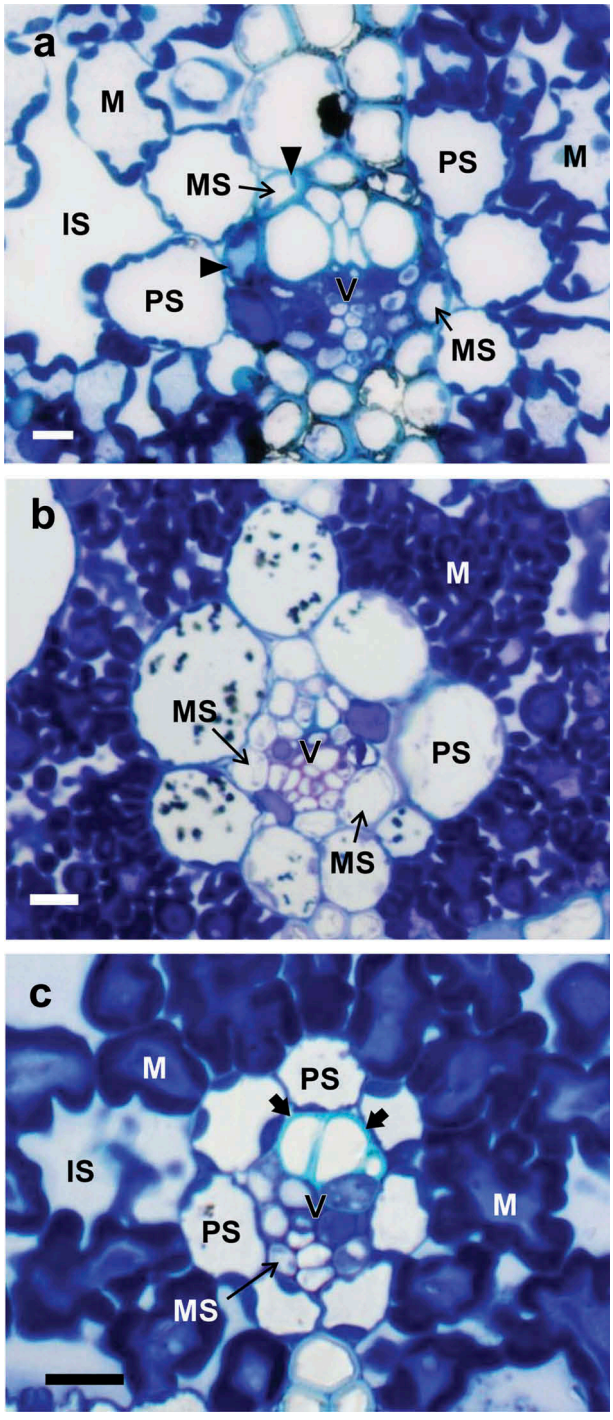

Figure 1. Transverse sections of parenchyma and mestome sheath cells surrounding small vascular bundles of C_3 grasses: (a) *Elymus tsukushiensis* (arrowheads show small chloroplasts in mestome sheath cells); (b) *Phragmites communis* (parenchyma sheath cells include dark-stained bodies in the vacuoles); (c) *Leersia japonica* (unlabeled arrows show lack of mestome sheath cells on the xylem side).
IS: intercellular space; M: mesophyll cell; MS: mestome sheath cell; PS: parenchyma sheath cell; V: vascular bundle. Scale bars = 10 µm.

Discussion

This is the first report that the MS cells of *E. tsukushiensis* contain granal chloroplasts. We have confirmed that leaves of *E. tsukushiensis* plants growing in Fukuoka, Japan, show the same structural trait (data not shown). These findings suggest that this structure is a general feature of *E. tsukushiensis*. Hattersley et al. (1986) reported that the MS cells of C_3 species of *Neurachne* and *Thyridolepis*, in the *Neurachne/Thyridolepis* clade (subtribe Neurachninae) of the subfamily Panicoideae, have chloroplasts. This clade includes closely related C_3, C_3–C_4 intermediate, and C_4 species (Christin et al., 2012). Ueno and Sentoku (2006) found that the MS cells of the grass *Alloteropsis semialata* ssp. *eckloniana* (a C_3-like form) have numerous chloroplasts. *Alloteropsis semialata*, in the subfamily Panicoideae, includes various photosynthetic forms (C_3, C_3-like, C_3–C_4 intermediate, C_4-like, and C_4;

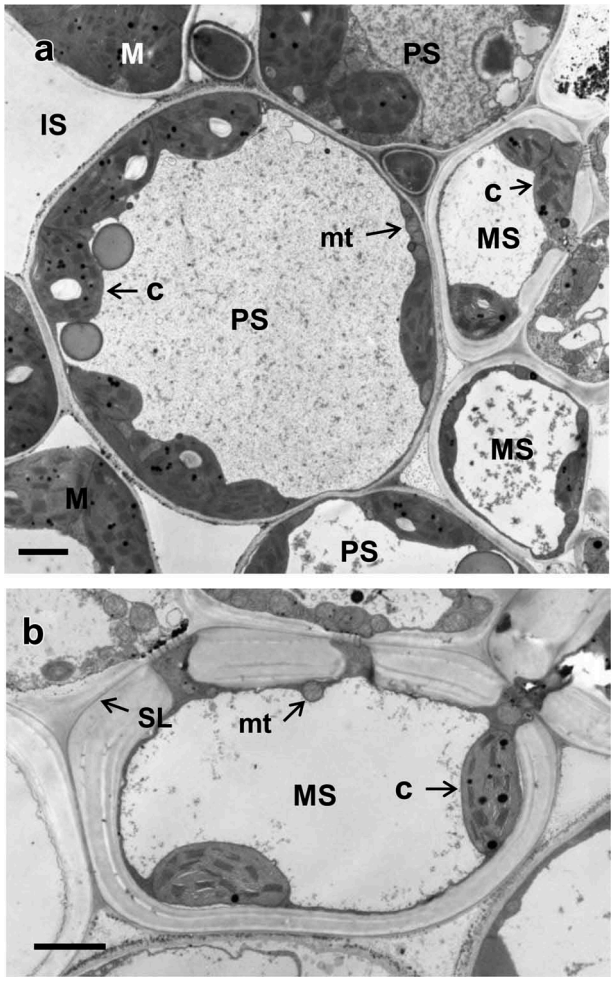

Figure 2. Ultrastructure of parenchyma and mestome sheath cells in leaves of *Elymus tsukushiensis*: (a) Parenchyma and mestome sheath cells; (b) mestome sheath cell.
IS: intercellular space; M: mesophyll cell; MS: mestome sheath cell; PS: parenchyma sheath cell; SL: suberized lamella; c: chloroplast; mt: mitochondrion. Scale bars = 2 μm.

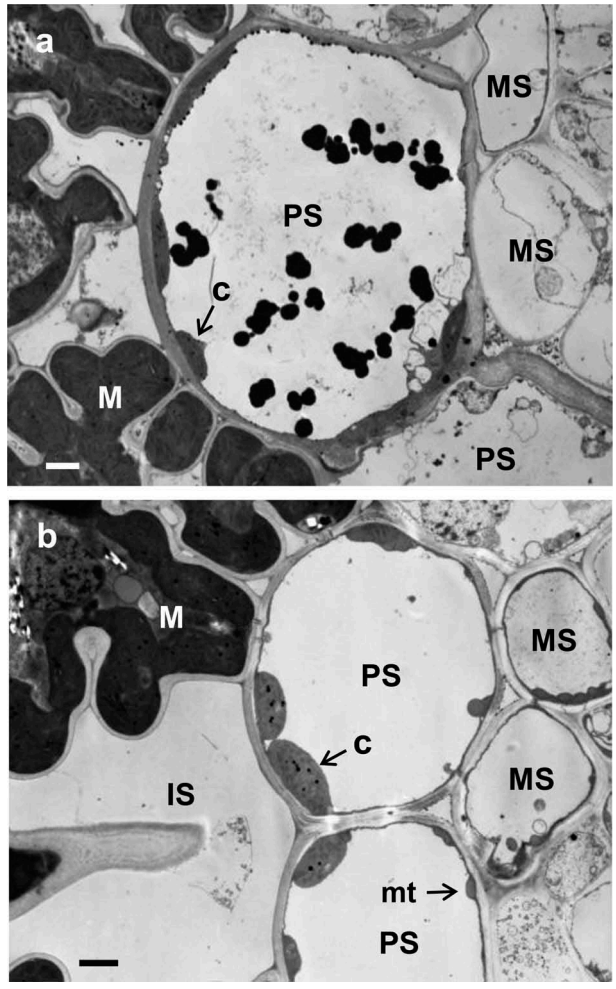

Figure 3. Ultrastructure of parenchyma and mestome sheath cells in leaves of (a) *Phragmites communis* and (b) *Leersia japonica*. The parenchyma sheath cell of *P. communis* includes many electron-dense bodies in the vacuole that are not chloroplasts.
IS: intercellular space; M: mesophyll cell; MS: mestome sheath cell; PS: parenchyma sheath cell; c: chloroplast; mt: mitochondrion. Scale bars = 2 μm.

Lundgren et al., 2016; Ueno & Sentoku, 2006). Accordingly, these C_3 forms with chloroplasts in the MS cells have high affinity to C_4 grasses. It is thought that the BS cells of most NADP-ME-type C_4 grasses originated from the MS cells of C_3 grasses (Brown, 1975; Dengler et al., 1985). It is important to note that *E. tsukushiensis*, in the subfamily Pooideae, has no affinity to C_4 taxa. The Pooideae, which include wheat and barley, are widespread in cool-temperate regions (Edwards & Smith, 2010), and all member species are thought to be C_3 type (Grass Phylogeny Working Group II, 2012). It would be intriguing to learn whether these MS cells represent an anatomical precondition to the BS cells of C_3–C_4 intermediate and C_4 grasses.

Table 1. Size and number of organelles in mesophyll, parenchyma sheath, and mestome sheath cells of *Elymus tsukushiensis*.

Organelle	Mesophyll cells	PS cells	MS cells
Chloroplasts			
Size (μm)	6.7 ± 0.8[a] (40)	4.9 ± 1.1[b] (40)	3.1 ± 0.1[c] (26)
Number per cell	11.2 ± 2.9 (20)	5.3 ± 2.6 (14)	1.3 ± 1.0 (20)
Mitochondria			
Size (μm)	0.37 ± 0.1[a] (40)	0.23 ± 0.07[c] (38)	0.31 ± 0.07[b] (21)
Number per cell	18.0 ± 8.5 (20)	8.9 ± 3.6 (14)	4.4 ± 3.9(20)

Values are means ± SD. The number of organelles or cells examined is given in parentheses.
The different letters indicate a significant difference at $p < 0.05$.

The physiological function of the MS chloroplasts in *E. tsukushiensis* is as yet unclear. In general, the MS cells of C_3 grasses lack chloroplasts, and their cell walls are thicker than those of the PS cells (Brown, 1975) and have suberized lamellae that are considered to be impermeable to H_2O and CO_2 (Mertz & Brutnell, 2014). Therefore, inward diffusion of CO_2

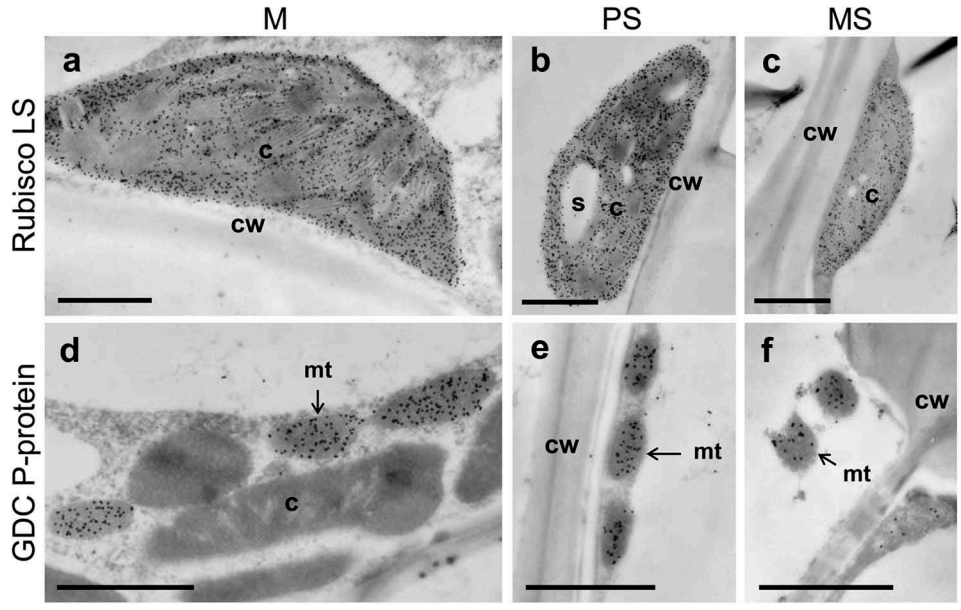

Figure 4. Immunogold labeling for (a–c) the Rubisco large subunit in chloroplasts and (d–f) glycine decarboxylase P-protein in mitochondria of mesophyll, parenchyma sheath, and mestome sheath cells of *Elymus tsukushiensis*: (a, d) Mesophyll cell; (b, e) parenchyma sheath cell; (c, f) mestome sheath cell.
M: mesophyll cell; MS: mestome sheath cell; PS: parenchyma sheath cell; c: chloroplast; cw: cell wall; mt: mitochondrion; s: starch grain. Scale bars = 1 μm.

Table 2. Immunogold labeling densities of the large subunit of Rubisco and the P-protein of glycine decarboxylase (GDC) in mesophyll, parenchyma sheath, and mestome sheath cells of *Elymus tsukushiensis*.

Enzyme	Mesophyll cells	PS cells	MS cells
Rubisco LS			
Chloroplasts (μm^{-2})	365 ± 40[a] (8)	357 ± 51[a] (9)	310 ± 72[a] (9)
Cytosol + others (μm^{-2})	1.0 ± 2.4 (6)	0.1 ± 0.4 (8)	0.3 ± 0.9 (8)
GDC P-protein			
Mitochondria (μm^{-2})	404 ± 73[a] (40)	285 ± 54[b] (38)	129 ± 64[c] (20)
Cytosol + others (μm^{-2})	1.1 ± 0.4 (10)	0.3 ± 0.7 (14)	0.3 ± 0.7(8)

Values are means ± SD. The number of organelles or cell profiles examined is given in parentheses.
The different letters indicate a significant difference at $p < 0.05$.

from the intercellular spaces in the mesophyll would be unexpected because of the positioning of the MS cells and the structure of their cell walls. In *E. tsukushiensis*, the MS chloroplasts were smaller than the mesophyll chloroplasts but accumulated Rubisco as densely as in the mesophyll chloroplasts. The MS mitochondria also accumulated GDC, although at a lower density than in the mesophyll mitochondria. Rubisco is a representative enzyme of the C_3 cycle, whereas GDC is a key enzyme of the glycolate (photorespiratory) pathway, which is involved in the decarboxylation of glycine in mitochondria (Schulze et al., 2016). Therefore, the MS cells of *E. tsukushiensis* could have photosynthetic and photorespiratory function, although the activities may be much lower than those in mesophyll cells. In chloroplast-containing vascular tissues in stems of C_3 eudicots, it is suggested that CO_2 in water taken up by roots may also be fixed (Hibberd & Quick, 2002). Whether a similar physiological mechanism functions in leaves of C_3 grasses as well is unknown.

There have been many studies of leaf anatomy of grasses since the discovery of C_4 photosynthesis, but these studies have focused mainly on grasses in clades including C_4 species, not in clades consisting only of C_3 species. To understand the origin and development of C_4-type BS cells, further extensive studies of leaf ultrastructure in grasses of C_3 clades will be required.

Disclosure statement

No potential conflict of interest was reported by the authors.

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