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REGULAR PAPER

Intracellular position of mitochondria in mesophyll cells differs between C_3 and C_4 grasses

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Abstract In C_3 plants, part of the CO_2 fixed during photosynthesis in chloroplasts is released from mitochondria during photorespiration by decarboxylation of glycine via glycine decarboxylase (GDC), thereby reducing photosynthetic efficiency. The apparent positioning of most mitochondria in the interior (vacuole side of chloroplasts) of mesophyll cells in C_3 grasses would increase the efficiency of refixation of CO_2 released from mitochondria by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in chloroplasts. Therefore, in mesophyll cells of C_4 grasses, which lack both GDC and Rubisco, the mitochondria ought not to be positioned the same way as in C_3 mesophyll cells. To test this hypothesis, we investigated the intracellular position of mitochondria in mesophyll cells of 14 C_4 grasses of different C_4 subtypes and subfamilies (Chloridoideae, Micraioideae, and Panicoideae) and a C_3–C_4 intermediate grass, *Steinchisma hians*, under an electron microscope. In C_4 mesophyll cells, most mitochondria were positioned adjacent to the cell wall, which clearly differs from the positioning in C_3 mesophyll cells. In *S. hians* mesophyll cells, the positioning was similar to that in C_3 cells. These results suggest that the mitochondrial positioning in C_4 mesophyll cells reflects the absence of both GDC and Rubisco in the mesophyll cells and the high activity of phospho*enol*pyruvate carboxylase. In contrast, the relationship between the mitochondrial positioning and enzyme distribution in *S. hians* is complex, but the positioning may be related to the capture of respiratory CO_2 by Rubisco. Our study provides new possible insight into the physiological role of mitochondrial positioning in photosynthetic cells.

Keywords C_3 plant · C_3–C_4 intermediate plant · C_4 plant · Mesophyll cell · Mitochondrion · Photorespiration

Introduction

Plants fix atmospheric CO_2 by photosynthesis and release CO_2 by photorespiration and respiration. In C_3 plants, atmospheric CO_2 is first fixed by carboxylase of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and is assimilated into sucrose through the Calvin-Benson (C_3) cycle. In the ambient atmosphere, oxygenase of Rubisco is also activated, and 2-phosphoglycolate is generated by oxidation of ribulose 1,5-bisphosphate. This C_2 compound is metabolized in the glycolate pathway, and the intermediate metabolite, glycine, is decarboxylated by glycine decarboxylase (GDC) in mitochondria, thereby releasing CO_2 (Bauwe 2011; Schulze et al. 2016). This photorespiratory CO_2 loss limits the photosynthetic efficiency in C_3 plants. However, some plants use CO_2-concentrating mechanisms, such as the C_4 cycle, to suppress photorespiration (Hatch 1987).

In leaf cells, the organelles involved in photosynthesis and photorespiration are chloroplasts, mitochondria, and

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peroxisomes. Plants take in CO_2 from the intercellular spaces within leaves (Terashima et al. 2011). At the same time, they must suppress the loss of CO_2 that has been fixed (Sage and Khoshravesh 2016). In C_3–C_4 intermediate plants, for example, GDC is restricted to the mitochondria of bundle sheath (BS) cells, and the mitochondria are centripetally positioned, adjacent to the vascular bundle and covered by chloroplasts. This positioning of organelles associated with the glycine shuttle would facilitate the capture of photorespiratory CO_2 released from mitochondria by chloroplasts in the BS cells (Hylton et al. 1988; Rawsthorne 1992; Sage et al. 2012; Schlüter and Weber 2016; Ueno et al. 2003).

In contrast to studies of BS cells, there have been few studies of the intracellular positioning of mitochondria in mesophyll cells. However, recent studies of the mesophyll cells of C_3 grasses have suggested that photorespired and respired CO_2 may be recycled in these cells by an elaboration of the positioning of chloroplasts and mitochondria without alterations in their biochemistry. In rice (C_3), the periphery of mesophyll cells is covered by many chloroplasts with protrusions (stromules), and mitochondria are positioned in the cell interior. This positioning of chloroplasts would facilitate the uptake of CO_2 from intercellular spaces and the scavenging of CO_2 released by mitochondrial photorespiration by Rubisco in the chloroplasts (Sage and Sage 2009). A subsequent study of rice and wheat (Busch et al. 2013) estimated that 24–38% of photorespired and respired CO_2 is reassimilated within mesophyll cells by this positional elaboration of the organelles. More recently, we investigated the intracellular position of mitochondria and chloroplasts in mesophyll and BS cells of leaves of 10 species of C_3 grasses (Hatakeyama and Ueno 2016); 61–92% of mitochondria in the mesophyll cells were positioned on the vacuole side of chloroplasts. These data revealed that the pattern of intracellular positioning of mitochondria is widespread among the mesophyll cells of C_3 grasses. However, it would not be easy to demonstrate the refixation of CO_2 photorespired and respired in C_3 leaves during photosynthesis.

In this respect, it would be important to know the positioning of mitochondria in the mesophyll cells of C_4 plants. In C_4 plants, atmospheric CO_2 is fixed by phospho*enol*pyruvate carboxylase (PEPC) in mesophyll cells; the C_4 acids generated by this process are transported to BS cells, where they are decarboxylated, and the released CO_2 is refixed by Rubisco of chloroplasts (Hatch 1987). The C_4 cycle concentrates CO_2 for Rubisco in the BS cells, resulting in reduced photorespiration. In contrast with mesophyll cells of C_3 plants, those of C_4 plants lack both GDC and Rubisco but accumulate PEPC (Edwards et al. 2001; Hatch 1987; Ohnishi and Kanai 1983; Ueno 1998; Yoshimura et al. 2004). These cellular distributions of photosynthetic and photorespiratory enzymes suggest that the mesophyll cells of C_4 plants would not require the particular positioning of mitochondria found in those of C_3 plants, because neither the loss of photorespired CO_2 from mitochondria nor the capture of CO_2 by chloroplasts occurs, whereas PEPC is capable of the refixation of respired CO_2 in the cytosol.

There are many studies of the ultrastructure of leaves in C_4 plants (e.g., Carolin et al. 1973; Hattersley and Browning 1981; Prendergast et al. 1986, 1987 for grass family; Bruhl and Perry 1995; Carolin et al. 1977; Ueno et al. 1988 for sedge family; Carolin et al. 1975, 1978; Kim and Fisher 1990; Marshall et al. 2007; Voznesenskaya et al. 2007, 2010, 2016 for dicot families). However, these studies focused mainly on the BS cells and did not mention the positioning of mitochondria in mesophyll cells. This is the case in previous reviews about C_4 leaf anatomy (Dengler and Nelson 1999; Edwards and Voznesenskaya 2011; Lundgren et al. 2014). A recent study of mesophyll cells of C_4 plants reported the number, size, and position of chloroplasts but not of mitochondria (Stata et al. 2014, 2016). To date, we have no available data on the positioning of mitochondria in mesophyll cells in C_4 plants. In this study, therefore, we investigated the intracellular position of mitochondria in mesophyll cells of C_4 grasses.

In addition, we examined mesophyll cells of the C_3–C_4 intermediate grass *Steinchisma hians* (formerly *Panicum milioides*; Duvall et al. 2003). This species shows Kranz-like leaf anatomy and reduced photorespiration without operation of the C_4 cycle (Brown and Brown 1975; Kanai and Kashiwagi 1975). The mesophyll cells lack GDC (Hylton et al. 1988) but have Rubisco (Hattersley et al. 1977; Ku et al. 1976). This pattern of enzyme localization differs from those in C_3 and C_4 plants. Therefore, this C_3–C_4 intermediate grass provides an opportunity to evaluate the physiological significance of intracellular positioning of mitochondria in mesophyll cells.

In this study, we examined the positioning of mitochondria in the mesophyll cells of 14 C_4 grasses of different C_4 subtypes and subfamilies, and found that it clearly differs between C_3 and C_4 grasses. The physiological implications are discussed, together with those of our data for the C_3–C_4 intermediate grass.

Materials and methods

Plant materials and growth

In the first experiment, we investigated 3 C_4 grass species representing 3 C_4 biochemical subtypes: *Zea mays* L. (NADP-malic enzyme [NADP-ME] type), *Panicum miliaceum* L. (NAD-ME type), and *Urochloa texana* (Buckley)

R. Webster (phospho*enol*pyruvate carboxykinase [PCK] type), all in the subfamily Panicoideae (Table 1). We also examined *Steinchisma hians* (Elliott) Nash of the subfamily Panicoideae as a representative C_3–C_4 intermediate species (Table 1). Seeds were sown in 5-L pots filled with sandy soil that contained 0.6 g each of N (as $(NH_4)_2SO_4$), P (as $Ca(H_2PO_4)_2$), and K (as KCl). Plants were grown in a growth chamber (natural sunlight, wherein photosynthetic photon flux density [PPFD] at midday exceeded 1000 μmol m^{-2} s^{-1}; 25 °C; 70% R.H.) for about 8 weeks in August and September. We examined leaves of 3 plants per species (1 leaf per plant) to identify the intracellular position of mitochondria in mesophyll cells.

In the second experiment, we investigated a wide range of species rather than few species in detail: 3 species of the NADP-ME type [*Digitaria ciliaris* (Retz.) Koeler, *Eriachne aristidea* F. Muell, and *Setaria italica* (L.) P. Beauv.], 5 species of the NAD-ME type [*Cynodon dactylon* (L.) Pers., *Eleusine coracana* (L.) Gaertn., *E. indica* (L.) Gaertn., *Leptochloa chinensis* (L.) Nees, and *Panicum dichotomiflorum* Michx.], and 3 species of the PCK type [*P. maximum* Jacq., *Sporobolus fertilis* (Steud.) W.D. Clayton, and *Zoysia japonica* Steud.] (Table 2). They belong to 3 subfamilies (GPWG II, 2012): 4 species of the Panicoideae, 1 species of the Micraroideae, and 6 species of the Chloridoideae (Table 2). Plants were grown from seeds in 5-L pots in in a greenhouse (natural sunlight: PPFD at midday >1500 μmol m^{-2} s^{-1}; temperature range 25–33 °C) for 4 to 5 weeks in August and September. One healthy plant per species was used for leaf ultrastructural observation.

The seeds of *S. hians* were provided by the National Institute of Agrobiological Resources, Tsukuba, Japan.

Table 1 Intracellular positions and numbers of mitochondria and chloroplasts in mesophyll cells of C_3–C_4 and C_4 grasses

Species	Photosynthetic type	Subfamily	Mitochondria			Chloroplasts (no. per cell profile)
			Outer position (%)	Inner position (%)	No. per cell profile	
Steinchisma hians	C_3–C_4	Panicoideae	22.6 ± 4.7	77.4 ± 4.7*	3.8 ± 0.6	4.9 ± 0.6
Zea mays	NADP-ME C_4	Panicoideae	97.4 ± 3.2	2.6 ± 3.2*	3.5 ± 0.9	5.4 ± 0.3
Panicum miliaceum	NAD-ME C_4	Panicoideae	94.0 ± 6.3	6.0 ± 6.3*	2.9 ± 0.8	4.0 ± 1.1
Urochloa texana	PCK C_4	Panicoideae	87.6 ± 7.2	12.4 ± 7.2*	5.6 ± 1.1	6.2 ± 0.4

Values are means ± SD of three plants

*Significantly different between the two positions of mitochondria ($P < 0.05$)

Subfamilies according to GPWG II (2012)

Table 2 Intracellular positions and numbers of mitochondria and chloroplasts in mesophyll cells of C_4 grasses

Photosynthetic type and species	Subfamily	Mitochondria			Chloroplasts (no. per cell profile)
		Outer position (%)	Inner position (%)	No. per cell profile	
NADP-ME C_4					
Digitaria ciliaris	Panicoideae	93.2 ± 8.2	6.8 ± 8.2	6.7 ± 1.1	6.7 ± 1.4
Eriachne aristidea	Micraroideae	92.3 ± 10.3	7.7 ± 10.3	5.5 ± 2.5	5.7 ± 1.4
Setaria italica	Panicoideae	92.6 ± 7.9	7.4 ± 7.9	8.5 ± 3.2	5.2 ± 1.5
NAD-ME C_4					
Cynodon dactylon	Chloridoideae	85.0 ± 33.7	15.0 ± 33.7	2.3 ± 1.5	4.3 ± 1.1
Eleusine coracana	Chloridoideae	93.8 ± 9.5	6.2 ± 9.5	4.8 ± 1.9	5.2 ± 1.7
E. indica	Chloridoideae	90.3 ± 20.4	9.7 ± 20.4	3.5 ± 0.7	3.8 ± 0.7
Leptochloa chinensis	Chloridoideae	82.5 ± 30.0	17.5 ± 30.0	2.5 ± 0.7	4.9 ± 1.6
Panicum dichotomiflorum	Panicoideae	93.5 ± 11.9	6.5 ± 11.9	3.5 ± 1.4	4.1 ± 0.9
PCK C_4					
P. maximum	Panicoideae	97.2 ± 9.6	2.8 ± 9.6	1.8 ± 0.6	2.8 ± 0.8
Sporobolus fertilis	Chloridoideae	100 ± 0.0	0.0 ± 0.0	2.2 ± 1.3	3.0 ± 1.0
Zoysia japonica	Chloridoideae	100 ± 0.0	0.0 ± 0.0	1.2 ± 0.9	1.9 ± 0.3

Values are means ± SD of 12 cells

Subfamilies according to GPWG II (2012)

Those of *Eriachne aristidea* F. Muell were provided by the Plant Introduction Station, ARS, USDA. Other seeds were purchased or collected in the field.

Ultrastructural observation

Leaf sections were prepared for ultrastructural observation as described in our previous study of C_3 grasses (Hatakeyama and Ueno 2016). Segments from the middle of fully expanded uppermost leaves collected at 09:00 to 10:00 on a sunny day were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 2 h, washed with phosphate buffer, post-fixed with 2% (v/v) OsO_4 in 50 mM phosphate buffer (pH 6.8) for 2 h, dehydrated through an acetone series, and embedded in Spurr's resin. Ultra-thin sections were sectioned with a diamond knife, stained with lead citrate, and observed under an electron microscope (JEM-100CX II K, JEOL Ltd., Tokyo, Japan) at 75 kV.

Quantification of mitochondria and chloroplasts

In the first experiment, we recorded the numbers of mitochondria and chloroplasts and the intracellular positions of mitochondria in 10 to 15 mesophyll cells in each leaf of three plants per species under the electron microscope. For intracellular positioning of mitochondria, we counted the mitochondria on the vacuole side of chloroplasts (inner position) and on the cell wall side, including isolated mitochondria not associated with chloroplasts but adjacent to the cell wall (outer position), as in our previous study (Hatakeyama and Ueno 2016); equal numbers of mesophyll cells were selected from abaxial and adaxial sides. In the second experiment, we recorded the same details in 12 mesophyll cells per leaf per species.

Statistical analysis

Student's *t*-test was used to test the significance ($P < 0.05$) of differences between the proportions of mitochondria in the inner and outer positions of mesophyll cells.

Results

In the first experiment, we examined 3 plants per species to confirm statistic difference in positioning of mitochondria in mesophyll cells of representative C_4 species of 3 C_4 subtypes and a C_3–C_4 intermediate (*S. hians*) (Table 1). In *S. hians*, the mesophyll consisted of small cells (Fig. S1A), and 77% of mitochondria were located in the inner position (Table 1; Fig. 1a, b). The chloroplasts developed protrusions, and mitochondria were often positioned between

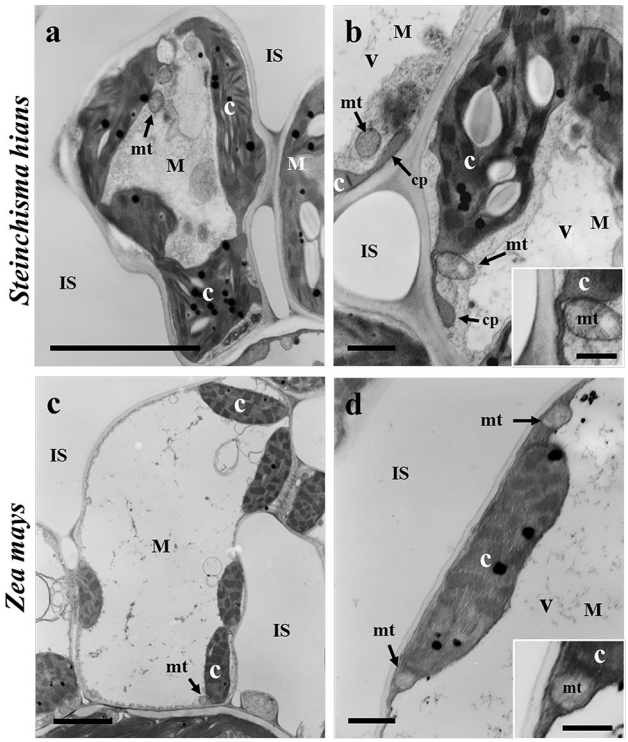

Fig. 1 Ultrastructure in mesophyll cells of **a, b** the C_3–C_4 intermediate grass, *Steinchisma hians*, and **c, d** the NADP-ME type C_4 grass, *Zea mays*. In **b, d**, enlarged images of mitochondria are shown. *IS* intercellular space, *M* mesophyll cell, *c* chloroplast, *mt* mitochondrion, *v* vacuole, *cp* chloroplast protrusion. *Bars* for **a** and **c** = 5 µm. *Bars* for **b** and **d** = 1 µm. *Bars* for enlarged images in **b** and **d** = 0.5 µm

those and the vacuole (Fig. 1b). On the other hand, the mesophyll cells of the 3 C_4 grasses, *Z. mays* (Fig. 1c; Fig. S1B), *Panicum miliaceum* (Fig. 2a; Fig. S2A), and *Urochloa texana* (Fig. 2c; Fig. S2B), were larger than those of *S. hians*. In contrast to those of *S. hians*, 88–97% of mitochondria in mesophyll cells of the C_4 grasses were positioned along the cell walls and sometimes lay between the cell walls and chloroplasts (outer position) (Table 1; Figs. 1d, 2b, d). In general, the mitochondria of the C_4 grasses were positioned near the chloroplasts, which had no visible protrusions (Figs. 1d, 2b, d). These data showed that mitochondria in mesophyll cells of the C_4 grasses are located exclusively in the outer position.

To confirm whether this positioning of mitochondria found in the C_4 grasses occurs in other C_4 grasses as well, in the second experiment, we expanded our survey to 11 C_4 grasses from different C_4 subtypes; *Digitaria ciliaris, Eriachne aristidea* and *Setaria italica* of the NADP-ME type, *Cynodon dactylon, Eleusine coracana, E. indica, Leptochloa chinensis*, and *Panicum dichotomiflorum* of the NAD-ME type, and *P. maximum, Sporobolus fertilis*, and *Zoysia japonica* of the PCK type (Table 2). In them

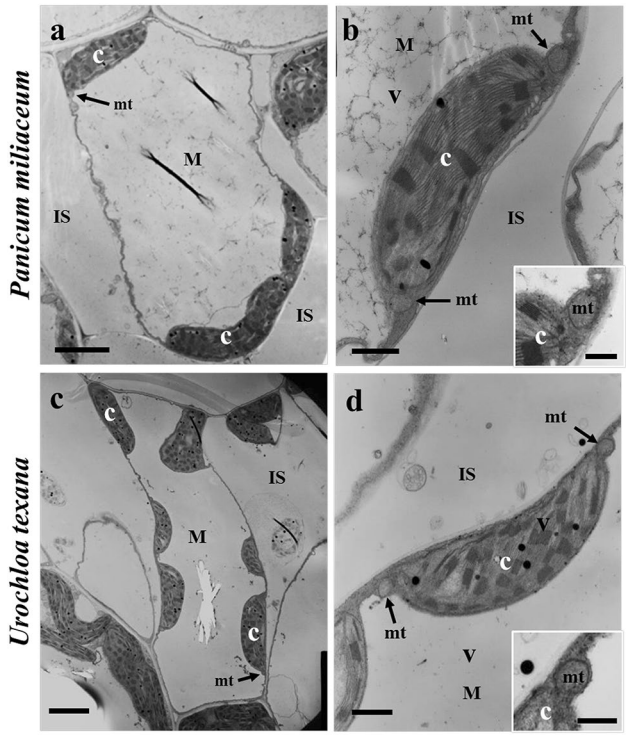

Fig. 2 Ultrastructure in mesophyll cells of **a, b** the NAD-ME type C_4 grass, *Panicum miliaceum*, and **c, d** the PCK type C_4 grass, *Urochloa texana*. In **b, d**, enlarged images of mitochondria are shown. *IS* intercellular space, *M* mesophyll cell, *c* chloroplast, *mt* mitochondrion, *v* vacuole. *Bars* for **a** and **c** = 5 μm. *Bars* for **b** and **d** = 1 μm. *Bars* for enlarged images in **b** and **d** = 0.5 μm

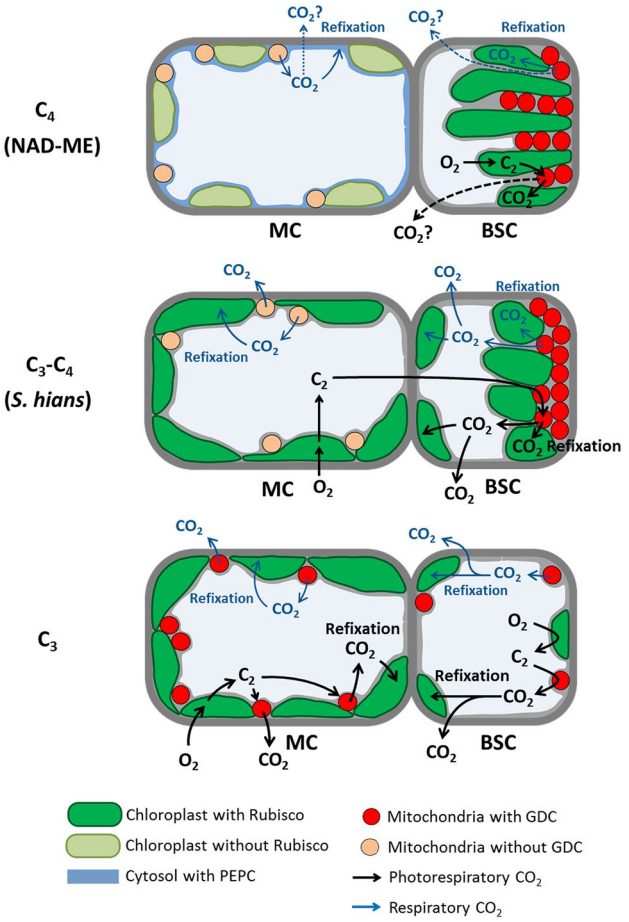

Fig. 3 Relationships between the positioning of mitochondria and chloroplasts and the localization of photosynthetic and photorespiratory enzymes in mesophyll cells (MC) of C_3, C_3–C_4 intermediate (*Steinchisma hians*), and C_4 (NAD-ME type) grasses. Structural features and enzyme localizations in bundle sheath cells (BSC) are also shown. The refixation and loss of photorespiratory and respiratory CO_2 are shown, but the photosynthetic fixation of CO_2 from intercellular spaces is omitted. *GDC* glycine decarboxylase, *PEPC* phospho*enol*pyruvate carboxylase, *Rubisco* ribulose 1,5-bisphosphate carboxylase/oxygenase

also, most mitochondria were located in the outer position (Table 2). In three NADP-ME type and five NAD-ME type C_4 grasses, 83–94% of mitochondria in mesophyll cells were located in the outer position. In three PCK type C_4 grasses, 97–100% of mitochondria in mesophyll cells were located in the outer position, but the number of mitochondria per cell profile was fewer than those of the NADP-ME and NAD-ME type C_4 grasses (Table 2). As in the C_4 grasses investigated in the first experiment, none of the C_4 grasses had chloroplast protrusions (data not shown).

Discussion

Our study is the first to report the positioning of mitochondria in mesophyll cells of C_4 plants. Figure 3 shows the schematic model of relationships between the intracellular positions of mitochondria and chloroplasts and the localization of GDC, Rubisco, and PEPC in mesophyll and BS cells in C_3, C_3–C_4 intermediate (*S. hians*), and C_4 grasses (represented by NAD-ME type). Our previous study (Hatakeyama and Ueno 2016) found that mitochondria in mesophyll cells of C_3 grasses were positioned mainly at the inner position (vacuole side of chloroplasts). This positioning in C_3 grasses may help the capture of photorespiratory (and respiratory) CO_2 released from mitochondria by chloroplasts (Busch et al. 2013; Hatakeyama and Ueno 2016; Sage and Sage 2009; Fig. 3). In contrast, we found that many mitochondria in the mesophyll cells of C_4 grasses were located at the outer position, along cell walls, but rarely at the inner position, irrespective of C_4 subtype or subfamily (Tables 1, 2; Fig. 3). Our observation supports the hypothesis that mesophyll mitochondria of C_4 grasses would not show the intracellular positioning of those of C_3 grasses.

As mentioned in the Introduction, we interpret that the subcellular localization of Rubisco, PEPC and GDC

permits this positioning of mitochondria in C_4 mesophyll cells. In C_4 leaves, Rubisco accumulates in the chloroplasts of BS cells only, not in those of mesophyll cells (Edwards et al. 2001; Hatch 1987; Ueno 1998; Fig. 3). As a result, the mesophyll chloroplasts cannot fix CO_2. It is worth pointing out that the chloroplasts in the mesophyll cells of C_4 grasses had no evident protrusions, unlike in C_3 grasses, in which protrusions are frequently observed (Busch et al. 2013; Hatakeyama and Ueno 2016; Sage and Sage 2009). These structures, also called stromules (Gray et al. 2001; Hanson and Sattarzadeh 2011), contain Rubisco, as in the stroma of chloroplasts (Bourett et al. 1999). Because they increase the surface area of chloroplasts facing intercellular spaces in mesophyll cells, they improve the capture of diffusive CO_2. It could also aid the scavenging of photorespiratory CO_2 released from mitochondria in the cell interior (Buchner et al. 2015; Busch et al. 2013; Hatakeyama and Ueno 2016; Moser et al. 2015; Sage and Sage 2009). The absence of the protrusion in the mesophyll chloroplasts of C_4 grasses may be related to the absence of Rubisco in those chloroplasts. In C_4 leaves, GDC activity also is absent in the mesophyll mitochondria, although it is present in the BS mitochondria (Ohnishi and Kanai 1983; Yoshimura et al. 2004; Fig. 3). Therefore, photorespiratory CO_2 loss does not occur in the mesophyll mitochondria of C_4 grasses. As a result, the mesophyll mitochondria would not require the inner positioning found in the mesophyll cells of C_3 grasses.

Plants perform mitochondrial respiration as well as photorespiration. In C_3 grasses, the positioning of mesophyll mitochondria would also contribute to the capture of CO_2 from mitochondrial respiration under light (Busch et al. 2013; Hatakeyama and Ueno 2016; Fig. 3). In C_4 grasses, the respiratory CO_2 could be fixed by PEPC in the cytosol of mesophyll cells (Fig. 3).

We also examined the C_3–C_4 intermediate grass *S. hians*. This species has almost no functional C_4 cycle (Kanai and Kashiwagi 1975) and is classified as a type I C_3–C_4 intermediate (Edwards and Ku 1987). The intracellular position of mitochondria in the mesophyll cells was similar to that of C_3 grasses. Rubisco occurs in the chloroplasts of both mesophyll and BS cells (Hattersley et al. 1977; Khoshravesh et al. 2016; Ku et al. 1976; Fig. 3), as in other type I C_3–C_4 intermediate species (Ueno 2011). The chloroplast protrusion occurred in the mesophyll cells. On the other hand, GDC is lacking in the mesophyll mitochondria but accumulates greatly in the BS mitochondria (Hylton et al. 1988; Fig. 3). In *S. hians*, PEPC activity is low, reflecting the absence of a functional C_4 cycle (Ku et al. 1976). Therefore, the relationship between the positioning of mitochondria and the accumulation pattern of Rubisco, GDC and PEPC in *S. hians* appears to be more complex than those in C_3 and C_4 grasses. In mesophyll cells of *S. hians*, many chloroplasts are distributed along the cell wall (Fig. 1a). Similar positioning of chloroplasts in mesophyll cells occurs in the type I C_3–C_4 intermediate species of *Flaveria* as well (Stata et al. 2016). As a result of the occupation of chloroplasts, mitochondria may be located in the cell interior. This positioning would facilitate the capture of CO_2 from mitochondrial respiration by Rubisco of chloroplasts.

Our results reveal that the intracellular positioning of mitochondria in mesophyll cells differs between C_3 and C_4 grasses. The difference may reflect differences in biochemical traits of photosynthesis and photorespiration between them. It will be of great interest to know whether the positioning we found here generally occurs in leaves of other C_3 and C_4 groups as well. Further analyses of other C_3–C_4 intermediate species will be required, including intermediates of different types, such as *Flaveria* species (Edwards and Ku 1987; McKown and Dengler 2007; Stata et al. 2016).

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