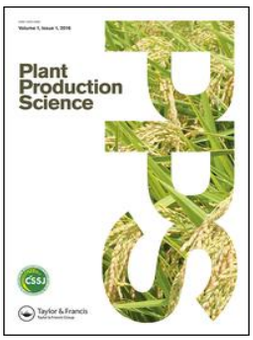

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# Structural and physiological responses of the $C_4$ grass *Sorghum bicolor* to nitrogen limitation

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**ABSTRACT**

Nitrogen (N) is one of the major nutrients influencing photosynthesis and productivity of $C_4$ plants as well as $C_3$ plants. $C_4$ photosynthesis operates through close coordination between mesophyll (M) and bundle sheath (BS) cells. However, how the development of structural and physiological traits in leaves of $C_4$ plants is regulated under N limitation remains uncertain. We investigated structural and physiological responses of leaves of the NADP-ME-type $C_4$ grass *Sorghum bicolor* to N limitation. Plants were grown under four levels of N supply (.05 to .6 g N per 5-L pot). Decreasing N supply resulted in decreases in net photosynthetic rate, stomatal conductance, leaf N and chlorophyll contents, and the activity ratio of phospho*enol*pyruvate carboxylase to ribulose 1,5-bisphosphate carboxylase/oxygenase and increases in $\delta^{13}C$ values and photosynthetic N use efficiency. Low-N leaves were thinner and had smaller photosynthetic cells, especially in M, resulting in lower M/BS tissue area ratio, and contained smaller and fewer chloroplasts. The BS chloroplasts in the low-N leaves accumulated abundant starch grains. The number of thylakoids per granal stack was reduced in M chloroplasts but not in BS chloroplasts. The low-N leaves had thicker cell walls, especially in the BS cells, which might be associated with less negative $\delta^{13}C$ values, and fewer plasmodesmata in the BS cells. These data reveal structural and physiological responses of $C_4$ plants to N limitation, most of which would be related to cellular N allocation, light use, $CO_2$ diffusion and leakiness, and metabolite transport under N limitation.

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**CLASSIFICATION**
Crop morphology

## Introduction

Nitrogen (N) is one of the most important nutrients for the growth of plants and strongly influences the productivity and yield of crops (Xu et al., 2012). The effects of N on physiological traits, including photosynthesis, have been well investigated in both $C_3$ and $C_4$ species. N limitation reduces both photosynthetic rate and growth, largely because a great amount of leaf N is allocated to soluble and thylakoid proteins involved in photosynthesis (Evans, 1983; Ghannoum et al., 2005, 2011; Makino et al., 2003; Sage et al., 1987).

In general, $C_4$ plants show higher photosynthetic efficiency than $C_3$ plants under high light intensity and high temperature (Ehleringer & Monson, 1993). The $C_4$ cycle concentrates $CO_2$ for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) of the $C_3$ cycle. The leaves of most $C_4$ plants show a particular anatomical structure, called Kranz leaf anatomy, characterized by two concentric layers consisting of mesophyll (M) and bundle sheath (BS) cells surrounding the vascular bundle. Atmospheric $CO_2$ is fixed by phospho*enol*pyruvate carboxylase (PEPC) in the M cells, and the resultant $C_4$ compounds are transported to the BS cells, in which they are decarboxylated by $C_4$-acid decarboxylating enzymes such as NADP-malic enzyme (NADP-ME). As a result, the concentration of $CO_2$ within the BS cells is elevated to several times atmospheric level. Under these conditions, Rubisco can efficiently fix $CO_2$, whereas its oxygenase activity and therefore photorespiration are suppressed. The $C_3$ compounds generated in the decarboxylation of $C_4$ acids return to the M cells and are used to reproduce the $CO_2$ acceptor, PEP (Hatch, 1987; Kanai & Edwards, 1999). The biochemical advantages of $C_4$ photosynthesis also confer $C_4$ plants higher water and N use efficiencies in photosynthesis and biomass production than in $C_3$ plants (Brown, 1978; Ghannoum et al., 2005, 2011; Tsutsumi et al., 2017). These physiological traits of $C_4$ plants are beneficial for agriculture, especially in tropical and subtropical regions and stressful environments.

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N limitation affects structural, biochemical, and physiological traits of leaves. For instance, it reduces the size and number of chloroplasts in leaves (Chonan et al., 1977), reflecting the reduced synthesis of proteins of the photosynthetic machinery such as Rubisco (Evans, 1983). In $C_4$ plants, the differentiation of the two photosynthetic cell types makes the structural responses of leaves to N limitation more complex than in $C_3$ plants, yet few studies have focused on these responses (Aviovich & Cresswell, 1983; Ma et al., 2017; Tazoe et al., 2006). It is thought that a quantitative balance between M and BS cells is required for the close coordination of the $C_4$ and $C_3$ cycles and the efficient operation of $C_4$ photosynthesis (Dengler et al., 1994; Hattersley, 1984; Ueno, 1996; Yoshimura et al., 2004). However, how the N level regulates the differentiation of both M and BS cells in $C_4$ plants remains uncertain. When plants grow under N limitation, they often experience photoinhibition (Kumagai et al., 2014; Lu & Zhang, 2000; Verhoeven et al., 1997). We have almost no information about how the thylakoid system of M and BS chloroplasts responds to N limitation.

Recent studies suggest that $CO_2$ leakage from BS cells is one of the factors determining photosynthetic efficiency of $C_4$ plants, because the maintenance of a high $CO_2$ concentration ($[CO_2]$) within BS cells is essential for the operation of $C_4$ photosynthesis (Hatch et al., 1995; Kromdijk et al., 2014; von Caemmerer & Furbank, 2003). Cell wall thickness influences mesophyll conductance ($g_m$) (Evans et al., 2009; Scafaro et al., 2011; Terashima et al., 2011). For efficient operation of $C_4$ photosynthesis, thinner cell walls would allow the M cells to increase $g_m$, whereas thicker cell walls would be required for the BS cells to minimize the leakage of $CO_2$ and to elevate the $[CO_2]$. How the cell wall thickness of the M and BS cells in $C_4$ plants responds to N levels in reaching a compromise on the conflicting requirements of $CO_2$ diffusion remains unknown.

It is thought that in $C_4$ photosynthesis, metabolites are exchanged by symplastic diffusion through plasmodesmata at the interface between M and BS cells (Hatch, 1987; Kanai & Edwards, 1999). The end photosynthetic products are then transported through plasmodesmata from BS cells to vascular cells (Botha, 1992; Russin et al., 1996). However, whether the plasmodesmatal density in these cells is modulated in reflection of photosynthetic capacities at different N levels is also unknown.

The aim of this study was to elucidate the structural responses of M and BS cells of the NADP-ME-type $C_4$ grass *Sorghum bicolor* (L.) Moench in relation to physiological responses of photosynthetic traits. We characterized physiological responses of sorghum plants grown under 4 different N levels, and analyzed responses of leaf structural traits in plants grown under the highest and lowest N levels.

## Materials and methods

### Plant materials and growth conditions

Seeds of *Sorghum bicolor* (L.) Moench 'Haretaka' were provided by the National Livestock Breeding Center, Saku, Nagano, Japan. They were germinated on perforated multi-well nursery boxes filled with loam soil granules in July 2013 (summer) for 1 week in a greenhouse at Kyushu University. The seedlings were then transplanted into 5-L pots (1 plant per pot) holding sandy loam soil containing .05, .2, .4, or .6 g of N (as ammonium nitrate), 1.0 g of phosphorus (as calcium superphosphate), and 1.0 g of potassium (as potassium chloride). The .6 g N treatment was approximately equivalent to the standard amount of N application in field cultivation of sorghum. The plants were grown in the greenhouse for 4 weeks, and the uppermost fully expanded leaf from each of 4 plants at each N level was sampled for measurements. During the growth period, the minimum and maximum temperatures in the greenhouse were 25 and 35 °C, and the maximum light intensity was ca. 1600 µmol $m^{-2}$ $s^{-1}$.

### Gas exchange measurements

Gas exchange was measured with an infrared $CO_2$/$H_2O$ gas analyzer (Li-6262, Li-COR, Inc., Lincoln, NE, U.S.A) installed in an open gas-exchange system, as described in Kumagai et al. (2014), at a photosynthetic photon flux density (PPFD) of 1500 µmol $m^{-2}$ $s^{-1}$, a leaf temperature of 30 °C, 60% relative humidity, and atmospheric $[CO_2]$. Net photosynthetic rate ($P_N$) and stomatal conductance ($g_s$) were calculated as described in Long and Hallgren (1985).

### Chl content and specific leaf weight (SLW)

The same leaves as used for photosynthesis measurements were used for determination of Chl content and specific leaf weight (SLW). Leaf samples (2.5 $cm^2$ from the middle of a leaf) were collected and immersed in 80% acetone for 1 day in the dark until the leaf pieces became colorless. Then the Chl content in the acetone solution was determined according to Arnon (1949). Other leaf samples were air-dried at 80 °C for 1 day and weighed. SLW was calculated as the dry weight divided by the leaf area.

### Leaf N content and photosynthetic N use efficiency (PNUE)

The N content of the same leaves used above was measured. If a leaf was too small, the next lower leaf was added. The leaf samples were air-dried at 80 °C for 1 to 2 days and milled to a fine powder. The N content of .3 g of powder was determined by using a micro-Kjeldahl procedure

(Tsutsumi et al., 2017). PNUE was calculated as $P_N$ divided by leaf N content.

### *Carbon isotope ratio*

For determination of $\delta^{13}C$ values, leaf samples were air-dried at 60 °C, and ground with a mortar and pestle. The $^{12}C$ and $^{13}C$ contents were determined from 2 mg samples as described in Ueno (2013).

### *Assay of enzyme activities*

The middle of leaves was collected in the morning (09:00 to 10:00) and immediately frozen in liquid N. The samples (.25 g fresh weight) were ground with a pestle in a mortar (on ice) containing .5 g sea sand, 25 mg polyvinylpyrrolidone, and 1 mL of grinding medium containing 50 mM HEPES-KOH (pH 7.5), .2 mM EDTA, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM dithiothreitol, and .7% (w/v) bovine serum albumin. Homogenates were filtered through gauze. Part of each filtrate was taken for determination of Chl content (Arnon, 1949). The filtrates were centrifuged at 10,000 × *g* for 5 min at 4 °C, and the supernatants were used for enzymatic assays. Activities of PEPC and Rubisco were measured spectrophotometrically in 1 mL reaction mixtures as reported by Ueno and Sentoku (2006) at 30 °C, the same temperature as used in the leaf photosynthesis measurements.

### *Stomatal size and density*

The stomatal response to N application level was examined in plants grown with .05 and .6 g N per pot. Leaf fragments (ca. .5 $cm^2$) were sampled from the middle of the same leaves as used for the photosynthesis measurements, fixed in formalin – acetic acid – alcohol, and cleared in chloral hydrate – saturated ethanol according to the method of Ueno et al. (2006). Guard cell length was measured for 20 cells per leaf. The stomatal density was measured at 4 points per leaf at × 400 magnification.

### *Quantification of anatomical traits of leaves*

The structural responses of leaves were examined in plants grown with .05 and .6 g N. Leaf segments were sampled from the middle of leaves in the morning (about 08:00). They were immediately fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h, washed with phosphate buffer, and post-fixed in 2% (v/v) $OsO_4$ in phosphate buffer for 2 h. They were then dehydrated through an acetone series and embedded in Quetol resin (Nisshin-EM Co. Ltd., Shinjuku, Tokyo, Japan). Transverse sections (ca. 1 µm thick) were cut with a glass knife on an ultramicrotome, stained with toluidine blue O, and observed under a light microscope.

Leaf thickness and interveinal distance (the distance between the centers of adjacent small vascular bundles) in transverse sections were measured at 3 points per leaf. The sizes of M and BS cells surrounding small vascular bundles were measured for 10 cells per leaf. The size of M cells was represented by the length of the long axis, and that of BS cells by the diameter. The profile areas of epidermis, M, intercellular space, BS, and vascular bundles were measured by NIH Image software (US National Institutes of Health) in 4 transverse sections for each N level. The constitution ratios (proportions of each tissue) were calculated from these data. In addition, the profile areas of 10 M and 10 BS cells per leaf were measured. Simultaneously, the outline length of M cells exposed to intercellular spaces was measured, and exposed cell wall length per M cell (M profile area) was calculated.

### *Quantification of ultrastructural traits of leaves*

Transverse ultrathin sections were cut with a diamond knife from the same leaf samples embedded in resin, stained with lead citrate, and observed under an electron microscope (JEM-100CX IIK, JEOL, Tokyo, Japan) at 75 kV.

The number of chloroplasts in each of 10 M and 10 BS cells per leaf was counted. The length of the long axis of the chloroplasts was measured for 5 chloroplasts per leaf on electron micrographs. The profile area of 5 chloroplasts per leaf was measured by NIH Image. Since BS chloroplasts of the .05 g N plants accumulated many starch grains, the area of starch grains was excluded from the estimation of the chloroplast profile area. The number of thylakoids per granal stack in each of 4 chloroplasts of the M and BS cells was counted under an electron microscope.

The cell wall thickness of each of 3 M cells and 3 BS cells per leaf was measured on electron micrographs. Cell walls that were cut perpendicular and showed a clear edge were carefully selected for measurement to avoid overestimation of the thickness. The thickness of BS cell walls exposed to intercellular spaces and at the interface between M and BS cells was measured. Only the thickness of M cell walls exposed to intercellular spaces was measured. The number of plasmodesmata per BS cell was counted at the interfaces between M and BS cells, between adjacent BS cells, and between BS and vascular cells surrounding 5 small vascular bundles per leaf.

### *Total dry weight*

After the above measurement and sampling, all leaves, stems and roots were air-dried at 80 °C for 3 days and weighed. The dry weight of leaves sampled for the analysis

of N content was also added to total dry weight of each plant.

### Statistical analyses

The statistical significance of differences in leaf physiological traits among treatments was assessed by Tukey's multiple comparison test at $p < .05$. That of differences in structural traits between lowest and highest N treatments were determined by Student *t*-test at $p < .05$.

## Results

### Physiological traits

When sorghum plants were grown at different N levels, the total dry weight (all tissues excluding sampled leaf tissue but including roots) and the leaf N and Chl contents decreased with decreasing N level (Figure 1(A)–(C)). SLW also decreased slightly, but the difference was significant only in .05 g N plants (Figure 1(D)). $P_N$ and $g_s$ (Figure 1(E) and (F)) decreased and PNUE (Figure 1(G)) increased with decreasing N level. There were high positive correlations of leaf N content with $P_N$ (Figure 2(A)) and a positive correlation between SLW and $P_N$ (Figure 2(B)). The $\delta^{13}C$ values of all plants were within the range typical of $C_4$ plants (Cernusak et al., 2013) and increased with decreasing N level (Figure 1(H)).

Activities of both PEPC and Rubisco decreased with decreasing N level, and were positively correlated with leaf N content (PEPC, $r = .901$, $p < .001$; Rubisco, $r = .811$, $p < .001$) and $P_N$ (Figure 2(C) and (D)). The PEPC to Rubisco activity ratio also decreased with decreasing N level (Figure 3).

### Anatomical traits of leaves

The N responses of leaf structural traits of plants grown at the lowest and highest N levels (.05 and .6 g N) were investigated. The leaf thickness and interveinal distance in .05 g N plants decreased to 86% and 85%, respectively, of those in .6 g N plants (Table 1). Stomatal density increased and guard cell length decreased in .05 g N plants (Table 1).

The leaves in both treatments showed a Kranz-type anatomy typical of NADP-ME type $C_4$ grasses, in which a single BS surrounds the vascular bundle (Figure (4)). The BS cells contained centrifugally arranged chloroplasts in both treatments. Unlike the BS chloroplasts of .6 g N plants (Figure 4(D)), those of .05 g N plants accumulated many starch grains (Figure 4(C); described again in Figure 6). As expected, the cell length and cell profile area (size parameters) decreased in both M and BS cells of .05 g N plants, more so in the M cells (Table 1).

The constitution ratio of each tissue in the total leaf profile area was similar between treatments in epidermis, BS tissue, and vascular bundle (Figure (5)). However, .05 g N plants had a lower ratio of M tissue (excluding intercellular space) than .6 g N plants. As a result, the area ratio of M to BS tissues in .05 g N plants (2.44) was 81% of that in .6 g N plants (3.00; Table 1). The ratio of intercellular space (Figure (5)) and the exposed cell wall length per M cell (Table 1) was higher in .05 g N plants than in .6 g N plants.

### Ultrastructural traits of leaves

The number of chloroplasts per cell decreased in .05 g N plants in both cell types, more so in the BS cells (Table 1). In .05 g N plants, the M chloroplasts were slender but the BS chloroplasts were round owing to accumulation of many starch grains (Figure 6). As a result, the reduction in chloroplast length was smaller in the M chloroplasts than in the BS chloroplasts, but the reduction in chloroplast profile area (excluding starch grains) showed the reverse (Table 1). The BS chloroplasts had few grana, as reported previously (e.g. Yoshimura et al., 2004), in both treatments (Table 1). In the M chloroplasts, the number of thylakoids per granal stack was lower in .05 g N plants (Table 1).

In both treatments, the cell walls were much thicker in the BS cells than in the M cells (Figure 6; Table 1). The cell walls of both cell types were thicker in .05 g N plants (Table 1). The increase was greater in the BS cells (Table 1). The degree of thickening of cell walls differed between the BS cell walls exposed to intercellular space (221%) and the interface between M and BS cells (156%). The ratio of thickness between the BS cell walls exposed to intercellular space and the M cell walls was higher in .05 g N plants (5.73) than in .6 g N plants (3.16; Table 1). The BS cell walls in both treatments had suberized lamellae in the outer tangential and radial walls (Figure 6(C)–(F)), which are considered to be a barrier to $CO_2$ diffusion (Mertz & Brutnell, 2014), and sometimes in the inner tangential walls also (data not shown).

Total number of plasmodesmata per BS cell profile was significantly lower in .05 g N plants (Table 1). The numbers of plasmodesmata penetrating the cell walls between adjacent BS cells (radial walls) and between BS cells and vascular cells (inner tangential walls) were almost the same in the two treatments. On the other hand, that between M cells and BS cells (outer tangential walls; Figure 6(E) and (F)) decreased non-significantly in .05 g N plants (Table 1).

## Discussion

### Physiological responses

The total plant dry weight and the N and Chl contents of leaves decreased greatly and SLW decreased slightly

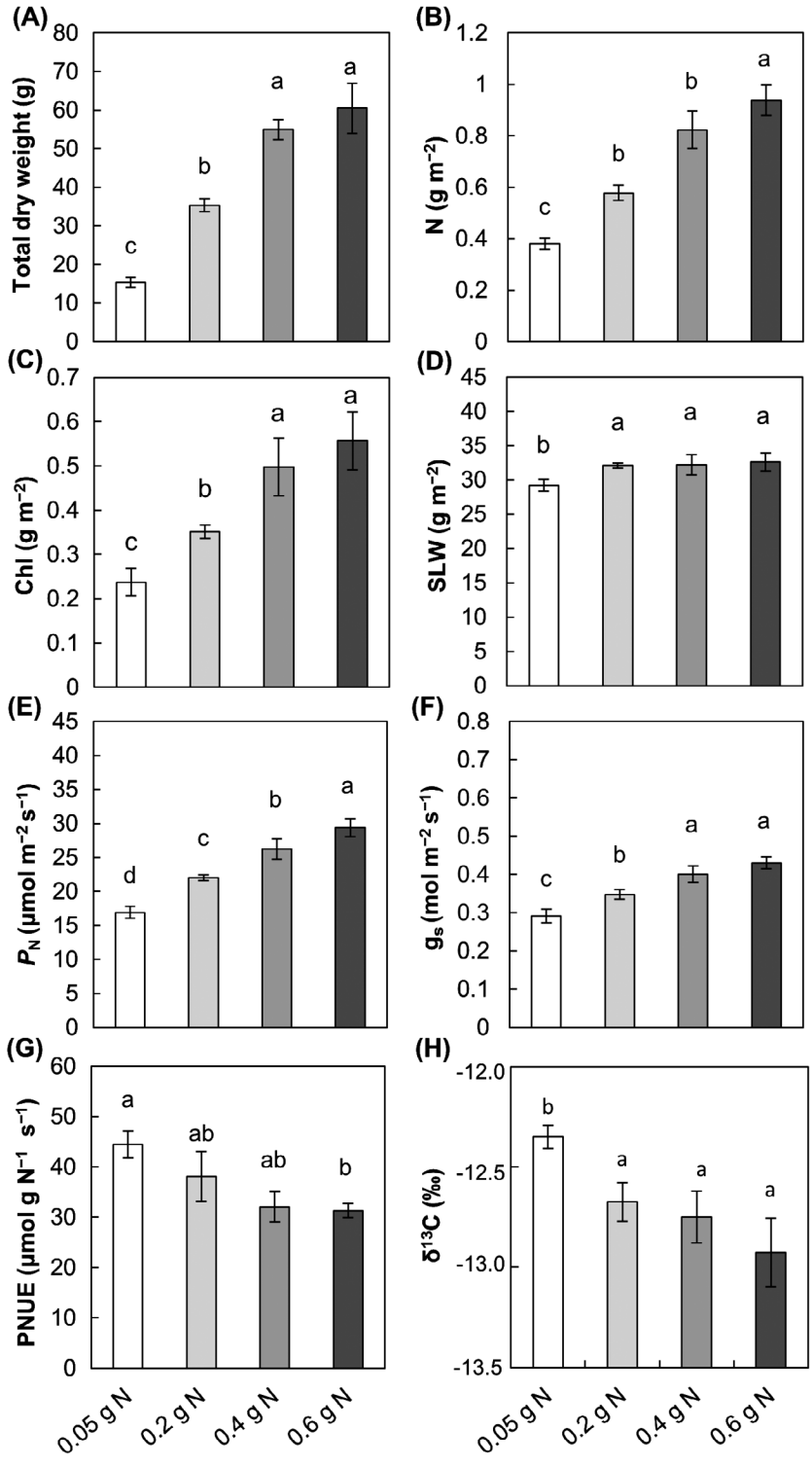


**Figure 1.** N responses of (A) total dry weight, (B) leaf N content, (C) Chl content, (D) specific leaf weight (SLW), (E) net photosynthetic rate ($P_N$), (F) stomatal conductance ($g_s$), (G) photosynthetic nitrogen use efficiency (PNUE), and (H) $\delta^{13}C$ values of leaves in sorghum plants grown in 5-L pots containing .05, .2, .4, or .6 g N. Data are means ± SD of 4 plants in each N treatment. Bars with the same letter are not significantly different ($p < .05$).

with decreasing N level (Figure 1(A)–(D)). $P_N$ also decreased (Figure 1(E)) and was highly correlated with leaf N content (Figure 2(A)), as reported in many plants (e.g. Brown, 1978; Makino et al., 2003). Correspondingly, $g_s$ also decreased (Figure 1(F)) and was highly correlated with leaf N content ($r = .957$; $p < .01$) and $P_N$ ($r = .944$; $p < .01$). Therefore, the decreased $g_s$ may account in part for the reduced $P_N$. In .05 g N plants, the guard cell length decreased and

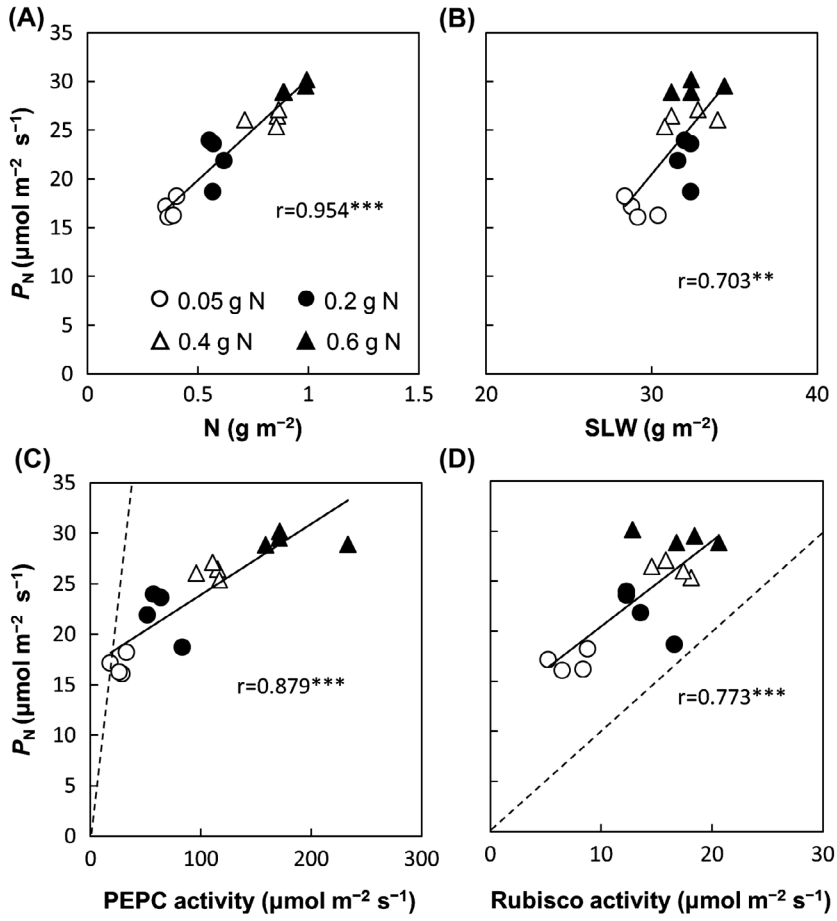


**Figure 2.** Relationships between net photosynthetic rate ($P_N$) and (A) leaf N content, (B) specific leaf weight (SLW), (C) PEPC activity, and (D) Rubisco activity in sorghum plants grown in 5-L pots containing .05, .2, .4, or .6 g N. $^{**}p < .01$, $^{***}p < .001$. Broken lines ($y = x$) show enzyme activities that would be required for equal $P_N$.

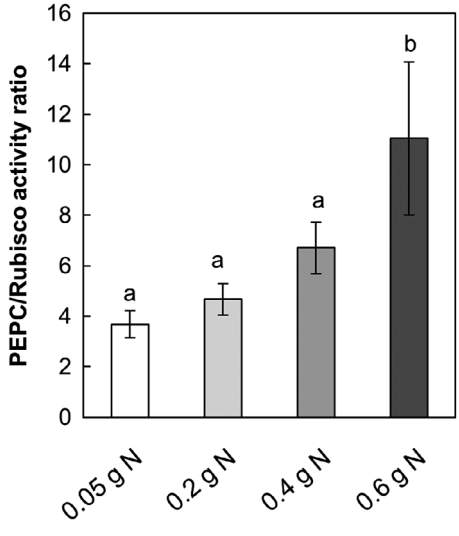


**Figure 3.** Ratio of PEPC activity to Rubisco activity in sorghum plants grown in 5-L pots containing .05, .2, .4, or .6 g N. Data are means ± SD of 4 plants in each N treatment. Bars with the same letter are not significantly different ($p < .05$).

stomatal density increased (Table 1). This negative relationship between the two has been found in other plants also (Franks et al., 2009; Tsutsumi et al., 2017; Yabiku & Ueno, 2017). It appears superficially that these morphological responses of stomata offset each other. However, whether the two parameters are involved in changing $g_s$ remains an intriguing issue because of the complex relationship between pore size and stomatal density (Fanourakis et al., 2015).

Previous studies have found a positive correlation between $P_N$ and SLW in $C_3$ leaves (reviewed in Ghannoum et al., 2011). Because the photosynthetic tissue of $C_3$ plants is constituted of just M cells, the leaves can simply stack M cells with increasing N level, resulting in increased SLW and thicker leaves. However, the SLW of $C_4$ species falls within a narrow range, because $C_4$ leaves need both M and BS cells, which impose an anatomical constraint (Ghannoum et al., 2011; Tsutsumi et al., 2017; Yabiku & Ueno, 2017). In sorghum also, the magnitude of the N response of SLW was small (Figure 1(D)), but there was a positive correlation between $P_N$ and SLW (Figure 2(B)). This suggests that

**Table 1.** Comparison of leaf thickness and structural traits (means ± SD) in leaves of sorghum plants grown under different N levels.

| Trait | .05 g N | .6 g N | .05/.6 g N |
|---|---|---|---|
| Leaf thickness (μm) | 128 ± 7 | 149 ± 6* | .86 |
| Interveinal distance (μm) | 102 ± 6 | 120 ± 11* | .85 |
| Stomatal density (No. $mm^{-2}$) | 245 ± 10 | 200 ± 11* | 1.23 |
| Guard cell length (μm) | 32.7 ± 1.0 | 37.5 ± .8* | .87 |
| Cell length | | | |
| M cell (μm) | 25.7 ± 3.4 | 31.0 ± 3.8* | .83 |
| BS cell (μm) | 19.2 ± 2.4 | 21.4 ± 3.4* | .90 |
| Cell profile area | | | |
| M cell ($\mu m^2$ per cell) | 191 ± 39 | 254 ± 56* | .75 |
| BS cell ($\mu m^2$ per cell) | 226 ± 56 | 268 ± 69* | .84 |
| M/BS tissue area ratio | 2.44 ± .16 | 3.00 ± .76 | .81 |
| Intercellular space ratio in M tissue (%) | 18.2 ± 1.3 | 13.9 ± 1.3* | 1.31 |
| Exposed cell wall length per M cell area (μm $\mu m^{-2}$) | .27 ± .02 | .19 ± .02 * | 1.42 |
| M chloroplasts | | | |
| No. per cell | 4.0 ± 1.0 | 5.4 ± 1.2* | .74 |
| Length (μm) | 5.3 ± .6 | 5.6 ± .8 | .95 |
| Area ($\mu m^2$)[a] | 5.8 ± 1.3 | 8.7 ± 1.9* | .67 |
| No. of thylakoids per granal stack | 14.6 ± 4.9 | 17.5 ± 6.2* | .83 |
| BS chloroplasts | | | |
| No. per cell | 3.1 ± .9 | 5.5 ± 1.3* | .56 |
| Length (μm) | 7.0 ± .9 | 8.8 ± 1.3* | .80 |
| Area ($\mu m^2$)[a] | 10.3 ± 2.3 | 11.9 ± 2.0* | .87 |
| No. of thylakoids per granal stack | 2.5 ± .8 | 2.4 ± .6 | 1.04 |
| Cell wall thickness | | | |
| M cell (μm)[b] | .164 ± .093 | .134 ± .049* | 1.22 |
| BS cell (μm)[c] | .939 ± .174 | .424 ± .056* | 2.21 |
| M cell/BS cell interface (μm) | .419 ± .037 | .269 ± .027* | 1.56 |
| BS cell/M cell ratio[d] | 5.73 | 3.16 | 1.81 |
| Density of plasmodesmata in BS cell | | | |
| M cell/BS cell interface (No. per cell) | 7.2 ± .8 | 8.5 ± 1.3 | .85 |
| BS cell/BS cell interface (No. per cell) | 1.6 ± .2 | 1.6 ± 1.0 | 1.00 |
| BS cell/vascular cell (No. per cell) | 2.0 ± .4 | 2.1 ± .5 | .95 |
| Total number per cell (No. per cell) | 10.8 ± .6 | 12.2 ± .6* | .89 |

The values are given as the mean ± SD of 4 plants in each N treatment.
*Significant difference between .05 g N and .6 g N plants at $p < .05$.; [a]Chloroplast area excluding starch grains.
[b]Thickness of M cell walls exposed to intercellular space.
[c]Thickness of BS cell walls exposed to intercellular space.
[d]Ratio between thickness of BS cell wall exposed to intercellular space and thickness of M cell.

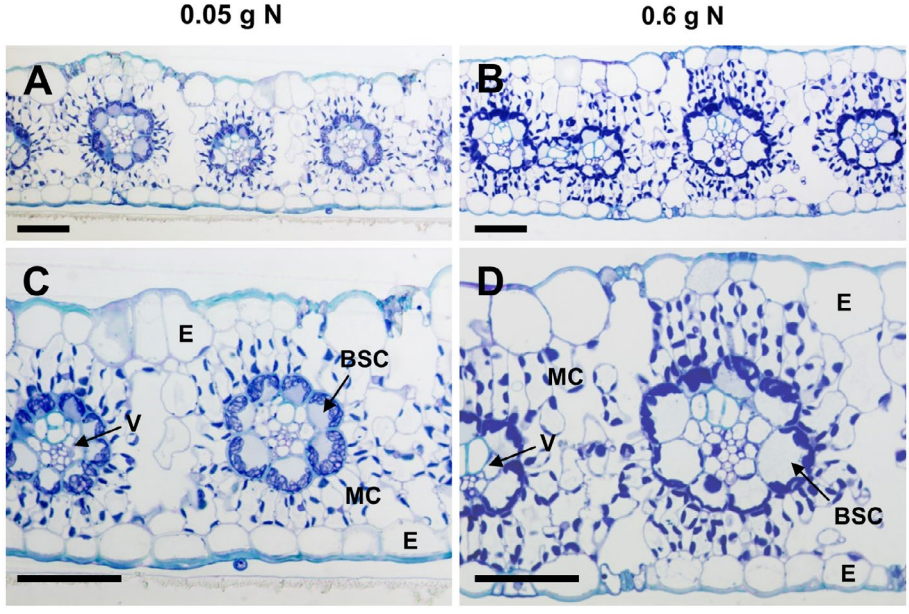


**Figure 4.** Comparison of leaf anatomy of sorghum plants grown in 5-L pots containing (A, C) .05 or (B, D) .6 g N. (A, B) Transverse sections. (C, D) Enlargement of A and B. Scale bars = 50 μm. BSC, bundle sheath cell; E, epidermis; MC, mesophyll cell; V, vascular bundle.

sorghum leaves cope with N limitation by intracellular modulation, keeping the fundamental anatomical framework required for $C_4$ photosynthesis.

The N limitation would affect $P_N$ through reduced amounts and activities of photosynthetic enzymes in leaves. In fact, activities of PEPC and Rubisco were reduced with decreasing N, although it seems that activities of Rubisco are underestimated probably due to degradation and/or inactivation in extraction, since the activities were lower than those would be required for equal $P_N$ (Figure 2(D)). Nevertheless, activities of PEPC and Rubisco were significantly correlated with leaf N content ($p < .001$) and $P_N$ (Figure 2(C) and (D)). In $C_4$ photosynthesis, PEPC is responsible for primary carboxylation in M cells, and Rubisco for secondary carboxylation in BS cells. The ratio of PEPC to Rubisco activity declined with decreasing N (Figure 3). This means that PEPC activity was reduced more than Rubisco activity with decreasing leaf N content. Similar N responses of the enzymes were reported in some $C_4$ species such as maize (Sugiyama et al., 1984) and *Amaranthus retroflexus* (Sage et al., 1987). In another NADP-ME-type $C_4$ grass maize, Rubisco is a rate-limiting factor of photosynthesis but PEPC is not so (Usuda, 1984; Yabiku & Ueno, 2017). Therefore, it is reasonable that sorghum plants also cope with N limitation by lowering the allocation of N to PEPC more than to Rubisco.

The sorghum plants had high PNUE (30 to 44 μmol g $N^{-1}$ $s^{-1}$). These values fall within the range of PNUE previously

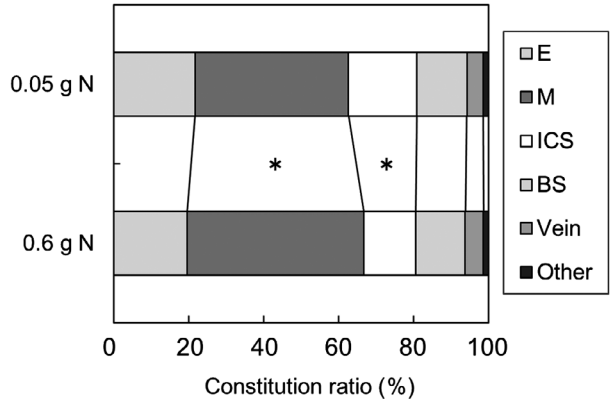


**Figure 5.** Ratios of tissues constituting leaves of sorghum plants grown in 5-L pots containing .05 or .6 g N. E, epidermis; M, mesophyll; ICS, intercellular space; BS, bundle sheath. *Significant difference between .05 g N and .6 g N plants at $p < .05$.

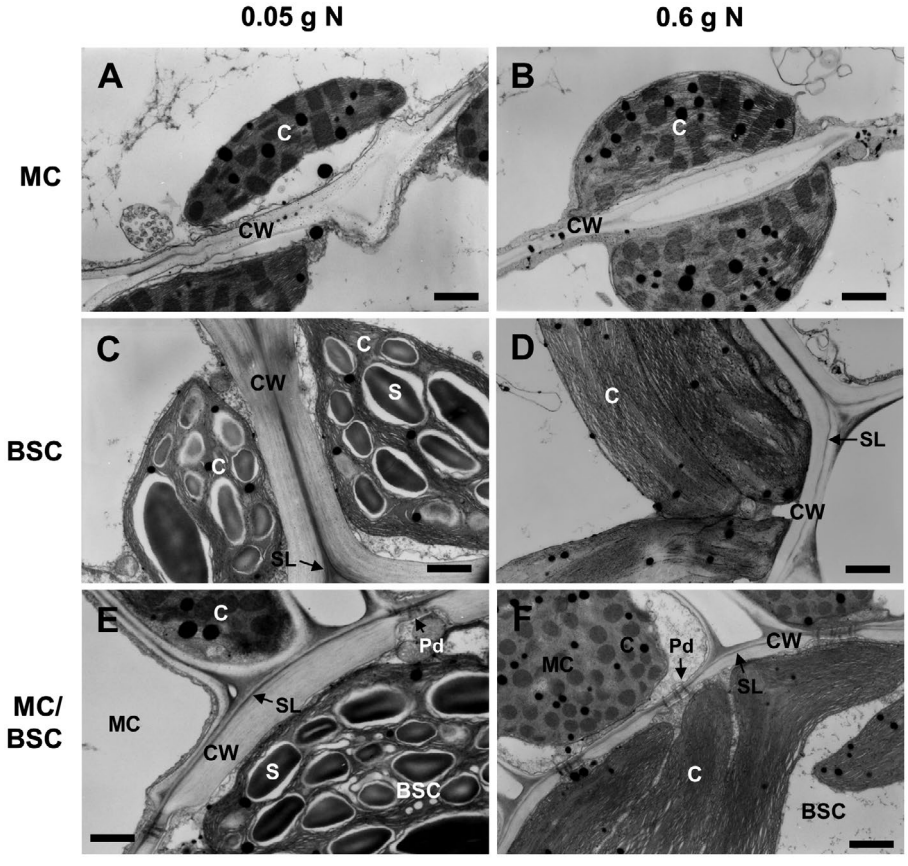


**Figure 6.** Comparison of leaf ultrastructure of sorghum plants grown in 5-L pots containing (A, C, E) .05 g N or (B, D, F) .6 g N. (A, B) Chloroplasts of M cells. (C, D) Chloroplasts of BS cells. (E, F) Interfaces between M and BS cells. Scale bars = 1 μm. BSC, bundle sheath cell; c, chloroplast; CW, cell wall; MC, mesophyll cell; Pd, plasmodesma; s, starch grain; SL, suberized lamella.

reported in NADP-ME-type $C_4$ grasses, which are known to have higher PNUE than NAD-ME type-$C_4$ grasses and $C_3$ grasses (Ghannoum et al., 2005, 2011). The PNUE of sorghum increased with decreasing N. This response was also observed in the $C_3$ grass rice in a parallel experiment, although PNUE was always lower in the rice (data not shown). Ghannoum et al. (2005) also reported higher PNUE under lower N in several $C_4$ grasses. Thus, this response might be a common feature in both $C_3$ and $C_4$ grasses, suggesting that plants use efficiently limited N under N deficiency to perform photosynthesis efficiently.

### *Quantitative responses of tissues and cells*

The constitution ratios of the BS and vascular bundle did not differ between the .05 and .6 g N plants, but that of the M decreased in the .05 g N plants (Figure 5). On the other hand, that of the epidermis did not differ between the two plants (Figure 5). This response of the epidermis differed from that in the $C_3$ grass barley (Van Arendonk et al., 1997), in which the epidermis thickens in high-N-level plants and becomes a major storage site for inorganic N. These data suggest that cellular N storage differs between grass species. In $C_4$ leaves, a quantitative balance between M and BS tissue volume allows efficient operation of $C_4$ photosynthesis (Dengler et al., 1994; Hattersley, 1984; Ueno, 1996). The M/BS tissue area ratio was lower in .05 g N plants than in .6 g N plants (Table 1). This means that the accumulation site of PEPC (M) is reduced relative to that of Rubisco (BS) in N limitation. This cellular response corresponds with the response of PEPC and Rubisco activities (Figure 3).

It is evident that N limitation results in the reduction in size of individual inner cells of leaves, as well as stomata (Table 1). Correspondingly, leaf thickness and interveinal distance were reduced in .05 g N plants. However, the reduction was greater in the M cells than in the BS cells, as seen in both cell length and sectional profile area (Table 1). In addition, the intercellular space ratio in M tissue increased in the .05 g N plants relative to the .6 g N plants (Table 1). This and the increase in exposed cell wall length per M cell (Table 1) may compensate for the reduction in $CO_2$ conductance by thicker cell walls of M cells, as discussed later.

### *Response of chloroplasts*

In .05 g N plants, the number and size of chloroplasts decreased together with the reduction of M and BS cells (Table 1). Such responses of chloroplasts are observed in N-limited rice plants (Chonan et al., 1977). The reduction of chloroplast number per cell was greater in the BS cells than in the M cells, whereas that of chloroplast size (profile area) showed the reverse. Thus, the manner of reduction of chloroplasts under N limitation differs between the M and BS cells. The BS chloroplasts accumulated abundant starch grains. This phenomenon was previously observed in a PEP carboxykinase-type $C_4$ grass, *Panicum maximum*, grown under N limitation and was considered to be due to that N starvation injures the process by which $C_3$ cycle intermediates are translocated between chloroplasts and cytosol (Aviovich & Cresswell, 1983).

It is intriguing that the number of thylakoids per granal stack decreased in the M chloroplasts but was unchanged in the BS chloroplasts in .05 g N plants (Table 1). In NADP-ME-type $C_4$ plants, the BS chloroplasts substantially lack grana (they are agranal) or have fewer thylakoids per granal stack than the M chloroplasts (Hatch, 1987; Ueno et al., 2005; Yoshimura et al., 2004). In these $C_4$ plants, reducing power (NADPH) necessary for driving the $C_3$ cycle in BS chloroplasts is provided through the decarboxylation of malate transported from the M chloroplasts (Hatch, 1987; Kanai & Edwards, 1999). Our data show that this fundamental mechanism of NADP-ME-type biochemistry is retained irrespective of N level. On the other hand, plants grown under low N levels may experience photoinhibition (Kumagai et al., 2014; Lu & Zhang, 2000; Verhoeven et al., 1997). The reduced grana in the M chloroplasts may be an acclimatization to N limitation.

### *Response of cell wall thickness*

In .05 g N plants, the cell walls thickened (Table 1). This feature is similar to that found in sclerophytes. The drawdown of $[CO_2]$ from intercellular spaces to the carboxylation site is greater in sclerophytes than in non-sclerophytes (Warren, 2008). In $C_3$ plants, $CO_2$ is fixed by Rubisco in the chloroplasts of M cells, and the cell wall thickness is one of the major factors determining $g_m$ (Evans et al., 2009; Scafaro et al., 2011; Terashima et al., 2011; Tomas et al., 2013). This would be the case in $C_4$ plants also (Evans & Loreto, 2000), although the process of diffusion of $CO_2$ into the M cells differs between $C_3$ and $C_4$ plants, reflecting the difference in biochemical traits (Hatch, 1987; Kanai & Edwards, 1999). Thicker cell walls in the M cells would reduce $g_m$ in $C_4$ plants as well. The $[CO_2]$ in the intercellular space within leaves is lower in $C_4$ plants than in $C_3$ plants (Evans & Loreto, 2000). By contrast, thicker BS cell walls would reduce $CO_2$ leakage from BS cells. The ratio of cell wall thickness between .05 and .6 g N plants was higher in the BS cells than in the M cells, particularly in the BS cell walls exposed to intercellular space (Table 1). These structural features might contribute to maintaining high $[CO_2]$ within the BS cells.

The $\delta^{13}C$ values became less negative with decreasing applied N (Figure 1(H)), suggesting lowered carbon isotope discrimination. This N response of $\delta^{13}C$ values may

reflect reduced $CO_2$ leakage from BS cells in low-N plants. However, there are conflicting data on the N response of $CO_2$ leakage assessed from carbon isotope discrimination: with decreasing N, BS leakiness increased (Ma et al., 2017; Meinzer & Zhu, 1998), decreased (Yin et al., 2011), or depended on species (Fravolini et al., 2002). More reliable evaluation of BS leakiness in sorghum would be required, because post-photosynthesis processes might also affect $\delta^{13}C$ values (Cernusak et al., 2013; von Caemmerer et al., 2014). In addition, we don't know how N level affects cell wall porosity, which is a critical factor influencing the diffusion of $CO_2$ through the cell wall, together with cell wall thickness (Evans et al., 2009; Tosens et al., 2012). Levels of applied N change the chemical composition of leaves, such as the content of cellulose and lignin in $C_3$ grasses (*Poa*; Van Arendonk et al., 1997) and that of rhamnos and galactose in the $C_4$ grass *Miscanthus* × *giganteus* (Ma et al., 2017). Furthermore, it cannot be ruled out that the N responses of $g_s$ and the balance of PEPC/Rubisco activity may also be involved in the change in $\delta^{13}C$ values.

### *Response of plasmodesmatal density*

The plasmodesmatal density of leaves changes during the sink–source transition (Roberts et al., 2001) and in response to environmental factors (Sowinski et al., 2007). Our results suggest that the plasmodesmatal density in the BS cells of sorghum was affected by N level (Table 1). It was lower (although not significantly) at the interface between the M and BS cells (but was unchanged in other regions of BS cells) in .05 g N plants than in .6 g N plants. On the other hand, total number of plasmodesmata per BS cell was significantly lower. This response may be associated with a modulation of the symplastic traffic of photosynthetic metabolites between the M and BS cells. Reduced $P_N$ at low N levels would coincide with a reduced intercellular flux of metabolites. On the other hand, it was almost unchanged at the interface between the BS and vascular cells. These plasmodesmata play a prominent role in the symplastic export of photosynthates from BS cells to vascular cells in the $C_4$ grass maize (Russin et al., 1996). Accordingly, it seems unlikely that this transport process is responsible for the abundant starch accumulation in BS chloroplasts of .05 g N sorghum plants.

## Conclusion

This study revealed the structural response of sorghum leaves to N limitation in relation to physiological responses of photosynthesis. $C_4$ photosynthesis is a highly regulated biochemical mechanism achieved by close coordination between M cells and BS cells. Our results show that leaf structural traits are altered along with physiological traits of $C_4$ photosynthesis. Most changes would be associated with cellular allocation of N, light use, $CO_2$ diffusion and leakiness, and metabolite transport under N limitation. Studies of different $C_4$ subtypes of grasses will be needed to show whether the structural and physiological responses of sorghum plants to N stress can be generalized to other $C_4$ plants.

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