Variations in structural, biochemical, and physiological traits of photosynthesis and resource use efficiency in *Amaranthus* species (NAD-ME-type C₄)

Nobuko Tsutsumia, Miyuki Tohyab, Taiken Nakashimaa and Osamu Uenoa,c

*a*Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka, Japan; *b*School of Agriculture, Kyushu University, Fukuoka, Japan; *c*Faculty of Agriculture, Kyushu University, Fukuoka, Japan

**ABSTRACT**

C₄ plants show higher photosynthetic capacity and productivity than C₃ plants owing to a CO₂-concentrating mechanism in leaves, which reduces photorespiration. However, which traits regulate the photosynthetic capacity of C₄ plants remains unclear. We investigated structural, biochemical, and physiological traits associated with photosynthesis and resource use efficiency in 20 accessions of 12 species of *Amaranthus*, NAD-malic enzyme-type C₄ dicots. Net photosynthetic rate (Pₙ) ranged from 19.7 to 40.5 μmol m⁻² s⁻¹. Pₙ was positively correlated with stomatal conductance and nitrogen and chlorophyll contents of leaves and was weakly positively correlated with specific leaf weight. Pₙ was also positively correlated with the activity of the C₃ enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, but not with the activities of the C₄ enzymes phosphoenolpyruvate carboxylase and NAD-malic enzyme. Structural traits of leaves (stomatal density, guard cell length, leaf thickness, interveinal distance, sizes of mesophyll and bundle sheath cells and the area ratio between these cells) were not significantly correlated with Pₙ. These data suggest that some of the biochemical and physiological traits involved in interspecific Pₙ variation, whereas structural traits are not directly involved. Photosynthetic nitrogen use efficiency ranged between 260 and 458 μmol mol⁻¹ N s⁻¹. Photosynthetic water use efficiency ranged between 5.6 and 10.4 mmol mol⁻¹. When these data were compared with previously published data of C₄ grasses, it is suggested that common mechanisms may determine the variations in resource use efficiency in grasses and this dicot group.

**Introduction**

Photosynthetic capacity is important for plant productivity and is a potential target to increase crop productivity (Evans, 2013; Zhu et al., 2010). In general, C₄ plants show higher photosynthetic capacity and productivity than C₃ plants (Brown, 1999) owing to a CO₂-concentrating mechanism, which provides a high-CO₂ environment around ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and thereby suppresses photorespiration (Hatch, 1987; Osmond et al., 1982). Many studies have documented the genetic variation of photosynthetic rate and its regulatory factors (Flood et al., 2011). However, fewer studies have been performed on C₄ crops than on C₃ crops (e.g. Baer & Schrader, 1985; Peng et al., 1991). The range of genetic variation in photosynthetic rate in C₄ crops and the traits of photosynthesis that control this variation remain unclear. The regulation of C₄ photosynthesis is more intricate than that of C₃ photosynthesis (von Caemmerer & Furbank, 2016; Wang et al., 2014), because C₃ photosynthesis occurs in mesophyll cells, whereas C₄ photosynthesis is achieved through a collaboration of mesophyll and bundle sheath (BS) cells. First, atmospheric CO₂ is fixed by phosphoenolpyruvate carboxylase (PEPC) of mesophyll cells and formed C₄ acids are transported to BS cells, where they are decarboxylated by C₄ acid decarboxylase; the released CO₂ is refixed by Rubisco in the BS cells and assimilated to carbohydrate in the C₄ cycle (Hatch, 1987). Some of these reactions are rate limiting in C₄ photosynthesis (Baer & Schrader, 1985; von Caemmerer et al., 1997; Usuda et al., 1984).

CO₂ diffusion through stomata to the carboxylation site of photosynthetic cells also regulates photosynthetic rate. Many studies reported a positive relationship between photosynthetic rate and stomatal conductance (gₛ) (Evans & Loreto, 2000; Flexas et al., 2012; Wong et al., 1979). Structural traits of leaves, such as size and density of...
stomata and photosynthetic cells, are also involved in CO₂ diffusion within leaves (Evans & Loreto, 2000; Giuliani et al., 2013). In C₄ plants, the quantitative balance of mesophyll and BS cells may be critical, because close coordination of the C₄ and C₃ cycles is required for efficient C₄ photosynthesis (Dengler et al., 1994; Lundgren et al., 2014; Ueno, 1996; Ueno et al., 2006).

In modern agriculture, efficient use of resources, such as nutrients and water, is of primary concern together with the increase in crop productivity (Ghannoum et al., 2011; Xu et al., 2012). Nitrogen (N) is the most important nutrient limiting plant productivity. C₄ plants use N more efficiently in photosynthesis and dry matter production than C₃ plants (Brown, 1977; Ghannoum et al., 2005, 2011; Taylor et al., 2010; Vogan & Sage, 2011). Photosynthetic N use efficiency (PNUE) is defined as net photosynthetic rate ($P_N$) per unit leaf N content. Water also limits plant growth and productivity, especially in rain-fed agriculture. C₄ plants also use water more efficiently in photosynthesis and dry matter production than C₃ plants (Ghannoum et al., 2011; Osmond et al., 1982; Taylor et al., 2010; Vogan & Sage, 2011). Photosynthetic water use efficiency (PWUE), which is defined as $P_N$ per unit of transpiration rate ($T_r$), represents instantaneous water use efficiency of leaves. Although a considerable number of studies on genetic variation in resource use efficiency are available for C₄ crops, data on C₄ crops are also limited to some major C₄ grass crops (Maranville & Madhavan, 2002; Uribelarra et al., 2009).

C₄ plants are divided into three biochemical subtypes depending on the difference in the mechanism of decarboxylation of C₄ acids in BS cells: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PCK) types (Hatch, 1987). Recent studies on C₄ grasses suggested differences in N use efficiency among the C₄ subtypes (Ghannoum et al., 2005; Togawa & Ueno, 2015), but we need more data to assess whether this conclusion can be extended to other C₄ groups.

*Amaranthus* is a genus in the Amaranthaceae family of dicots and includes many valuable grain and vegetable crops. Some *Amaranthus* species were widely consumed by prehistoric and modern Native Americans. The grains and leaves are rich in nutrients and minerals (Kachiguma et al., 2015). *Amaranthus* belongs to the NAD-ME type (El-Sharkawy, 2016; Ueno, 2001) and provides a unique opportunity to examine the genetic variation in photosynthetic traits and resource use efficiency in dicot crops of this type.

In this study, we investigated the structural, biochemical, and physiological traits of photosynthesis in 20 accessions of 12 species of *Amaranthus* to clarify the factors that affect genetic variation in $P_N$. In addition, we assessed the ranges of genetic variation of PNUE and PWUE in the *Amaranthus* species.

**Materials and methods**

**Plant materials and growth**

The accessions and species of *Amaranthus* examined in this study are listed in Table 1. Seeds were provided by the Plant Introduction Station, Agricultural Research Service, USDA, and by Dr M. Katsuta, National Institute of Crop Science, National Agriculture and Food Research Organization, Tsukuba, Japan. Seeds were germinated in perforated multiwell nursery boxes filled with loam soil granules and were grown for about 3 weeks in a greenhouse at the experimental field of Kyushu University in July, 2012. The seedlings were then transplanted to 5 L pots (one plant per pot) containing sandy loam soil containing nitrogen, phosphorus, and potassium fertilizers (1.57 g each) in the form of ammonium nitrate, calcium superphosphate, and potassium chloride, respectively. The plants were grown outdoors for 4 weeks (August to September; mean air temperature, 26 °C; relative humidity, 65%). Plants were irrigated twice a day. Fully expanded mature upper leaves were used (three plants per accession, but two plants for *Amaranthus tricolor* PI 604669). In most strains, sampling and measurements for structural, biochemical and physiological traits of leaves were carried out at vegetative stage. In several strains, however, plants at flowering stage were used (Table 1).

**Gas exchange and PWUE**

An infrared CO₂/H₂O gas analyzer (Li-6262, Li-COR, Inc., Lincoln, NE, USA) installed in an open gas-exchange system was used as reported in Nakashima et al. (2011). The measurements were made under 1,500 μmol m⁻² s⁻¹ of photosynthetic photon flux density, leaf temperature of 30 °C, relative humidity of 60%, and atmospheric CO₂ concentration. $P_N$, $g_s$, and $T_r$ were calculated according to Long and Hallgren (1985). PWUE was calculated from $P_N$ and $T_r$ values.

**N and chlorophyll contents, specific leaf weight, and PNUE**

The same leaves were used to measure gas exchange and parameters described in this subsection. For plants with small leaves, lower leaves (the first and second lower leaves from the uppermost leaf) were added for measurement of N content (Table 1). It was considered that there is almost no positional effect on N content of leaves, because chlorophyll (Chl) and soluble protein contents did not largely differ between mature uppermost leaves and middle
position-leaves (the seventh lower leaf) of Amaranthus plants (Nakashima et al., 2012). Samples were air dried at 80 °C for 1 to 2 days and milled to a fine powder. The N content of each leaf sample (0.3 g of powder) was determined using a micro-Kjeldahl procedure (Hashiba & Kanahama, 2002). The P NUE was calculated from P N and leaf N contents. Five leaf disks (6 mm diameter each) per plant were immersed in 80% acetone for 2 to 3 days in the dark until the color was leached, and Chl content in the acetone solution was measured spectrophotometrically according to Arnon (1949). Another five leaf disks were air dried at 80 °C for 1 day and weighed; specific leaf weight (SLW) was calculated by dividing dry weight by leaf area values.

Enzyme assays
Leaf samples were immediately frozen in liquid nitrogen and stored at about −80 °C. Leaves (0.25 g fresh weight) were ground on ice with a pestle in a mortar containing 0.5 g of sea sand, 25 mg of polyvinylpyrrolidone, 7 mg of bovine serum albumin, and 1 mL of grinding medium (50 mM HEPES-KOH (pH 7.5), 0.2 mM EDTA-2Na, 2.5 mM MgCl 2, 2.5 mM MnCl 2, 5 mM dithiothreitol, and 0.2% (v/v) Triton X-100). The homogenates were filtered through gauze, the filtrates were centrifuged at 10 000×g for 5 min at 4 °C, and the supernatants were used for the enzyme assays. An aliquot of the filtrate was taken for Chl content determination. Activities of PEPC, NAD-ME, and Rubisco were measured spectrophotometrically in 1 mL reaction mixtures (Ueno & Sentoku, 2006) at 30 °C (the same temperature as for gas exchange measurements). To measure total Rubisco activity, the supernatant was preincubated in the presence of 10 mM NaHCO 3 and 10 mM MgCl 2 at 25 °C for 10 min.

Quantification of structural traits in leaves
Cleared leaves were prepared as described in Ueno et al. (2006). Samples were obtained from the middle position of the leaves used for gas-exchange measurement. Leaf samples were fixed in a mixture of formaldehyde, acetic acid, and ethanol in water for 1 to 2 days; incubated in 70% ethanol at 80 °C for 36 h; and washed in distilled water several times. Then they were incubated in 80% lactic acid at 80 °C for 20 h and stored in chloral hydrate–saturated ethanol. Stomata on the adaxial and abaxial epidermis were observed under a light microscope (Biophot; Nikon, Tokyo, Japan). The guard cell length (GL) of five stomata selected randomly was measured at 300× magnification with an ocular micrometer with 4 replications for each surface of each leaf sample. The GL represented the mean of stomata on both leaf surfaces (40 stomata on adaxial and abaxial surfaces). Stomatal density (SD, sum of the number of stomata on both leaf surfaces per unit leaf area) was determined in a field of 0.385 mm 2 at 300× magnification with 4 replications for each surface of each leaf sample.

Samples taken from the middle position of the leaves used for gas-exchange measurement were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h. They were then washed with phosphate buffer and post-fixed in 2% OsO 4 in 50 mM sodium phosphate buffer for 2 h at room temperature. Samples were dehydrated through an acetone series, infiltrated with Quetol resin (Kushida & Kushida, 1982) for 1 day, embedded in fresh Quetol resin at 70 °C, and sectioned transversely at about 1 μm thickness with glass knives on an ultramicrotome (Porter-Blum MT-2B, Sorvall Inc., Nobwalk, Connecticut, USA). The sections were stained with 1% toluidine blue O. Structural traits were quantified in a representative leaf section from each leaf under the light microscope. Leaf thickness and chlorenchyma thickness (the thickest part of each vascular bundle sector) were measured at three points per section. Intervenial distance (IVD) was measured between the center of adjacent veins at 3 to 8 points per section. The length of the long axis (a parameter of the size of mesophyll cells) was measured for 10 palisade-like mesophyll cells (adaxial mesophyll cells) on vascular bundles per section. The diameter of BS cells (a parameter of the size of BS cells) was measured for 10 BS cells per section. The cross-sectional areas of mesophyll and BS cells surrounding vascular bundle (one vascular bundle per leaf section) were measured using the ImageJ software (Schneider et al., 2012), and the area (volume) ratio between mesophyll and BS cells (M/BS ratio) was
2.1 times the former. The mean \( P_N \) was 30.2 ± 6.1 μmol m\(^{-2}\) s\(^{-1}\) (Table 2). The intraspecific difference in \( P_N \) was small (Figure 1(A); Suppl. data 1). The value of \( g_s \) ranged from 165.7 to 245.6 mmol m\(^{-2}\) s\(^{-1}\); the latter was 1.5 times the former (Table 2). \( T_r \) ranged from 2.9 to 4.1 mmol m\(^{-2}\) s\(^{-1}\); the latter was 1.4 times the former (Table 2). PWUE varied from 5.6 mmol mol\(^{-1}\) in \( A. tricolor \) (Komena) to 10.4 mmol mol\(^{-1}\) in \( A. hyp \times hyb \), with the mean of 8.5 ± 1.3 mmol mol\(^{-1}\) (Figure 1(B); Table 2; Suppl. data 1). \( P_N \) was positively correlated with \( g_s \) (Figure 2(A); Table 3). PWUE was significantly correlated with \( P_N \) and \( g_s \), but not with \( T_r \) (Table 3).

\[ SLW, N, \text{ and Chl contents and PNUE} \]

SLW varied considerably among \( A. hyp \times hyb \) species, ranging from 20.0 to 34.2 g m\(^{-2}\) (Table 2). The N content of

Figure 1. Variations in (A) net photosynthetic rate (\( P_N \)), (B) photosynthetic water use efficiency (PWUE), and (C) photosynthetic N use efficiency (PNUE) in \( A. hyp \times hyb \) species. Notes: \( P_N \) is arranged from high to low mean values. Accessions of the same species are shown in the same tone. Mean ± SD (n = 3, except n = 2 for \( A. tricolor \) PI 604669). Statistical evaluation of the species and strain differences in these parameters is shown in Suppl. data 1.
of 344 ± 56 μmol mol⁻¹ N s⁻¹ (Figure 1(C); Table 2; Suppl. data 1). SLW was weakly correlated with PN (Figure 2(B)), but strongly correlated with leaf N content (Table 3). PN was positively correlated with N and Chl contents (Figure 2(C), (D)). PNUE was significantly correlated with PN and gs but not with leaf N content (Table 3). PWUE was significantly correlated with N and Chl contents (Table 3).

**Activities of photosynthetic enzymes**

PEPC activity showed a large variation, ranging from 25.1 to 136.0 μmol m⁻² s⁻¹ (Table 2). The lowest value was found in Ames 2177 and the highest in Tsurushin (both *A. hypochondriacus*), indicating that PEPC activity varies widely even within the same species. NAD-ME activity also showed a large variation, ranging from 12.8 to 62.7 μmol m⁻² s⁻¹ (Table 2). The variation in Rubisco activity (9.3–27.5 μmol m⁻² s⁻¹) was lower than the variations in PEPC and NAD-ME activities (Table 2). PN was positively correlated with Rubisco activity but not with PEPC and NAD-ME activities (Figure 3). Rubisco activity was positively correlated with SLW, N, and Chl contents, and with PWUE and NAD-ME activities (Table 3). PNUE was significantly correlated with N and Chl contents (Table 3).

**Structural traits of leaves**

The mean SD was 405 ± 105 mm⁻² (range: 204–607 mm⁻²; Table 2). The ratio of adaxial to abaxial SD was 0.9 ± 0.1 (data not shown). The mean GL was 25.7 ± 2.2 μm (range: 22.8–31.1 μm; Table 2). The ratio of adaxial to abaxial GL was 1.0 ± 0.1 (data not shown). The SD × GL, an index of 344 ± 56 μmol mol⁻¹ N s⁻¹ (Figure 1(C); Table 2; Suppl. data 1). SLW was weakly correlated with PN (Figure 2(B)), but strongly correlated with leaf N content (Table 3). PN was positively correlated with N and Chl contents (Figure 2(C), (D)). PNUE was significantly correlated with PN but not with leaf N content (Table 3). PWUE was significantly correlated with N and Chl contents (Table 3).

### Table 2. Comparison of physiological, biochemical and structural traits of photosynthesis and resource use efficiency in *Amaranthus* species.

| Trait                      | Mean ± SD | Minimum | Maximum | Max/min | F value |
|----------------------------|-----------|---------|---------|---------|---------|
| $P_N$ (μmol m⁻² s⁻¹)       | 30.2 ± 6.1| 19.7    | 40.5    | 2.1     | 9.70*** |
| $g_s$ (mmol m⁻² s⁻¹)       | 208.7 ± 20.0| 165.7 | 245.6   | 1.5     | 3.58*** |
| $T_r$ (mmol m⁻² s⁻¹)       | 3.5 ± 0.3 | 2.9     | 4.1     | 1.4     | 3.49*** |
| PWUE (μmol mol⁻¹)          | 8.5 ± 1.3 | 5.6     | 10.4    | 1.9     | 8.41*** |
| SLW (g m⁻²)                | 28.5 ± 4.1| 20.0    | 34.2    | 1.7     | 12.77***|
| N content (mmol m⁻²)       | 89.0 ± 15.9| 53.2  | 114.1   | 2.1     | 11.63***|
| Chl content (g m⁻²)        | 0.41 ± 0.1| 0.28    | 0.51    | 1.8     | 6.89*** |
| PNUE (μmol mol⁻¹ N s⁻¹)    | 344 ± 56  | 260     | 458     | 1.8     | 4.60*** |
| PEPC activity (μmol m⁻² s⁻¹)| 80.1 ± 33.3| 25.1  | 136.0   | 5.4     | 5.46*** |
| NAD-ME activity (μmol m⁻² s⁻¹)| 38.4 ± 12.2| 12.8  | 62.7    | 4.9     | 10.62***|
| Rubisco activity (μmol m⁻² s⁻¹)| 18.4 ± 4.7| 9.3    | 27.5    | 3.0     | 5.19*** |
| SD (No. mm⁻²)              | 405 ± 105 | 204     | 607     | 3.0     | 8.08*** |
| GL (μm)                    | 25.7 ± 2.2| 22.8    | 31.1    | 1.4     | 10.49***|
| SD × GL (mm mm⁻²)          | 10.2 ± 2.2| 6.4     | 14.2    | 2.2     | 9.25*** |
| Leaf thickness (μm)        | 185 ± 17  | 159     | 222     | 1.4     | 5.70*** |
| Chlorenchyma thickness (μm)| 156 ± 16  | 130     | 190     | 1.5     | 5.30*** |
| IVD (μm)                   | 136 ± 15  | 113     | 156     | 1.4     | 1.49NS  |
| MC length (μm)             | 34.3 ± 5.5| 28.2    | 49.9    | 1.8     | 6.68*** |
| BSC diameter (μm)          | 33.1 ± 3.5| 27.6    | 42.7    | 1.5     | 5.48*** |
| M/BS area ratio            | 2.8 ± 0.3 | 2.3     | 3.2     | 1.4     | 3.10*** |

Notes: Mean ± SD in 20 accessions of 12 species. Significance of F values at $P$: *** < 0.001; ** < 0.01; * < 0.05; NS – not significant.

**Figure 2.** Relationships between $P_N$ and (A) stomatal conductance ($g_s$), (B) specific leaf weight (SLW), (C) leaf N content, and (D) chlorophyll (Chl) content in *Amaranthus* species.

Notes: Mean ± SD ($n$ = 3, except $n$ = 2 for *A. tricolor* PI 604669). Significant at $P$: * < 0.05; ** < 0.01; *** < 0.001.
Table 3. Correlation coefficients (r) and their statistical significance for the relationships between physiological and biochemical parameters in *Amaranthus* species.

|                | $P_N$ | $g_s$ | $T_r$ | PWUE | SLW | N content | Chl content | PNUE | PEPC activity | NAD-ME activity | Rubisco activity |
|----------------|-------|-------|-------|------|-----|-----------|-------------|------|---------------|----------------|-----------------|
| $P_N$          | 1.000 |       |       |      |     |           |             |      |               |                |                 |
| $g_s$          | 0.780*** | 1.000|       |      |     |           |             |      |               |                |                 |
| $T_r$          | 0.710*** | 0.954*** | 1.000 |      |     |           |             |      |               |                |                 |
| PWUE           | 0.934*** | 0.532* | 0.417NS | 1.000 |     |           |             |      |               |                |                 |
| SLW            | 0.489*  | 0.400NS | 0.330NS | 0.487* | 1.000 |           |             |      |               |                |                 |
| N content      | 0.650** | 0.499* | 0.451* | 0.626** | 0.926*** | 1.000 |           |      |               |                |                 |
| Chl content    | 0.614** | 0.472* | 0.470* | 0.563** | 0.399NS | 0.550* | 1.000 |      |               |                |                 |
| PNUE           | 0.535*  | 0.454* | 0.418NS | 0.468* | -0.419NS | -0.280NS | 0.148NS | 1.000 |               |                |                 |
| PEPC activity  | 0.305NS | 0.097NS | 0.049NS | 0.349NS | -0.089NS | 0.101NS | 0.484* | 0.285NS | 1.000 |               |                 |
| NAD-ME activity| 0.249NS | 0.138NS | 0.240NS | 0.279NS | 0.577**  | 0.498*  | 0.143NS | -0.249NS | 0.099NS | 1.000 |               |
| Rubisco activity| 0.746** | 0.414NS | 0.312NS | 0.816*** | 0.640**  | 0.731*** | 0.561** | 0.124NS | 0.372NS | 0.531* | 1.000 |

Notes: Values represent r from linear regression. Significant at P: * < 0.05; ** < 0.01; *** < 0.001, NS – not significant.
and IVD (Table 5). IVD was negatively correlated with SD (Table 4).

The length of mesophyll cells ranged from 28.2 to 49.9 μm, and the diameter of BS cells ranged from 27.6 to 42.7 μm (Table 2). The M/BS ratio was between 2.3 and 3.2 (Table 2). These structural parameters also showed no significant correlation with the physiological traits (Figure 6(D); Suppl. data 2). The diameter of BS cells and the length of mesophyll cells were correlated negatively with SD and positively with GL (Figure 6(E) and (F); Table 4). Leaf and chlorenchyma thickness, and IVD (Table 5). IVD was negatively correlated with SD (Table 4).

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**Discussion**

**Variations in physiological and biochemical traits of photosynthesis**

To the best of our knowledge, our study is the first comprehensive report on the variation of $P_N$ in the species of an NAD-ME-type C₄ dicot crop. The mean value of $P_N$ was 20.2 μmol m⁻² s⁻¹, and the difference between the lowest and the highest values was 2.1 times. In our preliminary study performed in 2010 using 21 accessions of 9 species of *Amaranthus* (Tsutsumi et al., 2011), a similar mean
value of $P_N$ (29.7 μmol m$^{-2}$ s$^{-1}$) was found, with an interspecific difference of 2.5 times. *Amaranthus cannabinus*, which showed the highest $P_N$ value, grew vigorously and occasionally reached 3 m in height. The grain species (*A. caudatus, A. cruentus, A. hybridus*, and *A. hypochondriacus*) had intermediate $P_N$ values, whereas the vegetable species (*A. dubius, A. tricolor*, and *A. viridis*) had the lowest $P_N$ values (Table 1; Figure 1(A)).

$P_N$ was positively correlated with $g_s$ and $T_r$ (Figure 2(A); Table 3), as expected from previous studies of C$_3$ and C$_4$ plants (Evans & Loreto, 2000; Wong et al., 1979); $P_N$ was also positively correlated with Chl and N contents of leaves (Figure 2(C), (D)). This suggests that some photochemical and biochemical reactions of photosynthesis are closely involved in the variation in $P_N$ of *Amaranthus* species. SLW was weakly positively correlated with $P_N$ (Figure 2(B)). Positive correlation was also found between $P_N$ and SLW ($r = 0.726, p < 0.01$) in our preliminary study (Tsutsumi et al., 2011). Positive correlation between $P_N$ and SLW has been found in leaves of some C$_3$ species, but not in leaves of C$_4$ species (Ghannoum et al., 2011). Thus, *Amaranthus* seems to have an unusual relationship between $P_N$ and SLW.

C$_4$ photosynthesis is achieved by cooperation of the C$_4$ and C$_3$ cycles. Our data confirm that all *Amaranthus* species examined here are NAD-ME-type C$_4$ plants, because they have high NAD-ME activity. A positive correlation was found between $P_N$ and Rubisco activity but not between $P_N$ and PEPC or NAD-ME activity (Figure 3). It remains unknown the activities of which enzymes are rate limiting in NAD-ME-type C$_4$ photosynthesis (von Caemmerer & Furbank, 2016). In maize, an NADP-ME-type C$_4$ grass, Rubisco, and pyruvate, Pi dikinase, an enzyme responsible for the regeneration of PEP, are suggested to be the rate-limiting enzymes, because their activities were strongly correlated with $P_N$ (Baer & Schrader, 1985; Usuda et al., 1984). Using antisense RNA, von Caemmerer et al. (1997) demonstrated that Rubisco is the rate-limiting enzyme for C$_4$ photosynthesis in *Flaveria bidentis*, a transformable NADP-ME-type C$_4$ dicot. Our data also suggest that Rubisco is the rate-limiting enzyme of NAD-ME-type C$_4$ photosynthesis.

Figure 5. Transverse sections of leaves of representative species of *Amaranthus*. (A) *A. cannabinus* (PI 641042; $P_N = 41$ μmol m$^{-2}$ s$^{-1}$), (B) *A. hyp × A. hyb* (Plainsman; 39 μmol m$^{-2}$ s$^{-1}$), (C) *A. caudatus* (Ames 5301; 33 μmol m$^{-2}$ s$^{-1}$), (D) *A. cruentus* (Ames 5276; 29 μmol m$^{-2}$ s$^{-1}$), (E) *A. viridis* (PI 536439; 23 μmol m$^{-2}$ s$^{-1}$), and (F) *A. tricolor* (Komena; 20 μmol m$^{-2}$ s$^{-1}$). Notes: BSC, bundle sheath cell; MC, mesophyll cell; VB, vascular bundle. Bars = 50 μm.
activation state) and total Rubisco activity (fully activated state) in C₄ grasses, although total activity was higher than initial activity in wheat (C₃). By contrast, Baer and Schrader (1985) found that total activity of Rubisco in maize cultivars is lower than the initial activity. In our study, Rubisco was preincubated with Mg²⁺ and CO₂ to measure total activity. Therefore, the behavior of Rubisco activity in Amaranthus may resemble that observed in maize, and the initial activity may be higher than the total activity. On the other hand, it could not be ruled out that some inactivation and degradation of Rubisco may occur during extraction procedure (Usuda & Shimogawara, 1994).

In Amaranthus species, PEPC activity was higher than NAD-ME activity (Table 2). This is recognized in other study of Amaranthus as well (Bailey et al., 2000). In our study, there was no significant correlation between PEPC and NAD-ME activity and between PEPC and Rubisco activity in Amaranthus species. In our study, Rubisco activity was somewhat lower than that required to equal \( P_N \) (Figure 3(C)). The reason for this is unknown. Usuda et al. (1984) reported no difference between the initial Rubisco activity (\( \text{in vivo} \) activation state) and total Rubisco activity (fully activated state) in C₄ grasses, although total activity was higher than initial activity in wheat (C₃). By contrast, Baer and Schrader (1985) found that total activity of Rubisco in maize cultivars is lower than the initial activity. In our study, Rubisco was preincubated with Mg²⁺ and CO₂ to measure total activity. Therefore, the behavior of Rubisco activity in Amaranthus may resemble that observed in maize, and the initial activity may be higher than the total activity. On the other hand, it could not be ruled out that some inactivation and degradation of Rubisco may occur during extraction procedure (Usuda & Shimogawara, 1994).

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### Table 4. Correlation coefficients \((r)\) and their statistical significance for the relationships between stomatal and structural traits in Amaranthus species.

| Trait                | Stomatal density (SD) | Guard cell length (GL) | SD × GL |
|----------------------|----------------------|------------------------|---------|
| Leaf thickness       | –0.399**             | 0.665**                | –0.241**|
| Chlorenchyma thickness | –0.439**             | 0.688**                | –0.294**|
| IVD                  | –0.659**             | 0.436**                | –0.618**|
| MC length            | –0.610**             | 0.773**                | –0.507* |
| BSC diameter         | –0.666**             | 0.794**                | –0.530* |
| M/BS ratio           | 0.114NS              | –0.195NS               | 0.046NS |

Notes: Values represent \( r \) from linear regression. Significant at \( P: \) ** < 0.01; *** < 0.001; NS – not significant.

### Table 5. Correlation coefficients \((r)\) and their statistical significance for the relationships between structural parameters in Amaranthus species.

| Trait                | Leaf thickness | Chlorenchyma thickness | IVD | MC length | BSC diameter | M/BS ratio |
|----------------------|----------------|------------------------|-----|-----------|--------------|------------|
| Leaf thickness       | 1.000          | 0.977****              | 0.635** | 0.606** | 0.831***      | –0.278**   |
| Chlorenchyma thickness | 0.977****      | 1.000                  | 0.635** | 0.661** | 0.873***      | –0.250**   |
| IVD                  | 0.630**        | 0.635**                | 1.000 | 0.585**   | 0.680***      | –0.014**   |
| MC length            | 0.606**        | 0.661**                | 0.585** | 1.000    | 0.788***      | –0.051**   |
| BSC diameter         | 0.831***       | 0.873***               | 0.680*** | 0.046**  | 1.000         | –0.377**   |
| M/BS ratio           | –0.278**       | –0.250**               | –0.014** | –0.051** | –0.377**      | 1.000      |

Notes: Values represent \( r \) from linear regression. Significant at \( P: \) ** < 0.01; *** < 0.001; NS – not significant.
In NADP-ME-type $C_4$ grasses, a positive correlation is found between PEPC and NADP-ME activity and between PEPC and Rubisco activity (Usuda et al., 1984). In a mutant of *Amaranthus edulis* with reduced activity of either PEPC or NAD-ME, however, activities of other photosynthetic enzymes were not down-regulated (Bailey et al., 2000; Dever et al., 1998). Therefore, these data and our study suggest that the regulatory mechanism of $C_4$ photosynthesis in *Amaranthus* species may differ from that in NADP-ME-type $C_4$ grasses. In our study, PEPC and NAD-ME activities did not correlate with $P_N$ (Figure 3(A), (B)). In the *Amaranthus* mutants, a 55% reduction of PEPC activity resulted in slight decrease (ca. 12%) in $P_N$ (Bailey et al., 2000), whereas about 50% reduction of NAD-ME activity had no effect on $P_N$ (Dever et al., 1998). These data suggest that these enzymes, especially NAD-ME, have little control over $P_N$ in *Amaranthus* species.

**Variations in structural traits of photosynthesis**

Stomata are a critical structural trait in photosynthesis and transpiration, because atmospheric CO$_2$ enters and water leaves through stomata. Although we expected some significant relationships between stomatal and gas-exchange ($P_N$, $g_s$, and $T_i$) parameters, we could not find them (Figure 4(B), (C); Suppl. data 2). In Arabidopsis, genetically increased SD resulted in enhanced $P_N$ (Tanaka et al., 2013). In a grass (*Leymus chinensis*), SD was positively correlated with $P_N$ and $g_s$ (Xu & Zhou, 2008). In our study, $P_N$ was positively correlated with $g_s$ in the *Amaranthus* species. Therefore, it seems likely that the degree of stomatal opening, together with the GL and SD, is involved in a complex way in the variation in $P_N$, because these stomatal parameters are potentially involved in the maximum stomatal conductance (Lawson & Blatt, 2014). There was a negative correlation between GL and SD (Figure 4(A)); it appears that a decrease in GL is compensated by an increase in SD and vice versa, as in other species (Büssis et al., 2006; Franks et al., 2009). The interspecific difference in SD in *Amaranthus* was much greater than that of GL (Table 2). This indicates that there are physical and genetic limitations on the range of changes of GL, whereas SD has much greater flexibility. The physiological significance of the difference between variability of GL and SD remains an intriguing issue.

In general, if CO$_2$ diffusion within the leaf is not a limiting factor, $P_N$ of thicker leaves would be higher than that of thinner leaves, because thicker leaves accumulate larger amounts of proteins involved in photosynthesis per unit leaf area. In some $C_4$ species, thicker leaves with higher SLW show higher $P_N$ (Ghannoum et al., 2011). The thickness of $C_4$ leaves is restricted to a narrow range (Ghannoum et al., 2011), because under high light intensity $C_4$ leaves stack mesophyll cells, whereas $C_4$ leaves need to maintain quantitative balance between the two cell types. In *Amaranthus* species, SLW (Figure 2(B)) but not leaf or chlorenchyma thickness (Figure 6(A); Suppl. data 2) was positively correlated with $P_N$. These data show that increased leaf and chlorenchyma thicknesses do not result in higher SLW in *Amaranthus* (Suppl. data 2).

The M/BS ratio is a structural parameter associated with quantitative balance of the metabolic functions of $C_4$ and $C_3$ cycles (Dengler et al., 1994; Lundgren et al., 2014; Ueno, 1996). The IVD is a structural parameter involved in photosynthetic transport and water flow within leaves and in exchange of metabolites between mesophyll and BS cells in $C_4$ leaves (Dengler et al., 1994; Lundgren et al., 2014; Ueno et al., 2006). In *Amaranthus*, these two parameters were not significantly correlated with $P_N$ (Suppl. data 2). This was also the case for the size of mesophyll and BS cells (Figure 6(B), (D); Suppl. data 2). In general, small mesophyll cells would result in a large mesophyll surface area exposed to intercellular spaces per unit leaf area and thereby high CO$_2$ fluxes into mesophyll cells. However, Baer and Schrader (1985) reported that in maize cultivars, higher $P_N$ is associated with large leaf cell size, which was estimated from DNA content. This appears to be in conflict with the general relationship between cell size and $P_N$ in leaves. Our data in *Amaranthus* species indicate that structural traits of leaves, such as the M/BS ratio, IVD, and cell size, do not account for the variation of $P_N$.

Leaf and chlorenchyma thicknesses were positively correlated with IVD (Figure 6(C); Table 5). Positive relationships were also found between leaf and chlorenchyma thicknesses and the sizes of mesophyll and BS cells (Table 5). The sizes of mesophyll and BS cells were correlated negatively with SD but positively with GL (Figure 6(E) and (F); Table 4). These data suggest that the sizes of leaf cells change in concert with each other, which might permit smooth functioning of $C_4$ photosynthesis in *Amaranthus* species.

In this study, we failed to find structural factors primarily associated with the variation in $P_N$. The CO$_2$ leakage from BS cells influences photosynthetic efficiency of $C_4$ plants (Kromdijk et al., 2014; von Caemmerer & Furbank, 2016). Structural factors associated with mesophyll conductance might also be related to the variation in $P_N$ (Evans & Loreto, 2000; Flexas et al., 2012). Detailed analysis of leaf structural traits, such as the surface areas of photosynthetic cells exposed to intercellular spaces and their cell wall thickness, is required for understanding the genetic variation in $P_N$.

**Variations in resource use efficiency**

In previous studies, mean values of PNUE in $C_3$ and $C_4$ species ranged from 170 to 260 and 300 to 580 μmol mol$^{-1}$ N
s−1, respectively (Ghannoum et al., 2005, 2011; Togawa & Ueno, 2015; Vogan & Sage, 2011). As expected, PNUE values of Amaranthus species were higher than those of C3 species. Although PNUE of C4 grasses has been investigated (Brown, 1977; Ghannoum et al., 2005; Taylor et al., 2010; Togawa & Ueno, 2015), our study revealed a large genetic variation in PNUE among closely related C4 dicot species (Figure 1(C)). The PNUE is determined by leaf N content and the traits involved in $P_N$; the variation in PNUE was affected more by $P_N$ than by leaf N content (Table 3). Leaf structural traits were not correlated with the variation in PNUE. In leaves, N is allocated to cell walls and storage pools, as well as photosynthetic proteins (Poorter & Evans, 1998). Therefore, we could not rule out that the species difference in N allocation distorts the genetic variation in PNUE of Amaranthus. There was no clear difference in PNUE between grain and vegetable species of Amaranthus (Figure 1(C)).

Ghannoum et al. (2005) performed 3 separate experiments on PNUE of C4 grasses and found that mean values of PNUE range from 390 to 525 μmol mol−1 N s−1 in NADP-ME type and from 300 to 351 μmol mol−1 N s−1 in NAD-ME type. We found that PNUE of Amaranthus was close to those of the grasses of the NAD-ME type, which suggests that a common mechanism may determine PNUE of C4 subtypes of monocot and dicot plants. Greater turnover rate of Rubisco in NADP-ME than in NAD-ME-type C4 grasses was suggested as one of the main causes of the differences in PNUE (Ghannoum et al., 2005). The catalytic properties of Rubisco in Amaranthus species remain unknown.

In previous studies, mean values of PWUE in C3 and C4 species ranged from 2.2 to 5.2 and 6.6 to 12.0 mmol mol−1, respectively (Osmond et al., 1982; Togawa & Ueno, 2015). The PWUE of Amaranthus species was higher than those of C3 species. It is thought that higher PWUE in C4 species is due to their higher $P_N/g_s$ which is achieved by a CO2-concentrating mechanism (Ghannoum et al., 2011). The $P_N/g_s$ ratio (often called A/gs) is often used as another index of water use efficiency in leaves; its mean value (0.145 μmol mmol−1) was close to those reported in C4 grasses (Ghannoum et al., 2001; Taylor et al., 2010). We found a large genetic difference in PWUE among Amaranthus species (Figure 1(B)). PWUE is determined by the traits involved in $P_N$ and $T_r$; the effect of $P_N$ appeared to be stronger than that of $T_r$ (Table 2). Vegetable species tended to have lower PWUE than grain species (Figure 1(B)), as found for $P_N$. In contrast to PNUE, no significant differences have been found in PWUE and $P_N/g_s$ among C4 subtypes of grasses (Ghannoum et al., 2001, 2011; Taylor et al., 2010; Togawa & Ueno, 2015). Our data on PWUE and PNUE of Amaranthus species suggest that common mechanisms may determine the variations in resource use efficiency in grasses and this dicot group.

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