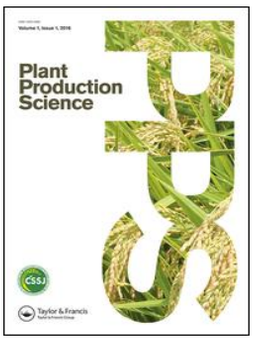

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Variations in structural, biochemical, and physiological traits of photosynthesis and resource use efficiency in *Amaranthus* species (NAD-ME-type C_4)

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ABSTRACT

C_4 plants show higher photosynthetic capacity and productivity than C_3 plants owing to a CO_2-concentrating mechanism in leaves, which reduces photorespiration. However, which traits regulate the photosynthetic capacity of C_4 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 C_4 dicots. Net photosynthetic rate (P_N) ranged from 19.7 to 40.5 μmol m^{-2} s^{-1}. P_N was positively correlated with stomatal conductance and nitrogen and chlorophyll contents of leaves and was weakly positively correlated with specific leaf weight. P_N was also positively correlated with the activity of the C_3 enzyme ribulose-1,5-bisphoshate carboxylase/oxygenase, but not with the activities of the C_4 enzymes phospho*enol*pyruvate 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 P_N. These data suggest that some of the biochemical and physiological traits are involved in interspecific P_N 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 C_4 grasses, it is suggested that common mechanisms may determine the variations in resource use efficiency in grasses and this dicot group.

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CLASSIFICATION
Crop Physiology

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, C_4 plants show higher photosynthetic capacity and productivity than C_3 plants (Brown, 1999) owing to a CO_2-concentrating mechanism, which provides a high-CO_2 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 C_4 crops than on C_3 crops (e.g. Baer & Schrader, 1985; Peng et al., 1991). The range of genetic variation in photosynthetic rate in C_4 crops and the traits of photosynthesis that control this variation remain unclear. The regulation of C_4 photosynthesis is more intricate than that of C_3 photosynthesis (von Caemmerer & Furbank, 2016; Wang et al., 2014), because C_3 photosynthesis occurs in mesophyll cells, whereas C_4 photosynthesis is achieved through a collaboration of mesophyll and bundle sheath (BS) cells. First, atmospheric CO_2 is fixed by phospho*enol*pyruvate carboxylase (PEPC) of mesophyll cells and formed C_4 acids are transported to BS cells, where they are decarboxylated by C_4 acid decarboxylase; the released CO_2 is refixed by Rubisco in the BS cells and assimilated to carbohydrate in the C_3 cycle (Hatch, 1987). Some of these reactions are rate limiting in C_4 photosynthesis (Baer & Schrader, 1985; von Caemmerer et al., 1997; Usuda et al., 1984).

CO_2 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 (g_s) (Evans & Loreto, 2000; Flexas et al., 2012; Wong et al., 1979). Structural traits of leaves, such as size and density of

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stomata and photosynthetic cells, are also involved in CO_2 diffusion within leaves (Evans & Loreto, 2000; Giuliani et al., 2013). In C_4 plants, the quantitative balance of mesophyll and BS cells may be critical, because close coordination of the C_4 and C_3 cycles is required for efficient C_4 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. C_4 plants use N more efficiently in photosynthesis and dry matter production than C_3 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. C_4 plants also use water more efficiently in photosynthesis and dry matter production than C_3 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 C_3 crops, data on C_4 crops are also limited to some major C_4 grass crops (Maranville & Madhavan, 2002; Uribelarrea et al., 2009).

C_4 plants are divided into three biochemical subtypes depending on the difference in the mechanism of decarboxylation of C_4 acids in BS cells: NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and PEP carboxykinase (PCK) types (Hatch, 1987). Recent studies on C_4 grasses suggested differences in N use efficiency among the C_4 subtypes (Ghannoum et al., 2005; Togawa & Ueno, 2015), but we need more data to assess whether this conclusion can be extended to other C_4 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 CO_2/H_2O 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 CO_2 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

Table 1. *Amaranthus* species examined in this study.

Species	Use	Accession No./ strain	Locality
A. australis J.D. Sauer		PI 553076	Florida, USA
		PI 553077	Florida, USA
A. cannabinus L.		PI 641042[a,b]	New Jersey, USA
A. caudatus L.	G	Ames 5301	Peru
A. cruentus L.	G	Ames 2092	Nepal
		Ames 5276	Guatemala
		PI 628793	Zaire
A. dubius Mart. ex Thell.	V	PI 605352[a,b]	Jamaica
A. hybridus L.	G	Ames 5361	Pennsylvania, USA
		Ames 5605	Greece
A. hyp × *A. hyb*		Plainsman[a]	USA
A. hypochondriacus L.	G	New Aztec	Japan
		Tsurushin	Japan
		Ames 2177	Nepal
A. palmeri S. Wats.		PI 633587[a]	Senegal
A. quitensis Kunth.		Ames 15316[a]	Argentina
A. tricolor L.	V	Komena	Japan
		PI 527321	China
		PI 604669	Taiwan
A. viridis L.	V	PI 536439[a,b]	Maldives

Notes: Use: G, grain; V, vegetable.
[a]Plants at flowering stage were examined.
[b]Lower leaves were added for measurements of N content and enzyme activities.

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 PNUE 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. Interveinal 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

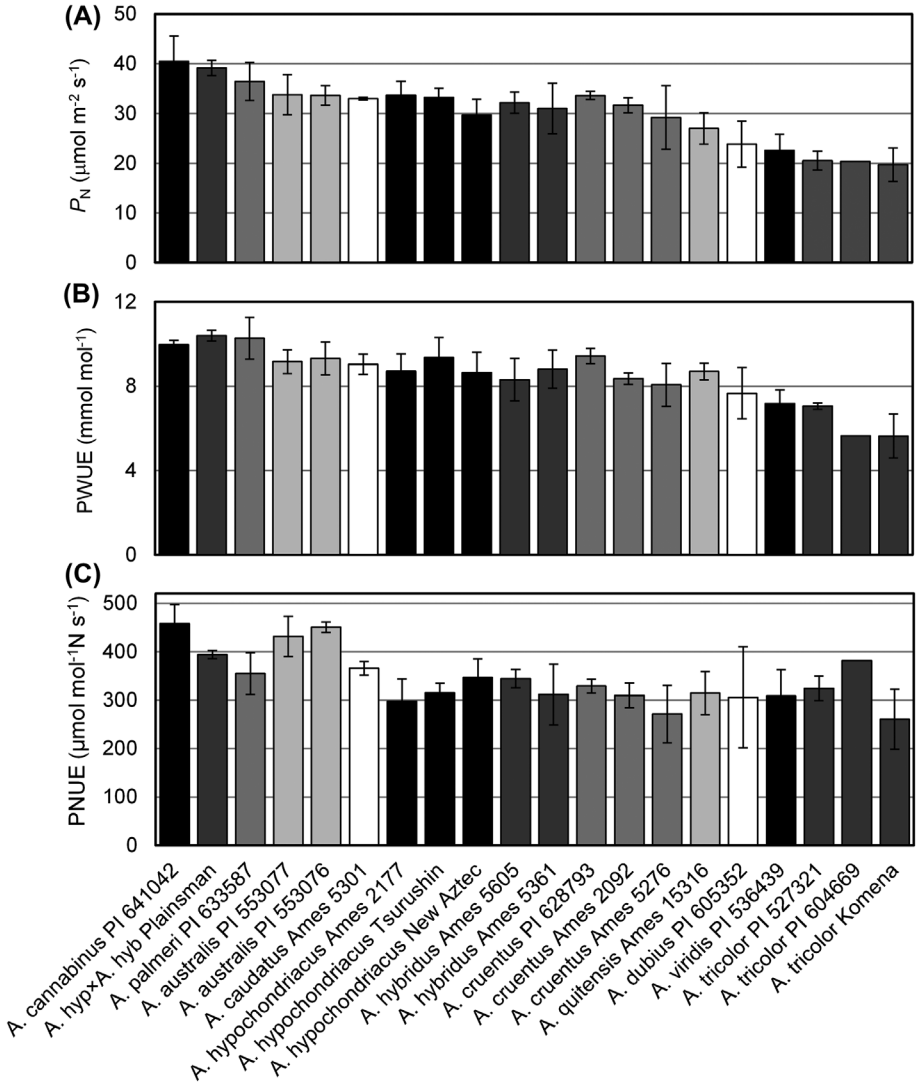

Figure 1. Variations in (A) net photosynthetic rate (P_N), (B) photosynthetic water use efficiency (PWUE), and (C) photosynthetic N use efficiency (PNUE) in *Amaranthus* species.

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.

calculated. This parameter represents the relative proportion of mesophyll and BS cells.

Statistical analyses

The data are presented as means ± SD (n = 3, except n = 2 for *Amaranthus tricolor*, PI 604669). They were statistically evaluated by analysis of variance (ANOVA). The species/strain differences in Pn, PNUE and PWUE were assessed with Tukey's test.

Results

Gas exchange and PWUE

A large variation in P_N was found among *Amaranthus* species (Figure 1(A)). P_N ranged from 19.7 μmol m^{-2} s^{-1} in *A. tricolor* (Komena) to 40.5 μmol m^{-2} s^{-1} in *A. cannabinus*; the latter 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* × *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) and T_r (Table 3). PWUE was significantly correlated with P_N and g_s, but not with T_r (Table 3).

SLW, N, and Chl contents and PNUE

SLW varied considerably among *Amaranthus* species, ranging from 20.0 to 34.2 g m^{-2} (Table 2). The N content of

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 (mmol 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	1.49^{NS}
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; ^{NS} – not significant.

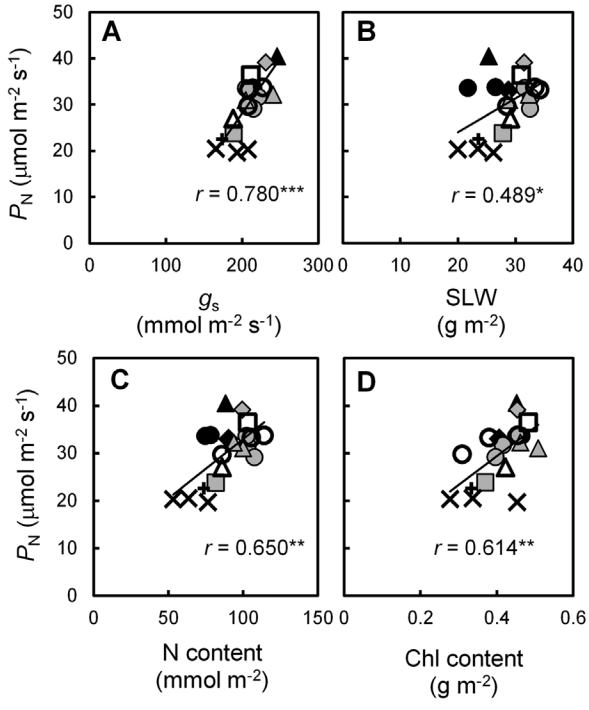

Figure 2. Relationships between P_N and (A) stomatal conductance (g_s), (B) specific leaf weight (SLW), (C) leaf N content, and (D) chlorophyll (Chl) content in *Amaranthus* species.
Notes: Mean ± SD (n = 3, except n = 2 for *A. tricolor* PI 604669). Significant at *P*: *<0.05; **<0.01; *** < 0.001.

leaves varied from 53.2 to 114.1 mmol m^{-2}, and Chl content varied from 0.28 to 0.51 g m^{-2} (Table 2). The PNUE varied from 260 μmol mol^{-1} N s^{-1} in *A. tricolor* (Komena) to 458 μmol mol^{-1} N s^{-1} in *A. cannabinus*, with the mean of 344 ± 56 μmol mol^{-1} N s^{-1} (Figure 1(C); Table 2; Suppl. data 1). SLW was weakly correlated with P_N (Figure 2(B)), but strongly correlated with leaf N content (Table 3). P_N was positively correlated with N and Chl contents (Figure 2(C), (D)). PNUE was significantly correlated with P_N and g_s 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). P_N 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). NAD-ME activity was positively correlated with SLW and N content; PEPC activity was positively correlated with Chl content (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

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*	0.417^{NS}	1.000							
SLW	0.489*	0.400^{NS}	0.330^{NS}	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**	0.395^{NS}	0.550*	1.000				
PNUE	0.535*	0.454*	0.418^{NS}	0.468*	-0.419^{NS}	-0.286^{NS}	0.148^{NS}	1.000			
PEPC activity	0.305^{NS}	0.097^{NS}	0.049^{NS}	0.349^{NS}	-0.089^{NS}	0.101^{NS}	0.484*	0.285^{NS}	1.000		
NAD-ME activity	0.240^{NS}	0.138^{NS}	0.240^{NS}	0.279^{NS}	0.577**	0.498*	0.143^{NS}	-0.249^{NS}	0.097^{NS}	1.000	
Rubisco activity	0.746***	0.414^{NS}	0.312^{NS}	0.816***	0.640**	0.731***	0.561**	0.124^{NS}	0.372^{NS}	0.531*	1.000

Notes: Values represent *r* from linear regression. Significant at *P*: * < 0.05; ** < 0.01; *** < 0.001, ^{NS} – not significant.

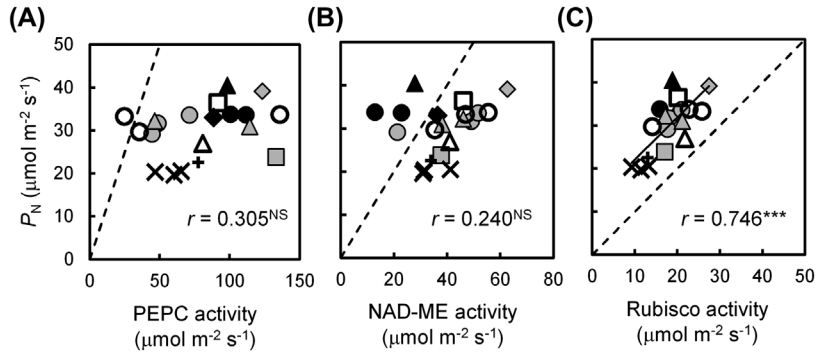

Figure 3. Relationships between P_N and (A) PEP carboxylase (PEPC) activity, (B) NAD-malic enzyme (NAD-ME) activity, and (C) Rubisco activity in leaves of *Amaranthus* species.
Notes: Mean ± SD (n = 3, except n = 2 for *A. tricolor* PI 604669). Significant at P: *** < 0.001; NS not significant. Symbols for *Amaranthus* species are as in Figure 2. Broken lines ($y = x$) show enzyme activities that would be required for equal P_N.

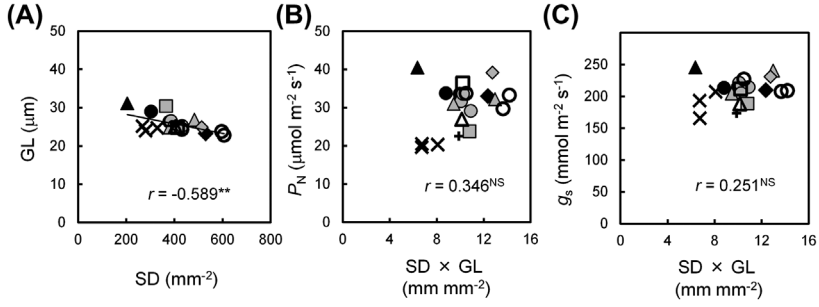

Figure 4. Relationships between (A) stomatal density (SD) and guard cell length (GL), (B) GL × SD and P_N, and (C) GL × SD and g_s in leaves of *Amaranthus* species.
Notes: Mean ± SD (n = 3, except n = 2 for *A. tricolor* PI 604669). Significant at P: ** < 0.01; NS not significant. Symbols for *Amaranthus* species are as in Figure 2.

indicating total stomatal pore length per unit leaf surface, ranged from 6.4 to 14.2 mm mm^{-2} (Table 2). There was a negative correlation between SD and GL (Figure 4(A)). P_N (Figure 4(B)), g_s (Figure 4(C)) and T_r were not significantly correlated with SD, GL, or SD × GL (Suppl. data 2). SLW was positively correlated with SD and SD × GL (Suppl. data 2). PWUE and PNUE were not correlated with any stomatal parameters (Suppl. data 2).

All *Amaranthus* species examined showed typical Kranz-type leaf anatomy (Figure 5). The BS cells contained many centripetally located chloroplasts. A layer of mesophyll cells surrounded the BS, and the mesophyll cells had a palisade-like structure at the adaxial side and a spongy one at the abaxial side, in agreement with our previous study (Ueno, 2001).

Leaf thickness ranged from 159 to 222 μm, whereas chlorenchyma thickness ranged from 130 to 190 μm (Table 2). The IVD also showed a large variation from 113 to 156 μm. The smallest and largest values of IVD were found in two accessions of *A. tricolor*. These structural parameters of leaves showed no significant correlation with the gas exchange parameters such as P_N, g_s, and T_r (Figure 6(A), (B); Suppl. data 2). Leaf and chlorenchyma thicknesses were positively correlated with GL (Table 4) and IVD (Figure 6(C); 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).

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 C_4 dicot crop. The mean value of P_N was 30.2 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 11 species of *Amaranthus* (Tsutsumi et al., 2011), a similar mean

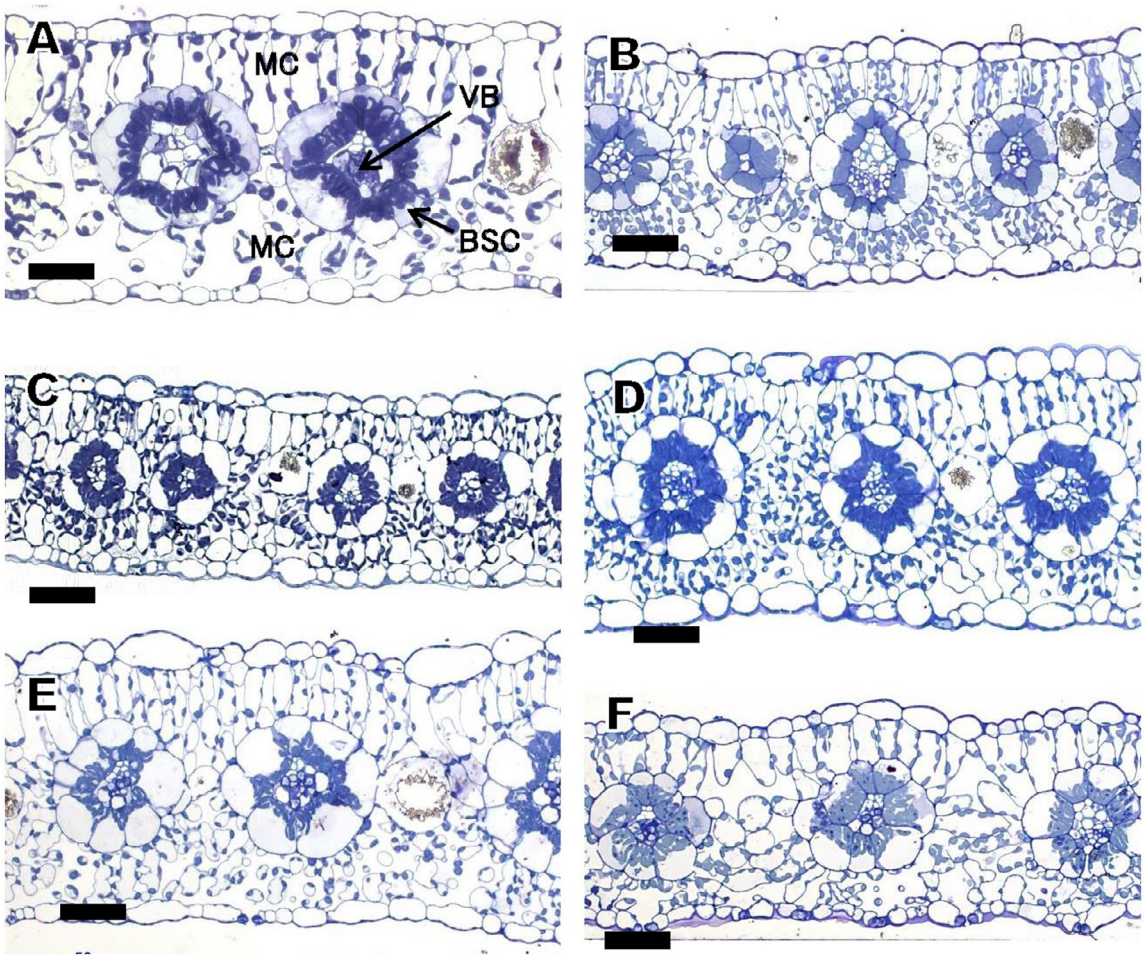

Figure 5. Transverse sections of leaves of representative species of *Amaranthus*. (A) *A. cannabinus* (PI 641042; P_N = 41 μmol m^{-2} s^{-1}), (B) *A. hyp* × *A. hyb* (Plainsman; 39 μmol m^{-2} s^{-1}), (C) *A. caudatus* (Ames 5301; 33 μmol m^{-2} s^{-1}), (D) *A. cruentus* (Ames 5276; 29 μmol m^{-2} 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.

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

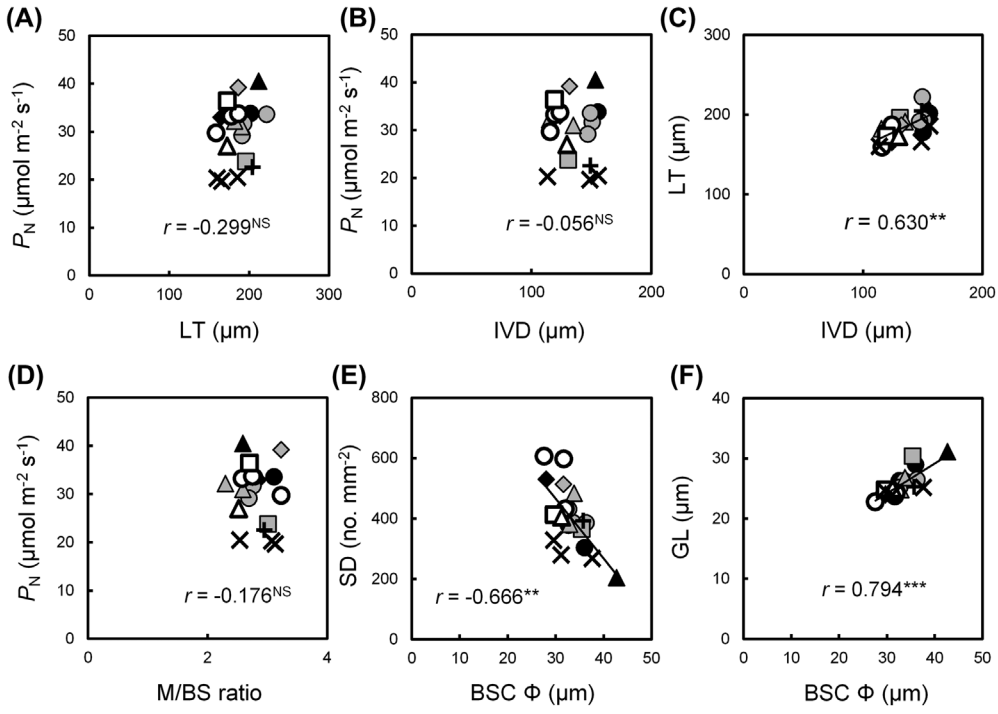

Figure 6. Relationships between (A) leaf thickness (LT) and P_N, (B) interveinal distance (IVD) and P_N, (C) IVD and LT, (D) M/BS ratio and P_N, (E) diameter of BS cells (BSC Φ) and SD, and (F) BSC Φ and GL in leaves of *Amaranthus* species.
Notes: Mean ± SD (n = 3, except n = 2 for *A. tricolor* PI 604669). Significant at P: ** < 0.1; ***< 0.01; NS not significant. Symbols for *Amaranthus* species are as in Figure 2.

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^{NS}	0.665**	–0.241^{NS}
Chlorencyma thickness	–0.439^{NS}	0.688***	–0.294^{NS}
IVD	–0.659**	0.436^{NS}	–0.618**
MC length	–0.610**	0.773***	–0.507*
BSC diameter	–0.666**	0.794***	–0.530*
M/BS ratio	0.114^{NS}	–0.195^{NS}	0.046^{NS}

Notes: Values represent r from linear regression. Significant at P: * < 0.05; ** < 0.01; *** < 0.001; NS – not significant.

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 (*in vivo* activation state) and total Rubisco activity (fully activated state) in C_4 grasses, although total activity was higher than initial activity in wheat (C_3). 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 Mg^{2+} and CO_2 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 *Amarantus* 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

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

Trait	Leaf thickness	Chlorencyma thickness	IVD	MC length	BSC diameter	M/BS ratio
Leaf thickness	1.000					
Chlorenchyma thickness	0.977***	1.000				
IVD	0.630**	0.635**	1.000			
MC length	0.606**	0.661**	0.585**	1.000		
BSC diameter	0.831***	0.873***	0.680***	0.788***	1.000	
M/BS ratio	–0.278^{NS}	–0.250^{NS}	–0.014^{NS}	–0.051^{NS}	–0.377^{NS}	1.000

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

(Table 3). 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_r) 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_3 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_3 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 photosynthate 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 leakiness 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 C_3 species. Although PNUE of C_4 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 C_4 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 C_4 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 C_4 subtypes of monocot and dicot plants. Greater turnover rate of Rubisco in NADP-ME than in NAD-ME-type C_4 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 C_3 and C_4 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 C_3 species. It is thought that higher PWUE in C_4 species is due to their higher P_N, which is achieved by a CO_2-concentrating mechanism (Ghannoum et al., 2011). The P_N/g_s ratio (often called A/g_s) 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 C_4 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 C_4 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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Disclosure statement

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