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Effects of growth light and nitrogen nutrition on the organization of the photosynthetic apparatus in leaves of a C_4 plant, *Amaranthus cruentus*

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ABSTRACT

Properties of C_4 photosynthesis were examined in *Amaranthus cruentus* L. (NAD-malic enzyme (ME) subtype, dicot) grown under different light and nitrogen (N) conditions, from the viewpoint of N investment into their photosynthetic components. In low-light (LL) leaves, chlorophyll content per leaf area was greater and chlorophyll *a*/*b* ratio was lower than in high-light (HL) leaves. These indicate that LL leaves invest more N into their light-harvesting systems. However, this N investment did not contribute to the increase in the quantum yield of photosynthesis on the incident photon flux density (PFD) basis (Q_i) in LL leaves. N allocation to ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) was significantly higher in HL-high N (HN) leaves than in other leaves. On the other hand, N allocation to C_4 enzymes [phospho*enol*pyruvate carboxylase (PEPC) and pyruvate P_i dikinase (PPDK)] was unaffected by the growth conditions. Maximum photosynthetic rates (P_{max}) per Rubisco content were similar irrespective of the growth light treatments. Carbon isotope ratios ($\delta^{13}C$) in the leaf dry matter were more negative in LL leaves than in HL leaves (LL = –19.3‰, HL = –16.0‰) and independent of leaf N. Vein density was highest in HL-HN leaves, and leaf thickness was unaffected by the growth light treatments. From these results, we conclude that *A. cruentus* leaves would not acclimate efficiently to low growth light.

Key-words: carbon isotope ratio; phospho*enol*pyruvate carboxylase (PEPC); quantum yield; ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco).

INTRODUCTION

C_4 leaves change their photosynthetic characteristics in response to growth light conditions. *Zea mays* plants (NADP-malic enzyme (ME) subtype, monocot) grown in high light developed thicker leaves with higher maximum net photosynthetic rates (P_{max}) per unit leaf area than those grown in low light (Louwerse & Zweerde 1977). In *Paspalum conjugatum* (NADP-ME subtype, monocot), which naturally occurs in the understoreys of rain forests, chlorophyll content per unit leaf area was increased by shading (Ward & Woolhouse 1986). In *Amaranthus retroflexus* (NAD-ME subtype, dicot), grana in both mesophyll and bundle sheath (BS) chloroplasts in the leaves grown in low light were more stacked than those grown in high light (Fisher & Evert 1982). These responses are similar to those known for C_3 plants (Björkman 1981). However, the responses of the C_4 plants appear to differ depending on the decarboxylation subtype, whether the plants are monocots or dicots, or even on the species. For example, unlike *P. conjugatum*, *Z. mays* showed no increase in chlorophyll content in response to low growth light (Ward & Woolhouse 1986), although both are NADP-ME and belong to the Gramineae family.

For C_3 leaves, photosynthesis models with respect to nitrogen (N) use have been developed to explain acclimation mechanisms of photosynthesis in response to growth light (Evans & Seemann 1989; Hikosaka & Terashima 1995). These models are based on theoretical predictions of the optimum N investment into photosynthetic components under various growth light and N levels. The same approach can be applied to the C_4 plants. Actually, there are some studies that have examined the effects of growth light on N investment into the photosynthetic enzymes in C_4 leaves. Sugiyama, Mizuno & Hayashi (1984) showed that *Z. mays* leaves grown in high light showed greater N allocation to the C_4 enzymes [phospho*enol*pyruvate carboxylase (PEPC) and pyruvate P_i dikinase (PPDK)]. On the other hand, the proportion of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) to the soluble protein fraction did not respond to growth light – in contrast with the tendency known for C_3 plants (Hikosaka & Terashima 1995). Makino *et al.* (2003) compared the N allocation to photosynthetic components between *Z. mays* and a C_3 plant, rice (*Oryza sativa*). They found that *Z. mays* leaves allocated more N into the thylakoid components and showed greater photosynthetic rates per unit leaf N than *O. sativa*, even at the saturating CO_2 level. Their data clearly indicate that the *Z. mays* leaves realize their greater

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ribulose 1,5-bisphosphate (RuBP) regeneration capacity by saving the N investment into Rubisco. Very recently, Ghannoum *et al*. (2005) compared the N allocation to photosynthetic components between NAD-ME and NADP-ME grasses grown at various N nutrition levels. From these studies, however, it is still difficult to elucidate the trends in the changes in N allocation in response to growth light levels.

The CO_2 leak from BS cells is an essential factor in C_4 photosynthesis efficiency. Henderson, von Caemmerer & Farquhar (1992) found that in *Amaranthus edulis* and *Z. mays,* CO_2 leakiness, the ratio of the rate of CO_2 leakage from BS cells to the rate of CO_2 fixation by PEPC, increased with the decrease in irradiance. The increase in CO_2 leakiness in low light is considered to be due to the decrease in Rubisco activity relative to that of PEPC caused by the decrease in ATP and NADPH production (von Caemmerer & Furbank 1999). The Rubisco/PEPC activity balance may also be disturbed by N stress (Meinzer & Zhu 1998). Furthermore, anatomical characteristics, including interveinal distance (IVD) and conductance of BS cells, have been argued to be important determinants of C_4 photosynthetic efficiency (Brown & Byrd 1993; Hatch, Agostino & Jenkins 1995; Ogle 2003).

To comprehensively understand the responses of C_4 photosynthesis to growth conditions in terms of N use, we need more information about N investment into photosynthetic proteins, not only in *Z. mays* but also in various types of C_4 plants. Therefore, we used a dicot, *Amaranthus cruentus* L. (NAD-ME subtype), in this study. We grew the plants under different light and N conditions and measured the contents of various photosynthetic proteins. We then examined the relationships between the contents of these proteins and photosynthetic performance to understand to what extent the changes in N investment affect photosynthetic performance. We also examined the stable carbon isotope ratio ($\delta^{13}C$) and leaf anatomy to obtain insight into the CO_2 leak from BS cells.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *A. cruentus* L. were sown in vermiculite in pots (120 mm in diameter and 200 mm in height, one plant per pot). They were placed in a growth chamber (30 °C, 14 h day length, 360 μmol quanta m^{-2} s^{-1}). After germination, they were grown in the growth chamber for a week and given 100 mL of a Hoagland's solution containing 2 mM KNO_3, 2 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.665 mM NaH_2PO_4, 25 μM Fe-EDTA, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 25 μM H_3BO_4, 0.25 μM Na_2MoO_4, 50 μM NaCl and 0.1 μM $CoSO_4$ everyday. Then, they were transferred to a compartment (*c*. 40 m^3) in a natural-light greenhouse. All the pots were placed on a bench whose top was a coarse wire net 50 cm above the ground. Light level was either high or low. For the low light level, a frame box (about a cubic meter) whose top and three lateral sides (except the northern side) were covered with layers of black shade cloth, was placed on the bench. In this shade treatment, the midday photon flux density (PFD) on clear days was ≈ 200 μmol quanta m^{-2} s^{-1} just above the low-light (LL) plants; PFD for the high-light (HL) plants was 1100 μmol quanta m^{-2} s^{-1}. In this compartment of the greenhouse, a large electric fan was used for ventilation and air circulation and the windows were all open. The average day and night temperatures during plant growth in the greenhouse were 31 and 24 °C, respectively. Every other day, the plants were given 100 mL of a double-strength Hoagland's solution that contained either 12, 3 or 0.6 mM of N. In the N-deficient solutions, NO_3^- was replaced by Cl^- (Hikosaka 1996). The plants grown at 12, 3 and 0.6 mM N are called high-N (HN), medium-N (MN) and low-N (LN) plants, respectively.

Gas exchange measurements

Gas exchange measurements were conducted on fully expanded leaves of 2- to 3-month-old plants. The gas exchange rate was measured with a portable gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). Before the measurements, the plants were kept at 34 °C in a darkened growth chamber. After 18 h of the dark pre-treatment, the photosynthesis light-response curve was obtained, with a stepwise increase in PFD from 0 to 1500 μmol quanta m^{-2} s^{-1}. Light was provided by a high-power metal halide lamp (PCS-UMX250, NPI, Tokyo, Japan). The CO_2 concentration within the cuvette was maintained at 360 μL L^{-1}, and leaf temperature was kept at 34 °C. P_{max} was measured at 1500 μmol quanta m^{-2} s^{-1}. After the gas exchange measurements, leaf discs measuring 1 cm in diameter were obtained and frozen in a –80 °C freezer.

The quantum yield of CO_2 uptake on an incident PFD basis (Q_i) was estimated as the slope of the photosynthetic rate versus PFD obtained by the least squares method (within the PFD range from 0 to 90 μmol quanta m^{-2} s^{-1}). The quantum yield on an absorbed PFD basis (Q_a) was calculated from Q_i and leaf absorptance (Abs):

$$Q_a = Q_i/Abs. \quad (1)$$

The leaf absorptance was measured with an integrating sphere and a quantum sensor (LI-190, Li-Cor) using the high-power metal halide lamp that was used for the gas exchange measurement. Measurements of leaf absorptance were made using HL ($n = 20$) and LL leaves ($n = 21$) that were different from those used for the Q_i measurement. After the leaf absorptance measurement, leaf discs measuring 1 cm in diameter were obtained for chlorophyll determination (see below). The leaf absorptance depended on chlorophyll content and this dependence was expressed by a rectangular hyperbola (Evans 1996):

$$Abs = 0.94\ Chl/(0.065 + Chl), \quad (2)$$

where Chl is the chlorophyll content per unit leaf area (mmol m^{-2}; $r^2 = 0.77$). The data for HL and LL leaves were pooled. Using Eqn 2, leaf absorptance was estimated from

the chlorophyll content for each of the leaves used for the Q_i measurement.

Measurements of chlorophyll and protein contents

Chlorophyll was extracted from the leaf discs with *N*, *N*-dimethylformamide, and chlorophyll contents and chlorophyll *a*/*b* ratios were determined according to Porra, Thompson & Kriedemann (1989).

Rubisco, PEPC and PPDK contents were determined using the method of Makino *et al*. (2003) with minor modifications. The frozen leaf discs were homogenized in an extraction buffer containing 0.2 mM EDTA-2Na, 10 mM dithiothreitol, 2 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, the Complete Protease Inhibitor (Roche Applied Science, Mannheim, Germany), 0.1% Triton X-100 and 100 mM Tris-HCl at pH 6.8. The homogenate was centrifuged at 12 000 *g* for 5 min at 4 °C. A part of the supernatant was used for the measurement of the soluble protein content using a protein assay kit based on the Bradford method (Bio-Rad Protein Assay, Bio-Rad, CA, USA). The supernatant was mixed with an equal amount of a double-strength loading buffer (for ingredients, see below), treated at 100 °C for 5 min and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Because Rubisco was bound to thylakoid membranes as shown by Makino & Osmond (1991), the membrane-bound Rubisco was extracted from the pellet. The pellet was solubilized with the loading buffer [5% (w/v) lithium dodecylsulfate, 5% β-mercaptoethanol, 0.1% bromophenol blue, 5% glycerol and 25 mM Tris-HCl at pH 6.8], heated at 100 °C for 5 min and centrifuged at 12 000 *g*. The supernatant obtained was analysed by SDS-PAGE. SDS-PAGE was performed with a 12.5% polyacrylamide gel according to Laemlii (1970). The gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 and then de-stained. The bands of Rubisco large subunit (LS), small subunit (SS), PEPC and PPDK in *A. cruentus* leaves were identified by their abundance, taking account of the molecular masses of maize proteins at 52, 15, 99 (Uedan & Sugiyama 1976) and 94 kDa (Sugiyama 1973), respectively. Small pieces of the gel containing these bands were excised with a razor blade, and the stained proteins were eluted in formamide at 50 °C for 4 h. The absorbance at 595 nm of the formamide extraction was measured with a spectrophotometer (U-3310, Hitachi, Tokyo, Japan). The protein concentration was calculated using bovine serum albumin as the standard (Makino, Mae & Ohira 1986; Fukayama *et al*. 2001).

N contents and $\delta^{13}C$

The leaf sample was dried in an oven at 80 °C for about 48 h. The dried leaf was well ground, and the total leaf N was analysed with an NC analyser (Vario EL III, Elementar, Hanau, Germany). $\delta^{13}C$ of the leaf dry matter was measured with a stable isotope ratio mass spectrometer (Delta-S, Finnigan MAT, Bremen, Germany). $\delta^{13}C$ was expressed as permil (‰) deviation from the PeeDee belemnite (PDB) standard.

Leaf structure

Leaf segments (1 × 2 mm) were taken from the basal parts of the fully expanded leaves with a razor blade, avoiding major veins. The segments were vacuum-infiltrated in 2.5% glutaraldehyde in a 25 mM cacodylate buffer (pH 7.2) and fixed overnight at 4 °C. They were then postfixed by 2% osmium tetroxide for 3 h and dehydrated in the graded acetone series and the propylene oxide series. The dehydrated segments were embedded in Spurr's resin (Spurr 1969).

Leaf cross sections 1 μm thick were obtained with glass knives on an ultramicrotome (Reichert Ultracut S, Leica, Vienna, Austria). Sections were stained with 0.5% toluidine blue and observed under a light microscope (BX-50, Olympus, Tokyo, Japan). Light micrographs were taken with a digital camera (C-3030 Zoom, Olympus) as images of 2048 × 1536 pixels.

The venation pattern was analysed using the leaf segments embedded in Spurr's resin; the leaf segments were not sectioned, as the samples were transparent. The veins were traced using the micrographs of the leaf segments embedded in Spurr's resin. The total vein length (L_v) and leaf area (A_{leaf}) in the leaf segment were measured with image analysis software (NIH Image, public domain software, developed at US National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/). Leaf vein density per unit leaf area (D_v) was calculated as follows:

$$D_v \text{ (mm mm}^{-2}) = L_v \text{ (mm)}/A_{leaf} \text{ (mm}^2). \quad (3)$$

Statistical analyses

Relationships between various photosynthetic characteristics and leaf N content in HL and LL leaves were expressed as linear regression lines. When both of the slopes of the regression lines for HL and LL leaves were significantly different from zero ($P < 0.05$), analysis of covariance (ANCOVA) was used to analyse differences in the slope and intercept of these regression lines. When either of the slopes was not statistically different from zero, Student's *t*-test was used. For comparison of mean values of leaf vein density and those of Rubisco N among the growth conditions, the Tukey–Kramer's multiple comparison test was used.

RESULTS

Chlorophyll and quantum yield

Chlorophyll contents per leaf area are plotted against total leaf N (Fig. 1a). In both HL and LL leaves, the chlorophyll content increased with the increase in leaf N. Although slopes of the regression lines were not different, the intercepts were significantly different ($P < 0.001$). At any leaf N,

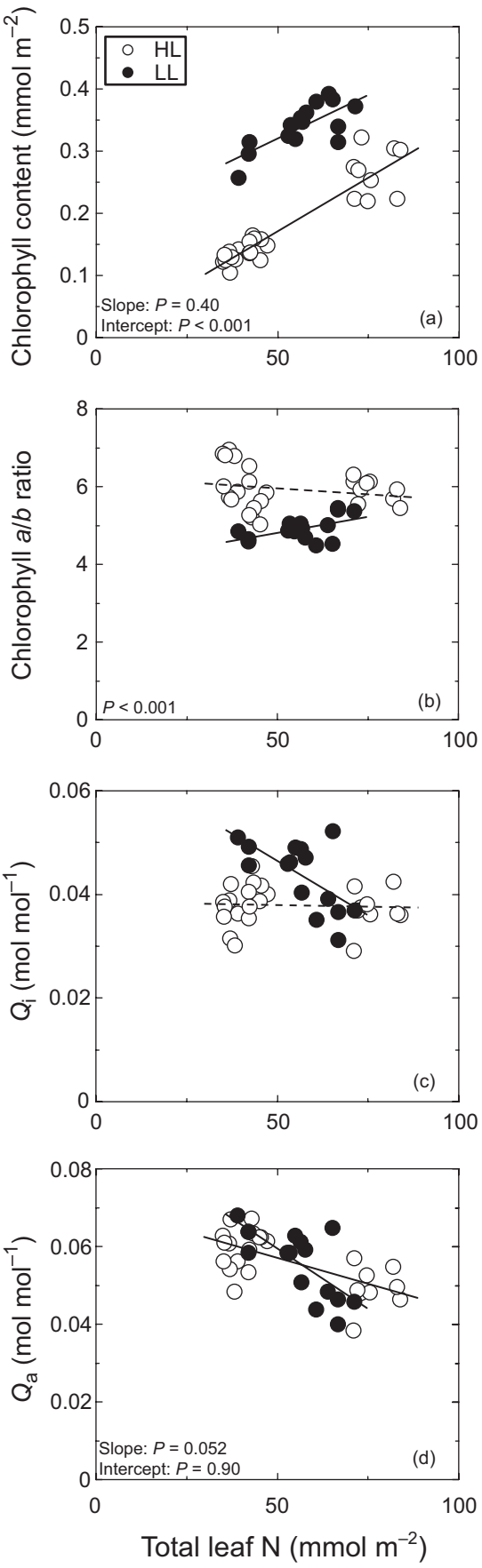


Figure 1. Relationship between total leaf N and (a) chlorophyll contents on a leaf area basis, (b) chlorophyll *a*/*b* ratio, (c) Q_i and (d) Q_a, respectively. Open and closed circles denote *Amaranthus cruentus* leaves grown under high-light (HL) and low-light (LL) conditions, respectively. Solid and dashed lines represent the regression lines, whose slopes were either significantly ($P < 0.05$) or not significantly different from zero, respectively. To analyse the differences between HL and LL leaves, analysis of covariance (ANCOVA) (a, d) and Student's *t*-test (b) were use. Results of the statistical analyses are shown as *P*-values. N, nitrogen; PFD, photon flux density; Q_i, quantum yield of photosynthesis on the incident PFD basis; Q_a, quantum yield of photosynthesis on the absorbed PFD basis.

the chlorophyll content was greater by 1.5–2 times in LL leaves than in HL leaves.

The chlorophyll *a*/*b* ratio was significantly lower in LL leaves than in HL leaves (Student's *t*-test, $P < 0.001$; Fig. 1b). In LL leaves, there was a positive relationship between chlorophyll *a*/*b* ratio and leaf N, while in HL leaves, chlorophyll *a*/*b* ratio was independent of leaf N.

The Q_i decreased with the increase in leaf N in LL leaves, while in HL leaves, they were not dependent on leaf N (Fig. 1c). The Q_a decreased with the increase in leaf N in both HL and LL leaves, and the dependencies were not statistically different (Fig. 1d). The mean Q_a were around 0.056 mol mol^{-1}, which corroborates the previous observations with the *Amaranthus* species (0.053 mol mol^{-1}: Ehleringer & Pearcy 1983; Hunt, Weber & Gates 1985a).

Rubisco, C_4 enzymes and P_{max}

The contents of soluble protein, Rubisco, PEPC and PPDK are plotted against leaf N (Fig. 2). Here, the Rubisco content denotes the sum of the Rubisco LS and SS contents in the soluble and membrane-bound fractions. The proportion of membrane-bound Rubisco varied with growth conditions. In HL-HN leaves, it attained as much as 83% of the total Rubisco, whereas, it was about 14% in LL-HN leaves (data not shown). The soluble protein content denotes the amount of soluble protein assayed by the Bradford method plus the membrane-bound Rubisco. The soluble protein content increased with the increase in leaf N in both HL and LL leaves, although the relationship between the soluble protein and leaf N was weak in LL leaves ($P = 0.12$). For the same leaf N, the contents of soluble protein and C_4 enzymes did not differ between HL and LL leaves (Fig. 2a, c & d). On the other hand, the Rubisco content was significantly lower in LL leaves than in HL leaves (the difference in the intercept, $P < 0.001$; Fig. 2b).

The allocation of leaf N to Rubisco in HL-HN leaves was significantly greater than that in other leaves ($P < 0.05$; Table 1). However, the N allocation to PEPC and PPDK were not altered with growth conditions. The observed N allocation to Rubisco, PEPC and PPDK were similar to those in *Z. mays* (8.5, 2.8 and 1.5%: Makino *et al*. 2003).

The P_{max} linearly increased with leaf N in both HL and LL leaves (Fig. 3a), similar to previous observations on

Table 1. Nitrogen (N) allocation to photosynthetic enzymes

Growth conditions	Leaf N allocation (%)		
	Rubisco	PEPC	PPDK
High light			
High N (12 mM N)	12.5 ± 1.5[a]	2.7 ± 0.5	1.8 ± 0.4
Medium N (3 mM N)	9.1 ± 2.0[b]	2.6 ± 0.7	1.7 ± 0.3
Low N (0.6 mM N)	9.5 ± 1.0[b]	2.2 ± 0.4	1.4 ± 0.4
Low light			
High N	9.2 ± 2.0[b]	2.5 ± 0.9	1.7 ± 0.3
Medium N	8.9 ± 1.5[b]	2.4 ± 0.8	1.8 ± 0.3
Low N	8.0 ± 0.8[b]	2.2 ± 0.4	1.8 ± 0.3

Proportions of leaf N in Rubisco, PEPC and PPDK were calculated by assuming that the N content of proteins is 16% of the mass of proteins (Makino *et al*. 2003). Data are presented as means ± SD ($n = 3$–8). Different letters denote the significant difference between the treatments by Tukey–Kramer's multiple comparison test ($P < 0.05$).
Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PEPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate P_i dikinase.

Amaranthus powelli (Hunt, Weber & Gates 1985b) and *A. retroflexus* leaves (Sage & Pearcy 1987). When compared at the same leaf N, P_{max} were slightly – but significantly – greater in HL leaves than in LL leaves (the difference in the intercept; $P < 0.005$). However, when plotted against Rubisco contents, the regression lines were almost identical (Fig. 3b).

$\delta^{13}C$ and leaf anatomy

The difference in $\delta^{13}C$ between HL and LL leaves was significant (Student's *t*-test, $P < 0.001$), and $\delta^{13}C$ values were independent of leaf N in both HL and LL leaves (Fig. 4). The average $\delta^{13}C$ value in HL leaves was –16.0‰, while that in LL leaves was –19.3‰.

A. cruentus leaves showed a typical Kranz anatomy of the NAD-ME subtype. However, anatomical characteristics were different depending on the growth conditions (Fig. 5). HL-HN leaves appeared to have more veins per unit width of the cross section, thus the shorter IVD. The trend was quantitatively confirmed (Table 2): HL-HN leaves showed the highest vein densities among the other leaves. The cell size appeared to be smallest in HL-HN leaves. Leaf thickness was not affected by the growth light but rather by the leaf N. When HL-LN and LL-HN leaves having similar leaf N were compared, anatomical characteristics were very similar (cf. Fig. 5b & c).

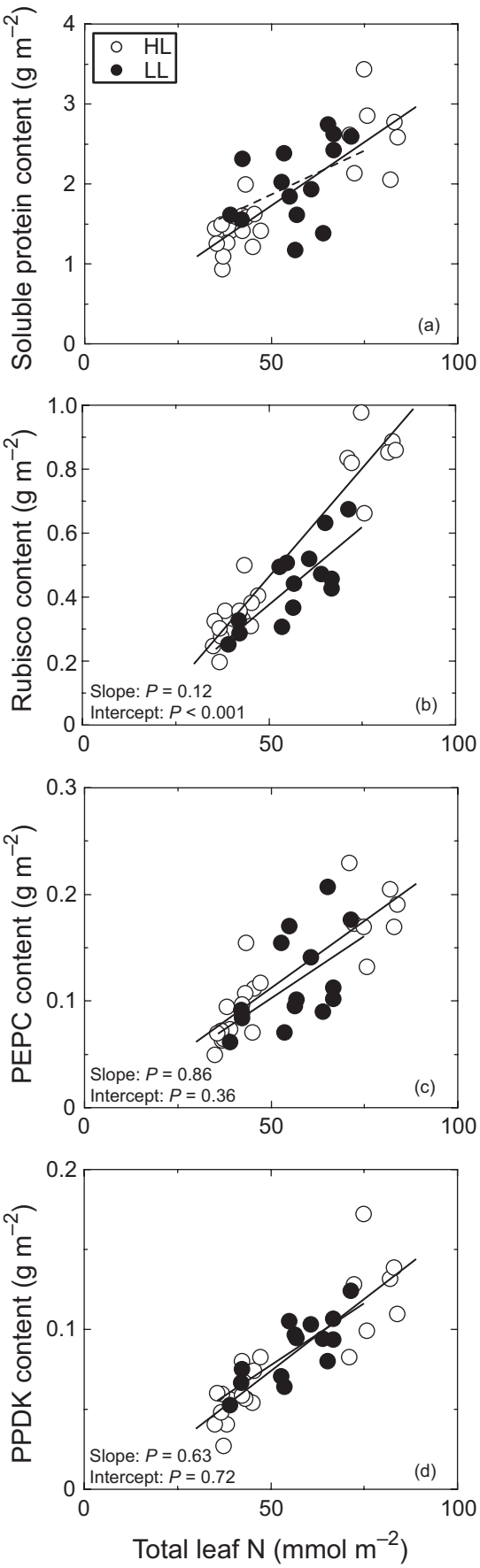


Figure 2. Relationship between total leaf N and the amounts of (a) soluble protein, (b) Rubisco, (c) PEPC and (d) PPDK, respectively. Symbols and other details are similar to Fig. 1. Results of an analysis of covariance (ANCOVA) (b–d) are shown as *P*-values. N, nitrogen; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PEPC, phospho*enol*pyruvate carboxylase; PPDK, pyruvate P_i dikinase.

Table 2. Effects of growth conditions on vein density per unit leaf area (D_v) in leaves of *Amaranthus cruentus*

Growth conditions	D_v (mm mm^{-2})
High light	
High N (12 mM N)	12.1 ± 1.0[a]
Low N (0.6 mM N)	9.8 ± 1.3[b]
Low light	
High N	9.6 ± 0.5[b]
Low N	9.2 ± 0.4[b]

Data are presented as means ± SD ($n = 6$ or 7). Different letters denote the significant difference between the treatments by Tukey–Kramer's multiple comparison test ($P < 0.01$).

DISCUSSION

In this study, we compared photosynthetic properties, in particular, the N investment into the photosynthetic components among the leaves of *A. cruentus* plants grown at two light and three N nutrition levels. We repeated the same experiments in 2002 and 2003, and very similar results were obtained. In this paper, we present only the data obtained in 2003 unless otherwise stated.

Chlorophyll and quantum yield

In healthy C_3 plants, the Q_a is almost constant irrespective of the chlorophyll content, and the difference in the Q_i between sun and shade leaves is largely attributed to the difference in leaf absorptance (Björkman & Demmig 1987). C_3 shade leaves preferentially invest their N into the light-harvesting complexes; thus, shade leaves show greater leaf absorptance and Q_i than sun leaves having the same leaf N (Evans & Seemann 1989; Hikosaka & Terashima 1995). Such responses were also reported for a shade-adapted C_4 grass, *P. conjugatum* (Ward & Woolhouse 1986). In *A. cruentus* plants, the chlorophyll content per leaf area was greater, and chlorophyll *a*/*b* ratio was lower in LL leaves than in HL leaves having the same leaf N (Fig. 1a & b). These indicate that LL leaves invest more N into chlorophyll-protein complexes, the light-harvesting ones in particular. However, in *A. cruentus*, Q_a was not constant; it decreased with the increase in leaf N in both HL and LL leaves (Fig. 1d). Thus, the difference in Q_i in *A. cruentus* leaves cannot be explained by the leaf absorptance. Probably, other factors such as distribution of photosystem I and II in mesophyll and BS chloroplasts (Pfündel, Nagel & Meister 1996), CO_2 leak from BS cells (Meinzer & Zhu 1998) and leaf anatomy (Ogle 2003) are involved.

N investment into photosynthetic enzymes

C_3 sun leaves preferentially invest their N into Calvin cycle enzymes, electron carriers and ATPase; such N investment contributes to the increase in P_{max} (Terashima & Evans 1988; Evans & Seemann 1989; Hikosaka & Terashima 1995). To achieve a given P_{max}, C_4 leaves need much less Rubisco than C_3 leaves; however, they should invest their N into the C_4 enzymes that are involved in the CO_2 concentrating cycle (Schmitt & Edwards 1981; Makino *et al.* 2003). Given that the balance between C_3 and C_4 cycles is important in carrying out an efficient C_4 photosynthesis, the N investment into Rubisco and C_4 enzymes may be coordinately regulated. Contrary to this expectation, in *A. cruentus*, HL-HN leaves allocated more N to Rubisco than other leaves, but the allocation to PEPC and PPDK was not different from those in other leaves (Fig. 2b–d and Table 1). In other words, *A. cruentus* leaves would not markedly change N investment into the C_4 enzymes in response to growth conditions. These results differ from the observations in *Z. mays* plants that the N allocation to Rubisco was almost constant irrespective of the leaf N, whereas that to PEPC increased with the increase in leaf N (Sugiyama *et al.* 1984; Makino *et al.* 2003). The difference in the N allocation pattern between *A. cruentus* and

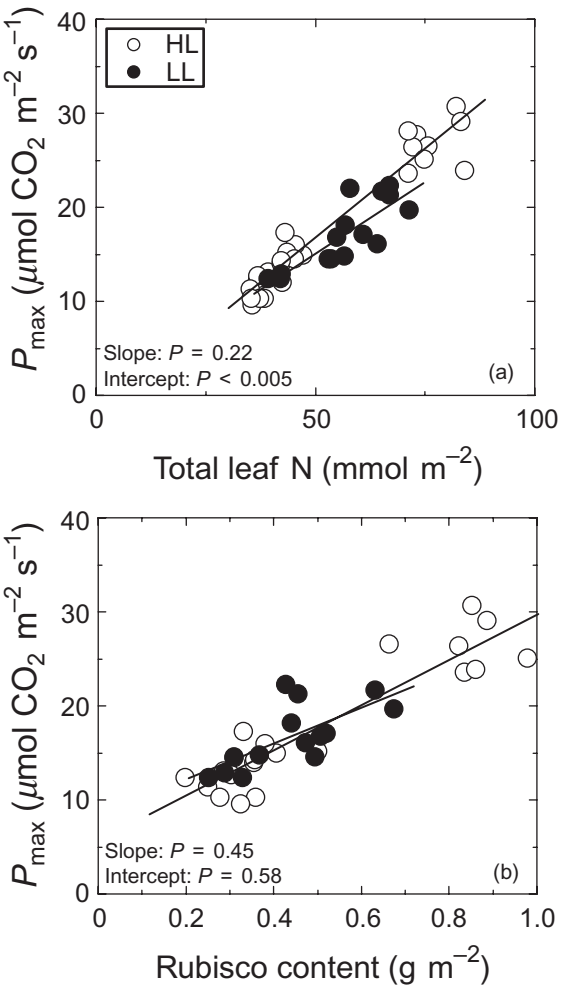


Figure 3. Relationship between P_{max} and (a) total leaf N and (b) Rubisco content, respectively. Symbols and other details are similar to Fig. 1. Results of an analysis of covariance (ANCOVA) are shown as *P*-values. P_{max}, maximum rate of net photosynthesis; N, nitrogen; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase.

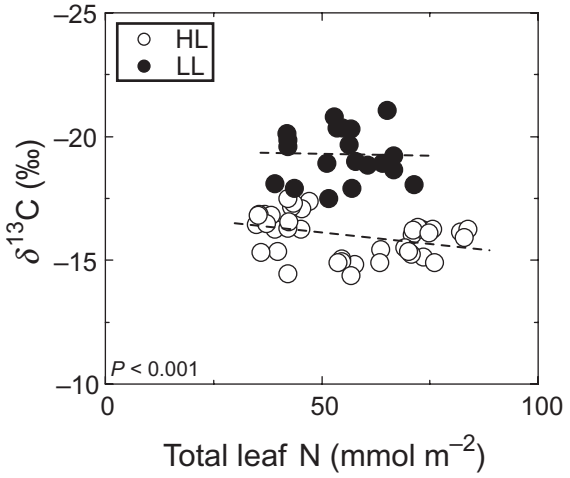


Figure 4. Relationship between carbon isotope ratio (δ^{13}C) and total leaf nitrogen (N). These data include the data obtained both in 2002 and 2003. Symbols are as shown in Fig. 1. The result of Student's *t*-test is shown as a *P*-value.

Z. mays could be due to the difference in the regulation of photosynthetic gene expression (Langdale & Nelson 1991; Wang, Klessig & Berry 1992; Sheen 1999).

P_{max} was somewhat greater in HL leaves than in LL leaves having the same leaf N (Fig. 3a). When P_{max} was plotted against the Rubisco content, the regression lines were almost identical between HL and LL leaves (Fig. 3b). This result indicates that P_{max} was dependent on the Rubisco content. It is noteworthy that the ratio of P_{max} to the Rubisco content decreased with the increase in Rubisco content. Possibly, this result indicates that the efficiency of Rubisco activity might be reduced in HN leaves. In LL leaves, the observed N investment into Rubisco appears to be wasteful because these leaves seldom receive high light. Alternatively, this trait may be regarded as an insurance in situations wherein there is a sudden improvement of the growth light environment.

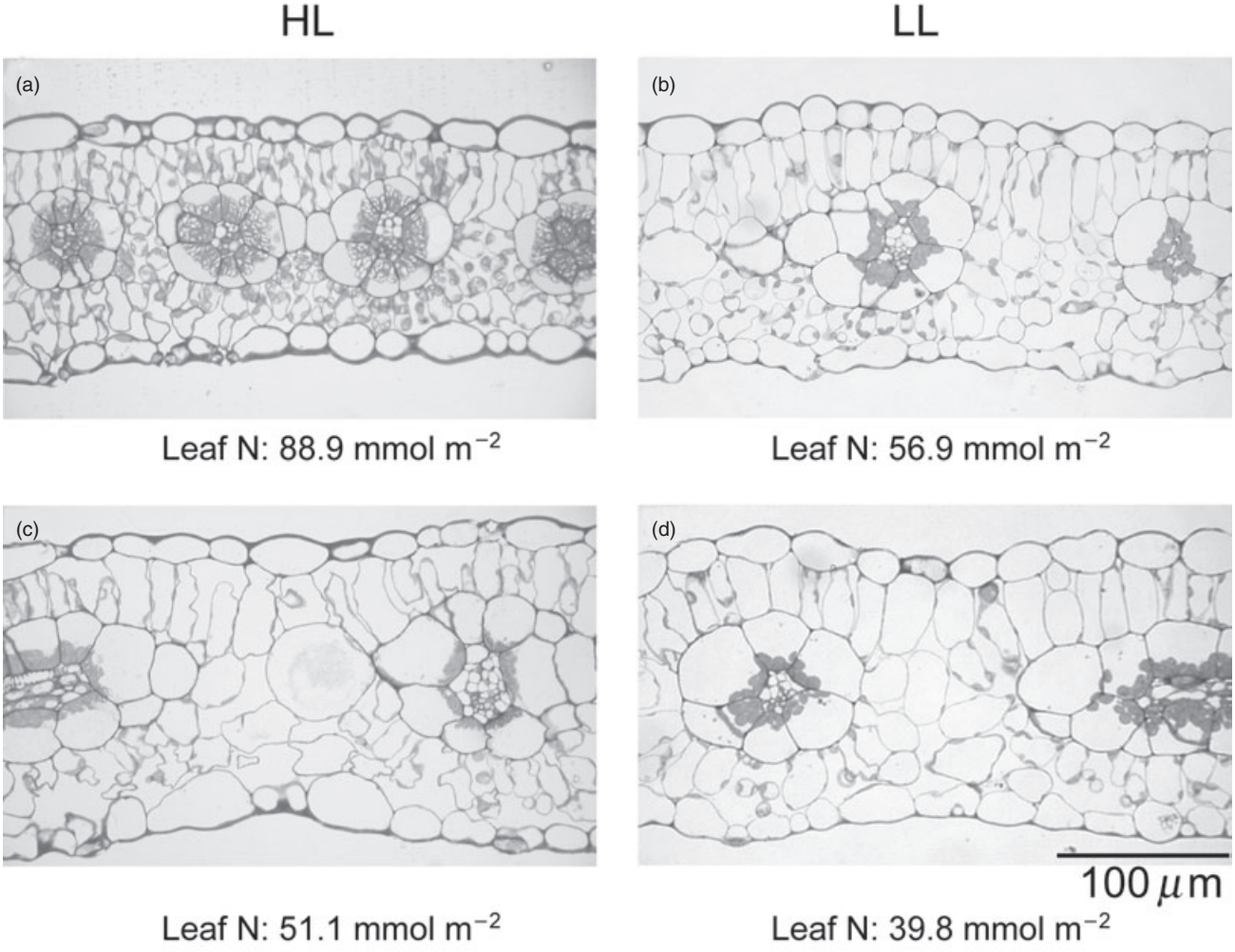


Figure 5. Cross sections of mature leaves of *Amaranthus cruentus* grown under (a) high light-high nitrogen (N) (HL-HN), (b) low light-high N (LL-HN) high light-low N (HL-LN), (c) and (d) low light-low N (LL-LN) conditions. A scale bar denotes 100 μm. Total leaf N are shown for each leaf.

$\delta^{13}C$ and CO_2 leak

In the present study, $\delta^{13}C$ values were more negative in LL leaves than in HL leaves and they were unaltered by leaf N (HL = –16.0‰; LL = –19.3‰; Fig. 4). The more negative $\delta^{13}C$ in shade leaves than in sun leaves was reported in Buchmann *et al*. (1996), and the absence of leaf N-dependence in $\delta^{13}C$ was reported in Henderson *et al*. (1994). However, it is new that the $\delta^{13}C$ values, particularly those of LL leaves, were more negative than the typical values for C_4 plants (–10 to –14‰; Smith & Epstein 1971). Perhaps, the more negative $\delta^{13}C$ values were attributable to the $\delta^{13}C$ value of the ambient air, which was somewhat more negative than –8‰ due to a well-known effect of fossil fuel combustion as our campus is located in the city (Widory & Javoy 2003). In addition to the effect of the ambient air, water stress could also lower the $\delta^{13}C$ values (Farquhar 1983; von Caemmerer & Furbank 2003). However, in NAD-ME species, the effect of water stress has been claimed to be much smaller than that of growth light (Buchmann *et al*. 1996). Therefore, the markedly more negative $\delta^{13}C$ values in the present LL leaves could be mostly attributed to the effect of low light, and it might be due to the increased CO_2 leakage under low light conditions as was observed (Henderson *et al*. 1992). Possibilities that the difference in $\delta^{13}C$ values between HL and LL leaves was partly affected by a difference in the composition of leaf materials and/or in the storage of end products could not be ruled out (O'Leary 1981; Henderson *et al*. 1994; von Caemmerer & Furbank 2003). More detailed measurements using online carbon isotope methods are necessary to quantify the degree of CO_2 leakage under low light.

It has been proposed that the relative amounts and activities of PEPC to Rubisco are important determinants of CO_2 leakiness (Ranjith *et al*. 1995; von Caemmerer *et al*. 1997; Meinzer & Zhu 1998). In the present study, the N allocation to Rubisco and those to the C_4 enzymes were almost constant, except for HL-HN leaves in which N allocation to Rubisco was larger. The relative amounts of these enzymes did not explain the trends in the changes in $\delta^{13}C$ in response to growth light. A detailed analysis focusing on the activity balance between C_3 and C_4 cycles is needed.

Relationship between leaf anatomy and photosynthesis

In the present study, leaf thickness was not affected by the growth light (Fig. 5), which is different from the observations with C_3 leaves (Björkman 1981). C_3 sun leaves are thicker and have more Rubisco per leaf area, thereby they achieve greater P_{max} than the shade leaves. On the other hand, C_4 leaves have a CO_2 concentrating mechanism and do not have much Rubisco; therefore, they would not need to have thick leaves, having greater mesophyll surface area. However, the increase in leaf thickness under high light was reported for C_4 monocots (Louwerse & Zweerde 1977; Ward & Woolhouse 1986). When *Z. mays* plants were grown in high light, leaf thickness, size of BS cells and P_{max} increased (Louwerse & Zweerde 1977). Thus, *Z. mays* and *A. cruentus* are different not only in their N allocation pattern to photosynthetic enzymes, but also in their anatomical characteristics.

The anatomical characteristics of C_4 leaves might affect Q_a (Ogle 2003). Based on a strong tendency that Q_a increases with the decrease in IVD in C_4 grasses, Ogle (2003) argued that photon capture increased with the increase in vein density. In the present study, HL-HN leaves showed the highest vein densities per leaf area (Table 2). However, Q_a in HL-HN leaves was lowest (Fig. 1d). The result is different from the above-mentioned tendency for C_4 grasses (Ogle 2003). Unlike in C_4 grasses, the effect of vein density on Q_a might not be large in *A. cruentus*.

CONCLUSIONS

Few studies have specifically examined the effects of growth light on N investment into photosynthetic enzymes in C_4 dicots leaves. In the present study, we have shown that *A. cruentus* leaves increased N allocation to chlorophyll-protein complexes when grown in low light. However, this did not lead to the increase in Q_i. The N allocation to Rubisco was greatest in HL-HN leaves, and this contributed to the increase in P_{max}. In contrast, the N allocation to the C_4 enzymes was unaffected by the growth conditions. $\delta^{13}C$ values of the leaf dry matter were not affected by leaf N, but rather by growth light, and the values were more negative in LL leaves, possibly indicating a substantial CO_2 leak from BS cells under the low growth light. $\delta^{13}C$ values did not reflect the balance of N investments into Rubisco and C_4 enzymes. Probably, the *in vivo* activity balance was different from the ratio of the amounts of these enzymes.

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