Faster Rubisco Is the Key to Superior Nitrogen-Use Efficiency in NADP-Malic Enzyme Relative to NAD-Malic Enzyme C_4 Grasses[1]

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In 27 C_4 grasses grown under adequate or deficient nitrogen (N) supplies, N-use efficiency at the photosynthetic (assimilation rate per unit leaf N) and whole-plant (dry mass per total leaf N) level was greater in NADP-malic enzyme (ME) than NAD-ME species. This was due to lower N content in NADP-ME than NAD-ME leaves because neither assimilation rates nor plant dry mass differed significantly between the two C_4 subtypes. Relative to NAD-ME, NADP-ME leaves had greater in vivo (assimilation rate per Rubisco catalytic sites) and in vitro Rubisco turnover rates (k_{cat}; 3.8 versus 5.7 s^{-1} at 25°C). The two parameters were linearly related. In 2 NAD-ME (*Panicum miliaceum* and *Panicum coloratum*) and 2 NADP-ME (*Sorghum bicolor* and *Cenchrus ciliaris*) grasses, 30% of leaf N was allocated to thylakoids and 5% to 9% to amino acids and nitrate. Soluble protein represented a smaller fraction of leaf N in NADP-ME (41%) than in NAD-ME (53%) leaves, of which Rubisco accounted for one-seventh. Soluble protein averaged 7 and 10 g (mmol chlorophyll)$^{-1}$ in NADP-ME and NAD-ME leaves, respectively. The majority (65%) of leaf N and chlorophyll was found in the mesophyll of NADP-ME and bundle sheath of NAD-ME leaves. The mesophyll-bundle sheath distribution of functional thylakoid complexes (photosystems I and II and cytochrome *f*) varied among species, with a tendency to be mostly located in the mesophyll. In conclusion, superior N-use efficiency of NADP-ME relative to NAD-ME grasses was achieved with less leaf N, soluble protein, and Rubisco having a faster k_{cat}.

C_4 photosynthesis involves the close collaboration of two photosynthetic cell types, the mesophyll (M) and bundle sheath (BS). A key characteristic of the C_4 syndrome is the operation of a CO_2 concentrating mechanism, which serves to raise the CO_2 concentration in the BS around Rubisco to levels high enough to suppress photorespiration and almost saturate photosynthesis in air (Hatch, 1987). This explains the commonly observed high photosynthetic rates of C_4 relative to C_3 leaves, when comparisons are made under high light and temperature. C_4 plants also have greater photosynthetic rates and accumulate more biomass than C_3 plants for less leaf nitrogen (N) and Rubisco (Bolton and Brown, 1978; Brown, 1978; Schmitt and Edwards, 1981; Ghannoum et al., 1997; Ghannoum and Conroy, 1998; Makino et al., 2003). The C_4 photosynthetic pathway is divided into three biochemical subtypes following the major C_4 acid decarboxylation enzyme (NAD-malic enzyme [ME], NADP-ME, and phospho*enol*pyruvate carboxykinase; Hatch, 1987). C_4 grasses with different biochemical subtypes have characteristic leaf anatomy (Hattersley, 1992) and different geographic distribution according to rainfall, such as seen in Australia (Hattersley, 1992) and South Africa (Ellis et al., 1980). With increasing rainfall, NADP-ME grasses increase in abundance, whereas NAD-ME grasses become less abundant. The aforementioned observations triggered our interest in the comparative physiology of the C_4 subtypes, especially because little is understood about the physiological significance of the biochemical diversification of C_4 photosynthesis. Consequently, we undertook a number of studies comparing NAD-ME and NADP-ME grasses because they represent the two most contrasting and floristically abundant subtypes (Hattersley, 1992). Restricting our study to two subtypes also allowed us to include more species in our comparisons. Previously, we showed that water stress enhances water use efficiency to a greater extent in NAD-ME than NADP-ME grasses (Ghannoum et al., 2002). While carrying out these experiments, we observed that NADP-ME grasses tended to have a lower

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leaf N content and greater whole-plant (NUE) and photosynthetic (PNUE) N-use efficiencies than their NAD-ME counterparts.

Differences in PNUE are mainly brought about by differences in photosynthetic capacity or foliar N allocation either within the photosynthetic apparatus or to nonphotosynthetic pools (e.g. cell walls, nitrate; Field and Mooney, 1986; Poorter and Evans, 1998). In C_3 plants, photosynthetic N allocation is well established (Evans and Seemann, 1989). Recently, Makino et al. (2003) constructed a leaf N budget for maize (*Zea mays*), a NADP-ME monocot. However, foliar N allocation in C_4 leaves is complicated by the presence of three biochemical subtypes and two photosynthetic cell types. There is indirect evidence that the C_4 cycle has a greater N requirement in the NAD-ME relative to the NADP-ME subtype. For example, the C_4 cycle of the NAD-ME relative to the NADP-ME pathway involves more enzymatic steps and amino acids (Kanai and Edwards, 1999). Also, it is well known that the BS chloroplasts of NADP-ME grasses lack PSII activity (Hatch, 1987), which may translate into reduced thylakoid N cost of NADP-ME leaves. In addition, differences in PNUE may be due to variations in the efficiency of the photosynthetic apparatus, such as enzyme kinetics. The superior PNUE of C_4 relative to C_3 leaves is not only due to higher photosynthetic rates per leaf N and Rubisco content but also to a Rubisco with faster turnover rate (k_{cat}; von Caemmerer and Quick, 2000; Sage, 2002). Which of these factors best explain our observed differences in leaf N and PNUE between NAD-ME and NADP-ME grasses? To answer this question, we carried out a series of experiments addressing three specific questions. (1) How widespread are the differences in leaf N, PNUE, and NUE between NAD-ME and NADP-ME grasses? Do they persist under low N supply? (See experiments 1–3.) (2) Are these differences due to Rubisco amount and/or specific activity? (See experiment 3.) (3) Are these differences related to N partitioning in the M and BS tissues? (See experiment 4.)

Table I. *List of 13 NAD-ME and 15 NADP-ME C_4 grass species used in 4 experiments*

Experiments 1 to 3 were used for growth, gas exchange, and biochemical analysis. Experiment 4 was used for BS and M N budget analysis.

Species	Experiment			
	1	2	3	4
NAD-ME	*n = 10*	*n = 9*	*n = 7*	*n = 2*
Astrebla lappacea (Lindl.) Domin	*	*	*	
Astrebla pectinata (Lindl.) F. Muell. ex Benth.	*	*	*	
Astrebla squarrosa C.E. Hubbard	*	*		
Cynodon dactylon	*	*		
Enteropogon acicularis Nees	*			
Eleusine coracana Gaertn		*		
Eragrostis setifolia N.M. Wolf	*			
Eragrostis superba N.M. Wolf	*	*	*	
Leptochloa dubia P. Beauv.	*	*		
Panicum coloratum	*	*	*	*
Panicum decompositum R.Br.	*	*	*	
Panicum miliaceum			*	*
Panicum virgatum			*	
NADP-ME	*n = 14*	*n = 9*	*n = 7*	*n = 2*
Bothriochloa biloba Kuntze	*	*	*	
Bothriochloa bladhii Kuntze	*	*	*	
Bothriochloa pertusa	*			
Cenchrus ciliaris	*	*	*	*
Cymbopogon ambiguus Spreng.	*	*		
Cymbopogon bombycinus Spreng.	*			
Dichanthium aristatum (R.Br.) A. Camus	*			
Dichanthium sericeum (R.Br.) A. Camus	*	*	*	
Digitaria brownii (R&S) Hughes	*	*		
Digitaria smutsi Haller	*			
Paspalum dilatatum	*	*	*	
Paspalum notatum	*	*	*	
Pennisetum alopecuroides Rich.	*			
Pennisetum clandestinum Hochst. ex Chiov.	*	*	*	
Sorghum bicolor cv pop sorghum				*

RESULTS

Leaf *A*, N, PNUE, and NUE (Experiments 1–3)

In three separate experiments, different combinations of 13 NAD-ME and 14 NADP-ME C_4 grasses were grown under adequate (experiments 1 and 2) and/or deficient (experiment 3) N supplies (Table I). In each case, average PNUE and NUE were significantly greater in NADP-ME than in NAD-ME grasses (Fig. 1, C and D; Table II). This difference was associated with lower leaf N in NADP-ME grasses, whereas average CO_2 assimilation rates (A) were similar for the two subtypes (Fig. 1, A and B; Table II). NADP-ME species accumulated more biomass than NAD-ME counterparts in only one out of three experiments (Table II). Low N supply reduced leaf A and N, biomass accumulation, and tillering, whereas it increased PNUE, NUE, and biomass allocation to roots in all species (Fig. 1; Table II). N deficiency affected measured parameters equally in both subtypes (Tables II and III). Whole-plant N concentration per dry mass was not significantly different between the two subtypes. Lower shoot (leaf and stem) N in NADP-ME relative to NAD-ME grasses was counterbalanced by higher root N (Table II). Of all measured parameters, leaf N and PNUE had the most consistent and significant difference, and species values showed the greatest range separation between the two subtypes (Fig. 1; Table II).

Rubisco Amount and Activity (Experiment 3)

N deficiency reduced leaf A, chlorophyll (Chl), N, and Rubisco content in seven NAD-ME and seven NADP-ME C_4 grasses, and this effect was not significantly different between the two subtypes (Fig. 2; Table III). Leaf N and A as well as leaf N and Rubisco sites were well correlated (r^2 between 0.62–0.74) across species and treatments (Fig. 2, A and B). The slopes of the former (A versus N; Fig. 2A) but not the latter (Rubisco versus N; Fig. 2B) relationships were significantly different between the two subtypes ($P < 0.05$). In vitro Rubisco catalytic turnover rate (k_{cat}) was assayed at 25°C, for better comparison with the literature, and 10 mM $NaHCO_3$ by measuring Rubisco activity and content of catalytic sites on the same extract of leaves harvested under high light. In vitro k_{cat}, thus measured, was well correlated with calculated in vivo k_{cat} at 30°C (A/Rubisco sites; Fig. 2C). k_{cat} values were significantly greater in NADP-ME than in NAD-ME species (Fig. 3; Table IV).

Leaf N Partitioning in the BS and M (Experiment 4)

The two NAD-ME species (*Panicum miliaceum* and *Panicum coloratum*) allocated relatively more of their leaf N to Rubisco (8%) and other soluble proteins (45%) compared with the two NADP-ME species (*Sorghum bicolor* and *Cenchrus ciliaris*; 5% and 36%, respectively; Fig. 4). In the larger survey of 14 C_4 grasses, Rubisco N fraction also tended to be slightly larger in the NAD-ME than NADP-ME grasses (Table III). For the 4 grasses, 30% of leaf N was recovered in the thylakoids, 8% in amino acids, and 5% as nitrate.

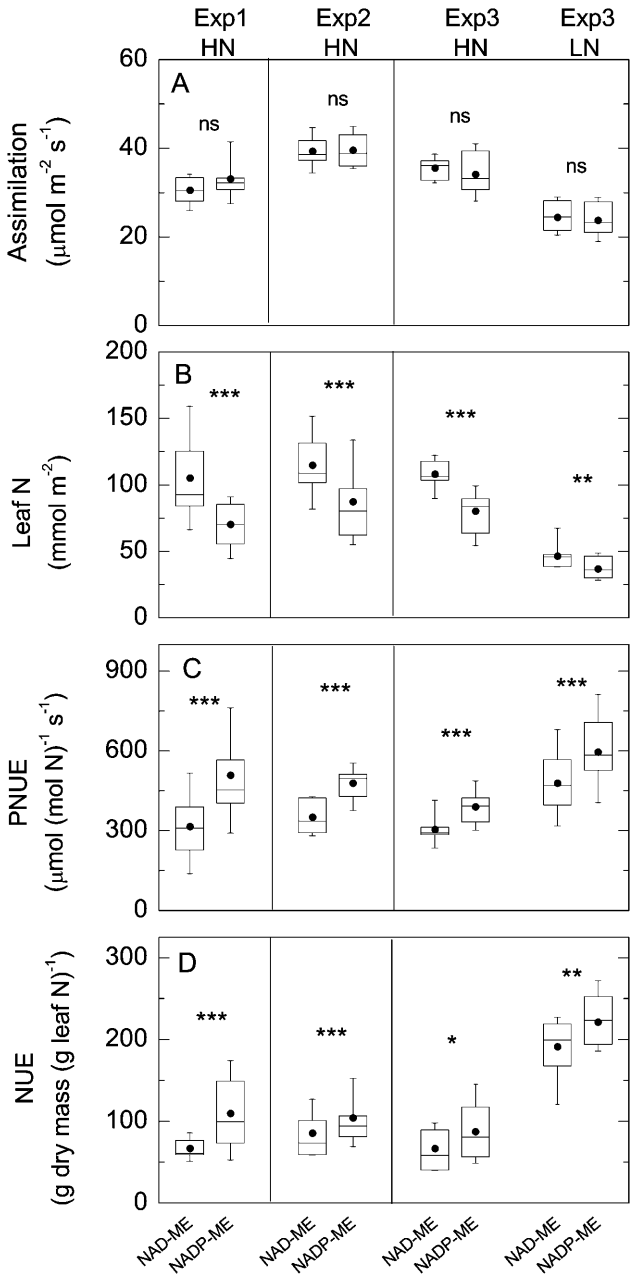

Figure 1. Box-whisker plots of assimilation rates (A), N content (B), PNUE (C), and NUE (D) in 13 NAD-ME and 14 NADP-ME C_4 grasses (experiments 1–3; Table I). The box and whisker represent the 25 to 75 percentile and minimum-maximum distributions of the data, respectively. Means (●) and significance levels for the subtype effect calculated as described in "Materials and Methods" are shown and are: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ns, not significant ($P \geq 0.05$). HN, High N soil supply; LN, low N soil supply.

Table II. *Summary of average steady-state gas exchange parameters, leaf, stem (including sheath), root, and whole-plant N, leaf mass per area, PNUE, NUE, and some growth parameters of NAD-ME and NADP-ME C_4 grasses from three independent experiments (experiments 1–3; Table I)*

Maximal phospho*enol*pyruvate carboxylase ($V_{p\ max}$) and Rubisco ($V_{c\ max}$) activities were estimated from A/C_i curves (experiment 2) using the C_4 photosynthesis model of von Caemmerer (2000). Within the same experiment, values followed by the same letter are not significantly different ($P > 0.05$). −, Not determined. HN, High N soil supply; LN, low N soil supply.

Parameter	Experiment 1		Experiment 2		Experiment 3			
	HN		HN		HN		LN	
	NAD-ME	NADP-ME	NAD-ME	NADP-ME	NAD-ME	NADP-ME	NAD-ME	NADP-ME
A (μmol m^{-2} s^{-1})	30a[a]	33a[a]	39a[b]	40a[b]	35b	35b	25a	24a
g (mol m^{-2} s^{-1})	0.25a[a]	0.28a[a]	0.26a[b]	0.28a[b]	0.22b	0.23b	0.16a	0.16a
C_i/C_a	0.37a[a]	0.38a[a]	0.31a[b]	0.33a[b]	0.26a	0.26a	0.29a	0.33b
$V_{p\ max}$ (μmol m^{-2} s^{-1})	–	–	81a	82a	–	–	–	–
$V_{c\ max}$ (μmol m^{-2} s^{-1})	–	–	47a	46a	–	–	–	–
$V_{p\ max}/V_{c\ max}$	–	–	1.72a	1.78a	–	–	–	–
Leaf N (mmol m^{-2})	119b	70a	115b	88a	108d	81c	47b	37a
Leaf N (mg g^{-1})	39b	32a	34b	31a	41c	37b	18a	18a
Stem N (mg g^{-1})	–	–	21b	19a	–	–	–	–
Root N (mg g^{-1})	–	–	16a	19b	–	–	–	–
Plant N (mg g^{-1})	–	–	24a	23a	–	–	–	–
Leaf mass per area (g m^{-2})	44b[a]	31a[a]	42b	37a	36c	31b	35c	28a
A/N (μmol CO_2 [mol N]$^{-1}$ s^{-1})	313a	525b	351a	478b	299a	388b	445c	611d
NUE (g dry mass [g leaf N]$^{-1}$)	67a	111b	95a	116b	65a	88b	191c	218d
Tiller no. (plant^{-1})	–	–	–	–	10b	16c	4a	5a
Plant dry mass (g plant^{-1})	9.2a[a]	14.6b[a]	9.9a[b]	10.7a[b]	4.4b	4.5b	1.5a	1.3a
Leaf mass fraction (% plant dry mass)	39b[a]	33a[a]	35b[b]	31a[b]	41d	35c	32b	28a
Stem mass fraction (% plant dry mass)	50a[a]	50a[a]	47a[b]	54b[b]	35c	44d	26a	28a
Root mass fraction (% plant dry mass)	11a[a]	17b[a]	18b[b]	15a[b]	24b	21a	42c	44c

[a]Data taken from Ghannoum et al. (2001). [b]Data taken from Ghannoum et al. (2002).

Up to 4% and 17% of total leaf N were unaccounted for in the NAD-ME and NADP-ME leaves, respectively (Fig. 4). About 60% to 67% of total leaf N and Chl were allocated to the BS tissue of NAD-ME species compared with 33% to 38% for NADP-ME species (Table V). This uneven and opposite N and Chl distribution between BS and M tissues was corroborated by the pattern of Chl *a* autofluorescence (Fig. 5). Most of the Chl *a* autofluorescence signal came from the BS of the two NAD-ME species (Fig. 5, A and B), whereas

Table III. *Leaf N, Chl, Rubisco sites, and the fraction of leaf N invested in Rubisco of seven NAD-ME and seven NADP-ME grasses grown under high (HN) and low (LN) soil N supply (experiment 3; Table I)*

Values are means ± SE. Significance levels are as described in the legend of Figure 1.

Species	Leaf N		Chl		Rubisco		Rubisco N	
	HN	LN	HN	LN	HN	LN	HN	LN
	mmol m^{-2}		*μmol m^{-2}*		*μmol sites m^{-2}*		*% leaf N*	
NAD-ME								
Astrebla lappacea	103 ± 6	30 ± 1	389 ± 27	215 ± 0	11.1 ± 0.6	4.9 ± 0.3	8.4 ± 0.0	12.8 ± 1.4
Astrebla pectinata	126 ± 0	90 ± 6	453 ± 19	277 ± 0	6.8 ± 0.7	4.6 ± 0.3	4.2 ± 0.4	4.0 ± 0.0
Eragrostis superba	118 ± 1	48 ± 3	423 ± 11	263 ± 11	8.5 ± 0.2	4.7 ± 0.1	5.6 ± 0.2	7.7 ± 0.3
Panicum coloratum	119 ± 16	53 ± 2	473 ± 21	296 ± 27	11.0 ± 1.1	4.8 ± 0.3	7.3 ± 0.2	7.2 ± 0.3
Panicum decompositum	128 ± 5	60 ± 10	605 ± 60	383 ± 45	10.4 ± 1.2	6.9 ± 1.9	6.4 ± 0.5	8.8 ± 1.1
Panicum miliaceum	143 ± 7	47 ± 9	452 ± 25	224 ± 8	9.9 ± 1.9	3.6 ± 0.1	5.5 ± 1.3	6.1 ± 1.1
Panicum virgatum	132 ± 6	73 ± 2	646 ± 6	402 ± 10	8.8 ± 0.4	5.0 ± 0.3	5.3 ± 0.5	5.3 ± 0.2
NADP-ME								
Bothriochloa biloba	66 ± 4	44 ± 0	503 ± 69	326 ± 23	4.0 ± 0.6	2.4 ± 0.4	4.7 ± 0.4	4.3 ± 0.7
Bothriochloa bladhi	81 ± 3	60 ± 3	488 ± 25	512 ± 81	4.8 ± 0.1	4.6 ± 0.8	4.6 ± 0.3	6.0 ± 0.9
Cenchrus ciliaris	98 ± 1.4	27 ± 3	488 ± 16	203 ± 7	6.4 ± 0.4	2.0 ± 0.2	5.1 ± 0.2	5.7 ± 0.1
Dichanthium sericeum	83 ± 7	38 ± 1	586 ± 80	281 ± 9	7.4 ± 0.3	2.5 ± 0.2	7.0 ± 0.4	5.2 ± 0.5
Paspalum dilatatum	100 ± 12	40 ± 3	442 ± 71	268 ± 22	7.2 ± 0.6	4.5 ± 0.3	5.8 ± 1.2	8.8 ± 0.1
Paspalum notatum	87 ± 1	37 ± 1	536 ± 62	223 ± 10	8.2 ± 0.1	3.9 ± 0.4	7.4 ± 0.2	8.3 ± 0.5
Pennisetum clandestinum	108 ± 1	37 ± 1	462 ± 10	241 ± 14	6.0 ± 0.8	2.6 ± 0.0	4.4 ± 0.5	5.7 ± 0.1
Average (NAD-ME)	124 ± 5	57 ± 7	492 ± 36	294 ± 28	9.5 ± 0.6	4.9 ± 0.4	6.1 ± 0.5	7.4 ± 1.1
Average (NADP-ME)	89 ± 5	41 ± 4	501 ± 18	293 ± 39	6.3 ± 0.6	3.2 ± 0.4	5.6 ± 0.5	6.3 ± 0.6
P (subtype)	***	***	ns	ns	***	***	**	**
P (N)	***	***	***	***	***	***	**	**
P (N × subtype)	ns	ns	ns	ns	ns	ns	ns	ns

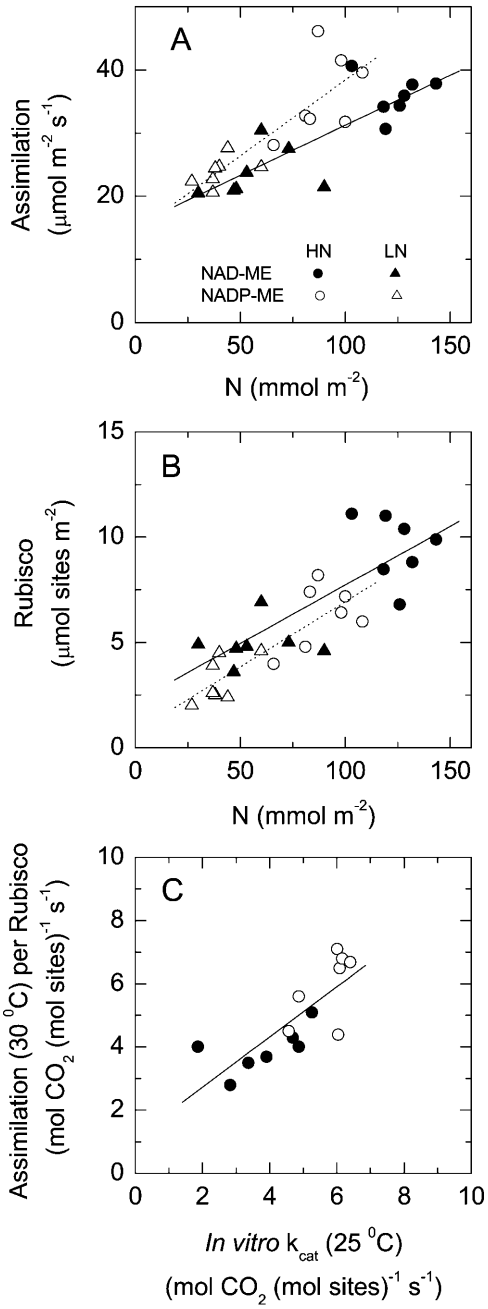

Figure 2. Relationships between assimilation rates (A) or Rubisco sites (B) and N content and between assimilation rates per Rubisco sites (in vivo k_{cat} at 30°C) and Rubisco in vitro k_{cat} at 25°C (C) for 7 NAD-ME (●, ▲) and 7 NADP-ME (○, △) C_4 grasses grown under high (●, ○; HN) and low (▲, △; LN) soil N supplies (experiment 3; Table I). Lines represent linear fits for NAD-ME (solid line, A: y = 15 + 0.16x, r^2 = 0.70; B: y = 2.18+ 0.05x, r^2 = 0.62), NADP-ME (dotted line, A: y = 14 + 0.24x, r^2 = 0.72; B: y = 0.74+ 0.06x, r^2 = 0.72), and all data (solid line, C: y = 1.14+ 0.79x, r^2 = 0.62).

the strongest signal emanated from the surrounding M tissue in the two NADP-ME species (Fig. 5, C and D). The distribution of thylakoid N followed that of Chl. About 48% to 60% and 32% to 37% of total thylakoid N were found in the BS of NAD-ME and NADP-ME leaves, respectively (Table V). About 62% and 55% of soluble protein were found in the BS of NAD-ME and NADP-ME leaves, respectively (Fig. 4; Table V). More than 70% of amino acids were found in the M of all 4 species. Nitrate was evenly distributed between the two tissues except in *S. bicolor*, in which most of it was in the M (Fig. 4; Table V).

Thylakoid N Partitioning in the BS and M (Experiment 4)

The content of functional PSII, PSI, and cytochrome (Cyt) *f* complexes approached 1 μmol m^{-2} in all 4 C_4 grasses, except for a significantly higher PSII content in *S. bicolor* (Table V). Although thylakoids accounted for a similar fraction of leaf N (approximately 30%) in the four species ($P > 0.05$), vast differences were found in thylakoid composition between the BS and M (Fig. 6). Relative to M, BS tissues of NAD-ME species had lower functional PSI and PSII reaction centers and Cyt *f*, all expressed per unit Chl (Fig. 6). Per unit Chl, functional PSI did not differ between M and BS in NADP-ME species, whereas both PSII and Cyt *f* were

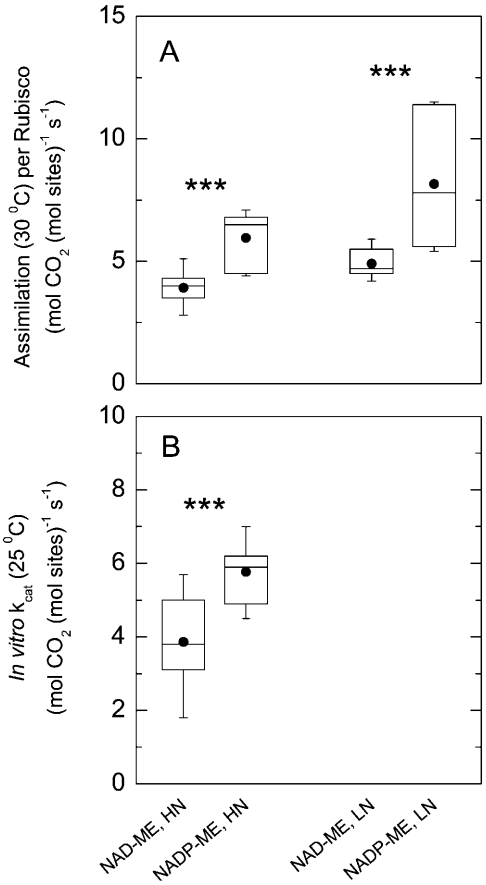

Figure 3. Box-whisker plots of assimilation rates per Rubisco sites (in vivo k_{cat} at 30°C; A) and Rubisco in vitro k_{cat} at 25°C (B) for 7 NAD-ME and 7 NADP-ME C_4 grasses grown under high (HN) and low (LN) soil N supplies (experiment 3; Table I). Other details are similar to Figure 1.

Table IV. *In vivo (assimilation rate [30°C] per Rubisco sites) and in vitro (25°C) k_{cat} (mol CO_2 [mol sites]$^{-1}$ s^{-1}) of 7 NAD-ME and 7 NADP-ME grasses grown under high (HN) and low (LN) soil N supply (experiment 3; Table I)*

Other details are as described for Table III.

Species	*A*/Rubisco (30°C)		In Vitro k_{cat} (25°C)
	HN	LN	HN
NAD-ME			
Astrebla lappacea	3.7 ± 0.3	4.2 ± 0.1	3.9 ± 0.1
Astrebla pectinata	5.1 ± 0.5	4.7 ± 0.2	5.3 ± 0.3
Eragrostis superba	4.0 ± 0.3	4.5 ± 0.2	4.9 ± 0.3
Panicum coloratum	2.8 ± 0.2	4.9 ± 0.3	2.8 ± 0.2
Panicum decompositum	3.5 ± 0.3	4.6 ± 0.7	3.4 ± 0.0
Panicum miliaceum	4.0 ± 0.9	5.9 ± 0.1	1.9 ± 0.0
Panicum virgatum	4.3 ± 0.2	5.5 ± 0.1	4.7 ± 0.6
NADP-ME			
Bothriochloa biloba	7.1 ± 0.5	11.5 ± 1.6	6.0 ± 0.3
Bothriochloa bladhi	6.8 ± 0.5	5.4 ± 0.1	6.2 ± 0.3
Cenchrus ciliaris	6.5 ± 0.4	11.4 ± 1.1	6.1 ± 0.3
Dichanthium sericeum	4.4 ± 0.0	9.6 ± 0.3	6.1 ± 0.1
Paspalum dilatatum	4.5 ± 0.9	5.6 ± 0.5	4.6 ± 0.1
Paspalum notatum	5.6 ± 0.0	5.8 ± 0.0	4.9 ± 0.1
Pennisetum clandestinum	6.7 ± 0.7	7.8 ± 0.1	6.4 ± 0.2
Average (NAD-ME)	3.9 ± 0.3	4.9 ± 0.2	3.8 ± 0.5
Average (NADP-ME)	5.9 ± 0.4	8.2 ± 1.0	5.7 ± 0.3
P (subtype)	***	***	***
P (N)	*	*	
P (N × subtype)	ns	ns	

lower in the BS (Fig. 6). Diminished PSII activity in BS of NADP-ME leaves resulted in reduced photochemical efficiency (F_v/F_m; Table V). Thylakoid N can be divided into N pools associated either with pigment-protein complexes or with ATP synthesis and electron transport complexes. When expressed on a Chl basis, measured thylakoid N increased linearly with Cyt *f* content (Fig. 7). Values measured on the C_4 grasses here scattered around those reported for C_3 leaves. Calculated values were generally less than measured values for the BS tissues (Fig. 7).

DISCUSSION

N-Use Efficiency and Rubisco in NAD-ME and NADP-ME C_4 Grasses

This study was sparked by our observation that C_4 grasses belonging to the NADP-ME subtype have higher NUE and PNUE and lower leaf N content than NAD-ME grasses. Since then, we consolidated this finding in 3 separate experiments using various combinations of 27 NAD-ME and NADP-ME grasses grown under adequate and deficient soil N supplies (Fig. 1; Table II). Along the same line, Bowman (1991) found that two NADP-ME Panicum species accumulated more biomass per total shoot N than four NAD-ME species. When Taub and Lerdau (2000) compared the response of *A* to leaf N in three NAD-ME and three NADP-ME grasses, they concluded that species variations were greater than differences in PNUE between the two C_4 subtypes. It is likely that the small number of species used in the latter study hindered the emergence of a clear trend. Here, we report a consistent and convincing difference ($P < 0.05$) in NUE, PNUE, and leaf N for a large number of NAD-ME and NADP-ME C_4 grasses (Fig. 1). Differences in leaf N concentration were counterbalanced by changes in stem and root N, such that the same amount of total N was required to produce a gram of whole-plant dry mass in both types of C_4 grasses (Table II). Our subsequent investigation focused on dissecting leaf N in order to identify the N pool(s), which may contribute to the difference between the two subtypes in leaf N and PNUE.

Using four C_4 grasses, we found that NAD-ME species invest a greater proportion of leaf N in soluble protein and Rubisco relative to NADP-ME species. In a larger survey, NADP-ME grasses achieved the same photosynthetic rates as NAD-ME counterparts with less leaf N and Rubisco contents. Greater *A* per Rubisco sites (in vivo k_{cat}) in NADP-ME than in NAD-ME species suggests, among other things, that there is an intrinsic difference in Rubisco k_{cat} between the two subtypes. This was confirmed by in vitro k_{cat} measurements. Under the high light and temperature conditions used during our gas exchange

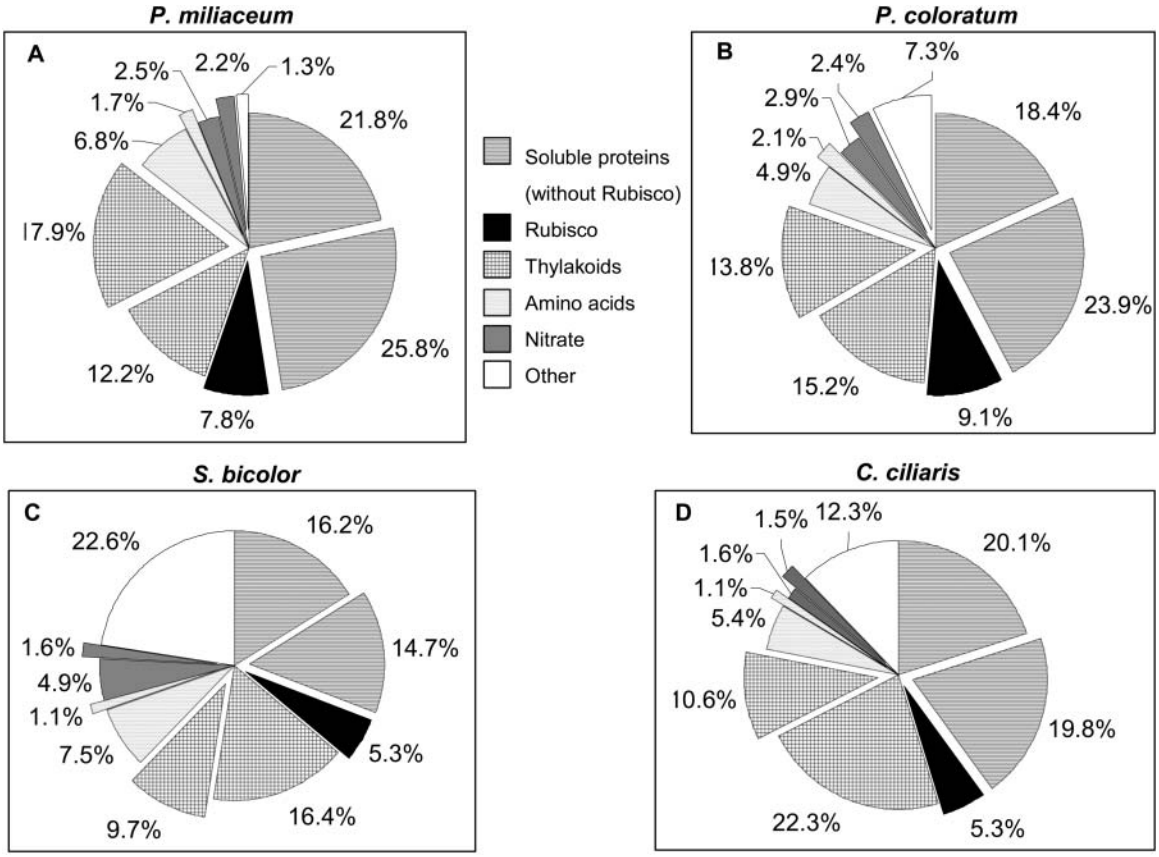

Figure 4. Total leaf N budget for *P. miliaceum* (A), *P. coloratum* (B), *S. bicolor* (C), and *C. ciliaris* (D; experiment 4; Table I). The contributions of soluble proteins without Rubisco, Rubisco, thylakoids, amino acids, nitrate, and others are calculated as described in ''Materials and Methods.'' The popped-out slices represent the BS fractions, whereas the remaining slices represent the M fractions. The percentage of total leaf N is shown next to each slice.

measurements, C_4 photosynthesis operates near maximal Rubisco activity. Therefore, the close correlation between Rubisco's in vivo and in vitro k_{cat} indicates that most variations in photosynthetic efficiency per Rubisco and, hence, per leaf N between grasses of the NAD-ME and NADP-ME subtypes are related to differences in Rubisco k_{cat} (Fig. 2C). In accordance with our results, Seemann et al. (1984) measured Rubisco k_{cat} (at 30°C) for a number of C_3 and C_4 species, obtaining values of 4.8 (s^{-1}) for 1 NAD-ME and 6.4 to 7.6 (s^{-1}) for 4 NADP-ME grasses. Comparisons of Rubisco kinetics reveal that the higher k_{cat} of C_4 relative to C_3 Rubisco entails a greater Michaelis-Menten constant for CO_2 ($K_{m\ [CO2]}$; Yeoh et al., 1980, 1981; Seemann et al., 1984; Wessinger et al., 1989; Sage, 2002). Given that Rubisco operates near CO_2 saturation in C_4 plants, C_4 photosynthesis is more sensitive to increases in Rubisco k_{cat} than $K_{m\ (CO2)}$ (von Caemmerer, 2000). In contrast with the C_3 versus C_4 comparison, differences in k_{cat} between the two C_4 subtypes do not appear to have had implications on $K_{m\ (CO2)}$. In an extensive survey, Yeoh et al. (1980) found no significant difference in $K_{m\ (CO2)}$ between NAD-ME and NADP-ME grasses. Although a link between Rubisco k_{cat} and $K_{m\ (CO2)}$ is theoretically justified (Morell et al., 1992), the two parameters have been shown to vary independently in Rubisco from different origins or when subjected to site-directed mutagenesis (Whitney et al., 1999, 2001). There is suggestion that changes in Rubisco k_{cat} are accompanied by a trade off in specificity for CO_2 relative to O_2 ($S_{c/o}$; Zhu et al., 2004). However, $S_{c/o}$ does not appear to differ much among higher plants (Kane et al., 1994). This argument remains constrained by the scarcity of kinetic data for C_4 Rubisco (von Caemmerer and Quick, 2000). In brief, NADP-ME grasses achieved similar photosynthetic capacity to NAD-ME counterparts with less leaf N and Rubisco having higher k_{cat}, mirroring the well known differences between C_3 and C_4 plants.

N Partitioning in the Leaves of NAD-ME and NADP-ME C_4 Grasses

When grown under high light, C_3 leaves typically allocate 22% of leaf N to thylakoids and 60% to soluble protein, one-third of which is Rubisco (Evans and

Table V. *Total leaf and BS Chl and N, thylakoid N, soluble protein, amino acids, nitrate, Rubisco, PSII, PSI, and Cyt f concentrations of two NAD-ME (P. miliaceum and P. coloratum) and two NADP-ME (S. bicolor and C. ciliaris) C_4 grasses (experiment 4; Table I)*

Dark respiration (R_d) and dark-adapted F_v/F_m are also shown. Values are means ± SE. Significance levels are as described in the legend of Figure 1. Within the same row, values followed by the same letter are not significantly different ($P > 0.05$).

Parameter	Fraction	*P. miliaceum*	*P. coloratum*	*S. bicolor*	*C. ciliaris*
Chl *a*/*b*	Leaf (n = 12)	4.2a ± 0.1	4.3ab ± 0.1	4.4b ± 0.1	4.4ab ± 0.1
	BS (n = 4)	4.1a ± 0.1	4.1a ± 0.2	6.3b ± 0.3	6.4b ± 0.5
	$P_{Fraction}$	ns	ns	**	**
Chl (μmol m^{-2})	Leaf (n = 12)	424a ± 26	425a ± 24	579b ± 36	464a ± 37
	BS (n = 4)	284b ± 18	256b ± 26	206a ± 15	164a ± 21
	BS/Leaf ($P_{Fraction}$)	0.60 (*)	0.59 (*)	0.33 (**)	0.38 (**)
Total N (mmol m^{-2})	Leaf (n = 6)	78a ± 10	87a ± 8	116b ± 3	87a ± 3
	BS (n = 3)	47b ± 5	51b ± 3	39ab ± 4	33a ± 2
	BS/Leaf ($P_{Fraction}$)	0.67 (*)	0.60 (***)	0.36 (***)	0.35 (**)
Thylakoid N (mmol N m^{-2})	Leaf (n = 3)	23.5a ± 1.7	25.2ab ± 0.4	30.3c ± 0.5	28.6bc ± 0.5
	BS (n = 2)	14.0c ± 0.3	12.0b ± 0.7	11.2b ± 0.3	9.2a ± 0.3
	BS/Leaf ($P_{Fraction}$)	0.60 (*)	0.48 (**)	0.37 (***)	0.32 (**)
Soluble protein (g m^{-2})	Leaf (n = 3)	3.8a ± 0.4	4.6a ± 0.3	3.7a ± 0.4	3.4a ± 0.2
	BS (n = 3)	2.3a ± 0.3	3.2a ± 1.1	2.0a ± 0.2	1.9a ± 0.1
	BS/Leaf ($P_{Fraction}$)	0.61 (*)	0.64 (*)	0.55 (*)	0.55 (*)
Amino acids (mmol m^{-2})	Leaf (n = 3)	6.6a ± 0.6	6.1a ± 0.9	10.0a ± 1.7	5.7a ± 0.8
	BS (n = 2)	1.3a ± 0.1	1.8a ± 0.5	1.3a ± 0.1	0.90a ± 0.1
	BS/Leaf ($P_{Fraction}$)	0.20 (**)	0.30 (*)	0.13 (*)	0.16 (*)
Nitrate (mmol m^{-2})	Leaf (n = 3)	3.7a ± 0.1	4.6b ± 0.4	7.5bb ± 1.2	2.7a ± 0.4
	BS (n = 2)	1.7a ± 0.3	2.1a ± 0.5	1.9a ± 0.1	1.3a ± 0.1
	BS/Leaf ($P_{Fraction}$)	0.46 (**)	0.45 (*)	0.25 (*)	0.49 (*)
Rubisco (μmol m^{-2})	Leaf (n = 12)	0.97b ± 0.06	1.25c ± 0.08	0.97b ± 0.06	0.73a ± 0.04
PSII (μmol m^{-2})	Leaf (n = 6)	1.03a ± 0.06	0.99a ± 0.08	1.35b ± 0.06	1.15a ± 0.06
	BS (n = 2)	0.18b ± 0.02	0.35c ± 0.01	0.02a ± 0.02	0.05a ± 0.01
	BS/Leaf ($P_{Fraction}$)	0.17 (***)	0.35 (***)	0.01 (***)	0.04 (***)
PSI (μmol m^{-2})	Leaf (n = 2)	1.06a ± 0.07	0.95a ± 0.05	1.01a ± 0.01	0.93a ± 0.13
	BS (n = 2)	0.26a ± 0.07	0.44b ± 0.02	0.37ab ± 0.01	0.36ab ± 0.03
	BS/Leaf ($P_{Fraction}$)	0.24 (*)	0.46 (*)	0.37 (***)	0.39 (*)
Cyt *f* (μmol m^{-2})	Leaf (n = 2)	1.03a ± 0.02	1.00a ± 0.05	1.00a ± 0.04	1.05a ± 0.16
	BS (n = 2)	0.39c ± 0.02	0.41c ± 0.03	0.20a ± 0.01	0.30b ± 0.01
	BS/Leaf ($P_{Fraction}$)	0.37 (**)	0.38 (*)	0.20 (**)	0.34 (*)
R_d (μmol m^{-2} s^{-1})	Leaf (n = 3)	−1.7a ± 0.12	−1.9a ± 0.08	−1.9a ± 0.10	−1.8a ± 0.40
F_v/F_m	Leaf (n = 3)	0.75a ± 0.01	0.81a ± 0.01	0.80a ± 0.01	0.80a ± 0.01
	BS (n = 3)	0.75b ± 0.01	0.81b ± 0.01	0.40a ± 0.01	0.45a ± 0.02
	$P_{Fraction}$	ns	ns	***	***

Poorter, 2001). In our study, C_4 leaves allocated 30% of leaf N to thylakoids, and 41% (NADP-ME) and 53% (NAD-ME) to soluble protein, one-seventh of which is Rubisco. This distribution is close to what Makino et al. (2003) reported for maize (NADP-ME), in which 34% and 33% of leaf N were found in thylakoids and soluble protein, respectively. The lower soluble protein fraction of C_4 relative to C_3 leaves increases the apparent allocation of N to thylakoids in C_4 leaves. Hence, the amount of soluble protein per Chl is greater for C_3 (10–20 g [mmol Chl]$^{-1}$) than C_4 leaves (6.3–7.4 NADP-ME, 8.9–10.8 NAD-ME). Using available literature values, Evans and von Caemmerer (2000) calculated the soluble protein cost of the C_3 and C_4 cycles. They estimated similar costs for C_3 and NAD-ME C_4 leaves (6.4 g soluble protein [mmol Chl]$^{-1}$) and a slightly lower value for NADP-ME leaves (6.1 g soluble protein [mmol Chl]$^{-1}$). The C_3 monocots wheat (*Triticum aestivum*; Evans, 1983) and rice (*Oryza sativa*; Makino et al., 1997) have about 10 g of soluble protein (mmol Chl)$^{-1}$, which is equivalent to the NAD-ME grasses. The slightly higher protein cost of the NAD-ME than the NADP-ME C_4 subtype may reflect the high activity of Asp and Ala aminotransferases in NAD-ME leaves. The calculation of Evans and von Caemmerer (2000) was based on measured Rubisco content of *Amaranthus edulis* (C_4 dicot), in which Rubisco accounted for 8% of leaf N (Sage et al., 1987). This proportion is similar to what Makino et al. (2003) reported for maize and is close to the upper bound of values observed in our study (Figs. 2B and 4). Our reported values for the soluble protein cost per Chl is 12% (NADP-ME) and 55% (NAD-ME) above that estimated for just the C_3 and C_4 cycle enzymes by Evans and von Caemmerer (2000). In brief, C_4 (relative to C_3) and NADP-ME (relative to NAD-ME) leaves allocate a lower proportion of N to soluble protein relative to thylakoids.

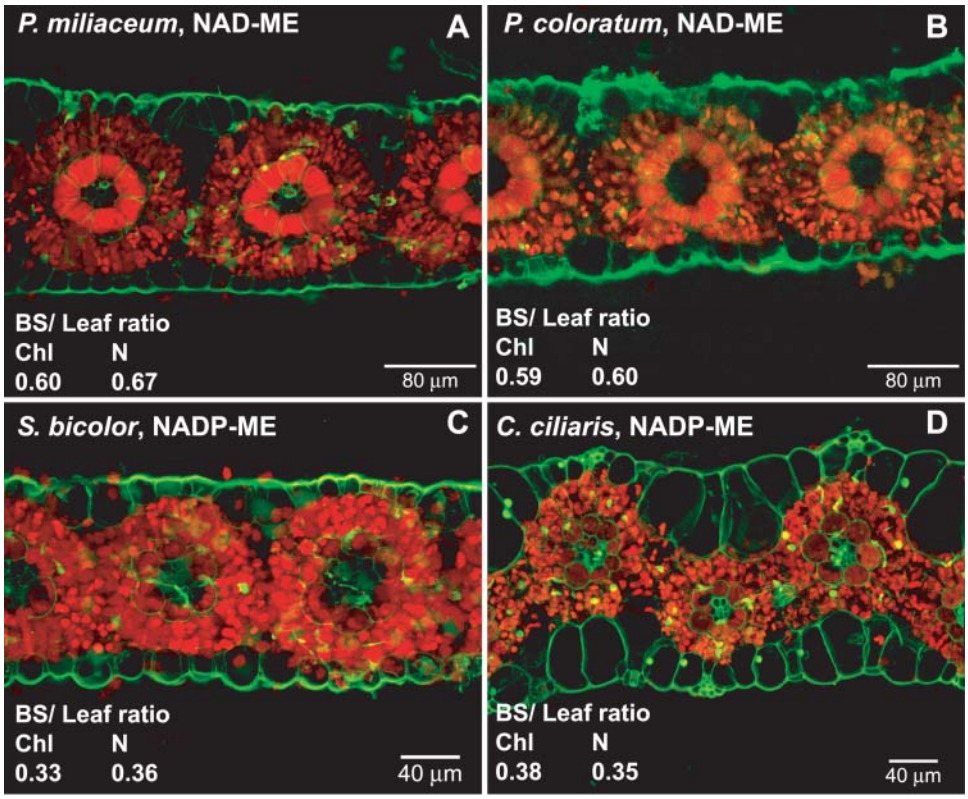

Figure 5. Chl *a* autofluorescence of leaf cross sections of two NAD-ME (*P. miliaceum* [A] and *P. coloratum* [B]) and two NADP-ME (*S. bicolor* [C] and *C. ciliaris* [D]) C_4 grasses (experiment 4; Table I). The images were obtained using confocal microscopy. Cell walls are shown in green and Chl *a* autofluorescence in red. The BS/leaf ratios of Chl and total N are shown for each species. Note the change of scale between the NAD-ME and NADP-ME images.

N Partitioning between the BS and M of NAD-ME and NADP-ME C_4 Grasses

The pattern of Chl distribution found in our study agrees with what has been reported for C_4 species (Mayne et al., 1975; Jenkins and Boag, 1985). The distribution of total leaf N between the M and BS mirrors that of Chl, indicating that most leaf N is linked to photosynthesis in the C_4 grasses. The intercellular compartmentation of the photochemical reactions of C_4 photosynthesis is less well documented than that of the CO_2 fixation reactions (Edwards et al., 1976; Hatch and Osmond, 1976; Hatch, 1987; Edwards and Krall, 1992). BS deficiency in PSII activity, as reported here and by other workers, represents the best documented example of the unequal composition of BS and M thylakoids in C_4 leaves (Mayne et al., 1975; Edwards et al., 1976; Ghirardi and Melis, 1984; Meierhoff and Westhoff, 1993). Suppressing PSII activity in the gas-tight BS cells serves to prevent the accumulation of detrimentally high O_2 concentrations. Our data indicate that the 2 NAD-ME grasses have also gone a long way in locating 65% to 83% of PSII activity in the M, away from the BS. This is higher than what has been reported for the NAD-ME grasses *P. miliaceum* and *Panicum capillare*: 46% based on the Hill reaction and 13% based on delayed light emission (Mayne et al., 1975; Edwards et al., 1976).

This discrepancy may be reconciled by recent evidence suggesting that BS thylakoids of NADP-ME species (e.g. maize and *S. bicolor*) contain incomplete and inactive PSII centers, devoid of the oxygen evolving complex (Meierhoff and Westhoff, 1993; Bassi et al., 1995). Data presented in Table V are consistent with there being inactive PSII complexes in the BS for two reasons. First, low PSII activity was observed in the BS of the NADP-ME species, whereas PSI content per unit Chl was similar for both BS and M thylakoids (Fig. 6B) and the Chl *a*/*b* ratio was higher in the BS, meaning that there could be no increase in the proportion of Chl associated with light-harvesting complexes (LHCs). Secondly, the observed amount of N per unit Chl in BS thylakoids exceeded that predicted on the basis of active PSII, PSI, and Cyt *f* complexes for all four species (Fig. 7). The N cost per Chl (mol N [mol Chl]$^{-1}$) of PSII is 83, whereas it is only 33 and 26 for PSI and LHCs, respectively. Any underestimate of PSII content reduces calculated thylakoid N content. Thus, it is likely that BS thylakoids of NAD-ME and NADP-ME species contain varying degrees of functional and nonfunctional PSII centers. PSII distribution between M and BS and active and inactive complexes varies among species and may be influenced by growth conditions.

The uneven PSII distribution necessitates a substantial 3-phosphoglycerate/triose phosphate (PGA/TP) shuttle service between the M and BS of NADP-ME and NAD-ME leaves. This is possible because the enzymes for PGA reduction are present in both cell types and subtypes (Hatch and Osmond, 1976). In

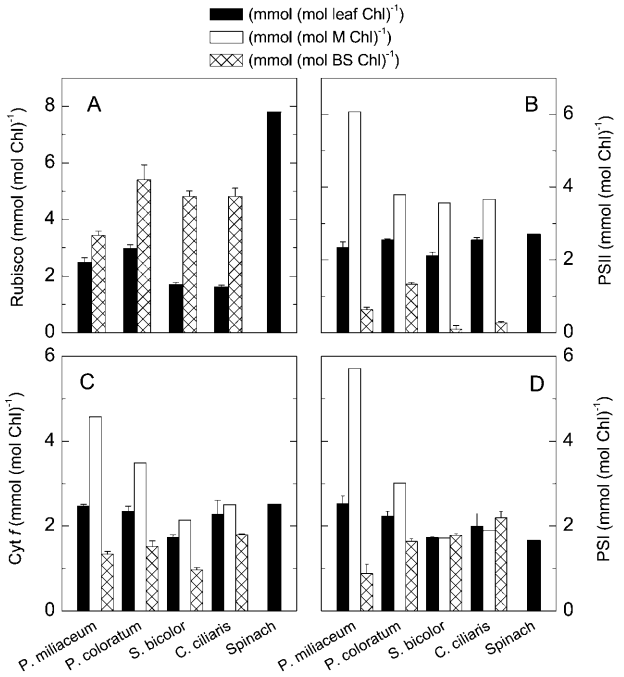

Figure 6. Concentration of Rubisco (A), PSII (B), Cyt *f* (C), and PSI (D) proteins expressed per leaf (black bars), M (white bars), or BS (hatched bars) Chl in four C_4 grasses (experiment 4; Table I) and spinach (Evans and Terashima, 1987). Values are means ± SE. See Table V for number of replications.

addition, NADP-ME and NAD-ME species possess sufficient PGA and TP concentration gradients between the M and BS, such that up to 50% of PGA produced in the BS can be reduced in the M (Hatch and Osmond, 1976; Leegood and von Caemmerer, 1988, 1989). Interestingly, Rubisco content per Chl in the BS is high and approximates those found in C_3 leaves (Fig. 4A). Thus, the presence of functional and nonfunctional PSII centers in BS tissue may enable the thylakoid membrane to better compact and make more room for Rubisco in the stroma (Chow, 1999). Although PSI was more equally distributed between the M and BS in 3 species, 76% of leaf PSI activity was found in the M of *P. miliaceum*. This departs from early reports (Mayne et al., 1975) and suggests that antenna size of the photosystems is smaller in the M than BS of *P. miliaceum*. The distribution of thylakoid complexes between the M and BS of C_4 grasses does not mirror closely the distribution of Chl and N.

The close match between measured and calculated thylakoid N contents (Fig. 7) suggests that N costs derived from C_3 plants are appropriate for C_4 plants. This is surprising given the differences in energetic requirements between the M and BS and the C_3 and C_4 cycles. The N cost of 8.85 mol N (mol Cyt *f*)$^{-1}$ suggested by Evans and Seemann (1989) and used here is considerably greater than that suggested by Hikosaka and Terashima (1995; 5.43 mol N [mol Cyt *f*]$^{-1}$). The uncertainty comes largely from there being few quantitative measurements of ATP synthase. Makino et al. (2003) compared leaf N budgets from rice with maize and measured both Cyt *f* and ATP synthase. The amount of N in ATP synthase relative to Cyt *f* was 4.7 and 5.3 for rice and maize, respectively, close to the value of 4.4 assumed here for the calculations (Fig. 7). The fraction of leaf N associated with ATP synthase and electron transport components was 5.7% and 9.4% for rice and maize, respectively, compared to 7.6% to 11.7% calculated for the 4 C_4 species here. The proportion calculated for 10 dicotyledonous C_3 species is similar, averaging 8.2% (Evans and Poorter, 2001).

CONCLUSION

In summary, NADP-ME grasses achieved similar photosynthetic capacity to NAD-ME counterparts with less leaf N and Rubisco having higher k_{cat}. Compared to NADP-ME, NAD-ME leaves allocate a greater fraction of leaf N to soluble protein and a similar fraction to thylakoids. Although the majority of leaf Chl and N were found in the BS of NAD-ME and M of NADP-ME grasses, the distribution of thylakoid complexes was species specific.

MATERIALS AND METHODS

This study reports on four experiments (Table I). Experiments 1, 2 (related to Ghannoum et al., 2001, 2002), and 3 were used for NUE and *A*/N calculations. Experiment 3 was also used for Rubisco activity assays. Experiment 4 was used for N budget analysis.

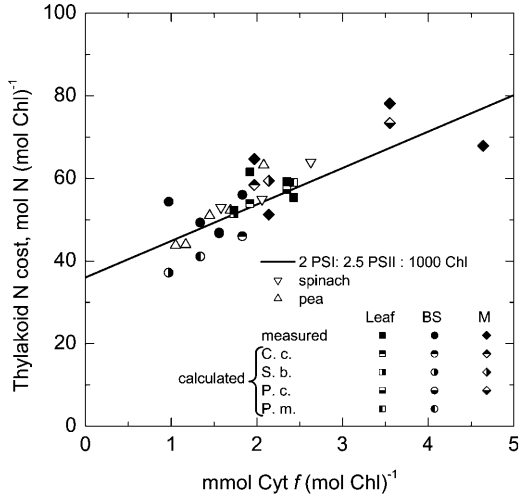

Figure 7. Thylakoid N cost as a function of Cyt *f* content per unit Chl. Closed symbols are values measured either on whole-leaf extracts (■) or BS (●) thylakoids, or by difference for the M thylakoids (♦). Half-filled symbols represent thylakoid N cost calculated from the contents of functional PSII, PSI, and Cyt *f* for each species and fraction. Open symbols denote thylakoids prepared from C_3 leaves (spinach [Terashima and Evans, 1988] and pea [Evans, 1987]). The solid line shows the calculated N cost for thylakoids with 2 mmol PSI (mol Chl)$^{-1}$, 2.5 mmol PSII (mol Chl)$^{-1}$, and varying Cyt *f* content, which represents the average complex concentrations observed for the C_4 leaves.

Plant Culture (Experiments 1–3)

Soil was supplemented with basal nutrients (excluding N) and added to 5- to 10-L pots (Ghannoum and Conroy, 1998; Ghannoum et al., 2001). Briefly, the soil was leached of readily available N by repeatedly washing it with water. Pots were transferred to a temperature-controlled glasshouse, where air temperatures ranged between 28°C to 30°C /22°C to 24°C day/night. Midday photosynthetic active radiation averaged 1,000 μmol m^{-2} s^{-1}. Seeds for 13 NAD-ME and 14 NADP-ME C_4 grasses (Table I) were obtained from local commercial suppliers or CSIRO Tropical Agriculture, St Lucia, Australia, and sown directly into the potted soil. Pots were watered to full capacity once daily with either water (low N treatment) or 60 mg N kg^{-1} soil (high N treatment) supplied as NH_4NO_3. After germination, plants were thinned to two to four seedlings per pot. There were three to four pots per species and N treatment.

Plant Culture (Experiment 4)

Seeds for two NAD-ME (*Panicum miliaceum* and *Panicum coloratum*) and 2 NADP-ME (*Sorghum bicolor* and *Cenchrus ciliaris*) C_4 grasses were sown directly in 4-L pots containing sterilized garden soil supplemented with 4 g of a controlled release fertilizer (15/4.8/10.8/1.2 nitrogen/phosphorus/potassium/magnesium plus trace elements [boron, copper, iron, manganese, molybdenum, and zinc]; Osmocot Plus; Scotts, Baulkam Hills, Australia). Plants were grown in controlled environment chambers (Phoenix, Adelaide, Australia) lit for 9 h with metal halide lamps supplying 800 μmol quanta m^{-2} s^{-1} followed by 1 h of incandescent lighting. Air temperature and relative humidity were maintained at 28/24°C and 60/80% day/night, respectively. Plants were watered daily and used for analysis 4 weeks after germination.

Growth and NUE Measurements (Experiments 1–3)

Plants were harvested 5 to 7 weeks after germination and separated into leaves, stems (including sheaths), and roots. Leaf area was determined by a digital image analyzer (Delta-T, Cambridge, UK). Roots were washed free of soil. Harvested samples were oven-dried at 80°C, weighed, and then ground to powder. Percentage of N was determined on the ground tissues using a flash combustion CNS analyzer (Fison NA1500; Fison Instruments, Milan, Italy). NUE was calculated as the ratio of plant dry mass to total leaf N content at harvest.

Gas Exchange Measurements (Experiments 1–4)

Measurements were made on the youngest fully expanded leaves 1 week before harvest using a portable photosynthesis system (LI-6400; LI-COR, Lincoln, NE), at a photosynthetic photon flux density of 1,500 (experiments 1 and 2) and 1,800 (experiments 3 and 4) μmol m^{-2} s^{-1} supplied by an in-built red/blue LED light source, a CO_2 partial pressure of 380 μbar, leaf temperature of 30°C, and leaf-to-air vapor pressure difference between 1.5 to 2.0 kPa. Dark respiration (R_d) was measured after 30 min of dark adaptation, before the light was turned on. In experiment 2, the response of A to step increases of intercellular CO_2 (C_i) was measured. A/C_i curves were fitted using the C_4 photosynthesis model of von Caemmerer (2000) to estimate maximal phospho*enol*pyruvate carboxylase ($V_{p\ max}$) and Rubisco ($V_{c\ max}$) activities. At the end of gas exchange measurements, leaves were cut under high light (>1,000 μmol m^{-2} s^{-1}) and either immersed in liquid N_2 then stored at −80°C for biochemical analysis or their area determined before being oven-dried, weighed, and their percentage of N measured as described above.

Preparation of BS Strands (Experiment 4)

The four grass species (Table I) were chosen based on their ability to produce highly pure BS strands by mechanical blending as checked under a light microscope (Agostino et al., 1989). The midrib was removed from the youngest fully expanded leaves, which were cut into 10- × 2-mm sections using a razor blade, soaked in distilled water, then blotted dry. About 7 g of fresh leaf sections were mixed with 100 mL of ice-cold blending buffer (50 mM Na-PO_4, 0.33 M sorbitol, 4 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 mM EDTA, pH 7.5) in a 250-mL cup of a Sorvall (Newton, CT) Omnimixer, and blended for 5 × 10 s at 60% speed. The homogenate was filtered through 1-mm, 0.5-mm, then 88-μm nylon mesh. The BS strands collected on the 88-μm mesh were washed copiously with blending buffer, then stored on ice.

Rubisco, Chl, Soluble Protein, Amino Acids, and Nitrate (Experiments 3 and 4)

Leaf sections (1–2 cm^2) or BS preparations (100 nmol Chl) were extracted in 1 mL of ice-cold buffer (50 mM EPPS-NaOH, 5 mM DTT, 15 mM $NaHCO_3$, 20 mM $MgCl_2$, 2 mM EDTA, 4% protease inhibitor cocktail [Sigma, St. Louis], 0.1% [w/v] polyvinylpolypyrrolidone, pH 8.0) and with or without 0.05% Triton X-100 using a 2-mL Potter-Elvehjem glass homogenizer kept on ice. Subsamples were taken from the crude homogenate for Chl determination in 80% acetone (Porra et al., 1989). The remaining homogenate was centrifuged at 16,100*g* for 0.5 min and the supernatant used for the following analysis. Total Rubisco content was estimated by the irreversible binding of [^{14}C]2-carboxy-D-arabinitol 1,5-bisphosphate to the fully carbamylated enzyme (Ruuska et al., 1998). Maximal Rubisco activity was assayed within 5 min of extraction, by incubating 20 μL of supernatant in 500 μL of reaction buffer (50 mM EPPS-NaOH, 20 mM $MgCl_2$, 1 mM EDTA, 10 mM $NaH^{14}CO_3$, pH 8.0) for 5 min at 25°C. Reaction was initiated by adding 0.5 mM ribulose 1,5-bisphosphate and stopped after 1 min with 7% formic acid. Acid-stable ^{14}C and specific activity of $NaH^{14}CO_3$ were then determined (Ruuska et al. 2000). In vitro k_{cat} was calculated as the ratio of Rubisco activity to content of [^{14}C]2-carboxy-D-arabinitol 1,5-bisphosphate binding sites, determined for the same leaf extract. Tobacco leaves were used as a reference with each run of activity assays, yielding k_{cat} between 3.2 to 3.4 s^{-1}. Soluble proteins were measured using a Coomassie Plus kit (Pierce). Free amino acids and nitrate were determined according to Moore (1968) and Cataldo et al. (1975), respectively.

Thylakoid Preparation and PSI and Cyt *f* Measurements (Experiment 4)

Leaf sections or BS preparations were extracted in a 7-mL Potter-Elvehjem glass homogenizer in ice-cold extraction buffer (50 mM Na-PO_4, 0.33 M sorbitol, 2 mM DTT, 5 mM $MgCl_2$, 2 mM EDTA, pH 6.5). Several extractions were carried out and pooled. The extracts were filtered as described above. The filtrate was centrifuged for 1 min at 1,000*g* and 4°C. The pellet, enriched in tissue debris and starch, was discarded. The supernatant was centrifuged for 4 min at 2,000*g*. The pellet was gently suspended and centrifuged twice in 40 mL of extraction buffer before being incubated for 5 min on ice in lysis buffer (extraction buffer devoid of sorbitol), then centrifuged. The pellet was finally washed, suspended in extraction buffer, snap frozen in liquid N_2, then stored at −80°C until used for PSI, Cyt *f*, or total N analysis. PSI was calculated as the amount of P_{700} using the absorbance change at 702 nm induced by flashes of blue-green light, after correcting for fluorescence (Evans, 1987). Cyt *f* complex was estimated from hydroquinol-reduced minus ferricyanide-oxidized difference spectra using a dual beam spectrophotometer (model 557; Perkin Elmer, Foster City, CA; Evans, 1987).

Functional Centers and Photochemical Efficiency of PSII (Experiment 4)

Functional PSII centers of leaf and BS tissue were quantified by the O_2 yield at 1% CO_2 and repetitive flashes at 10 Hz, with continuous background far-red light (Chow et al., 1989). BS preparations were layered on glass filter paper (Whatman GF/C; W&R Balston, Maidstone, England), and a gentle suction was applied to remove excess buffer. Photochemical efficiency of PSII was measured following 15 min of dark adaptation of leaf sections or BS preparations, using a portable fluorometer (Plant Efficiency Analyzer; Hansatech Instruments, Norfolk, UK).

Total Leaf, BS, and Thylakoid N (Experiment 4)

Total leaf N was determined on oven-dried, ground leaf sections taken from the same or matching leaves used for various analyses. BS fractions taken after Rubisco determination were oven-dried in preweighed tubes, weighed, then a subsample analyzed for total percentage of N. Similar results were obtained whether fractions were washed in buffer or distilled water before drying. To obtain pure thylakoids for N analysis, crude thylakoid preparations (0.5–1.0 μmol Chl) were layered over a centrifuge tube containing 30% Percol in washing buffer (25 mM Na-PO_4, 5 mM $MgCl_2$, pH 6.5) and centrifuged at 2,000*g* for 4 min and 4°C. The pure thylakoid bands situated about 1 cm from the top were sucked out and washed twice with the same washing buffer, then

suspended in distilled water. Subsamples with known Chl content were dried in tin cups at 80°C then analyzed for percentage of N.

Chl *a* Autofluorescence (Experiment 4)

Freshly hand cut, leaf cross sections were stained with 0.01 mg mL^{-1} propidium iodide to highlight cell walls, then mounted on a scanning confocal microscope (Leica SP2 LSCM; Leica Microsystems, Wetzlar, Germany). Sections were excited with weak laser beams at 488 nm for autofluorescence and 543 nm for the stain. Emission was collected between 690 to 740 nm and 550 to 620 nm for Chl *a* autofluorescence and the stain, respectively.

N Budget Calculation (Experiment 4) and Data Analysis

Leaf parameters were measured on area, Chl, and/or Rubisco basis. BS parameters were measured on a Chl and/or Rubisco basis. BS parameters (P) were converted to leaf area basis using the following formula (Rubisco exclusively located in BS):

$$\frac{[P]_{BS}}{[\text{Rubisco}]_{BS}} \times \frac{[\text{Rubisco}]_{\text{Leaf}}}{m^2}.$$

M proportion was calculated as the difference between leaf and BS fractions. Total N budgets for the BS and M were determined assuming that soluble protein (including Rubisco) contains 16% N by mass, molecular mass of 550,000g mol^{-1} for Rubisco, and that amino acids and nitrate contain 1 mol N per mol. Thylakoid N cost, T (mol N [mol Chl]$^{-1}$), was calculated according to Evans and Seemann (1989) and Hikosaka and Terashima (1995) from the following equation: T = [PSII] × 83.3 × 0.06 + [PSI] × 32.8 × 0.184 + [LHC] × 26 × 0.013 + [Cyt f] × 8.85, where [LHC] = (1,000 − [PSII] × 60 − [PSI] × 184)/13. [PSII], [PSI], [LHC], and [Cyt f] have the units mmol (mol Chl)$^{-1}$.

The pigment protein complexes have N costs of 83.3, 32.8, and 26 mol N (mol Chl)$^{-1}$ and bind 60, 184, and 13 mol Chl (mol complex)$^{-1}$ for PSII, PSI, and LHC, respectively. The N cost associated with Cyt f was 8.85 mol N (mol Cyt f)$^{-1}$.

The subtype effect (experiments 1 and 2) was calculated by two-way ANOVA (nested design, species nested in subtype) using a general linear model. In experiment 3, a three-way, nested ANOVA was used. Species and fraction effects (experiment 4) were analyzed by one-way ANOVA. For data presented in Tables II and V, a posthoc, Tukey HSD test was carried out on the grouped means.

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LITERATURE CITED

Agostino A, Furbank RT, Hatch MD (1989) Maximising photosynthetic activity and cell integrity in isolated bundle sheath cell strands from C_4 species. Aust J Plant Physiol **16:** 279–290

Bassi R, Marquardt J, Lavergne J (1995) Biochemical and functional properties of photosystem II in agranal membranes from maize mesophyll and bundle sheath chloroplasts. Eur J Biochem **233:** 709–719

Bolton J, Brown RH (1978) Effects of nitrogen nutrition on photosynthesis and associated characteristics in C_3, C_4 and intermediate grass species. Plant Physiol **61:** 38

Bowman WD (1991) Effect of nitrogen nutrition on photosynthesis and growth in C_4 *Panicum* species. Plant Cell Environ **14:** 295–301

Brown RH (1978) Difference in N use efficiency in C_3 and C_4 plants and its implications in adaptation and evolution. Crop Sci **18:** 93–98

Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal **6:** 71–80

Chow WS (1999) Grana formation: entropy-assisted local order in chloroplasts? Aust J Plant Physiol **26:** 641–647

Chow WS, Hope AB, Anderson JM (1989) Oxygen per flash from leaf disks quantifies photosystem II. Biochim Biophys Acta **973:** 105–108

Edwards GE, Huber SC, Ku SB, Rathnam CKM, Gutierrez M, Mayne BC (1976) Variation in photochemical activities of C_4 plants in relation to CO_2 fixation. *In* RH Burris, CC Black, eds, CO_2 Metabolism and Plant Productivity. University Park Press, Baltimore, pp 83–112

Edwards GE, Krall JP (1992) Metabolic interactions between organelles in C4 plants. *In* AK Tobin, ed, Plant Organelles. Cambridge University Press, Cambridge, pp 97–112

Ellis RP, Vogel JC, Fuls A (1980) Photosynthetic pathways and the geographical distribution of grasses in South West Africa/Namibia. S Afr J Sci **76:** 307–314

Evans JR (1983) Nitrogen and photosynthesis in the flag leaf of wheat (*Triticum aestivum* L). Plant Physiol **72:** 297–302

Evans JR (1987) The relationship between electron transport components and photosynthetic capacity in pea leaves grown at different irradiances. Aust J Plant Physiol **14:** 157–170

Evans JR, Poorter H (2001) Photosynthetic acclimation of plants to growth irradiance: the relative importance of specific leaf area and nitrogen partitioning in maximizing carbon gain. Plant Cell Environ **24:** 755–767

Evans JR, Seemann JR (1989) The allocation of nitrogen in the photosynthetic apparatus: costs, consequences and control. *In* WR Briggs, ed, Photosynthesis. Alan R. Liss, New York, pp 183–205

Evans JR, Terashima I (1987) Effects of nitrogen nutrition on electron transport components and photosynthesis in spinach. Aust J Plant Physiol **14:** 59–68

Evans JR, von Caemmerer S (2000) Would C_4 rice produce more biomass than C_3 rice? *In* B Hardy, ed, Redesigning Rice Photosynthesis to Increase Yield. International Rice Research Institute and Elsevier Science B.V., Amsterdam, pp 53–71

Field CB, Mooney HA (1986) The photosynthetic-nitrogen relationship in wild plants. *In* TJ Givinish, ed, On the Economy of Form and Function. Cambridge University Press, Cambridge, pp 25–55

Ghannoum O, Conroy JP (1998) Nitrogen deficiency precludes a growth response to CO_2 enrichment in C_3 and C_4 *Panicum* grasses. Aust J Plant Physiol **25:** 627–636

Ghannoum O, von Caemmerer S, Barlow EWR, Conroy JP (1997) The effect of CO_2 enrichment and irradiance on the growth, morphology and gas exchange of a C_3 (*Panicum laxum*) and a C_4 (*Panicum antidotale*) grass. Aust J Plant Physiol **24:** 227–237

Ghannoum O, von Caemmerer S, Conroy JP (2001) Plant water use efficiency of 17 Australian NAD-ME and NADP-ME C_4 grasses at ambient and elevated CO_2 partial pressure. Aust J Plant Physiol **28:** 1207–1217

Ghannoum O, von Caemmerer S, Conroy JP (2002) The effect of drought on plant water use efficiency of nine NAD-ME and nine NADP-ME Australian C_4 grasses. Funct Plant Biol **29:** 1337–1348

Ghirardi ML, Melis A (1984) Photosystem electron transport capacity and light harvesting antenna size in maize chloroplasts. Plant Physiol **74:** 993–998

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta **895:** 81–106

Hatch MD, Osmond CB (1976) Compartmentation and transport in C_4 photosynthesis. *In* CR Stocking, U Heber, eds, Encyclopedia of Plant Physiology, Vol 3. Springer-Verlag, Berlin, pp 144–184

Hattersley PW (1992) C_4 photosynthetic pathway variation in grasses (Poaceae): its significance for arid and semi-arid lands. *In* GP Chapman, ed, Desertified Grasslands: Their Biology and Management. Academic Press, London, pp 181–212

Hikosaka K, Terashima I (1995) A model of the acclimation of photosynthesis in the leaves of C_3 plants to sun and shade with respect to nitrogen use. Plant Cell Environ **18:** 605–618

Jenkins CLD, Boag S (1985) Isolation of bundle sheath cell chloroplasts from the NADP-ME type C_4 plant *Zea mays*. Capacities for CO_2 assimilation and malate decarboxylation. Plant Physiol **79:** 84–89

Kanai R, Edwards GE (1999) The biochemistry of C_4 photosynthesis. *In* RF Sage, RK Monson, eds, C_4 plant biology. Academic Press, San Diego, pp 49–87

Kane HJ, Viil J, Entsch B, Paul K, Morell MK, Andrews TJ (1994) An improved method for measuring the CO_2/O_2 specificity of ribulose bisphosphate carboxylase-oxygenase. Aust J Plant Physiol **21:** 449–461

Leegood RC, von Caemmerer S (1988) The relationship between contents of photosynthetic metabolites and the rate of photosynthetic carbon assimilation in leaves of *Amaranthus edulis* L. Planta **174:** 253–262

Leegood RC, von Caemmerer S (1989) Some relationships between contents of photosynthetic intermediates and the rate of photosynthetic carbon assimilation in leaves of *Zea mays* L. Planta **178:** 258–266

Makino A, Sakuma H, Sudo E, Mae T (2003) Differences between maize and rice in N-use efficiency for photosynthesis and protein allocation. Plant Cell Physiol **44:** 952–956

Makino A, Sato T, Nakano H, Mae T (1997) Leaf photosynthesis, plant growth and nitrogen allocation in rice under different irradiances. Planta **203:** 390–398

Mayne BC, Dee AM, Edwards GE (1975) Photosynthesis in mesophyll protoplasts and bundle sheath cells of various type of C4 plants. III. Fluorescence emission spectra, delayed light emission, and P700 content. Z Pflanzenphysiol **74:** 275–291

Meierhoff K, Westhoff P (1993) Differential biogenesis of photosystem II in mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme type C_4 plants: the nonstoichiometric abundance of the subunits of photosystem II in the bundle-sheath chloroplasts and the translational activity of the plastome-encoded genes. Planta **191:** 23–33

Moore S (1968) Amino acid analysis: aqueous dimethyl sulfoxide as solvent for ninhydrin reaction. J Biol Chem **243:** 6281–6283

Morell MK, Paul K, Kane HJ, Andrews TJ (1992) Rubisco: maladapted or misunderstood? Aust J Bot **40:** 431–441

Poorter H, Evans JR (1998) Photosynthetic nitrogen-use efficiency of species that differ inherently in specific leaf area. Oecologia **116:** 26–37

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the chlorophyll standards by atomic absorption spectroscopy. Biochim Biophys Acta **975:** 384–394

Ruuska SA, Andrews TJ, Badger MR, Hudson GS, Laisk A, Price GD, von Caemmerer S (1998) The interplay between limiting processes in C_3 photosynthesis studied by rapid-response gas exchange using transgenic tobacco impaired in photosynthesis. Aust J Plant Physiol **25:** 859–870

Ruuska SA, Andrews TJ, Badger MR, Price GD, von Caemmerer S (2000) The role of chloroplast electron transport and metabolites in modulating Rubisco activity in tobacco. Insights from transgenic plants with reduced amounts of cytochrome b/f complex or glyceraldehyde 3-phosphate dehydrogenase. Plant Physiol **122:** 491–504

Sage RF (2002) Variation in the k_{cat} of Rubisco in C_3 and C_4 plants and some implications for photosynthetic performance at high and low temperature. J Exp Bot **53:** 609–620

Sage RF, Pearcy RW, Seemann JR (1987) The nitrogen use efficiency of C_3 and C_4 plants. III. Leaf nitrogen effects on the activity of carboxylating enzymes in *Chenopodium album* (L.) and *Amaranthus retroflexus* (L.). Plant Physiol **85:** 355–359

Schmitt MR, Edwards GE (1981) Photosynthetic capacity and nitrogen use efficiency of maize, wheat, and rice: a comparison between C_3 and C_4 photosynthesis. J Exp Bot **32:** 459–466

Seemann JR, Badger MR, Berry JA (1984) Variations in the specific activity of ribulose-1,5-bisphosphate carboxylase between species utilizing differing photosynthetic pathways. Plant Physiol **74:** 791–794

Taub DR, Lerdau MT (2000) Relationship between leaf nitrogen and photosynthetic rate for three NAD-ME and three NADP-ME C_4 grasses. Am J Bot **87:** 412–417

Terashima I, Evans JR (1988) Effects of light and nitrogen nutrition on the organization of the photosynthetic apparatus in spinach. Plant Cell Physiol **29:** 143–155

von Caemmerer S (2000) Biochemical Models of Leaf Photosynthesis. CSIRO Publishing, Melbourne

von Caemmerer S, Quick WP (2000) Rubisco: physiology in vivo. *In* RC Leegood, TD Sharkey, S von Caemmerer, eds, Photosynthesis: Physiology and Metabolism, Vol 9. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 85–113

Wessinger ME, Edwards GE, Ku MSB (1989) Quantity and kinetic properties of ribulose 1,5-bisphosphate carboxylase in C_3, C_4, and C_3-C_4 intermediate species of *Flaveria* (Asteraceae). Plant Cell Physiol **30:** 665–671

Whitney SM, Baldett P, Hudson GS, Andrews TJ (2001) Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. Plant J **26:** 535–547

Whitney SM, von Caemmerer S, Hudson GS, Andrews TJ (1999) Directed mutation of the Rubisco large subunit of tobacco influences photorespiration and growth. Plant Physiol **121:** 579–588

Yeoh HH, Badger MR, Watson L (1980) Variations in $k_m(CO_2)$ of ribulose-1,5-bisphosphate carboxylase among grasses. Plant Physiol **66:** 1110–1112

Yeoh HH, Badger MR, Watson L (1981) Variations in kinetic properties of ribulose-1,5-bisphosphate carboxylases among plants. Plant Physiol **67:** 1151–1155

Zhu XG, Portis AR, Long SP (2004) Would transformation of C_3 crop plants with foreign Rubisco increase productivity? A computational analysis extrapolating from kinetic properties to canopy photosynthesis. Plant Cell Environ **27:** 155–165