Annals of Botany **97**: 611–621, 2006
doi:10.1093/aob/mcl010, available online at www.aob.oxfordjournals.org

Leaf Vascular Systems in C_3 and C_4 Grasses: A Two-dimensional Analysis

OSAMU UENO[1],*, YUKIKO KAWANO[2], MASATAKA WAKAYAMA[1],† and TOMOSHIRO TAKEDA[2]

[1]*Plant Physiology Department, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8602, Japan and*
[2]*Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka, 812-0053, Japan*

Received: 13 September 2005 Returned for revision: 20 October 2005 Accepted: 6 December 2005 Published electronically: 7 February 2006

- *Background and Aims* It is well documented that C_4 grasses have a shorter distance between longitudinal veins in the leaves than C_3 grasses. In grass leaves, however, veins with different structures and functions are differentiated: large longitudinal veins, small longitudinal veins and transverse veins. Thus, the densities of the three types of vein in leaves of C_3 and C_4 grasses were investigated from a two-dimensional perspective.
- *Methods* Vein densities in cleared leaves of 15 C_3 and 26 C_4 grasses representing different taxonomic groups and photosynthetic subtypes were analysed.
- *Key Results* The C_4 grasses had denser transverse veins and denser small longitudinal veins than the C_3 grasses (1·9 and 2·1 times in interveinal distance), but there was no significant difference in large longitudinal veins. The total length of the three vein types per unit area in the C_4 grasses was 2·1 times that in the C_3 grasses. The ratio of transverse vein length to total vein length was 14·3 % in C_3 grasses and 9·9 % in C_4 grasses. The C_3 grasses generally had greater species variation in the vascular distances than the C_4 grasses. The bambusoid and panicoid C_3 grasses tended to have a denser vascular system than the festucoid C_3 grasses. There were no significant differences in the interveinal distances of the three vein types between C_4 subtypes, although the NADP-malic enzyme grasses tended to have a shorter distance between small longitudinal veins than the NAD-malic enzyme and phospho*enol*pyruvate carboxykinase grasses.
- *Conclusions* It seems that C_4 grasses have structurally a superior photosynthate translocation and water distribution system by developing denser networks of small longitudinal and transverse veins, while keeping a constant density of large longitudinal veins. The bambusoid and panicoid C_3 grasses have a vascular system that is more similar to that in C_4 grasses than to that in the festucoid C_3 grasses.

Key words: C_3 and C_4 photosynthesis, interveinal distance, longitudinal vein, photosynthetic type, Poaceae, transverse vein.

INTRODUCTION

The leaves of C_4 plants have different anatomical features from those of C_3 plants. Usually, C_4 leaves are characterized by Kranz-type anatomy, in which the vascular bundle is surrounded by organelle-rich bundle sheath (BS) cells, and this tissue layer is further surrounded by radially arranged mesophyll (M) cells. In contrast, in C_3 leaves, the M cells are well developed relative to the BS cells, which include only a few organelles (Dengler and Nelson, 1999). In C_4 photosynthesis, atmospheric CO_2 is initially fixed in the M cells, then decarboxylation and refixation of CO_2 occur in the BS cells (Hatch, 1987).

C_4 plants are divided into three C_4 subtypes differing in the process of decarboxylation of C_4 acids: the NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME) and phospho*enol*pyruvate carboxykinase (PCK) types (Hatch, 1987). The difference in biochemical function is associated with that in structural features of leaves. In C_4 grasses, in general, the NADP-ME grasses have the BS that originated from the mestome sheath, whereas both the NAD-ME and PCK grasses have the BS that originated from the parenchyma sheath (Dengler and Nelson, 1999). The BS cells of the C_4 subtypes also differ in the structure, intracellular position and amount of the chloroplasts and mitochondria (Hatch, 1987; Prendergast *et al*., 1987; Yoshimura *et al*., 2004). The quantitative balance of photosynthetic tissues (Hattersley, 1984; Ohsugi and Murata, 1986; Dengler *et al*., 1994) and organelles (Yoshimura *et al*., 2004) between the M and BS cells reflects the difference in biochemical function of the photosynthetic subtypes.

In the early stage of C_4 plant studies, it was reported that C_4 leaves have a denser vascular system than C_3 leaves (Takeda and Fukuyama, 1971). This is clearly seen in leaves of grasses, which possess parallel venation. This distinctive difference between C_3 and C_4 leaves is usually expressed as a difference in the interveinal distance (distance between vein centres) (Takeda and Fukuyama, 1971; Crookston and Moss, 1974). For the efficient operation of C_4 photosynthesis, a short distance between the M and BS cells is a prerequisite for rapid diffusion of photosynthetic metabolites (Hatch, 1987). The proximity of veins may also be needed for the proper expression of photosynthetic enzymes in the M and BS cells (Langdale and Nelson, 1991; but see Wakayama *et al*., 2003).

C_4 plants have higher photosynthetic rates under high irradiance and at high temperatures than C_3 plants (Ehleringer and Monson, 1993). In general, greater photosynthetic rates would result in greater rates of photosynthate export, to remove recently formed photosynthate from leaves rapidly and so avoid end-product inhibition of photosynthesis (Roth-Nebelsick *et al*., 2001). C_4 plants have been reported to show higher export rates of photosynthate

* For correspondence. E-mail uenoos@nias.affrc.go.jp

† Permanent address: Department of Biochemistry and Molecular Biology, Saitama University, Saitama 338-8570, Japan

than C_3 plants (Hofstra and Nelson, 1969; Gallaher *et al*., 1975; Lush, 1976; Grodzinski *et al*., 1998; Leonardos and Grodzinski, 2000). In support of higher rates of translocation, some researchers have pointed to the denser vascular system (Crookston and Moss, 1974) and larger cross-sectional area of phloem (Gallaher *et al*., 1975) in C_4 leaves. The vascular bundle is composed of two kinds of conducting tissues: the xylem and phloem. Thus, it appears that C_4 leaves have a denser hydraulic network than C_3 leaves.

The CO_2-concentrating mechanism of the C_4 pathway gives C_4 plants an efficient photosynthetic mechanism under low stomatal conductance and thus a higher water use efficiency and photosynthetic ability under environments of low water availability than is the case for C_3 plants (Ehleringer and Monson, 1993; Sage, 2004). It is generally accepted that C_4 plants evolved from C_3 plants, accompanied by modifications to anatomical and biochemical features of leaves. A change in vein density of leaves undoubtedly occurred during the evolution from C_3 to C_4 plants (Sage, 2004; Ueno and Sentoku, 2006).

Leaf veins show a hierarchical order and have different structures. In grass leaves, the differentiation of transverse and longitudinal veins of different sizes allows a division of labour (Yamazaki, 1960; Lush, 1976; Altus and Canny, 1982, 1985; Altus *et al*., 1985; Fritz *et al*., 1989). The large longitudinal veins run from the leaf blade into the sheath. However, most of the small longitudinal veins in the leaf blade terminate at the junction of the blade and sheath (Chonan *et al*., 1974; Colbert and Evert, 1982; Russell and Evert, 1985; Dannenhoffer and Evert, 1994). The large longitudinal veins serve primarily in longitudinal transport of photosynthate outside the leaf blade. The small longitudinal veins serve primarily in collecting photosynthate from nearby photosynthetic cells. The transverse veins connect the longitudinal veins, and play important roles in the lateral transport of photosynthate from the small to the large longitudinal veins (Altus and Canny, 1982).

Water moves in the three types of vein opposite to the direction of photosynthate. The network system for water movement matches the structural demands of an efficient irrigation system (Pelletier and Turcotte, 2000; Roth-Nebelsick *et al*., 2001). Water absorbed in the root rises through the large longitudinal veins from the leaf base to the tip of the leaf blade. In the leaf blade, water moves laterally from the large longitudinal veins via the transverse veins to the small longitudinal veins, and is distributed to the M or transpired from stomata (Altus and Canny, 1985; Altus *et al*., 1985; Canny, 1990).

In order to relate the vascular architecture to physiological functions of the leaves, we need to consider the localization and functional partitioning of different types of veins. To our knowledge, however, most previous studies of the vascular density of C_3 and C_4 grass leaves have neglected vein types: only the distance between longitudinal veins has been measured (Takeda and Fukuyama, 1971; Crookston and Moss, 1974; Kawamitsu *et al*., 1985; Dengler *et al*., 1994). Exceptionally, Oguro *et al*. (1985) investigated the densities of transverse veins in leaves of some *Panicum* species.

Here, we analysed the densities of the three types of vein in leaves of various C_3 and C_4 grasses from a two-dimensional perspective. This family includes all three C_4 subtypes and has been investigated sufficiently for the photosynthetic types (Hattersley and Watson, 1992; GPWG, 2001). In addition, C_3 grasses consist of phylogenetically different groups such as the festucoid, bambusoid and panicoid grasses, which differ in their temperature requirements for growth. Thus, this family provides an ideal subject for comparative analysis of leaf vascular systems in plants that differ in photosynthetic types and ecological characteristics.

MATERIALS AND METHODS

Plant materials

Table 1 lists the grass species examined in this study: 15 C_3 species and 26 C_4 species. We divided the C_3 grasses into two subgroups depending on growth and flowering period: the panicoid and bambusoid C_3 grasses and the festucoid C_3 grasses. The bambusoid and panicoid C_3 grasses grow in summer, and the flowering period is late summer to early autumn. The festucoid C_3 grasses grow in spring, and the flowering period is late spring. Taxonomically, the panicoid, bambusoid and festucoid grasses belong to the subfamilies Panicoideae, Bambusoideae and Pooideae, respectively (Clayton and Renvoize, 1992). The C_4 species were divided into three subgroups depending on the C_4 biochemical subtypes: eight NADP-ME-type species, nine NAD-ME-type species and nine PCK-type species. All NADP-ME-type grasses, the three *Panicum* species in the NAD-ME-type grasses, and the four *Brachiaria* species, *Panicum maximum* and *Urochloa texana* in the PCK-type grasses belong to the subfamily Panicoideae. The other grasses of the NAD-ME and PCK types belong to the subfamily Chloridoideae (Clayton and Renvoize, 1992).

Seeds of 13 species (four *Brachiaria* species, *Chloris gayana*, *Eleusine coracana*, *Oryza sativa*, *Panicum coloratum*, *P. dichotomiflorum*, *P. maximum*, *P. miliaceum*, *Sorghum sudanense* and *Urochloa texana*) were sown in pots filled with fertilized field soil, and plants were raised outdoors in Tsukuba and Fukuoka, Japan, in summer. The plants were watered daily. Plants of the remaining 29 species growing naturally in the field in Fukuoka and Tsukuba were used.

Leaf samples were collected in May for the festucoid C_3 grasses and in August to September for the panicoid and bumbsoid C_3 grasses and all C_4 grasses. No significant differences in the interveinal distances occur among different leaf positions in rice plants, except that those in the primary leaves are somewhat smaller (Yamazaki, 1963). In barley leaves, the flag leaf and the first and second leaves below it tend to have similar leaf vein densities (Hanson and Rasmusson, 1975). Therefore, we used either flag leaves or the first leaves below the flag leaves for our experiments. Leaf blades were cut and immediately fixed in a mixture of formaldehyde, acetic acid and ethanol in water (FAA). The plants collected in the field were also retained as voucher specimens for exact identification.

TABLE 1. *The C_3 and C_4 grass species examined in this study, which are divided according to photosynthetic types and phylogenetic groups*

NADP-ME	NAD-ME	PCK
(a) C_4 species		
Panicoid	Panicoid	Panicoid
Digitaria sanguinalis (L.) Scopoli	*Panicum coloratum* L. var. *makarikariense* Goossens	*Brachiaria brizantha* (Hochst. Ex A. Rich) Stapf
D. violascens Link	*P. dichotomiflorum* Michaux	*B. decumbens* Stapf
Echinochloa crus-galli P. Beauv.	*P. miliaceum* L.	*B. humidicola* (Rendle) Schweick.
Paspalum distichum L.	Chloridoid	*B. mutica* (Forsk.) Stapf
Setaria glauca (L.) P. Beauv.	*Cynodon dactylon* (L.) Persoon	*Panicum maximum* Jacq.
S. viridis (L.) P. Beauv. var *minor* (Thunb.) Ohwi	*Eleusine coracana* (L.) Gaertner	*Urochloa texana* (Buckley) Webster
Sorghum sudanense Stapf	*E. indica* (L.) Gaertner	Chloridoid
Spodiopogon cotulifer (Thunb.) Hackel	*Eragrostis cilianensis* (Allioni) Vignolo-Lutati	*Chloris gayana* Kunth
	E. ferruginea (Thunb.) P. Beauv.	*Sporobolus indicus* R. Br. var. *purpureosuffusus* (Ohwi) T. Koyama
	Leptochloa chinensis (L.) Nees	*Zoysia tenuifolia* Willd.
Bambusoid	Panicoid	Festucoid
(b) C_3 species		
Leersia japonica Makino	*Hymenachne indica* Buse	*Agropyron tsukushiense* (Honda) Ohwi var. *transiens* (Hackel) Ohwi
Oryza sativa L. cv. Reiho	*Isachne globosa* O. Kuntze	*Alopecurus aequalis* Sobol. var. *amurensis* (Komar.) Ohwi
	Panicum bisulcatum Thunberg	*Avena fatua* L.
		Beckmannia syzigachne (Steud.) Fernald
		Briza minor L.
		Bromus catharticus Vahl
		B. rigidus Roth
		Dactylis glomerata L.
		Lolium multiflorum Lam.
		Poa acroleuca Steud.

Preparation of cleared leaves

Cleared leaf blades were prepared by a method described in Ueno (1995). The middle portions of fixed leaf blades were boiled in 70 % ethanol for about 10–20 min. After washing in distilled water several times, they were transferred to boiling 85 % lactic acid for 20 min, and then stored in chloral hydrate-saturated ethanol before analysis. The leaf vasculature was observed without staining under a light microscope.

Quantitative data of leaf vascular systems

Leaf veins were divided into three types: large longitudinal veins, small longitudinal veins and transverse veins (Chonan *et al*., 1974). The two types of longitudinal vein were distinguished by diameter in paradermal view under the light microscope (Fig. 1).

The distances between small longitudinal veins, between large longitudinal veins and between transverse veins were represented by means of 30 measurements of middle portions of 3–6 leaf blades taken from three plants. The distance between longitudinal veins was measured between the centres of adjacent veins. The transverse veins are usually curved, unlike the parallel longitudinal veins (Fig. 1). For the distance between transverse veins, therefore, the mean of the minimum and maximum distances between adjacent transverse veins running between a pair of longitudinal veins was calculated. The distance between small longitudinal veins was multiplied by that between transverse veins for each species to indicate areolar area, which represents the minimum area of photosynthetic tissue surrounded by veins.

The number of transverse veins per unit leaf area was measured on photomicrographs (70×) obtained from the middle portions of the 3–6 leaf blades. The lengths of longitudinal and transverse veins per unit leaf area were measured with a curvimeter on the same photomicrographs. The total vein length per unit leaf area and the ratio of transverse vein length to total vein length were calculated using these values.

Statistical analysis

We tested the significance at $P < 0{\cdot}05$ of any differences in mean values generated for each species between the C_4 and C_3 groups and between the five subgroups (the NADP-ME, the NAD-ME, the PCK, the C_3 bambusoids and panicoids, and the C_3 festucoids) using one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test (statistical software R 2.1.0, R Foundation of Statistical Computing, 2005). To test phylogenetic differences in the C_4 grasses, we also analysed any differences between the C_4 panicoids, the C_4 chloridioids, the C_3 bambusoids and panicoids, and the C_3 festucoids using the same statistical test.

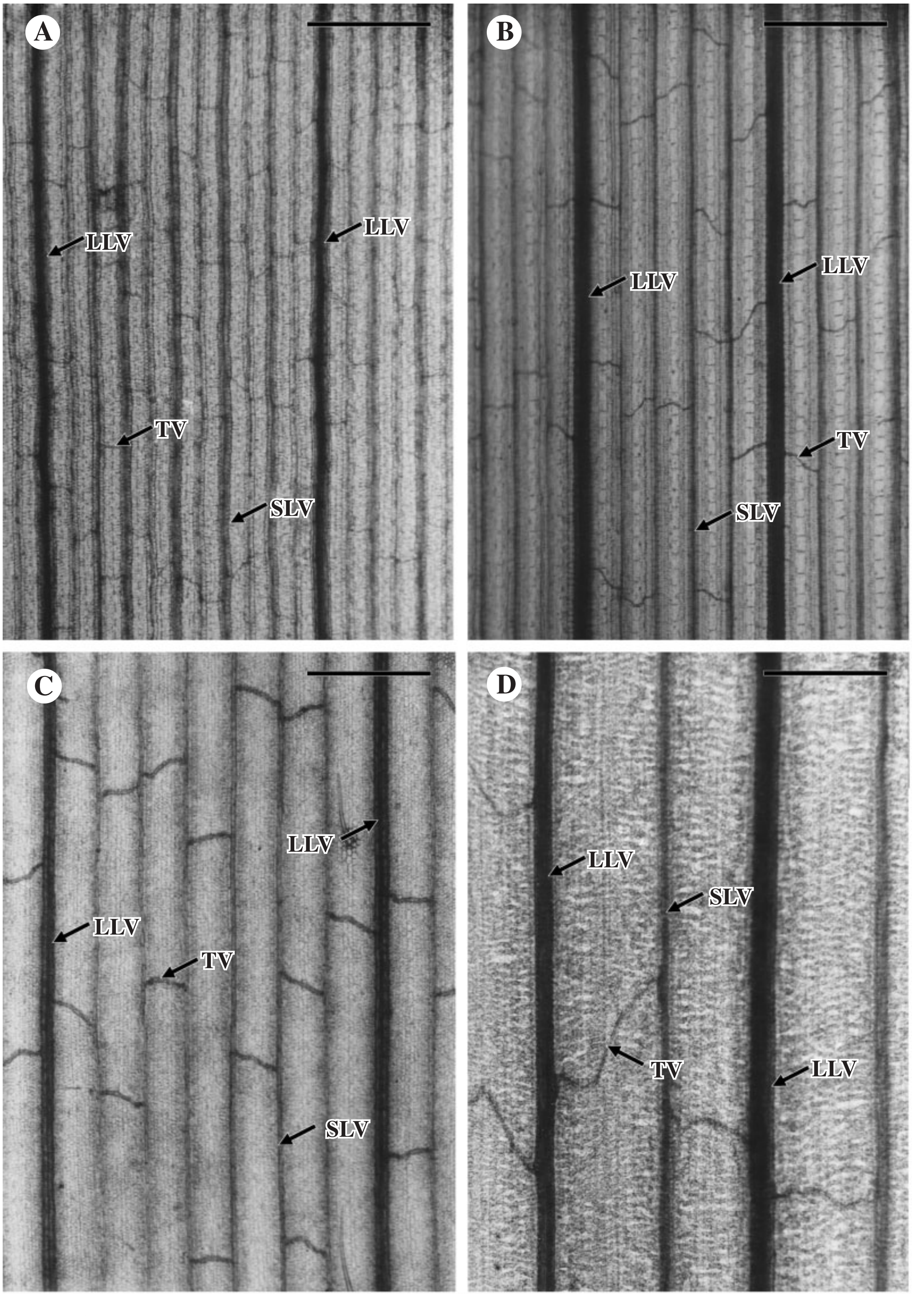

FIG. 1. Paradermal view of cleared leaf blades of C_3 and C_4 grasses. (A) *Digitaria sanguinalis*, an NADP-ME C_4 species. (B) *Eleusine indica*, an NAD-ME C_4 species. (C) *Panicum bisulcatum*, a panicoid C_3 species. (D) *Briza minor*, a festucoid C_3 species. LLV, large longitudinal vein; SLV, small longitudinal vein; TV, transverse vein. The magnification of the four photomicrographs is the same. Scale bars = 250 µm.

RESULTS

General features of leaf vascular system in grasses

In the paradermal view of cleared leaf blades, longitudinal veins run at regular intervals in parallel (Fig. 1). The size difference and the existence of strands of hypodermal sclerenchyma allowed the large longitudinal veins to be distinguished from the small longitudinal veins. The transverse veins connected two adjacent longitudinal veins, irrespective of size. The pattern of connection varied even

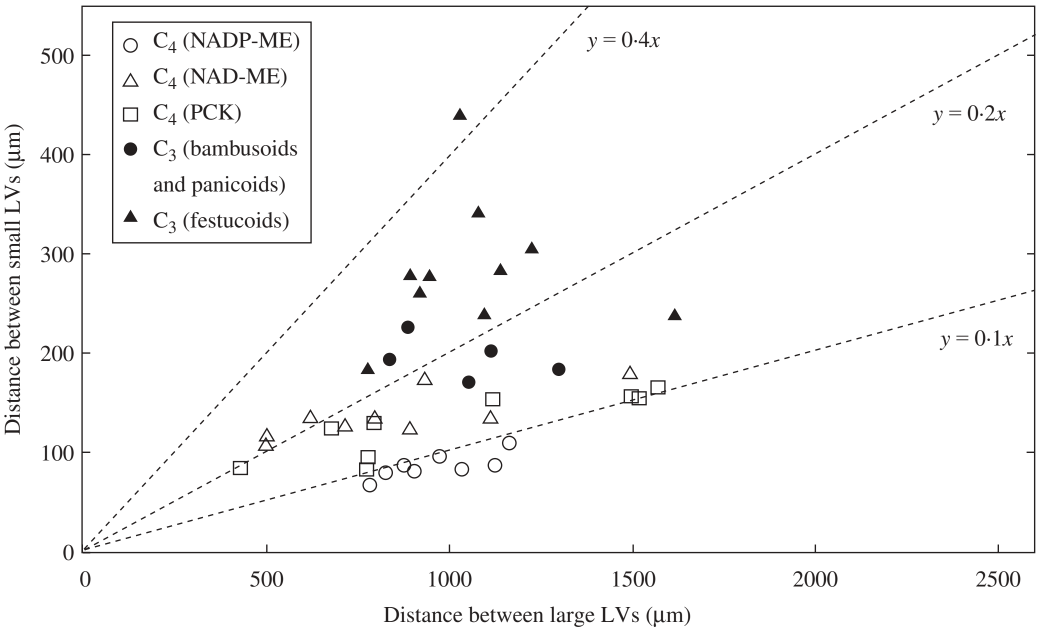

FIG. 2. Relationship of the distance between large longitudinal veins and that between small longitudinal veins in leaf blades of C_3 and C_4 grasses. Slopes of dotted lines show the ratio of the distance between small longitudinal veins (y) to that between large longitudinal veins (x). LV, longitudinal vein.

within a leaf: at a variety of angles and from straight to curved.

Interveinal distances of longitudinal and transverse veins

Figure 2 shows the relationship between large longitudinal veins and small longitudinal veins in the C_3 and C_4 grasses. The C_4 grasses had a significantly shorter distance between small longitudinal veins than the C_3 grasses: the mean value of the C_3 grasses was 2·2 times that of the C_4 grasses (Table 2; $P < 0{\cdot}05$). Within the C_4 grasses, the distance between small longitudinal veins in the NADP-ME grasses was shorter than those in the NAD-ME and PCK grasses (Table 2), although the difference was not significant. Within the C_3 grasses, there was a significant difference between the bambusoid and panicoid C_3 grasses and the festucoid C_3 grasses (Table 2). There was also a significant difference between the festucoid C_3 grasses and the three C_4 groups. When the C_4 grasses were compared between the phylogenetic groups, there was no significant difference between the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

With respect to the distance between large longitudinal veins (Fig. 2), the mean value of the C_3 grasses was 1·1 times that of the C_4 grasses, but this difference was not significant (Table 2). Likewise, there were no significant differences between the five subgroups (Table 2). No correlation was found between the density of large longitudinal veins and that of small longitudinal veins in the C_3 grasses ($r = 0{\cdot}045$, NS). Low positive correlations were found between the two densities in all grasses examined ($r = 0{\cdot}319$, $P < 0{\cdot}05$) and in the C_4 grasses ($r = 0{\cdot}526$, $P < 0{\cdot}01$). On the other hand, there were high positive correlations between the two densities in the NADP-ME ($r = 0{\cdot}773$, $P < 0{\cdot}05$), NAD-ME ($r = 0{\cdot}794$, $P < 0{\cdot}05$) and PCK ($r = 0{\cdot}864$, $P < 0{\cdot}01$) grasses. The ratio of the distance between small longitudinal veins to that between large longitudinal veins tended to be lower in C_4 grasses than in C_3 grasses (Fig. 2). Between the panicoid and chloridoid C_4 grasses, there was a tendency that chloridoid C_4 grasses had a somewhat shorter distance between large longitudinal veins than the panicoid C_4 grasses (Table 2).

Figure 3 shows the relationship between transverse veins and small longitudinal veins in the C_3 and C_4 grasses. There was a significant difference in the distance between transverse veins between the C_3 and C_4 grasses: the mean value of the C_3 grasses was 1·9 times that of the C_4 grasses (Table 2; $P < 0{\cdot}05$). The only significant difference between subgroups was that between the festucoid C_3 grasses and the other four subgroups as a whole (Table 2); the mean value was 2·0 times that of the bambusoid and panicoid C_3 grasses (Table 2). The values of all the bambusoid and panicoid C_3 grasses except *Hymenachne indica* (1491 µm) were comparable with those of C_4 grasses (Fig. 3). The bambusoid C_3 grass *O. sativa* showed the shortest distance between transverse veins (481 µm) among all grass species examined (Fig. 3). The festucoid C_3 grass *Bromus rigidus* showed the greatest distance (2574 µm; Fig. 3). There was a positive correlation between the distance between small longitudinal veins and that between transverse veins in all grasses (Fig. 3; $r = 0{\cdot}744$, $P < 0{\cdot}05$). There was no significant difference in the distance between transverse veins in the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

The mean value of the areolar area (the minimum area of photosynthetic tissue surrounded by veins) of the C_3 grasses was 4·3 times that of the C_4 grasses (Table 2; $P < 0{\cdot}05$). In the five subgroups, the festucoid C_3 grasses

TABLE 2. *Means of various measures of vascular density in the leaf blades of grasses of different groups and subgroups*

Group and subgroup	No. of species	Distance between small LVs (A) (μm)	Distance between large LVs (μm)	Distance between TVs (B) (μm)	Areolar area (A × B) ($\times 10^{-3}$ mm^2)	Total vein length per unit leaf area (mm mm^{-2})	Ratio of TV length to total vein length (%)	No. of TV per unit leaf area (mm^{-2})
C_4	26	118·5 ± 6·4^{a}	938·4 ± 62·2^{a}	765·6 ± 29·8^{a}	91·5 ± 6·5^{a}	[†]10·6 ± 0·6^{a}	[†]9·9 ± 0·6^{a}	9·8 ± 0·7^{a}
C_3	15	255·5 ± 18·5^{b}	1059·6 ± 54·5^{a}	1460·0 ± 154·5^{b}	395·3 ± 56·1^{b}	5·1 ± 0·3^{b}	14·3 ± 1·1^{b}	3·0 ± 0·6^{b}
NADP-ME	8	86·3 ± 4·3^{a}	960·5 ± 48·8^{a}	761·6 ± 54·1^{a}	64·3 ± 2·5^{a}	14·3 ± 0·7^{a}	7·2 ± 1·4^{a}	13·0 ± 2·6^{a}
NAD-ME	9	138·2 ± 7·9^{ab}	840·2 ± 106·0^{a}	725·9 ± 30·6^{a}	100·8 ± 7·7^{a}	8·9 ± 0·4^{b}	10·6 ± 2·4^{ab}	8·0 ± 1·7^{b}
PCK	9	127·3 ± 11·0^{a}	1017·0 ± 140·8^{a}	808·8 ± 66·0^{a}	105·1 ± 14·4^{a}	[‡]8·7 ± 0·8^{bc}	[‡]11·8 ± 1·4^{ab}	8·8 ± 1·2^{b}
C_3 bambusoids and panicoids	5	195·0 ± 9·4^{b}	1037·2 ± 82·7^{a}	883·6 ± 168·5^{a}	176·5 ± 42·3^{a}	6·1 ± 0·3^{cd}	15·0 ± 1·9^{b}	5·6 ± 0·9^{b}
C_3 festucoids	10	285·8 ± 21·8^{c}	1070·8 ± 73·3^{a}	1748·0 ± 147·1^{b}	504·7 ± 54·3^{b}	4·5 ± 0·3^{d}	14·0 ± 1·4^{b}	1·8 ± 0·3^{c}
C_4 panicoids	17	115·4 ± 8·6^{a}	1049·8 ± 75·7^{ab}	782·2 ± 42·6^{a}	90·6 ± 9·1^{a}	11·2 ± 0·9^{ab}	9·8 ± 0·9^{ab}	10·2 ± 0·9^{ab}
C_4 chloridoids	9	124·3 ± 9·4^{a}	728·0 ± 69·8^{ac}	734·1 ± 31·1^{a}	92·0 ± 8·8^{a}	[‡]9·2 ± 0·6^{abc}	[‡]10·2 ± 0·9^{abc}	9·1 ± 0·9^{abc}
C_3 bambusoids and panicoids	5	195·0 ± 9·4^{b}	1037·2 ± 82·7^{abc}	883·6 ± 168·5^{a}	176·5 ± 42·3^{a}	6·1 ± 0·3^{bcd}	15·0 ± 1·9^{ac}	5·6 ± 0·9^{bcd}
C_3 festucoids	10	285·8 ± 21·8^{c}	1070·8 ± 73·3^{ab}	1748·0 ± 147·1^{b}	504·7 ± 54·3^{b}	4·5 ± 0·3^{cd}	14·0 ± 1·4^{ac}	1·8 ± 0·3^{cd}

Values are given as mean ± s.e.

[†] $n = 25$ species; [‡] $n = 8$ species.

Values followed by the same letter are not significantly different at $P < 0·05$.

LV, longitudinal vein; TV, transverse vein.

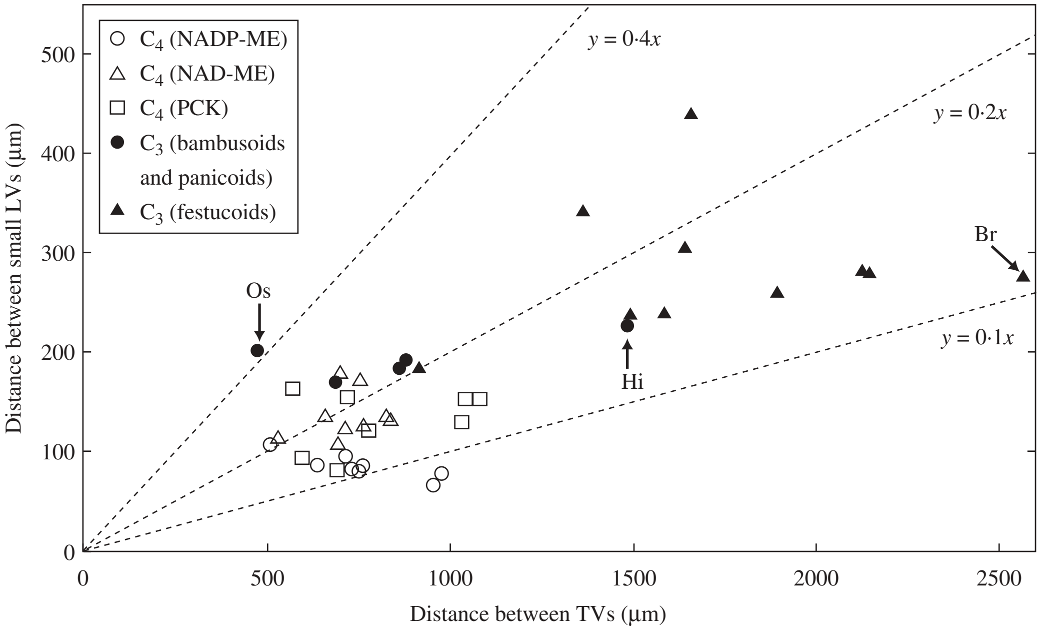

FIG. 3. Relationship of the distance between transverse veins and that between small longitudinal veins in leaf blades of C_3 and C_4 grasses. Slopes of dotted lines show the ratio of the distance between small longitudinal veins (y) to that between transverse veins (x). LV, longitudinal vein; TV, transverse vein; Br, *Bromus rigidus*; Hi, *Hymenachne indica*; Os, *Oryza sativa*.

had a significantly higher value than the other four subgroups (Table 2). Exceptionally, the festucoid C_3 grass *Dactylis glomerata* showed a low value (170×10^{-3} mm^2), whereas the panicoid C_3 grass *Hymenachne indica* showed a high value (337×10^{-3} mm^2; Fig. 4). Although there was no statistical difference between the other four subgroups, the mean value was lowest in the NADP-ME grasses and highest in the bambusoid and panicoid C_3 grasses (Table 2). There was no significant difference in the mean value between the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

Vein densities per unit leaf area

Several measures of leaf vein densities were calculated (Table 2). The mean value of total vein length per unit leaf area in C_4 grasses was 2·1 times that in C_3 grasses (Table 2). In the five subgroups, it was significantly higher in the NADP-ME grasses than in the other four subgroups (Table 2; Fig. 5). There was no significant difference between the NAD-ME and PCK grasses, between the PCK and the bambusoid and panicoid C_3 grasses, or between the bambusoid and panicoid C_3 and the festucoid C_3 grasses (Table 2). There was no significant difference in the mean

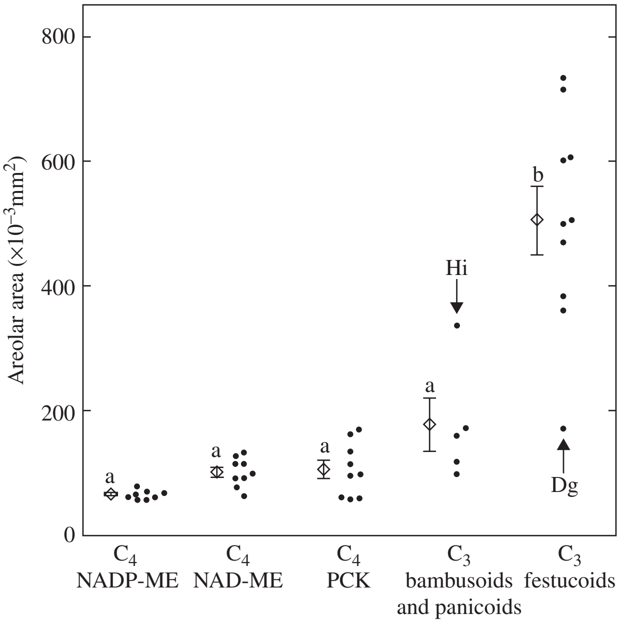

FIG. 4. Comparison of the areolar area (the minimum photosynthetic tissue area surrounded by veins) in leaf blades of C_3 and C_4 grasses. The values were calculated from the distances between small longitudinal veins and the distances between transverse veins. The mean and s.e. are shown for the respective subgroups. Values followed by the same lower case letter are not significantly different at $P < 0{\cdot}05$. Dg, *Dactylis glomerata*; Hi, *Hymenachne indica.*

value between the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

The ratio of transverse vein length to total vein length was 1·4 times higher in the C_3 grasses than in the C_4 grasses (Table 2). There was no significant difference in the mean values between the three C_4 subgroups, although the mean value in the NADP-ME grasses was significantly lower than those in the two C_3 subgroups (Table 2; Fig. 6). There was no significant difference in the mean value between the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

The number of transverse veins per unit leaf area in the C_4 grasses was 3·3 times that in the C_3 grasses (Table 2). The mean value of the NADP-ME grasses was significantly higher than the other five subgroups, and that of the festucoid C_3 grasses was significantly the lowest (Table 2; Fig. 7). There was no large difference in the mean value between the panicoid C_4 and the chloridoid C_4 grasses (Table 2).

DISCUSSION

Functional implications of differences of leaf vascular systems between C_3 and C_4 grasses

Our study confirmed that C_4 grasses have a denser system of small longitudinal veins than C_3 grasses, as reported in previous work (e.g. Takeda and Fukuyama, 1971; Crookston and Moss, 1974; Kawamitsu *et al*., 1985; Dengler *et al*., 1994). Our study is the first to demonstrate that the statistically significant differences observed between C_3 and C_4 grasses are due to the differences in small longitudinal veins, not large longitudinal veins. Furthermore, this study demonstrates that the leaves of C_4 grasses tend to develop transverse veins more densely than do those of C_3 grasses, although some C_3 grasses also had a short distance between transverse veins. Oguro *et al*. (1985) reported that in some *Panicum* species, C_4 species tend to have shorter distance between transverse veins than C_3 species.

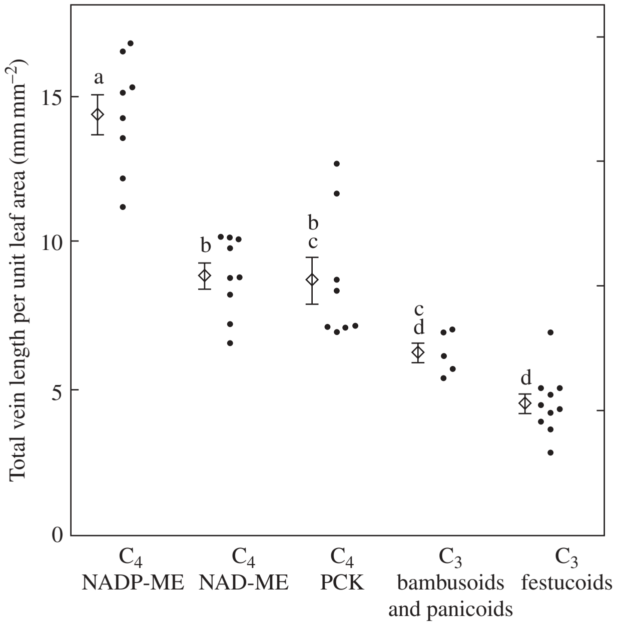

FIG. 5. Comparison of total vein length per unit leaf area in leaf blades of C_3 and C_4 grasses. The mean and s.e. are shown for the respective subgroups. Values followed by the same lower case letter are not significantly different at $P < 0.05$.

The large longitudinal veins contain a greater cross-sectional area of phloem than the small longitudinal veins (Altus and Canny, 1982; Colbert and Evert, 1982; Russell and Evert, 1985). The small longitudinal vein has about the same phloem area throughout the length of the blade, whereas the phloem area of a large longitudinal vein increases from the tip to the base (Altus and Canny, 1982). The transverse veins, which have a single sieve tube (Kuo *et al*., 1972; Chonan *et al*., 1985; Tiba and Frean, 1989), connect the longitudinal veins. The transverse veins and small longitudinal veins play a vital role in lateral transport of photosynthate from the small to the large longitudinal veins (Altus and Canny, 1982). Thus, denser development of transverse veins will probably be effective for movement of photosynthate from the small to the large longitudinal veins. Our study demonstrates that C_4 grasses have acquired a superior photosynthate translocation system by developing denser networks of small longitudinal and transverse veins, while keeping a constant density of large longitudinal veins.

This feature is also shown by the small areolar area. If we attempt to correlate structural features of leaves to photosynthate translocation, the leaf thickness should also be considered, because the volume of photosynthetic tissue would be more strongly involved in translocation than the

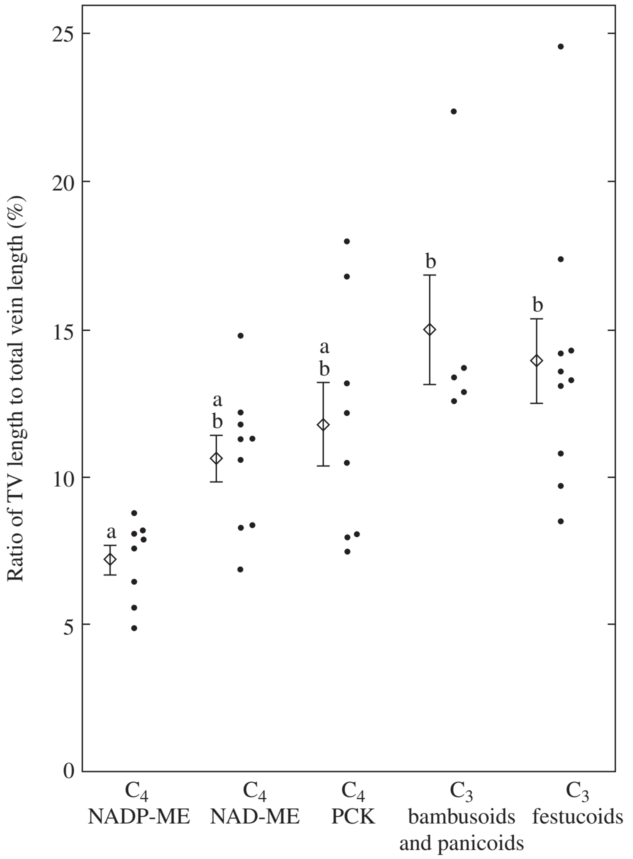

FIG. 6. Comparison of the ratio of transverse vein length to total vein length per unit leaf area in leaf blades of C_3 and C_4 grasses. The mean and s.e. are shown for the respective subgroups. Values followed by the same lower case letter are not significantly different at $P < 0{\cdot}05$.

areolar area. Dengler *et al.* (1994) reported that the leaves of C_4 grasses are significantly thinner than those of C_3 grasses, if C_3 bambusoid grasses, which have the thinnest leaves, are not included. Within C_4 grasses, there were no significant differences in the leaf thickness between NADP-ME, NAD-ME and PCK grasses, but NAD-ME C_4 grasses with PCK-like leaf anatomy have thinner leaves than other C_4 subtypes (Dengler *et al.*, 1994). In the NAD-ME C_4 grasses examined in our study, only *P. dichotomiflorum* has this PCK-like anatomy (Ohsugi and Murata, 1986). Thus, the festucoid C_3 grasses would have greater photosynthetic tissue volume than the C_4 grasses, because they have thick leaves with high areolar area.

It should also be noted that biochemical mechanisms such as sugar transport (Williams *et al.*, 2000), plasmodesmatal distribution in the BS cells (Botha, 1992) and the architecture of the conducting tissue are involved in the process of photosynthate translocation. It has been reported that C_4 species usually show preferential localization of sucrose phosphate synthase in the M cells, although there is a wide variation in the relative abundance between the M and BS cells among C_4 species (Lunn and Furbank, 1999). Thus, trioses produced in the BS cells must move first to the M cells before being converted to sucrose. Newly formed sucrose must then be returned to the BS cells and loaded to the phloem, suggesting a more complicated pathway of photosynthates in C_4 species as compared with that in C_3 species.

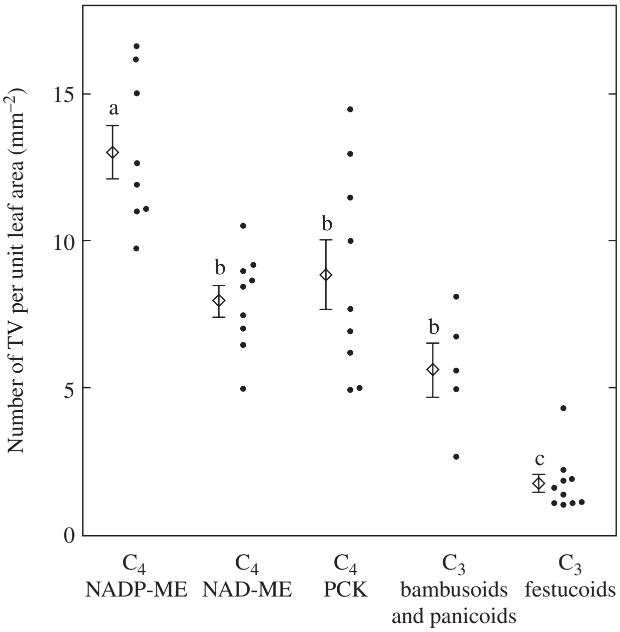

FIG. 7. Comparison of the number of transverse veins per unit leaf area in leaf blades of C_3 and C_4 grasses. The mean and s.e. are shown for the respective subgroups. Values followed by the same lower case letter are not significantly different at $P < 0{\cdot}05$.

Reflecting the different conducting roles, the large longitudinal veins contain vessels of larger diameter than the small longitudinal veins (Kuo *et al.*, 1974). The diameter of the largest vessel in the large longitudinal veins decreases with distance along the blade toward the tip, but that in the small longitudinal veins does not change (Altus *et al.*, 1985). The transverse veins possess only one vessel of similar diameter at all places in the leaf blade (Altus *et al.*, 1985). It appears that the leaves of C_4 grasses have structurally a better water distribution system than those of festucoid C_3 grasses by developing denser networks of the small longitudinal and transverse veins.

Leaf vascular system in C_4 grasses

Kawamitsu *et al.* (1985) reported that the C_4 grasses with BS cells that originated from mestome sheath cells (NADP-ME type) have a shorter interveinal distance in leaves than those with BS cells that originated from parenchyma sheath cells (NAD-ME and PCK types). They found no difference in the interveinal distance between the NAD-ME and PCK grasses. Other workers have reported the following order for the interveinal distance: NADP-ME < PCK < NAD-ME in *Panicum* C_4 grasses (Ohsugi and Murata, 1986) and NADP-ME = PCK ⩽ NAD-ME in more divergent C_4 grasses (Dengler *et al.*, 1994). We found no significant differences in the distance between small longitudinal veins between the three C_4 subgroups, although the mean value of the NADP-ME grasses was lower than those of the NAD-ME and PCK grasses. Thus, our data roughly correspond to the results of Kawamitsu *et al*. (1985), although the previous study did not distinguish the two types of longitudinal vein. It is unclear whether the features of the vascular system of C_4 subgroups are associated with some biochemical functions of photosynthesis, although it has been suggested that the interveinal distance could affect photosynthesis in C_4 grasses, resulting in differences in photon capture (quantum yield) (Ehleringer *et al*., 1997; Ogle, 2003). Fisher and Evert (1982) reported a vein density of 7·97 mm mm^{-2} for *Amaranthus retroflexus*, an NAD-ME C_4 dicot having a reticulated vascular system. This value is comparable with those of the NAD-ME C_4 grasses we examined.

When the measures of vascular density of the C_4 grasses were compared between the panicoid C_4 and the chloridoid C_4 grasses, there were no significant differences, except that the chloridoid C_4 grasses had a somewhat shorter distance between large longitudinal veins than the panicoid C_4 grasses. Thus, it seems that a similar change in the leaf vascular system occurred in parallel between the two phylogenetic lines, although further studies would be required for the density of large longitudinal veins.

Leaf vascular system in C_3 grasses

Considerable differences in leaf vascular systems within the C_3 grasses were revealed. The bambusoid and panicoid C_3 grasses generally had a shorter distance between small longitudinal veins than the festucoid C_3 grasses, but there were no differences in the distance between large longitudinal veins. The distance between transverse veins was generally shorter in the bambusoid and panicoid C_3 grasses than in the festucoid C_3 grasses. Thus, the bambusoid and panicoid C_3 grasses have leaves with a denser vascular system than the festucoid C_3 grasses. The two C_3 subgroups differ in the seasonal growth pattern: the former subgroup grows in summer, as do C_4 grasses, but the latter subgroup grows in spring. All the C_3 grasses we examined grow in sunny habitats; however, the bambusoid and panicoid C_3 grasses grow in wet habitats, whereas the festucoid C_3 grasses grow in mesic habitats. It remains unknown whether the gas exchange and water physiology of leaves differ between the two C_3 subgroups. Interestingly, the festucoid C_3 grasses tend to have larger stomata in the leaf blade than the bambusoid and panicoid C_3 grasses, but a lower density of stomata (Ashida and Sugino, 1984; Kawamitsu *et al*., 1996). For instance, *O. sativa* leaves show the highest density of stomata among grass species, but the stomatal size is very small (Kawamitsu *et al*., 1996). Such C_3 grasses, even though they grow in wet places, may have a high evaporative demand in order to lower the leaf temperature in the heat of the day. It has also been reported that even in paddy fields with enough water, rice plants are often subject to water stress at midday on a fine day because of intense transpiration over water absorption from roots, which is caused by a high vapour pressure deficit, accompanied by a decrease in leaf water potential and stomatal closure (Ishihara and Hirasawa, 1978; Ishihara and Saitoh, 1987). It would be interesting to study whether the two C_3 subgroups have developed different strategies in the vascular and stomatal architectures for water movement and transpiration in leaves.

Ecological and evolutionary implications of leaf vascular systems in C_3 and C_4 grasses

The vein density of leaves is influenced by various environmental factors. One of the factors that lead to higher vein densities is reduction of soil water availability. Likewise, high temperature induces a similar response in leaves (Uhl and Mosbrugger, 1999; Roth-Nebelsick *et al*., 2001). These environmental conditions are generally advantageous to the performance of C_4 plants, because C_4 photosynthesis is more efficient than C_3 photosynthesis under environments that promote photorespiration (Ehleringer and Monson, 1993). However, it seems that a change in atmospheric CO_2 concentration has no significant effect on the vein density of leaves in both the short and long term (Uhl and Mosbrugger, 1999), unlike the response of stomatal density, which declines as the CO_2 concentration increases (Woodward, 1987), although more data are needed to understand the effect of CO_2 concentration on the vein density (Roth-Nebelsick *et al*., 2001). An increase in the vein density of leaves might be an anatomical pre-conditioning to the evolution of C_4 plants from C_3 plants (Sage, 2004). Increasing vein density may initially have little effect on the performance of an effective CO_2 concentration mechanism, but may enhance the water status of leaves in hot environments (Sage, 2004). Kocacinar and Sage (2003) have found that C_4 dicots have a stem xylem structure and hydraulic function differing from that of C_3 dicots, reflecting their greater water use efficiency and lower water requirements. It is unknown whether C_4 grasses also have such characteristics.

Recent molecular phylogenetic studies on the grasses have demonstrated that C_4 photosynthesis originated multiple times among several closely related subfamilies (Kellogg, 2001). The earliest divergent branches in the grasses are the C_3 bambusoids and C_3 festucoids. The remainder of the family is in a large clade (the PACC clade) with a mix of C_3 and C_4 members. It includes the panicoids, the chloridoids, a lineage with *Aristida* and *Stipagrostis*, and a lineage with *Eriachne* (Sinha and Kellogg, 1996; Kellogg, 2001). Our study indicates that the festucoids have a sparse leaf vascular system, whereas a dense vascular system occurs in the two lineages, the panicoids and chloridoids, together with evolution of C_4 photosynthesis. A recent molecular phylogenetic study on the Panicoideae has demonstrated that C_3 photosynthesis is the ancestral condition in this subfamily and that C_4 photosynthesis arose at least eight times (Giussani *et al*., 2001). It is interesting to note that the panicoid C_3 grasses have a vascular system that is more similar to that in C_4 grasses than to that in the festucoid C_3 grasses. The bambusoid grasses we examined also had a relatively dense leaf vascular system, which is comparable with that of the panicoid C_3 grasses. The acquisition of this structural characteristic may partly be related to similar ecological features in these two groups. However, C_4 grasses with a denser leaf vascular system evolved within the panicoids but not within the bambusoids of old origin. A more extensive study would be required to understand the evolution of leaf vascular systems within the grass family, especially with respect to the bambusoids, the C_3 panicoids and the remaining two C_4 lineages including *Aristida* and *Eriachne*.

ACKNOWLEDGEMENTS

We thank Dr Y. Kawamoto (University of the Ryukyus) for his kind gift of seeds of *Brachiaria* species.

LITERATURE CITED

Altus DP, Canny MJ. 1982. Loading of assimilates in wheat leaves. I. The specialization of vein types for separate activities. *Australian Journal of Plant Physiology* **9**: 571–581.

Altus DP, Canny MJ. 1985. Water pathways in wheat leaves. I. The division of fluxes between different vein types. *Australian Journal of Plant Physiology* **12**: 173–181.

Altus DP, Canny MJ, Blackman DR. 1985. Water pathways in wheat leaves. II. Water conducting capacities and vessel diameters of different vein types, and the behaviour of the integrated network. *Australian Journal of Plant Physiology* **12**: 183–199.

Ashida K, Sugino M. 1984. Studies on the stomata in the leaf blades of gramineous weeds. *Weed Research (Japan)* **29**: 138–146.

Botha CEJ. 1992. Plasmodesmatal distribution, structure and frequency in relation to assimilation in C_3 and C_4 grasses in southern Africa. *Planta* **187**: 348–358.

Canny MJ. 1990. What becomes of the transpiration stream? *New Phytologist* **114**: 341–368.

Chonan N, Kawahara H, Matsuda T. 1974. Morphology of vascular bundles of leaves in gramineous crops. I. Observations on vascular bundles of leaf blades, sheaths and internodes in rice plants. *Proceedings of the Crop Science Society of Japan* **43**: 425–432.

Chonan N, Kawahara H, Matsuda T. 1985. Ultrastructure of transverse veins in relation to phloem loading in the rice leaf. *Japanese Journal of Crop Science* **54**: 160–169.

Clayton WD, Renvoize SA. 1992. A system of classification for the grasses. In: Chapman GP, ed. *Grass evolution and domestication*. New York: Cambridge University Press, 338–353.

Colbert JT, Evert RF. 1982. Leaf vasculature in sugarcane (*Saccharum officinarum* L.). *Planta* **156**: 136–151.

Crookston RK, Moss DN. 1974. Interveinal distance for carbohydrate transport in leaves of C_3 and C_4 grasses. *Crop Science* **14**: 123–125.

Dannenhoffer JM, Evert RF. 1994. Development of the vascular system in the leaf of barley (*Hordeum vulgare* L.). *International Journal of Plant Sciences* **155**: 143–157.

Dengler NG, Nelson T. 1999. Leaf structure and development in C_4 plants. In: Sage RF, Monson RK, eds. *C_4 plant biology*. San Diego: Academic Press, 133–172.

Dengler NG, Dengler RE, Donnelly PM, Hattersley PW. 1994. Quantitative leaf anatomy of C_3 and C_4 grasses (Poaceae): bundle sheath and mesophyll surface area relationships. *Annals of Botany* **73**: 241–255.

Ehleringer JR, Monson RK. 1993. Evolutionary and ecological aspects of photosynthetic pathway variation. *Annual Review of Ecology and Systematics* **24**: 411–439.

Ehleringer JR, Cerling TE, Helliker BR. 1997. C_4 photosynthesis, atmospheric CO_2, and climate. *Oecologia* **112**: 285–299.

Fisher DG, Evert RF. 1982. Studies on the leaf of *Amaranthus retroflexus* (Amaranthaceae): morphology and anatomy. *American Journal of Botany* **69**: 1133–1147.

Fritz E, Evert RF, Nasse H. 1989. Loading and transport of assimilates in different maize leaf bundles. Digital image analysis of ^{14}C-microautoradiographs. *Planta* **178**: 1–9.

Gallaher RN, Ashley DA, Brown RH. 1975. ^{14}C-photosynthate translocation in C_3 and C_4 plants as related to leaf anatomy. *Crop Science* **15**: 55–59.

Giussani LM, Cota-Sanchez JH, Zuloaga FO, Kellogg EA. 2001. A molecular phylogeny of the grass subfamily Panicoideae (Poaceae) shows multiple origins of C_4 photosynthesis. *American Journal of Botany* **88**: 1993–2012.

GPWG (Grass Phylogeny Working Group) 2001. Phylogeny and subfamilial classification of the grasses (Poaceae). *Annals of the Missouri Botanical Garden* **88**: 373–457.

Grodzinski B, Jiao J, Leonardos ED. 1998. Estimating photosynthesis and concurrent export rates in C_3 and C_4 species at ambient and elevated CO_2. *Plant Physiology* **117**: 207–215

Hanson JC, Rasmusson DC. 1975. Leaf vein frequency in barley. *Crop Science* **15**: 248–251.

Hatch MD. 1987. C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. *Biochimica et Biophysica Acta* **895**: 81–106.

Hattersley PW. 1984. Characterization of C_4 type leaf anatomy in grasses (Poaceae). Mesophyll:bundle sheath area ratios. *Annals of Botany* **53**: 163–179.

Hattersley PW, Watson L. 1992. Diversification of photosynthesis. In: Chapman GP, ed. *Grass evolution and domestication*. New York: Cambridge University Press, 38–116.

Hofstra G, Nelson CD. 1969. A comparative study of translocation of assimilated ^{14}C from leaves of different species. *Planta* **88**: 103–112.

Ishihara K, Hirasawa T. 1978. Relationship between leaf and xylem water potentials in rice plants. *Plant and Cell Physiology* **19**: 1289–1294.

Ishihara K, Saitoh K. 1987. Diurnal courses of photosynthesis, transpiration, and diffusive conductance in the single-leaf of the rice plants grown in the paddy field under submerged condition. *Japanese Journal of Crop Science* **56**: 8–17.

Kawamitsu Y, Hakoyama S, Agata W, Takeda T. 1985. Leaf interveinal distances corresponding to anatomical types in grasses. *Plant and Cell Physiology* **26**: 589–593.

Kawamitsu Y, Agata W, Hiyane S, Murayama S, Nose A, Shinjyo C. 1996. Relation between leaf gas exchange rate and stomata. I. Stomatal frequency and guard cell length in C_3 and C_4 grass species. *Japanese Journal of Crop Science* **65**: 626–633.

Kellogg EA. 2001. Evolutionary history of the grasses. *Plant Physiology* **125**: 1198–1205.

Kocacinar F, Sage RF. 2003. Photosynthetic pathway alters xylem structure and hydraulic function in herbaceous plants. *Plant, Cell and Environment* **26**: 2015–2026.

Kuo J, O'Brien TP, Zee S-Y. 1972. The transverse veins of the wheat leaf. *Australian Journal of Biological Science* **25**: 721–737.

Kuo J, O'Brien TP, Canny MJ. 1974. Pit-field distribution, plasmodesmatal frequency, and assimilate flux in the mestome sheath of wheat leaves. *Planta* **121**: 97–118.

Langdale JA, Nelson T. 1991. Spatial regulation of photosynthetic development in C_4 plants. *Trends in Genetics* **7**: 191–196.

Leonardos ED, Grodzinski B. 2000. Photosynthesis, immediate export and carbon partitioning in source leaves of C_3, C_3–C_4 intermediate and C_4 *Panicum* and *Flaveria* species at ambient and elevated CO_2 levels. *Plant, Cell and Environment* **23**: 839–851.

Lunn JE, Furbank RT. 1999. Sucrose biosynthesis in C_4 plants. *New Phytologist* **143**: 221–237.

Lush WM. 1976. Leaf structure and translocation of dry matter in a C_3 and a C_4 grass. *Planta* **130**: 235–244.

Ogle K. 2003. Implications of interveinal distance for quantum yield in C_4 grasses: a modeling and meta-analysis. *Oecologia* **136**: 532–542.

Oguro H, Hinata K, Tsunoda S. 1985. Comparative anatomy and morphology of leaves between C_3 and C_4 species in *Panicum. Annals of Botany* **55**: 859–869.

Ohsugi R, Murata T. 1986. Variations in the leaf anatomy among some C_4 *Panicum* species. *Annals of Botany* **58**: 443–453.

Pelletier JD, Turcotte DL. 2000. Shapes of river networks and leaves: are they statistically similar? *Philosophical Transactions of the Royal Society B: Biological Sciences* **355**: 307–311.

Prendergast HVD, Hattersley PW, Stone NE. 1987. New structural/biochemical associations in leaf blades of C_4 grasses (Poaceae). *Australian Journal of Plant Physiology* **14**: 403–420.

Roth-Nebelsick A, Uhl D, Mosbrugger V, Kerp H. 2001. Evolution and function of leaf venation architecture: a review. *Annals of Botany* **87**: 553–566.

Russell SH, Evert RF. 1985. Leaf vasculature in maize (*Zea mays* L.). *Planta* **164**: 448–458.

Sage RF. 2004. The evolution of C_4 photosynthesis. *New Phytologist* **161**: 341–370.

Sinha NR. Kellogg EA. 1996. Parallelism and diversity in multiple origins of C_4 photosynthesis in the grass family. *American Journal of Botany* **83**: 1458–1470.

Takeda T, Fukuyama M. 1971. Studies on the photosynthesis of the Gramineae. 1. Differences in photosynthesis among subfamilies and their relations with the systematics of the Gramineae. *Proceedings of the Crop Science Society of Japan* **40**: 12–20.

Tiba SD, Frean ML. 1989. A comparative study of the structure–function relationship of cross veins in leaves of *Digitaria eriantha* and *Zea mays*. *Annals of Botany* **63**: 433–439.

Ueno O. 1995. Occurrence of distinctive cells in leaves of C_4 species in *Arthraxon* and *Microstegium* (Andropogoneae-Poaceae) and the structural and immunocytochemical characterization of these cells. *International Journal of Plant Sciences* **156**: 270–289.

Ueno O, Sentoku N. 2006. Comparison of leaf structure and photosynthetic characteristics of C_3 and C_4 *Alloteropsis semialata* subspecies. *Plant, Cell and Environment* **28**: (in press), doi: 10.1111/j.1365–3040.2005.01418.x

Uhl D, Mosbrugger V. 1999. Leaf venation density as a climate and/or environmental proxy: a critical review and new data. *Palaeogeography, Palaeoclimatology, Palaeoecology* **149**: 17–30.

Wakayama M, Ueno O, Ohnishi J. 2003. Photosynthetic enzyme accumulation during leaf development of *Arundinella hirta*, a C_4 grass having Kranz cells not associated with veins. *Plant and Cell Physiology* **44:** 1330–1340.

Williams LE, Lemoine R, Sauer N. 2000. Sugar transporters in higher plants: diversity of roles and complex regulation. *Trends in Plant Science* **5**: 283–290.

Woodward FI. 1987. Stomatal numbers are sensitive to increase in CO_2 from pre-industrial levels. *Nature* **327**: 617–618.

Yamazaki K. 1960. Studies on the connecting strand of the vascular system in rice leaves. *Proceedings of the Crop Science Society of Japan* **29**: 400–403.

Yamazaki K. 1963. Studies on leaf formation in rice plants. II. The development of leaves in relation to their position on a stem. *Proceedings of the Crop Science Society of Japan* **32**: 81–88.

Yoshimura Y, Kubota F, Ueno O. 2004. Structural and biochemical bases of photorespiration in C_4 plants: quantification of organelles and glycine decarboxylase. *Planta* **220**: 307–317.