Plant, Cell & Environment

Plant, Cell and Environment (2009) **32**, 1833–1844 doi: 10.1111/j.1365-3040.2009.02043.x

Variations in the dorso-ventral organization of leaf structure and Kranz anatomy coordinate the control of photosynthesis and associated signalling at the whole leaf level in monocotyledonous species

ANA SOFIA SOARES-CORDEIRO[1,2], SIMON P. DRISCOLL[3], TILL K. PELLNY[2], ENRIQUE OLMOS[4], MARIA CELESTE ARRABAÇA[1] & CHRISTINE H. FOYER[3]

[1]Centro de Engenharia Biológica e Departamento de Biologia Vegetal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal, [2]Plant Science Department, Centre for Crop Genetic Improvement, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK, [3]Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, UK and [4]CEBAS-CSIC, Department of Abiotic Stress and Plant Pathology, P.O. Box 164, 30100-Murcia, Campus de Espinardo, Spain

ABSTRACT

Photosynthesis and associated signalling are influenced by the dorso-ventral properties of leaves. The degree of adaxial/abaxial symmetry in stomatal numbers, photosynthetic regulation with respect to light orientation and the total section areas of the bundle sheath (BS) cells and the surrounding mesophyll (M) cells on the adaxial and abaxial sides of the vascular bundles were compared in two C_4 [*Zea mays* (maize) and *Paspalum dilatatum*] and one C_3 [*Triticum turgidum* (Durum wheat)] monocotyledonous species. The C_3 leaves had a higher degree of dorso-ventral symmetry than the C_4 leaves. Photosynthetic regulation was the same on each side of the wheat leaves, as were stomatal numbers and the section area of the BS relative to that of the M cells (BS/M section area ratio). In contrast, photosynthetic regulation in maize and *P. dilatatum* leaves showed a marked surface-specific response to light orientation. Compared to the adaxial sides of the C_4 monocotyledonous leaves, the abaxial surfaces had more stomata and the BS/M section area ratio was significantly higher. Differences in dorso-ventral structure, particularly in Kranz anatomy, serve not only to maximize photosynthetic capacity with respect light orientation in C_4 monocotyledonous leaves but also allow adaxial and abaxial-specific signalling from the respective M cells.

Key-words: *Paspalum*, abaxial/adaxial leaf specification, C_3 photosynthesis, C_4 photosynthesis, Durum wheat, Kranz anatomy, maize, stomata.

INTRODUCTION

Leaf morphology and anatomy play an important role in the regulation of photosynthesis, providing a structural intercellular framework for the diffusion of gases and the optimization of photosynthetic activity (Terashima & Inoue 1985a,b; Bolhàr-Nordenkampf & Draxler 1993). Dorso-ventral variations in epidermal structure, particularly the number and density of stomata on each leaf surface, are common in both monocotyledonous and dicotyledonous species (Driscoll *et al.* 2006; Soares *et al.* 2008). Differences in photosynthetic regulation on the adaxial and abaxial sides of dorso-ventrally flattened leaves are determined by factors related to leaf structure and anatomy. These include: (1) intercellular gradients in the profile of light absorption; (2) the distribution of absorbed quanta within the leaf as light arrives from different orientations; and (3) the diffusion of CO_2 within the leaf (Sharkey *et al.* 1982; Long *et al.* 1989; Ögren & Evans 1993; Vogelmann 1993; Evans & Vogelmann 2003, 2006). In monocotyledonous species, which can also show side-specific differences in photosynthetic regulation with respect to the orientation of incident light (Driscoll *et al.* 2006; Soares *et al.* 2008) the situation is complicated further by the need to balance absorbed quanta between bundle sheath (BS) and mesophyll (M) cells. Unlike the leaves of dicotyledonous species, where intracellular CO_2 concentrations (Ci) are very similar whether they are estimated from either the upper or lower surfaces (Sharkey *et al.* 1982), Ci values obtained for the adaxial surface of monocotyledonous species can be significantly lower than those of the abaxial surface (Long *et al.* 1989). The adaxial and abaxial sides of the leaves of C_4 monocotyledonous species such as maize are considered to comprise essentially two separate compartments as there are physical dorso-ventral restrictions to airspace continuity (Long *et al.* 1989).

Leaf dorso-ventral polarity, together with adaxial and abaxial patterns of cellular structure and composition, which determine the relationships between the stomata and the adjacent M, are fixed very early in leaf meristem development. The specification and development of leaf

Correspondence: C. H. Foyer. Fax +44 0 113 323 3144; e-mail c.foyer@leeds.ac.uk

dorso-ventral polarity is regulated by adaxializing transcription factors (Bowman, Eshed & Baum 2002) and microRNAs that act in the abaxial domain of the leaf primordia by silencing adaxializing transcription factors (Emery *et al.* 2003; Juarez *et al.* 2004; Kidner & Martienssen 2004). The adaxializing factors, which are produced by the meristem, confer the adaxial identity on the upper part of leaf primordia while the lower part adopts abaxial fate by default (Bowman *et al.* 2002). The vertical gradients in leaf photosynthesis observed in the leaves of dicotyledonous species are also entrained during development. However, these gradients are strongly influenced by the direction of incident light. Inversion of dicotyledonous leaves at an early developmental stage so that the abaxial surface is exposed to incident light, results in the inversion of the adaxial/abaxial light-response curves of photosynthesis (Terashima, Sakaguchi & Hara 1986).

In contrast to the dorso-ventral specification of leaf structure that is fixed during leaf development, the diffusion of CO_2 within the leaf is not a constant parameter. CO_2 diffusion is altered by the acclimation processes in leaves in response to developmental (Miyazawa & Terashima 2001) and environmental triggers (Piel *et al.* 2002). Acclimatory changes in CO_2 diffusion can be as rapid and reversible as stomatal conductance responses (Centritto, Loreto & Chartzoulakis 2003; Flexas *et al.* 2007). Although the mechanisms that are involved in the acclimation of CO_2 diffusion pathways remain to be identified, observed changes in parameters used to measure CO_2 diffusion can often be related to variations in the cell wall structure and composition (von Caemmerer & Evans 1991; Sharkey *et al.* 1991; Lloyd *et al.* 1992; Evans & Loreto 2000). The composition of cell wall is altered in response to environmental stimuli; rapid changes in wall fortification and composition occur as a result of plant interactions with both biotic (e.g. pathogens) and abiotic (e.g. ozone) triggers. Moreover, the extent of wall cross-linking and abundance of defence compounds such as ascorbate in the apoplast/cell wall compartment are important determinants of the amount of available airspace within leaves (Horemans, Foyer & Asard 2000; Veljovic-Jovanovic *et al.* 2001; Pignocchi & Foyer 2003). Furthermore, the stomata on each surface respond differently to environmental triggers such as light intensity, allowing surface-specific regulation of stomatal opening and gas exchange (Pemadasa 1979; Travis & Mansfield 1981; Pemadasa 1982; Turner & Singh 1984; Wang, Wu & Assmann 1998).

In comparison to the leaves of dicotyledonous C_3 species, vertical sections through the leaves from monocotyledonous species reveal a high degree of dorso-ventral anatomical symmetry in structure and cellular organization. Many members of the Gramineaceae (Poaceae) such as *Paspalum dilatatum*, *Zea mays* (maize) and *Triticum turgidum* (Durum wheat) have a uniform M structure, and it is impossible to distinguish between palisade and spongy M under the microscope. Photosynthesis takes place on the M cells in C_3 plants, but C_4 photosynthesis requires the participation of both M and BS cells. At least three types of M cells can be distinguished in the leaves of C_4 grasses such as maize. While bulliform cells are found just below the adaxial epidermis, hyaline-form cells reside in the middle section and between the veins, and isodiametric cells are found above the abaxial epidermis (Long *et al.* 1989). The main anatomical variations between the Panicoideae (such as *P. dilatatum* and maize) and Pooideae (such as wheat) are related to the presence of Kranz-type anatomy and C_4 photosynthesis in the C_4 species. The M and BS chloroplasts are arranged centrifugally in relation to the vascular bundles in the leaves of C_4 NADP-ME subtypes such as maize and *P. dilatatum*. However, the M chloroplasts are much smaller than the BS chloroplasts; the difference in chloroplast size is probably related to the presence of agranal chloroplasts in the BS cells, while the M chloroplasts contain thylakoid membranes with well-developed grana. The C_4-type vascular bundle is characterized by large xylem vessels on the adaxial side, with proto-phloem and meta-phloem sieve tubes present on the abaxial side. In the C_3 species like wheat, the xylem vessels are also present on the adaxial side of the vascular bundle with the phloem sieve tubes arranged on the abaxial side. In wheat leaves, an additional cell layer called the mestome sheath surrounds the vascular tissue between the BS and the vascular tissues beneath. The wheat leaf mestome sheath consists of thick-walled lignified cells, and the BS chloroplasts are small compared to the M chloroplasts.

Simultaneous measurements of CO_2 and water vapour fluxes in maize leaves have confirmed that the central midrib represents a significant physical barrier to gaseous diffusion (Long *et al.* 1989). Thus, the adaxial and abaxial sides of maize leaves are essentially two separate air-space systems, in which the photosynthetic M cells are connected to the stomata on each side of the leaf, but not across the leaf blade. This structure facilitates leaf side-specific differences in intercellular CO_2 concentrations (Long *et al.* 1989). The maize leaf two-compartment model contrasts markedly with the situation in dicotyledonous leaves, where the limitation to diffusion between the upper and lower M is insignificant (Sharkey *et al.* 1982). The leaves of another C_4 monocotyledonous species, *P. dilatatum*, also show a pronounced entrained dorso-ventral asymmetry in the regulation of photosynthesis (Soares *et al.* 2008). Other than maize (Driscoll *et al.* 2006) and *P. dilatatum* (Soares *et al.* 2008), there are no other similar reports in the literature concerning other C_3 or C_4 grasses, and it is not known whether a diffusion barrier exists in C_3 monocotyledonous species. In the following study, we compared dorso-ventral asymmetries in the regulation of photosynthesis in different monocotyledonous leaves in relation to key features of leaf anatomy in Durum wheat (C_3), and in maize and *P. dilatatum* (C_4). Our first objective was to determine whether abaxial/adaxial specification in the regulation of photosynthesis with respect to light orientation was unique to C_4 monocotyledonous species or was also present in the leaves of a C_3 monocotyledonous species. The second objective was to further our current understanding of the mechanisms that contribute to the side-specific regulation of

photosynthesis and associated signalling in C_4 monocotyledonous species.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of the C_3 monocotyledonous species: *T. turgidum* ssp. *durum* (Desf.) Husn. cv. Cham 1 (Durum wheat), and two C_4 nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME) species: *Z. mays* L. hybrid H99 (maize) and *P. dilatatum* Poiret cv. Raki, were germinated and grown in controlled environment cabinets (Sanyo SGC228.CFX.J, Sanyo, Osaka) as described previously (Driscoll *et al.* 2006; Soares *et al.* 2008). It is important to note that we used the H99 maize hybrid in these experiments as this genotype has the potential to be used in future plant transformation studies. The H99 maize hybrid has lower rates of photosynthesis compared to the more classic corn varieties (Driscoll *et al.* 2006). Wheat, maize and *P. dilatatum* plants were grown for 5–7 weeks at an irradiance of 600–650 μmol m^{-2} s^{-1} at pot height (400–700 nm), provided by Phillips Master TL5 HO 49w/830 fluorescent lamps (Koninklijke, the Netherlands), with a 16 h photoperiod. Water was provided continuously through a drip-feed system so the plants always received adequate water. The youngest fully expanded leaves only were used in the following analyses. The chambers were maintained at a CO_2 concentration of 350 ± 20 μL L^{-1} (Eurotherm Ltd., Worthing, UK) with 80% relative humidity and day/night temperatures of 25 °C (day) and 19 °C (night).

Determination of pigment and protein contents

Pigment (chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids) and protein contents were determined on the middle sections of the youngest fully expanded leaves. Samples were ground in liquid nitrogen with quartz sand. Pigments extracted in 96% (v/v) ethanol were determined according to Lichtenhaler & Wellburn (1983). The soluble protein content was determined according to the method of Bradford (1976) after extraction in sodium phosphate buffer as described by Soares *et al.* (2008).

Gas-exchange measurements

Photosynthetic gas-exchange measurements were performed using an infrared gas analyser (model wa-225-mk3; ADC, Hoddesdon, UK), either on the whole leaves using a custom-made standard Parkinson-type whole leaf chambers designed for CO_2 and water vapour analysis (Novitskaya *et al.* 2002) or on each leaf surface separately using a custom-made dual Parkinson-type chambers designed for CO_2 and water vapour analysis (Soares *et al.* 2008). These chambers were made in the workshops at Rothamsted Research under the supervision of Keith Parkinson.

Eighteen plants per species were measured per experiment in the studies on whole leaves, and 12 plants per species per experiment were analysed in the studies on the leaf surface-specific measurements. The plants were measured in batches (between four and six plants per week) either for the whole leaf or leaf surface measurements. In some experiments, both types of measurement were performed on the same plants but in over 50% of the cases, the measurements were performed on separate plants at different times. Because the two leaf surfaces represent resistors in parallel, the whole leaf stomatal conductance values equal the sum of the two surfaces. It is important to note that the sum of the conductance and flux values obtained for the two surfaces separately was approximately the same as whole leaf values (plus or minus 10%) when all measurements were performed on the same leaves at the same time. We have presented the mean values obtained from all of the data for whole leaf measurements and for each leaf surface, as well as the ratios between the intercellular CO_2 concentrations (Ci) and atmospheric (Ca) CO_2 concentrations (Ci/Ca ratio), with pooled data obtained in separate experiments on different batches of plants. Thus, the data shown in the tables and figures were obtained from the whole range of plants that had been grown and analysed over the experimental periods.

All measurements were performed on the middle section of the youngest fully expanded leaves of each plant with light oriented either to the adaxial surface or to the abaxial surface, by inverting the leaf in the chamber. For maize and wheat leaves, the youngest fully expanded was the fifth leaf. However, as the *P. dilatatum* leaves grow from multiple tillers, it was not possible to assign a leaf number. This may at least in part explain the higher degree of variability observed in the *P. dilatatum* data compared to the leaves of the other two species.

As required, gas-tight tape was used to seal each half of the chamber, preventing any flux of gas between the two sides, as described by Soares *et al.* (2008). Steady-state rates for CO_2 assimilation were obtained at a leaf temperature of 20 °C either on the whole leaf or on both the adaxial and abaxial surfaces, maintained by water jackets as previously described by Soares *et al.* (2008). Measurements were conducted at 50% relative humidity, an ambient CO_2 concentration (Ca) of 350 μL L^{-1} and at an irradiance of between 900 and 1000 μmol m^{-2} s^{-1}. The irradiance was provided by overhead broad-spectrum lamps and supplied only from the top of the chamber both for the whole leaf measurements and for each leaf surface measurements (Soares *et al.* 2008). Intrinsic water use efficiency (WUE) values were calculated as the ratios between CO_2 assimilation rates and the stomatal conductance values. The calculations used for these were as described previously (Veljovic-Jovanovic *et al.* 2001; Noctor *et al.* 2002; Dutilleul *et al.* 2003; Soares *et al.* 2008).

Determination of stomatal density

The epidermal tissue of each section was stripped from the adaxial and abaxial surfaces of the wheat and maize leaf sections, as described by Driscoll *et al.* (2006). This experiment was performed three times using one leaf per plant from three plants per species. The epidermal peels were

mounted in citrate phosphate buffer (0.1 M sodium citrate, 0.1 M sodium phosphate, pH 6.5). As it was not possible to obtain epidermal peels from *P. dilatatum* leaves, stomatal density was calculated from epidermal imprints as described by Soares *et al.* (2008). The epidermal peels or imprints were examined by light microscopy (Olympus BH-2; Olympus Optical Co. Ltd, Tokyo, Japan), and digitized images were taken at random. The stomatal densities of each species were calculated from 24 randomly selected digitized images from the adaxial or the abaxial epidermal peels and imprints in each case, using the Sigma ScanPro photographic analysis software, version 5 (Sigma Chemical Co., St Louis, MO, USA).

Fixation, embedding and sectioning of leaves for optical microscopy

Leaf sections (1 × 1 mm) were perfectly immersed in the fixative, which consisted of 3% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2.5 h at 4 °C. It was not necessary to use vacuum infiltration, because the leaf structure was well preserved and similar to that observed using transmission electron microscopy (TEM). The sections were then washed three times (15 min each) in the same buffer, and then post-fixated in osmium tetraoxide (1%, v/v) in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h and washed three times in the same buffer for 15 min. The samples were dehydrated at ambient temperature using a graded ethanol series [35% (v/v), 50% (v/v), 70% (v/v), 96% (v/v) and twice at 100% (v/v) ethanol] with 30 min exposure at each step. Samples were embedded in Spurr resin (Fluka, Switzerland) according to Olmos *et al.* (2006): 100% (v/v) oxide propylene I (30 min exposure at each step), 50% (v/v) oxide propylene + 50% (v/v) Spurr resin (1.5 h exposure) and 100% (v/v) Spurr resin (overnight). The samples were finally transferred to flat embedding moulds filled with Spurr resin and polymerized at 70 °C for 24 h. Transverse semi-thin leaf sections (0.5 μm) were prepared using a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany), stained with toluidine blue and observed using a Leica DMR light microscopy (Leica Microsystems). Between 25 and 40 different vascular bundles were analysed per species.

Determination of the total section area of BS and M cells

For these measurements, each side of the leaf was measured separately relative to the middle of the BS, which in general corresponded to the middle of the leaf. In each species, the total section areas of the BS cells and that of the first layer of surrounding M cells were measured on the adaxial and abaxial sides of the vascular bundles. For simplicity, examples of the cell types measured are shown in Fig. 1, where the black solid lines indicate the area of the M cells and the white dotted lines represent the area of BS cells measured. At least 25 different leaf vascular bundles were measured per species in nine transverse semi-thin Spurr resin sections. Measurements of the cell section area of BS (shown as the while dotted line in Fig. 1) and M (shown as the black solid in Fig. 1) cells were performed using imaging analysis (Olmos *et al.* 2006) on photomicrographs taken at

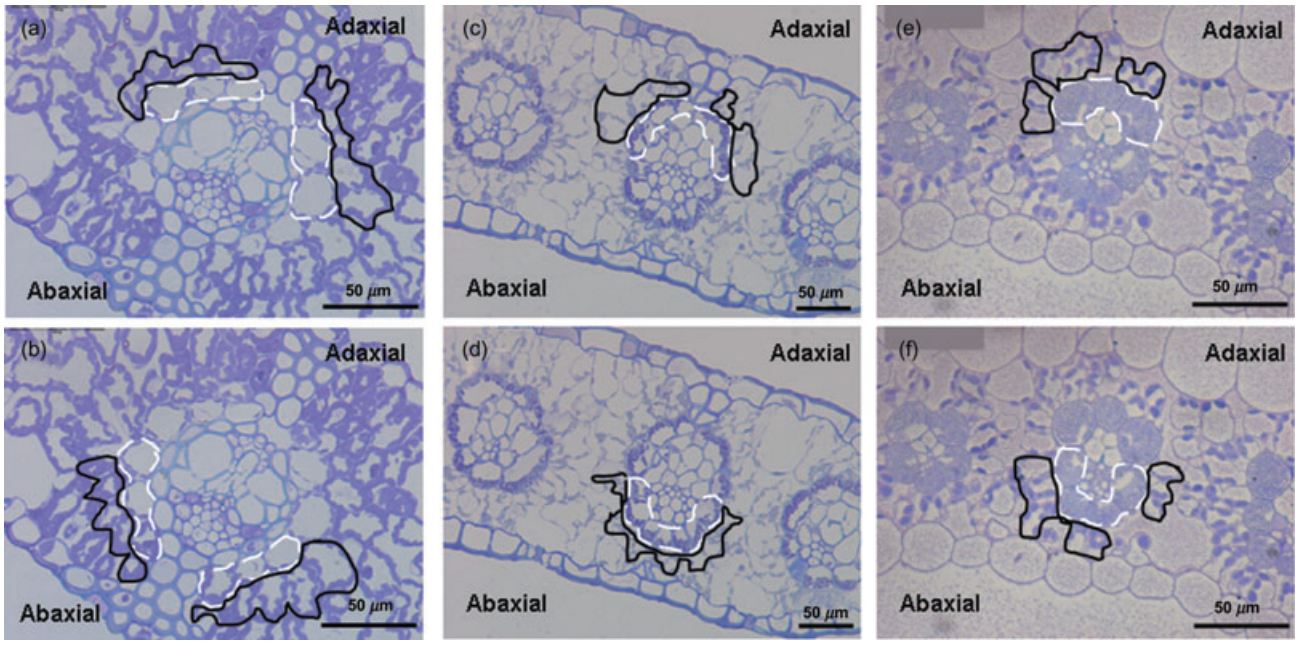

Figure 1. An illustration of the images used to calculate the total section area of the bundle sheath (BS) cells and the total section area of the surrounding first layer of mesophyll (M) cells, respectively. The BS cells that were measured are illustrated by the white dotted line while the M cells are shown as the black solid line on the adaxial (a, c, e) and abaxial (b, d, f) surfaces of wheat (a, b), maize (c, d) and *P. dilatatum* (e, f) leaves.

the same magnification using a Leica DMR light microscope. Image analysis was performed using Leica QM500 software (Leica Microsystems). Leica QM500 imaging analysis is a standard software package that provides quantitative data concerning the total section areas of the BS and MS cells (Olmos *et al.* 2006). The total section area of M on each side of the leaf is here defined as the total sum of cell section areas from M cells surrounding and in contact with the BS cells. Similarly, total section area of BS is defined as the total sum of cell section areas from BS cells in contact with the surrounding M cells. The calculations used in the Leica QM500 imaging analysis are as follows:

$$\text{Total section area BS} = \Sigma\, \text{BS}_{\text{area}}$$

$$\text{Total section area M} = \Sigma\, \text{M}_{\text{area}}$$

where:

BS_{area} = Area of each BS cell and M_{area} = Area of each M cell.

The BS/M section area ratio on each side of the leaf can thus be expressed as:

$$\text{Adaxial BS/M section area ratio} = \Sigma\, \text{BS}_{\text{area}} / \Sigma\, \text{M}_{\text{area}}; \text{ on the adaxial side}$$

$$\text{Abaxial BS/M section area ratio} = \Sigma\, \text{BS}_{\text{area}} / \Sigma\, \text{M}_{\text{area}}; \text{ on the abaxial side}$$

While the total section area data cannot be used directly to calculate the volumes of the BS and M cells because they have irregular shapes, cell section area data are considered to be a very useful for comparisons in morpho-metrical analyses. The BS/M section area ratio on each side of the leaf is here defined as the total section area of the BS cells divided by the total section area of the surrounding first layer of M cells on each leaf surface per vascular bundle.

Statistical analysis

Data were subjected to separate statistical analyses using parametric tests at the stringency of $P < 0.05$. Analysis of the data concerning pigments, protein contents and gas-exchange measurements was performed using the Statistical Package for Social Sciences (SPSS) 12.0, 2003 (SPSS Inc., Chicago, IL, USA). A standard *t*-test was used to analyse gas-exchange data. This test was also used to compare adaxial and abaxial stomatal densities, the total section area of the BS cells, the total section area of the surrounding first layer of M cells and the BS/M area ratios. The WUE data were analysed by comparing the effects of light orientation on each leaf surface using ANOVA and Tukey honestly significant difference (HSD) tests.

RESULTS

Leaf structure and composition

Wheat leaves had similar numbers of stomata on each leaf surface (Fig. 2a). In contrast, the leaves of maize and *P. dilatatum* plants grown under the same conditions had on average 30% more stomata on the abaxial surface than the adaxial surface (Fig. 2b,c). The leaves of all three monocotyledonous species showed pronounced structural dorso-ventral symmetry in cellular organization (Fig. 2). The structure of the leaves was well preserved in the fixation process; for example, in Fig. 2c, it is evident that the BS chloroplasts of *P. dilatatum* leaves are tightly packed and occupy almost the full volume of the cells.

The maize variety (H99) studied here had lower leaf chlorophyll, carotenoid and protein contents on a surface area basis than wheat or *P. dilatatum* leaves grown under the same conditions (Table 1).

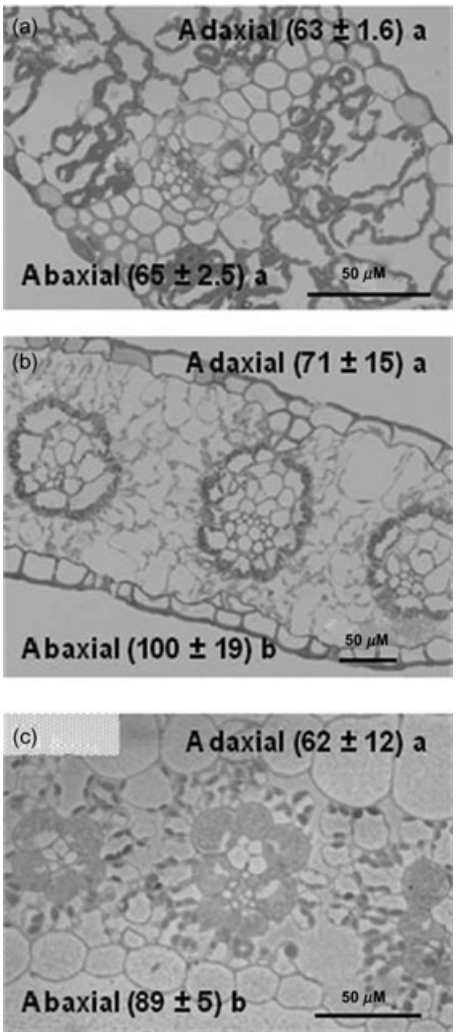

Figure 2. A comparison of the dorso-ventral structure of wheat (a), maize (b) and *P. dilatatum* (c) leaves. The stomatal density values (number mm^{-2}) for each surface are given in brackets. Values are the mean ± SD of $n = 24$ digitalized images from the adaxial and abaxial leaf surfaces of eight plants from each species. The statistical analysis ($P < 0.05$) compares data for each species separately, the different letters representing significant differences.

	T. turgidum	*Z. mays*	*P. dilatatum*
Chlorophyll *a* (mg m^{-2})	527 ± 6.3	302 ± 1.8	677 ± 5.6
Chlorophyll *b* (mg m^{-2})	250 ± 3.7	76 ± 0.7	177 ± 1.6
Chlorophyll *a* + *b* (mg m^{-2})	823 ± 6.0	378 ± 2.2	854 ± 7.1
Carotenoids (mg m^{-2})	86 ± 2.5	64 ± 0.6	135 ± 1.0
Soluble protein (mg m^{-2})	2953 ± 65.6	1621 ± 43.8	3604 ± 45.2

Table 1. Leaf pigment and protein contents of wheat (*Triticum turgidum*), maize (*Zea mays*) and *P. dilatatum* leaves

Whole leaf photosynthesis and intrinsic water use efficiencies

Whole leaf photosynthesis, stomatal conductance and Ci/Ca ratios in wheat were largely independent of light orientation (Fig. 3a–c), but this was not the case in either maize or *P. dilatatum* (Fig. 3a–c) leaves. In the leaves of these two C_4 species, the rates of CO_2 assimilation and stomatal conductance were higher under adaxial illumination than under abaxial illumination (Fig. 3a,b). There was a trend towards lower whole leaf Ci/Ca ratio when light was oriented to the adaxial surface in *P. dilatatum* and maize leaves (Fig. 3c). Whole leaf intrinsic WUE was largely independent of light orientation in wheat leaves (Table 2). However, the values were significantly higher ($P > 0.05$) in maize and *P. dilatatum* than wheat leaves (Table 2).

Effects of adaxial illumination on the dorso-ventral regulation of photosynthesis and stomatal conductance

The photosynthetic rates and the stomatal conductance values were highest on the adaxial surfaces of the wheat leaves under adaxial illumination (Fig. 4a,b). However, the abaxial surfaces had higher CO_2 assimilation rates than the adaxial surfaces in maize and *P. dilatatum* leaves under the same light illumination conditions, even though the stomatal conductance values were similar on the two leaf surfaces (Fig. 4b). Furthermore, the stomatal conductance values were much lower in the C_4 species than in wheat (Fig. 4b). While the Ci/Ca relationship was similar on both surfaces of wheat leaves, it was higher on the adaxial surfaces than the abaxial surfaces of the maize and *P. dilatatum* leaves (Fig. 4c).

Effects of abaxial illumination on the dorso-ventral regulation of photosynthesis and stomatal conductance

The responses of surface-specific photosynthesis to illumination via the abaxial surface (Fig. 5) were different than those observed upon adaxial illumination (Fig. 4). Wheat leaves had much higher photosynthetic rates and stomatal conductance values on the abaxial leaf surfaces under abaxial compared to adaxial illumination (Fig. 5a). Wheat abaxial surface photosynthetic rates were approximately 3.5 times higher under adaxial illumination than abaxial illumination (Figs 4a & 5a). However, there was a concomitant decrease (65%) in adaxial surface photosynthesis rates upon abaxial illumination, compared to adaxial illumination.

The leaves of the two C_4 species studied did not show the same surface-specific responses in CO_2 assimilation rates with respect to light orientation that were observed in wheat (Figs 4a & 5a). Like the wheat leaves, the abaxial surface of maize and *P. dilatatum* leaves showed high CO_2

Whole leaf – light to adaxial or abaxial surfaces

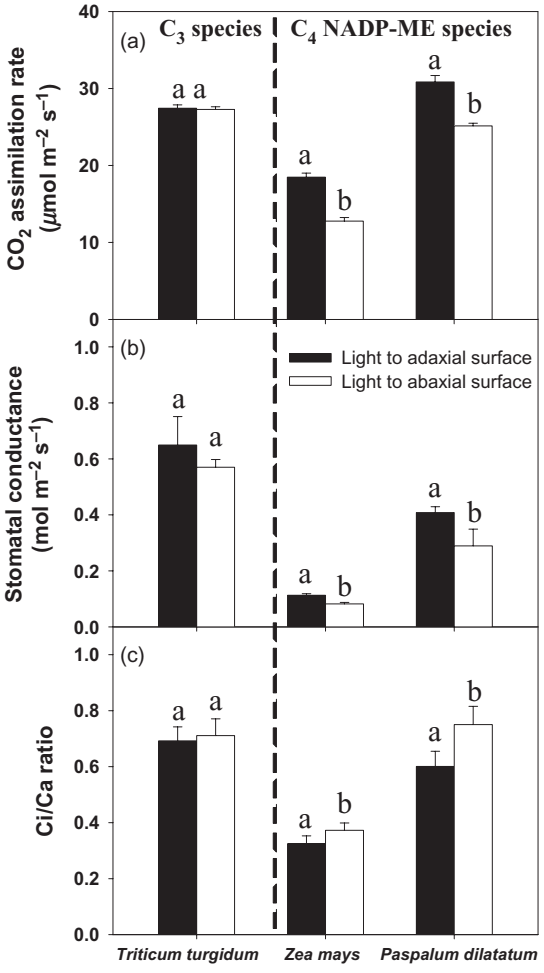

Figure 3. The effect of light orientation on the whole leaf CO_2 assimilation rate (a), stomatal conductance (b) and ratio between the intercellular (Ci) and the atmospheric (Ca) CO_2 concentrations (Ci/Ca ratio, c) in wheat, maize and *P. dilatatum* plants grown at 350 μL L^{-1} CO_2. Light was oriented to the adaxial (closed bars) or the abaxial (open bars) leaf surface. Data are the mean values ± SE of nine plants. The statistical analysis ($P < 0.05$) compares data for each species separately, the different letters representing significant differences.

Table 2. A comparison of the effects of light orientation on intrinsic WUE values determined in wheat (*Triticum turgidum*), maize (*Zea mays*) and *P. dilatatum* leaves

	Intrinsic WUE (μmol CO_2 mol H_2O^{-1})		
	T. turgidum	*Z. mays*	*P. dilatatum*
Light oriented to adaxial surface			
Whole leaf	42.25 ± 3.7 a	164.54 ± 14.6 a	75.63 ± 6.5 a
Adaxial surface	44.25 ± 4.1 a	160.58 ± 14.8 a	156.76 ± 15.2 b
Abaxial surface	42.55 ± 3.4 a	225.66 ± 20.6 b	310.45 ± 29.6 c
Light oriented to abaxial surface			
Whole leaf	47.85 ± 3.8 a	155.81 ± 15.3 a	87.02 ± 7.6 a
Adaxial surface	39.78 ± 3.3 a	–[a]	–[a]
Abaxial surface	68.89 ± 5.4 a	171.40 ± 16.3 ab	383.33 ± 35.6 b

The letters (a, b and c) indicate significant differences ($P < 0.05$) in the data (mean ± SE values for each species.

[a]Near-zero intrinsic WUE values are not considered here as the almost complete closure of the stomata can result in error regarding the determinations of stomatal conductance, which affects the values used to calculate the intrinsic WUE.

assimilation rates and stomatal conductance values under abaxial illumination (Fig. 5a,b). Photosynthesis rates were increased by approximately 50% in maize and *P. dilatatum* leaves under abaxial compared to adaxial illumination (compare data in Fig. 4a,b with that in Fig. 5a,b). However, the stomatal conductance and CO_2 assimilation rates on the adaxial surfaces of the maize and *P. dilatatum* fell to values below the level of detection under abaxial illumination (Fig. 5a). While, Ci/Ca relationships were similar on both surfaces of wheat leaves (Fig. 5c), the Ci/Ca parameter could not be determined for the adaxial surfaces of the maize and *P. dilatatum* leaves because of the very low stomatal conductance values.

Effects of light orientation on the dorso-ventral regulation of intrinsic water use efficiencies

The intrinsic WUE values were similar on both surfaces of wheat leaves and they were not significantly altered by light orientation (Table 2). In contrast, the intrinsic WUE values were higher on the abaxial surfaces than the adaxial surfaces under adaxial illumination in maize and *P. dilatatum* leaves (Table 2). As near-zero values were observed on the adaxial surfaces of the leaves of these two C_4 species under abaxial illumination, the effect of light orientation on intrinsic WUE values could only be determined for the abaxial surface. The abaxial surfaces of the *P. dilatatum* leaves showed significantly higher intrinsic WUE values under abaxial illumination (Table 2).

Dorso-ventral variations in the total section area of the BS cells and of the surrounding first layer of M cells

To determine whether the leaf side-specific differences in the regulation of photosynthesis were associated with dorso-ventral variations in Kranz anatomy, the cell section area of BS (shown as the white dotted line in Fig. 1) and M (shown as the black solid line in Fig. 1) cells was calculated for each species.

Wheat has two BS layers, an inner thicker walled sheath without chloroplasts and an outer layer with chloroplasts and similar wall thickness to the M cells. The inner BS of the wheat leaves my thus be considered to correspond to the mesotome or BS of the C_4 panicoid grass leaves. The mesotome is an endodermoid sheath that is important for the movement of metabolites and photosynthetic intermediates from the M to the vascular bundles. Therefore, in the present study, we have only considered the BS cells that are in direct contact with the M cells because the crucial role of the mestome cells in metabolite transfer associated with photosynthesis. Thus, the ratio of the section area of the BS cells relative to that of the M cells (BS/M section area ratio) was calculated because it is a measure of the BS/M interface. This parameter is thus related to the ability to transfer the metabolites that ensure the efficient operation of C_4 photosynthesis between the two cell types.

The total section area of BS and that of the first layer of surrounding M cells in the leaves was the similar on both sides of the wheat leaves (Table 3). While the total section area of BS cells was similar on both sides of the maize leaves, the area of the first layer of surrounding M cells was higher on the adaxial side than on the abaxial side of the leaf (Table 3). In contrast, the total section area of BS cells in *P. dilatatum* leaves was lower on the adaxial side than the abaxial side and the first layer of surrounding M cells had a relatively higher total section area than the abaxial side (Table 3). Thus, the BS/M section area ratios were lower on the adaxial sides of the vascular bundles in maize and *P. dilatatum* leaves compared to the abaxial sides, while the BS/M section area ratio was similar on both sides of the wheat leaves (Table 3).

DISCUSSION

The adaxial and abaxial sides of C_4 monocotyledonous leaves such as maize can be viewed as separate compartments in terms of CO_2 diffusion and assimilation (Long *et al.* 1989). The data presented here suggest that the two

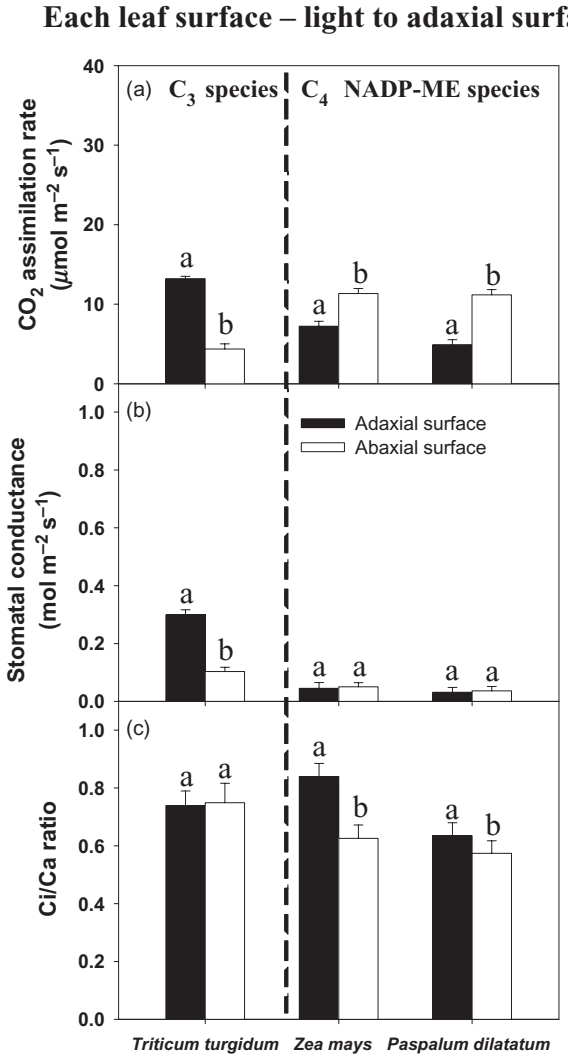

Figure 4. A comparison of the CO_2 assimilation rate (a), stomatal conductance (b) and ratio between the intercellular (Ci) and the atmospheric (Ca) CO_2 concentrations (Ci/Ca ratio, c) obtained for the adaxial (closed bars) and the abaxial (open bars) leaf surface in wheat, maize and *P. dilatatum* plants grown at 350 μL L^{-1} CO_2. Light was oriented to the adaxial leaf surface. Data are the mean values ± SE of nine plants. The statistical analysis ($P < 0.05$) compares data for each species separately, the different letters representing significant differences.

compartment model also applies to another C_4 grass species (*P. dilatatum*) but not to the C_3 monocotyledonous species, Durum wheat. The present comparison of the side-specific regulation of photosynthesis in wheat with that of two C_4 monocotyledonous plants shows that each species exhibits surface-specific responses of photosynthesis with respect to light orientation (Fig. 3). However, dorso-ventral variations were most marked when photosynthesis was measured on each leaf surface independently (Figs 4 & 5). The data show that there is also a dorso-ventral asymmetry in Kranz anatomy in the C_4 monocotyledonous leaves in addition to the two separate compartment system described by Long *et al.* (1989).

Leaves from all of the three monocotyledonous species studied here (Fig. 2) showed classic side-specific differences in the partitioning of the xylem and phloem, together with variations in the basic patterns of dorso-ventral M anatomy and structure that are characteristic of monocotyledonous leaves. However, the degree of dorso-ventral structural symmetry was much greater in the C_3 species than the two C_4 species. The stomatal numbers were the same on each surface of the wheat leaves (Fig. 2) and the BS/M section area ratio was the same on each side of the leaf (Table 3).

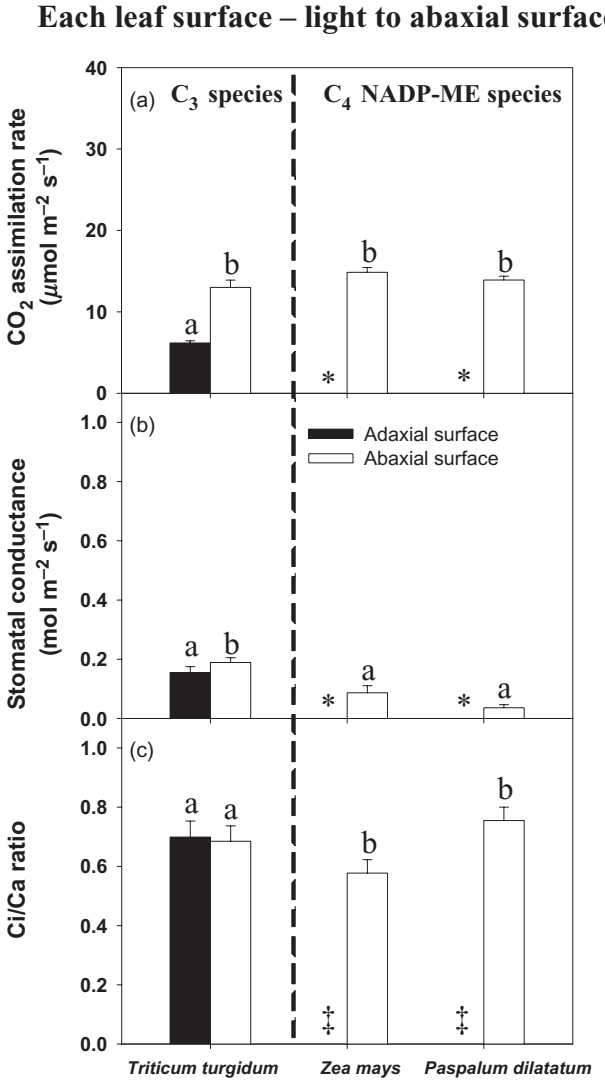

Figure 5. A comparison of the CO_2 assimilation rate (a), stomatal conductance (b) and ratio between the intercellular (Ci) and the atmospheric (Ca) CO_2 concentrations (Ci/Ca ratio, c) obtained for the adaxial (closed bars) and the abaxial (open bars) leaf surface in wheat, maize and *P. dilatatum* plants grown at 350 μL L^{-1} CO_2. Light was oriented to the abaxial leaf surface. Data are the mean values ± SE of nine plants. The statistical analysis ($P < 0.05$) compares data for each species separately, the different letters representing significant differences. * values below the level of detection. ‡ values too low for accurate calculation.

	T. turgidum	*Z. mays*	*P. dilatatum*
Adaxial leaf surface			
BS section area (μm^2)	1154 ± 77 a	2195 ± 164 a	1038 ± 41 a
M section area (μm^2)	2060 ± 121 a	4337 ± 419 a	2111 ± 88 a
BS/M section area ratio	0.58 ± 0.04 a	0.53 ± 0.03 a	0.50 ± 0.02 a
Abaxial leaf surface			
BS section area (μm^2)	1080 ± 59 a	2070 ± 125 a	1273 ± 72 b
M section area (μm^2)	1952 ± 104 a	3079 ± 157 b	1796 ± 91 b
BS/M section area ratio	0.57 ± 0.39 a	0.70 ± 0.04 b	0.71 ± 0.03 b

Data are mean ± SE of 25 vascular bundles per plant, with three plants analysed per species. The statistical analysis ($P < 0.05$) compares data for each species separately, the different letters representing significant differences.

Table 3. A comparison of the total section area of the bundle sheath (BS) cells and the first layer of surrounding mesophyll (M) cells and the BS/M section area ratios on each side of the vascular bundles of wheat (*Triticum turgidum*), maize (*Zea mays*) and *P. dilatatum* leaves

Moreover, the regulation of photosynthesis on the adaxial surface was similar to that on the abaxial surface with respect to light orientation (Figs 4 & 5). Regardless of adaxial/abaxial specification, the surface of the wheat leaf that was directly exposed to light showed similar high photosynthetic rates, with a 65% reduction in photosynthetic rates on the other non-illuminated surface. These observations suggest that there is a high conductance to CO_2 diffusion between the upper and lower sub-stomatal cavities in the wheat leaves studied here.

The leaves of the two C_4 monocotyledonous species showed a much lower level of dorso-ventral symmetry than the wheat leaves. There were larger numbers of stomata on the abaxial surfaces of maize and *P. dilatatum* leaves relative to the adaxial surfaces (Fig. 2). Moreover, the abaxial sides of the leaves had a significantly higher BS/M cell section area ratio (Table 3), and there was a remarkably rigid surface-specific regulation of photosynthesis with respect to light orientation (Figs 4 & 5). In particular, photosynthetic CO_2 assimilation rates on the adaxial sides of the maize and *P. dilatatum* leaves fell to values below detection because the stomata closed in the absence of direct illumination. In marked contrast, photosynthesis rates and stomatal closure on abaxial surfaces were much less affected by the absence of direct irradiation (Figs 4 & 5). We also observed similar patterns of dorso-ventral asymmetry with respect to light orientation in the leaves of another C_4 monocotyledonous species, sugarcane (*Saccharum officinarum*; data not shown). In all three C_4 monocotyledonous species, the abaxial surfaces exhibited higher rates of CO_2 assimilation under direct abaxial illumination but (unlike wheat) photosynthesis on the adaxial surfaces was not detectable in this situation.

The presence of a physical CO_2 diffusion barrier between the two sides of C_4 monocotyledonous leaves facilitates side-specific regulation of photosynthesis as discussed previously (Long *et al.* 1989; Driscoll *et al.* 2006; Soares *et al.* 2008). In the absence of the surface-specific effects on light absorptance, reflectance or transmission or the abundance of photosynthetic proteins (Cordón & Lagorio 2007; Soares *et al.* 2008), we are drawn to the conclusion that regulation of the BS/M section area ratio on each side of the leaf is an important mechanism contributing to the surface-specific regulation of photosynthesis in C_4 monocotyledonous leaves. We have no information as yet concerning the relative numbers of plasmodesmata connecting the M and BS cells on the upper and lower sides of the two C_4 monocotyledonous leaves studied here but the observed higher values for the BS/M section area ratios on the abaxial sides of the vascular bundles would tend to favour a more rapid metabolite transfer between the M and BS cells (Sowiński, Szczepanik & Minchin 2008). Metabolite transfer between the M and BS cells is not only central to the regulation of C_4 photosynthesis (von Caemmerer & Furbank 1999), but also facilitates a wide range of other process in C_4 leaves such as carbohydrate synthesis, nitrogen and sulphur metabolism (Leegood 2008). The ratios of the total section area of the BS cells to that of the surrounding M cells were lowest on the adaxial sides of the C_4 monocotyledonous leaves, where the stomatal numbers are lowest. Conversely, the ratios of the total section area of the BS cells to that of the surrounding M cells were highest on the abaxial sides of the C_4 monocotyledonous leaves, where the stomatal numbers were highest. It is thus tempting to suggest that there is a developmental relationship between stomatal numbers and the BS/M section area ratio, and possibly also the number of plasmodesmatal connections between the M and BS cells on each side of the leaf and that adaxialization factors in C_4 monocotyledonous leaves serve to reduce these parameters. Our present hypothesis considers that differing plasmodesmatal densities between cells on each side of the leaves might exert significant control over photosynthesis rates via regulation of metabolite transport rates between M and BS cells. This hypothesis was proposed because measurements of light absorption and transmission profiles and also of patterns of dorso-ventral distribution of key photosynthetic enzyme proteins showed no detectable variations between the two sides of the C_4 leaves (Soares *et al.* 2008). However, we have to acknowledge that while a given leaf could present similar reflectance, transmittance and absorptance profiles on the adaxial and abaxial surfaces, the internal distribution of absorbed quanta might be different when that leaf is irradiated from different orientations. Our hypothesis also assumes that the ratio of quanta absorbed in the BS and M chloroplasts is similar when light is directed to the adaxial compared to the abaxial surfaces, and that profiles of light absorption are the same in the adaxial and abaxial M cells.

It is important to consider the advantages conferred by having a two air-space system in the C_4 monocotyledonous grasses studied here that is not required in the C_3 species, Durum wheat. In addition to a precise surface-specific photosynthetic response to light orientation, the two air-space system will allow separation not only in the effects of stress factors (Long *et al.* 1989) but also in the perception and signalling of environmental change and stress. The surface-specific regulation of photosynthesis with respect to incident light may firstly provide a better adaptation to water deficits, as indicated by the presence of a significant effect of light orientation on intrinsic WUE in *P. dilatatum* leaves (Table 2) and secondly to accompanying light stress than that offered by a simple dorso-ventrally symmetrical structure. The Ci/Ca ratios of wheat leaves are similar on both surfaces (Figs 3 & 4) indicating no dorso-ventral differences in CO_2 assimilation rates. In contrast, the abaxial surfaces of the C_4 leaves maintain lower Ci/Ca ratios under adaxial illumination, consistent with the higher rates of photosynthesis on this side of the leaves (Fig. 3).

The two air-space system is useful not only in the optimization of whole leaf photosynthesis in relation to prevailing environmental conditions but it also allows the operation of surface-specific CO_2- and defence-signalling cascades. For example, the regulation of the stomata is not wholly autonomous but depends on the metabolic and signalling activities of the adjacent M. It is well established that stomatal opening is regulated in response to changes in CO_2 or light and that this involves a signal generated by the M cells (Mott, Sibbernsen & Shope 2008). While such effects have often been attributed to a reduction in Ci caused by light-induced photosynthetic CO_2 uptake, accumulating evidence suggests that M signal influencing stomatal opening is not primarily photosynthetic in origin (von Caemmerer *et al.* 2004; Baroli *et al.* 2008). Moreover, systemic light stress signalling involves many signalling cascades including salicylic acid, jasmonic acid and ethylene-dependent pathways (Karpinski *et al.* 1999; Rossel *et al.* 2007; Mühlenbock *et al.* 2008). Thus, a two-compartment system may facilitate separate dorso-ventral systemic stress signalling pathways such as those that participate or arise from leaf curling phenomena that are common in C_4 monocotyledonous leaves in response to environmental stresses such as drought (as illustrated in Fig. 6b). In leaves with an adaxial/abaxial two compartment system the diffusible signal from the M will not only exert effects on adjacent stomata opening (Mott *et al.* 2008) but also allow a high degree of dorso-ventral-specificity in many developmental and defence responses.

In conclusion, these data demonstrate that the dorso-ventral variations in Kranz anatomy participate in the control photosynthesis with respect to light orientation in C_4 monocotyledonous leaves. The presence of such dorso-ventral mechanisms that regulate metabolite transport between cells might not only be important in the regulation of photosynthesis and stomatal regulation but also in the control of side-specific cell signalling events in C_4 monocotyledonous leaves, particularly in relation to the direction of incident light (Fig. 6a).

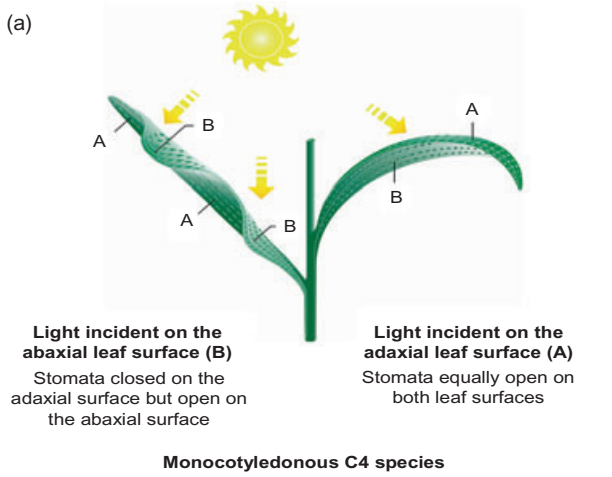

Figure 6. Diagrammatic representation of light orientation effects on stomatal closure (a) together with a photograph showing leaf rolling in maize plants deprived of water for 5 d (b). The white arrows on Fig. 6b indicate sections of the abaxial leaf surface that become exposed to adaxial irradiation upon rolling.

ACKNOWLEDGMENTS

Ana Sofia Soares-Cordeiro was supported by the Fundação para a Ciência e a Tecnologia (Ph.D. grant n° SFRH/13728/2003), the Fundação Calouste Gulbenkian and the Society for Experimental Biology. Rothamsted Research receives grant-aided support from the UK Biotechnology and Biological Sciences Research Council. Sample preparation for microscopy was undertaken in the Rothamsted Centre for Bioimaging. The authors thank Dr G.L. Lockett, Margot Forage Germplasm Centre, New Zealand for providing the *P. dilatatum* Poiret cv. Raki Seeds. We thank Soussanith Nokham, CSIRO, Canberra, for drawing the image in Fig. 6a and Marta Lopes for taking photo shown in Fig. 6b.

REFERENCES

Baroli I., Price G.D., Badger M.R. & von Caemmerer S. (2008) The contribution of photosynthesis to the red light response of stomatal conductance. *Plant Physiology* **146,** 737–747.

Bolhàr-Nordenkampf H.R. & Draxler G. (1993) Functional leaf anatomy. In *Photosynthesis and Productivity in a Changing Environment. A Field and Laboratory Manual* (eds D.O. Hall, J.M.O. Scurlock, H.R. Bolhar-Nordenkampf, R.C. Leegood & S.P. Long) pp. 91–112. Chapman & Hall, London, UK.

Bowman J.L., Eshed Y. & Baum S.F. (2002) Establishment of polarity in angiosperm lateral organs. *Trends in Genetics* **18,** 134–141.

Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry* **72,** 248–254.

von Caemmerer S. & Evans J.R. (1991) Determination of the average partial pressure of CO_2 in chloroplasts from leaves of several C_3 plants. *Australian Journal of Plant Physiology* **18,** 287–305.

von Caemmerer S. & Furbank R.T. (1999) Modeling C4 photosynthesis. In *C_4 Plant Biology* (eds R.F. Sage & R.K. Monson), pp. 313–373. Academic Press, San Diego, CA, USA.

von Caemmerer S., Lawson T., Oxborough K., Baker N.R., Andrews T.J. & Raines C.A. (2004) Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *Journal of Experimental Botany* **55,** 1157–1166.

Centritto M., Loreto F. & Chartzoulakis K. (2003) The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. *Plant, Cell & Environment* **26,** 585–594.

Cordón G.B. & Lagorio M.G. (2007) Optical properties of the adaxial and abaxial faces of leaves. Chlorophyll fluorescence, absorption and scattering coefficients. *Photochemical and Photobiological Sciences* **6,** 873–882.

Driscoll S.P., Prins A., Olmos E., Kunert K.J. & Foyer C.H. (2006) Specification of adaxial and abaxial stomata, epidermal structure and photosynthesis to CO_2 enrichment in maize leaves. *Journal of Experimental Botany* **57,** 381–390.

Dutilleul C., Driscoll S., Cornic G., De Paepe R., Foyer C.H. & Noctor G. (2003) Functional mitochondrial complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. *Plant Physiology* **131,** 264–275.

Emery J.F., Floyd S.K., Alvarez J., Eshed Y., Hawker N.P., Izhaki A., Baum S.F. & Bowman J.L. (2003) Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. *Current Biology* **13,** 1768–1774.

Evans J.R. & Loreto F. (2000) Acquisition and diffusion of CO2 in higher plant leaves. In *Photosynthesis: Physiology and Metabolism* (eds R.C. Leegood, T.D. Sharkey & S. von Caemmerer), pp. 321–351. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Evans J.R. & Vogelmann T.C. (2003) Photosynthesis within isobilateral *Eucalyptus pauciflora* leaves. *New Phytologist* **171,** 771–782.

Evans J.R. & Vogelmann T.C. (2006) Profiles of $^{14}CO_2$ fixation through spinach leaves in relation to light absorption and photosynthetic capacity. *Plant, Cell & Environment* **26,** 547–560.

Flexas J., Diaz-Espejo A., Galmés J., Kaldenhoff R., Medrano H. & Ribas-Carbo M. (2007) Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. *Plant, Cell & Environment* **30,** 1284–1298.

Horemans N., Foyer C.H. & Asard H. (2000) Transport and action of ascorbate at the plant plasma membrane. *Trends in Plant Science* **5,** 263–267.

Juarez M.T., Kui J.S., Thomas J., Heller B.A. & Timmermans M.C.P. (2004) MicroRNA-mediated repression of rolled *leaf1* specifies maize leaf polarity. *Nature* **428,** 84–88.

Karpinski S., Reynolds H., Karpinska B., Wingsle G., Creissen G. & Mullineaux P. (1999) Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. *Science* **284,** 654–657.

Kidner C.A. & Martienssen R.A. (2004) Spatially restricted microRNA directs leaf polarity through ARGONAUTE1. *Nature* **428,** 81–84.

Leegood R.C. (2008) Roles of the bundle sheath cells in leaves of C_3 plants. *Journal of Experimental Botany* **59,** 1663–1673.

Lichtenhaler H.K. & Wellburn A.R. (1983) Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions* **11,** 591–592.

Lloyd J., Syvertsen J.P., Kriedemann P.E. & Farquhar G.D. (1992) Low conductances for CO_2 diffusion from stomata to the sites of carboxylation in leaves of woody species. *Plant, Cell & Environment* **15,** 873–899.

Long S.P., Farage P.K., Bolhar-Nordenkampf H.R. & Rohrhofer U. (1989) Separating the contribution of the upper and lower mesophyll to photosynthesis in *Zea mays* L. leaves. *Planta* **177,** 207–216.

Miyazawa S.I. & Terashima I. (2001) Slow development of leaf photosynthesis in an evergreen broad-leaved tree, *Castanopsis sieboldii*: relationships between leaf anatomical characteristics and photosynthetic rate. *Plant, Cell & Environment* **24,** 279–291.

Mott K.A., Sibbernsen E.D. & Shope J.C. (2008) The role of the mesophyll in stomatal responses to light and CO_2. *Plant, Cell & Environment* **31,** 1299–1306.

Mühlenbock P., Szechyńska-Hebda M., Płaszczyca M., Baudo M., Mateo A., Mullineaux P.M., Parker J.E., Karpińska B. & Karpiński S. (2008) Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. *The Plant Cell* **20,** 2339–2356.

Noctor G., Veljovic-Jovanovic S., Driscoll S., Novitskaya L. & Foyer C.H. (2002) Drought and oxidative load in wheat leaves: A predominant role for photorespiration? *Annals of Botany* **89,** 841–850.

Novitskaya L., Trevanion S., Driscoll S.D., Foyer C.H. & Noctor G. (2002) How does photorespiration modulate leaf amino acid contents? A dual approach through modelling and metabolite analysis. *Plant, Cell & Environment* **25,** 821–836.

Ögren E. & Evans J.R. (1993) Photosynthetic light response curves. 1. The influence of CO_2 partial pressure and leaf inversion. *Planta* **189,** 182–190.

Olmos E., Kiddle G., Kumar S. & Foyer C.H. (2006) Ascorbic acid deficiencycauses changes in M cell structure and in root architecture in Arabidopsis thaliana. *Journal of Experimental Botany* **57,** 1645–1655.

Pemadasa M.A. (1979) Movements of abaxial and adaxial stomata. *New Phytologist* **82,** 69–80.

Pemadasa M.A. (1982) Abaxial and adaxial stomatal responses to light of different wavelengths and to phenylacetic acid on isolated epidermis of *Commelina communis* L. *Journal of Experimental Botany* **33,** 92–99.

Piel C., Frank E., Le Roux X. & Genty B. (2002) Effect of local irradiance on CO_2 transfer conductance of mesophyll in walnut. *Journal of Experimental Botany* **53,** 2423–2430.

Pignocchi C. & Foyer C.H. (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signaling. *Current Opinion in Plant Biology* **6,** 379–389.

Rossel J.B., Wilson P.B., Hussain D., Woo N.S., Gordon M.J., Mewett O.P., Howell K.A., Whelan J., Kazan K. & Pogson B.J. (2007) Systemic and intracellular responses to photooxidative stress in Arabidopsis. *The Plant Cell* **19,** 4091–4110.

Sharkey T.D., Imai K., Farquhar G.D. & Cowan I.R. (1982) A direct confirmation of the standard method for estimating partial pressure of CO_2. *Plant Physiology* **69,** 657–659.

Sharkey T.D., Vassey T.L., Vanderveer P.J. & Vierstra R.D. (1991) Carbon metabolism enzymes and photosynthesis in transgenic tobacco (*Nicotiana tabaccum* L.) having excess phytochrome. *Planta* **185,** 287–296.

Soares A.S., Driscoll S.P., Olmos E., Harbinson J., Arrabaça M.C. & Foyer C.H. (2008) Adaxial/abaxial specification in the regulation of photosynthesis and stomatal opening with respect to light orientation and growth with CO_2 enrichment in the C_4 species *Paspalum dilatatum*. *New Phytologist* **177,** 186–198.

Sowiński P., Szczepanik J. & Minchin E.H. (2008) On the mechanism of C_4 photosynthesis intermediate exchange between Kranz mesophyll and bundle sheath cells in grasses. *Journal of Experimental Botany* **59,** 1137–1147.

Terashima I. & Inoue Y. (1985a) Vertical gradient in photosynthetic properties of spinach chloroplasts dependent on intra-leaf light environment. *Plant and Cell Physiology* **24,** 781–785.

Terashima I. & Inoue Y. (1985b) Palisade tissue chloroplasts and spongy tissue chloroplasts in spinach: Biochemical and ultrastructural differences. *Plant and Cell Physiology* **26,** 63–75.

Terashima I., Sakaguchi S. & Hara N. (1986) Intra-leaf and intracellular gradients in chloroplast ultrastructure of dorsiventral leaves illuminated from the adaxial or abaxial side during their development. *Plant and Cell Physiology* **27,** 1023–1031.

Travis A.J. & Mansfield T.A. (1981) Light saturation of stomatal opening on the adaxial and abaxial epidermis of Commelina communis. *Journal of Experimental Botany* **32,** 1169–1179.

Turner N.C. & Singh D.P. (1984) Responses of adaxial and abaxial stomata to light and water deficits in sunflower and sorghum. *New Phytologist* **96,** 187–195.

Veljovic-Jovanovic S., Pignocchi C., Noctor G. & Foyer C.H. (2001) Low vitamin C in the vtc 1 mutant of *Arabidopsis thaliana* is associated with decreased growth and intracellular redistribution of the antioxidant system. *Plant Physiology* **127,** 426–435.

Vogelmann T.C. (1993) Plant tissue optics. *Annual Review of Plant Physiology and Plant Molecular Biology* **44,** 233–251.

Wang X.-Q., Wu W.-H. & Assmann S.M. (1998) Differential responses of adaxial and abaxial guard cell of broad bean to Abscisic acid and calcium. *Plant Physiology* **118,** 1421–1429.

Received 10 August 2009; accepted for publication 10 August 2009