Stomatal and pavement cell density linked to leaf internal CO₂ concentration

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INTRODUCTION

Stomata are pores, which can vary in aperture, on plant surfaces exposed to the air: they control water and CO₂ exchange between plants and the atmosphere. Globally, about 17 % of atmospheric CO₂ enters the terrestrial vegetation through stomata and is fixed as gross photosynthesis each year. Water in the earth’s atmosphere is replaced more than twice a year by stomata-controlled transpiration (Hetherington and Woodward, 2003). For plants to survive in a changing environment, stomata must be able to respond to environmental stimuli. Their long-term (days to millennia) response manifests itself through variations of stomatal density (SD, number of stomata per unit of leaf area) and/or size, whereas their short-term dynamics derive from changes in the width of the pores’ apertures, at a time scale of minutes. The effect of atmospheric CO₂ concentration on both SD and stomatal opening has been recognized (Woodward, 1987; Mott, 1988; Woodward and Bazzaz, 1988; Morison, 1998; Hetherington and Woodward, 2003; Franks and Beerling, 2009), used in reconstruction of paleoclimate (Retallack, 2001; Royer, 2001; Royer et al., 2001; Beerling and Royer, 2002; Beerling et al., 2002) and subjected to meta-analysis (Ainsworth and Rogers, 2007). It is well known that stomatal guard cells respond to the internal CO₂ concentration (Cᵢ) of the leaf rather than to the external CO₂ concentration: pores open when Cᵢ falls and close when it goes up (Mott, 1988; Willmer, 1988; Mott, 2009). It is tempting to speculate that similar sensing of Cᵢ may also act during the final setting of SD in mature leaves: it seems more plausible that the frequency of the supply ‘valves’ is controlled by chloroplast-mediated CO₂ demand than by globally modulated availability of CO₂ in the free atmosphere. However, to our knowledge, there are no experiments on the relationship of SD to internal CO₂.

Recently, our knowledge of how stomatal frequency is controlled at the genetic level (Nadeau and Sack, 2003; Coupe et al., 2006; Wang et al., 2007) and in response to CO₂ (Gray et al., 2000; Bergmann, 2006; Casson and Gray, 2008; Hu et al., 2010) has increased considerably, but the nature and location of the putative CO₂ sensor remain unresolved. Development of stomata considerably precedes leaf unfolding, and most stomata are already developed before the leaf reaches 10–20 % of its final area, when SD reaches its maximum (Tichá, 1982; Pantin et al., 2012). At that stage of ontogeny, stomata and their cell precursors experience a local atmosphere that is probably more humid and enriched in respired CO₂ than ambient...
air. Sensing of the free atmospheric environment is thus unlikely. In addition to atmospheric CO2, other external signals affect SD, among them irradiance, atmospheric and soil water content, temperature, and phosphorus and nitrogen nutrition (Schoch et al., 1980; Bakker, 1991; Abrams, 1994; Sun et al., 2003; Thomas et al., 2004; Lake and Woodward, 2008; Sekiya and Yano, 2008; Xu and Zhou, 2008; Fraser et al., 2009; Figueroa et al., 2010; Yan et al., 2012). It is useful to distinguish between the different steps of stomatal development that may be affected by the external cues, i.e. whether it is, on the one hand, the division of protodermal or meristemoid cells leading toward guard cell and pavement cell formation, or, on the other, the enlargement of already existing guard cells and pavement cells during leaf growth that is affected. Stimulation of the first group of processes usually leads to a change in the fraction of stomata among all epidermal cells [stomatal index (SI)], whereas the proportional enlargement of stomata and pavement cells during leaf expansion reduces SD and pavement cell density (PCD), and increases the maximal stomatal size and aperture. However, both developmental phases overlap (Asl et al., 2011).

In young leaves that are still metabolically supported by mature leaves, the signal about conditions in the free atmosphere may come from mature leaves (Lake et al., 2001; Miyazawa et al., 2006). A pivotal role in the signal has been attributed to abscisic acid (ABA) (Lake and Woodward, 2008) or the 13C content (δ13C) in assimilates (Sekiya and Yano, 2008). (For the sake of simplicity, we omit the 13C after δ here from on since carbon is the only element whose stable isotopes are considered here. Also, internal CO2 refers to the CO2 within the organ.) The degree of carbon isotope discrimination is considered a ‘fingerprint’ of the ratio of internal CO2 concentration over ambient CO2, Ci/Ca, which is negatively proportional to intrinsic water-use efficiency (WUE: Farquhar and Richards, 1984). However, what substitutes for the role of a signal derived from older leaves in the first ever phototrophic organ in a plant’s life, the cotyledon? The information on Cj/Ca experienced by the maternal generation could be delivered to cotyledons via assimilates stored in the seed. Alternatively, SD in cotyledons could be controlled by the local environment of primordial cotyledons.

Here, we tested (1) whether the density of stomata on fully developed true leaves is sensitive to ambient or internal CO2 concentration. We manipulated Cj by changing various environmental factors affecting photosynthetic rate and/or stomatal aperture (light quantity, ABA and osmotic stress) while keeping ambient CO2 constant during the plant’s growth. Four species were used for testing this question. In two species, we also investigated the Ci response of pavement cells and calculated the SI. Data from the literature for 16 plant species cultured at approximately 60 % relative air humidity, day/night temperatures of 25/20 °C and a 16 h photoperiod. The high-light and low-light treatments were species specific. Sunflower was grown at irradiances [photosynthetic photon flux density (PPFD)] of 700 μmol m–2 s–1 (high light) or 70 μmol m–2 s–1 (low light), both with the same spectral composition. After 5 weeks, the plants cultivated at each irradiance were divided into three sub-groups, and ABA [10–5 m, (+)-abscisic acid, Sigma-Aldrich, Germany] or PEG 6000 [5 % (w/w), Sigma-Aldrich, Germany] was added to the hydroponic solution in two sub-groups, leaving the third as the control. The solutions were renewed once a week. After 3 weeks, newly developed mature leaves were collected for carbon isotope analysis and estimation of SD. Arabidopsis plants were grown for 18 d at a PPFD of 200 or 80 μmol m–2 s–1 and the first rosette leaf was measured. Garden cress was grown from seeds in 100 mL pots in garden soil for 14 d at a PPFD of 500 μmol m–2 s–1 and the first rosette leaf was measured. Beech leaves were collected eight and ten times during the seasons of 2007 and 2009, respectively. The shaded leaves were sampled from a part of the crown exposed to the north and facing the forest; the sun-exposed leaves were collected from the opposite side, facing a meadow. The average PPFD at sampling time (1300–1500 h) was 1301 (±384) and 33 (±19) μmol m–2 s–1 in the sun-exposed and shaded environment, respectively (PAR sensors and data loggers Minikin R, EMS, Brno, Czech Republic).

Experiment 2: cotyledons and true leaves in air and helox at variable Cj. The SD and PCD on cotyledons and first leaves of garden cress were compared in a set of controlled-atmosphere experiments. We chose garden cress because of its fast growth rate allowing us to reduce costs for compressed gases, mainly helium. The plants were grown for 14 d from seed in an artificial atmosphere in 600 mL glass desiccators, through which a gas flow of 500 mL min–1 was maintained. There were 10–30 plants germinated on wet silica sand or perlite in each desiccator. Each harvest on the seventh and 14th day after seed watering (DAW) reduced the number of plants by ten. Plants were watered in 2- to 3-d intervals with tap water or with half-strength

**MATERIALS AND METHODS**

**Plants and growth conditions**

**Experiment 1: mature leaves at invariant Cj.** The SD of mature true leaves was estimated in three plant species (sunflower, Helianthus annuus; arabidopsis, Arabidopsis thaliana ecotypes Columbia and C24; and garden cress, Lepidium sativum) grown in a growth chamber (Fitotron, Sanyo, UK). Leaf samples of beech (Fagus sylvatica) were collected from a 60-year-old tree growing at a meadow–forest ecotone, from deeply shaded and sun-exposed parts of the crown. Sampling was organized in the course of two seasons (May–October 2007 and 2009) in 2- to 3-week intervals within a single tree in order to eliminate any genetic effects in the seasonal course of Ci. Groups of sunflower and garden cress plants were also treated with ABA or polyethylene glycol (PEG) added to the root medium in order to manipulate Ci via stomatal conductance. All plants were exposed to a free atmospheric CO2 concentration of about 390 μmol mol–1. Plants cultivated in growth chambers experienced 60 % relative air humidity, day/night temperatures of 25/20 °C and a 16 h photoperiod. The high-light and low-light treatments were species specific. Sunflower was grown at irradiances [photosynthetic photon flux density (PPFD)] of 700 μmol m–2 s–1 (high light) or 70 μmol m–2 s–1 (low light), both with the same spectral composition. After 5 weeks, the plants cultivated at each irradiance were divided into three sub-groups, and ABA [10–5 m, (+)-abscisic acid, Sigma-Aldrich, Germany] or PEG 6000 [5 % (w/w), Sigma-Aldrich, Germany] was added to the hydroponic solution in two sub-groups, leaving the third as the control. The solutions were renewed once a week. After 3 weeks, newly developed mature leaves were collected for carbon isotope analysis and estimation of SD. Arabidopsis plants were grown for 18 d at a PPFD of 200 or 80 μmol m–2 s–1 and the first rosette leaf was measured. Garden cress was grown from seeds in 100 mL pots in garden soil for 14 d at a PPFD of 500 μmol m–2 s–1 and the first rosette leaf was measured. Garden cress was grown from seeds in 100 mL pots in garden soil for 14 d at a PPFD of 500 μmol m–2 s–1 and the first rosette leaf was measured.
nutrient solution. Desiccators were placed in a growth chamber (Fitotron, Sanyo, UK) and attached to a computer-controlled gas mixing device (Tylan, USA and ProCont, ZAT Easy Control, Czech Republic). Plants were grown at a PPFD of 400 μmol m⁻² s⁻¹, with a 16 h photoperiod and day and night air temperatures of 23–25 °C. A mixture of He and O₂ at a v/v ratio of 79/21 (helox), or artificial air mixed from N₂ and O₂ at the same v/v ratio (air), with the addition of CO₂ at one of three concentrations (180, 400 and 800 μmol mol⁻¹) was fed into three parallel gas pathways to prepare one line with low humidity (60 ± 5%; LH) and two separate high humidity (90 ± 5%; HH) lines (using a two-channel dew point generator; Walz, Germany). The two gas mixtures differing in humidity flowed through two hermetically sealed desiccators with plants, arranged in parallel and having outlets open to the free atmosphere. The third pathway led the humid gas through a reducing valve into the third parallel desiccator with a vacuum pump attached to its outlet. This device allowed us to grow plants in the third desiccator at a total gas pressure reduced to one-half of that in the other two desiccators (hypobaric plants grown at pressure reduced to 450–500 hPa; RP). We included this hypobaric variant since plants grown at reduced total pressure operate at a C_i lower than what would have been expected for the given C_a (Körner et al., 1988). Gas mixtures were prepared from compressed He or N₂ (both with a purity of 4–6), oxygen (3.5) and CO₂ (20 % of CO₂ in N₂; all Messer, Czech Republic). The δ of the source CO₂ was −28.2 ‰. CO₂ and vapour concentrations were measured at the outlets of the desiccators with an IRGA (LiCor 6400; Li-Cor, Lincoln, NE, USA). The fractions of He and O₂ in the outlet atmosphere were measured with an He/O₂ analyser (Divesoft, Prague, Czech Republic) twice a day. Altogether, 18 repetitions with two different growth chambers (Sanyo, UK and Snijders Scientific, The Netherlands) were carried out. The carbon isotope composition of CO₂ in the chamber atmosphere was spatially homogeneous due to active ventilation (mean over the growth time δ = −11.0 ‰). Stomata were counted on fully expanded leaves of 6-week-old plants which were used for δ¹³C analysis. The designs of all four experiments are summarized in Table 1.

Stomatal density

The SD was estimated by light microscopy (Olympus BX61) on nail varnish imprints obtained directly from leaf surfaces (negatives) of adaxial and abaxial sides of mature leaves (only the abaxial side in beech). Cotyledons and first true leaves of 7-, 14- and 21-day-old garden cress and first rosette leaves of arabidopsis were investigated. Stomata and epidermal cells were counted on three plants per treatment, in each plant on adaxial and abaxial leaf and cotyledon sides. The cells on each side were counted in ten fields of 0.13 mm² each, randomly distributed across the leaf (apex, middle part and base). The results were expressed as counts of stomata or pavement cells per mm² of projected leaf area (total of adaxial and abaxial leaf sides), SD and PCD, respectively. The SI was calculated, where SD and PCD data were available, as SI(%) = SD/(SD + PCD) × 100.

Carbon isotope composition

The relative abundances of ¹³C over ¹²C (δ) were measured in leaf dry matter and, in expt 2 (garden cress in desiccators), also in seeds with testa removed (δ_a) and in the source CO₂ (δ_i) used for mixing the artificial atmospheres. δ_a and δ_i were −28.19 ‰ and −28.13 ‰, respectively. δ of growth chamber air was also estimated but was not used in the calculation of the treatment-induced changes of C_i (see later). Leaves were oven-dried at 80 °C, ground to a fine powder, packed in tin capsules and oxidized in a stream of pure oxygen by flash combustion at 950 °C in the reactor of an elemental analyser (EA) (NC 2100 Soil, ThermoQuest CE Instruments, Rodano, Italy). After CO₂ separation, the ¹³C/¹²C ratio (R) was detected via a continuous flow stable isotope ratio mass spectrometer (IRMS) (Delta plus XL, ThermoFinnigan, Bremen, Germany) connected on-line to the EA. The δ expressed in ‰ was calculated as the relative difference of sample and standard R: δ = (R sample/R standard − 1) × 1000. VPDB (IAEA, Vienna, Austria) was used as the standard. Cellulose (IAEA-C3) and graphite (USGS 24) were also included to ascertain the reliability of the results. Standard deviations of δ estimated in laboratory standard were < 0.05 ‰.

Leaf intercellular CO₂ concentration, C_i

We used the δ of leaf dry mass to evaluate the intercellular CO₂ concentration integrated over the leaf’s life time, C_i. The ¹³C discrimination during photosynthetic CO₂ fixation, Δ, is related to C_i as (Farquhar et al., 1989):

\[
\Delta = a + [(b - a) C_i/C_a]
\]
where $a$ and $b$ are $^{13}$CO$_2$ fractionations due to diffusion in the gas phase [4.4‰ in air and 2.0‰ in helox; see Farquhar et al. (1982) for derivation of $a$ based on reduced molecular masses] and carboxylation by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), excluding CO$_2$ dissolution and intracellular diffusion (27 ‰), respectively, and $C_a$ is the ambient CO$_2$ concentration. Contributions of (photo)respiration to $\Delta$ were considered to be small and were neglected. $\Delta$ is related to $^{13}$C abundance in the plant, $\delta_p$, and in air, $\delta_a$, as $\Delta = (\delta_a - \delta_p)/[(\delta_p/1000) + 1]$ where both $\delta$ and $\Delta$ are expressed in per mil ‰. The value of the denominator $(\delta_p/1000) + 1$ does not deviate much from 1 and can be omitted here; therefore $\Delta \approx (\delta_a - \delta_p)$ and rearrangement of eqn (1) for $C_i$ yields:

$$C_i = C_a \left( \frac{\delta_a - \delta_p - a}{b - a} \right)$$  (2)

In calculations of $C_i$ in leaves grown in the free atmosphere and in artificial mixed atmospheres, we used $\delta_a = -8.0$ ‰ and $-28.2$ ‰, respectively. Since the air in growth chambers can be contaminated with the $^{13}$C-depleted air exhaled by people handling the plants, we calculated and plotted differences in $C_i$ between the control and treated plants, rather than $C_i$ itself. The advantage of this procedure is that the differences do not depend on $\delta_a$ provided that the control and treated plants grew in the same mixed atmosphere, such as in our growth chamber experiments:

$$C_{i,t} - C_{i,c} = C_a \left( \frac{\delta_{p,c} - \delta_{p,t}}{b - a} \right)$$  (3)

where the subscripts $t$ and $c$ denote the treatment and control, respectively.

To calculate $C_i$ in semi-autotrophic cotyledons, it was necessary to determine the fraction $f$ of the cotyledons’ carbon originating from the heterotrophic source (seed). We grew garden cress in artificial air or in helox with $\delta$ significantly different from free air. Our seeds, which were produced in free air conditions at $\delta_a$.
close to \(-8\%\) and had \(\delta_p = -28.13\%\), grew in glass desiccators supplied with artificial air or helox with \(\delta_p = -28.19\%\). With an increasing proportion of autotrophy and \(f\) decreasing below 1, \(\delta_p\) decreased to values more negative than \(\delta_a\), with the sigmoid kinetics approaching a limit in fully autotrophic tissue with \(f = 0\). Typically, 14- to 21-day-old true leaves had not yet changed (4) (derived in Appendix) as:

\[
\frac{\Delta C}{C} \approx \frac{\delta_p - \delta_a}{f - 1 - a} \frac{C_a}{b - a}
\]

Statistical evaluation, meta-analysis

Descriptive statistics (mean, s.d.), Pearson correlation and linear regression were calculated using SigmaPlot v. 11.0 (SigmaPlot for Windows, Systat Software, Inc.). Normality was checked using normal probability plots and with Shapiro–Wilk tests. Linear correlations and confidence intervals were analysed using probability thresholds of 5 % and 95 %, respectively. Standard error of the mean of SI (SEM_{SI}) was calculated as the square root of the sum of squares of both partial derivatives of SI, each multiplied by the respective SD and PCD standard errors:

\[
\text{SEM}_{SI} = \sqrt{\left(\frac{\partial SI}{\partial SD} \text{SEM}_{SD}\right)^2 + \left(\frac{\partial SI}{\partial PCD} \text{SEM}_{PCD}\right)^2}
\]

After substituting the derivatives this becomes

\[
\text{SEM}_{SI} = \sqrt{\left(\frac{PCD \times 100}{(SD + PCD)^2} \text{SEM}_{SD}\right)^2 + \left[-\frac{SD \times 100}{(SD + PCD)^2} \text{SEM}_{PCD}\right]^2}
\]

where the horizontal lines above SD and PCD denote the mean SD and PCD values. STATISTICA v. 8 (StatSoft Ltd., Tulsa, OK, USA) was used in the meta-analysis. The SD and related \(\delta\) values were extracted from the available literature. Only those multifactorial studies were included in the meta-analysis where the environmental treatments were applied in a fully factorial design. Also, all studies on monocotyledons (grasses) were excluded from the meta-analysis as their SD can be governed by specific mechanisms.

RESULTS

The \(C_i\) response of stomatal density at invariant \(C_a\)

All investigated plant species responded to sub-optimal growth conditions with a change in the internal CO\(_2\) concentration of the leaf. Figure 1A shows that reduced irradiance increased \(C_i\) in shaded leaves compared with high-light controls in sunflower, beech and arabidopsis, and that there was a concomitant decrease in SD (see the points in the bottom right quadrant). The additional application of ABA or PEG to low-light-grown sunflower slightly modulated the \(C_i\) and SD deviations (points 2 and 3). Application of ABA or PEG to high-light-grown sunflower resulted in the opposite changes of \(C_i\) and SD to shading (points 4 and 5 in the upper left quadrant). The slope of the \(\Delta SD \sim \Delta C_i\) regression line is 5.23 (stomata) mm\(^{-2}\) \(\mu\)mol\(^{-1}\) (CO\(_2\)) mol. It indicates the sensitivity of the apparent SD response to \(C_i\). Since SD cannot reach zero or infinitely high values in a real plant, the \(C_i\) sensitivity applies only to the observed range of SD and should not be extrapolated. In order to overcome this limitation, we normalized the deviation of SD from the control (Fig. 1B). The relative decrement in SD with increasing \(C_i\) was attenuated exponentially, reaching an asymptote.
of –81.1 % for C<sub>i</sub> approaching a theoretical limit 10<sup>6</sup> (pure CO<sub>2</sub>) and setting the limit of phenotypic plasticity in lowering SD to about 20 % of the original value.

Comparison of the C<sub>i</sub> response in cotyledons and true leaves of garden cress

The results shown above were obtained at stable C<sub>a</sub>. In controlled-atmosphere experiments with garden cress, we used ambient (400 µmol mol<sup>−1</sup> as a control), sub-ambient (180 µmol mol<sup>−1</sup>) or super-ambient (800 µmol mol<sup>−1</sup>) CO<sub>2</sub> concentrations to grow cress plants for 14 d (or 21 d for sub-ambient CO<sub>2</sub>) in air or in helox atmosphere, each with two different gas humidities and also under reduced total pressure. Variations in C<sub>i</sub> were induced primarily by changing C<sub>a</sub>, using helox instead of air [CO<sub>2</sub> diffuses 2.3 times faster in helox than in air; see Parkhurst and Mott (1990)] and by reduction of total pressure, and only marginally by reduced air humidity. The time courses of developmental changes in δ and SD in all 18 experimental treatments are shown in Supplementary Data Figs. S1 and S2. The values in the bottom right quadrant of Fig. 2A and B (green triangles) show that the true leaves of super-ambient CO<sub>2</sub> plants had fewer stomata and pavement cells than the control plants. Conversely, in sub-ambient CO<sub>2</sub> plants, the SD and PCD increased (upper left quadrants of the plots). This effect was common for plants grown in air and helox, for plants grown at reduced atmospheric pressure (RP) and for both humidities (HH and LH). Therefore, for the regression analyses we analysed the six treatments (HH, LH and RP, each in helox and air) together and compared the C<sub>i</sub> response of true leaves (in 14-day-old plants) and cotyledons (at 7 and 14 DAW). In contrast to the true leaves, cotyledons did not alter the SD and PCD in response to C<sub>i</sub>. The slopes of the regression lines approached zero in cotyledons, while in the first leaves they significantly deviated from zero (Fig. 2; Table 2). The pavement cells in true leaves changed their density at 3.2 times the rate of stomata (see the ratio of slopes of the respective regression lines in Fig. 2A, B), which translates to an average reduction of 3.2 pavement cells for each stoma less in response to increasing C<sub>i</sub>.

The fraction of stomata among all epidermal cells (the SI) was almost invariant over the whole investigated range of C<sub>i</sub> in cotyledons and did not change during their development (SI ± SEM = 25.2 ± 1.4 % and 25.8 ± 2.4 % in 7- and 14-day-old plants, respectively). In true leaves, SI was higher than in cotyledons (29.4 ± 2.0 %) and changed only marginally with C<sub>i</sub> (slope = 0.0069 % mol µmol<sup>−1</sup>, R<sup>2</sup> = 0.32; see Table 2). However, standard errors of SI means determined from SD and PCD data were high and the SI changes with SD and PCD in all 18 experimental treatments are shown in Supplementary Data Figs. S1 and S2.

In order to verify the cotyledons’ insensitivity to C<sub>i</sub> in a way unbiased by estimation of the seed carbon fractions (see Appendix), we grew garden cress for a longer period (3 weeks)
confirmed that cotyledons are less sensitive to CO₂ relates to the changes in SD and putatively exerts its feed-

our experiments suggest that the internal rather than ambient CO₂ concentration of the leaf (Cᵢ) simultaneously under eight irradiances and equal Cᵢ. The results confirmed that cotyledons are less sensitive to Cᵢ than the first true leaves or are insensitive (Fig. 4A, B). The SD and PCD on the true leaves increased progressively at reduced Cᵢ. The SI in cotyledons did not respond to Cᵢ. However, in contrast to the previous experiment, SI significantly (P < 0.001) decreased with increasing Cᵢ in true leaves (Fig. 4C). The same data as in Fig. 4 but plotted in the form of differences from the ‘control’ irradiance (310 μmol m⁻² s⁻¹) and shown separately for abaxial and adaxial leaf sides are presented in Supplementary Data Fig. S4. CO₂ response of SI was steeper for the abaxial (lower) than the adaxial (upper) leaf side.

DISCUSSION

In this study we demonstrate for the first time that the internal CO₂ concentration of the leaf, Cᵢ, correlates strongly with the development of stomatal and pavement cells expressed in terms of their densities. We applied several independent environmental factors to manipulate Cᵢ. Therefore, the experimental results suggest, although they do not prove, that Cᵢ or a Cᵢ-related cue integrates the effects of those environmental parameters and conveys their signal into the machinery controlling SD. Our tests with cotyledons, the first assimilatory organs in a plant’s life, support the previous evidence that adult leaves are essential in generating the signal (Lake et al., 2001, 2002 Miyazawa et al., 2006). The results show that photosynthesis- and stomata-

controlled Cᵢ correlates with the number of stomatal and epidermal cells per unit of leaf surface: both insufficient stomatal conduc-
tance gₛ and/or an enhanced photosynthetic rate reduce Cᵢ, linking these processes directly or indirectly to the development of a new, acclimated leaf having more smaller stomata and pavement cells per mm² of the leaf surface. Conversely, elevated Cᵢ, caused for example by shading or elevated ambient CO₂ correlates with fewer stomata and pavement cells per mm² of the leaf surface and, consequently, larger epidermal cells develop (for examples of negative relationships between size and density of stomata, see Franks and Beerling, 2009; Franks et al., 2012). As this mechanism also operates at invariant Cᵢ, our experiments suggest that the internal rather than ambient CO₂ relates to the changes in SD and putatively exerts its feed-

back control over both the photosynthetic rate and stomatal conductance by modulating the SD and stomatal size, in analogy to the controls on stomatal aperture that were recognized long ago (Mott, 1988; Morison, 1998).

Lines of evidence for the involvement of a Cᵢ-related factor in stomatal density signalling

We present several pieces of indirect evidence indicating the involvement of Cᵢ or a Cᵢ-linked stimulus in adjustment of SD. First, coefficients of determination indicating the apparent role of Cᵢ in driving SD were fairly high (R² = 0.95) in our experiments with four different species and several environmental factors; 0.55 and 0.70 for stomata and pavement cells, respective-

ly, in garden cress treated with sub- and super-ambient Cᵢ and a number of environmental factors; 0.73 and 0.61 in garden cress grown at eight different PPFD (see Figs 1, 2 and 4). Secondly, when shifting the axes in such a way that the values of SD and Cᵢ in controls are set to zero as was done in Figs 1 and 2, the points representing the different treatments fall near to each other on a line through the origin. This near [0,0] intercept, shown in Figs 1A, 2A and 5A, suggests to us a dominant role for the Cᵢ-related factor and only relatively minor effects of other factors.

In our estimates, sun-exposed beech leaves showed a 13.7 % increase in SD per 1 % of ¹³C enrichment, when compared with shaded leaves. Sunflower plants grown under high-light or low-light conditions yielded values of 13·2 (control), 16·3 (PEG-stressed) and 18·9 (ABA-fed) % (SD) %‰⁻¹ (δ). A 1 % difference in δ is equivalent to a change in Cᵢ of about 17 and 15 μmol mol⁻¹ in air and helox, respectively. Similar values of SD sensitivity to Cᵢ (derived from δ) have been observed (though not analysed) by other authors for a wide spectrum of plants and growth conditions [e.g. leaves of Vigna sinensis (Sekiya and Yano, 2008); fossil cuticles of the Cretaceous conifer Frenelopsis (Aucour et al., 2008); and the complex en-

vironmental factors acting along altitudinal gradients (Körner et al., 1988)]. Another line of evidence linking Cᵢ with frequency of stomata was obtained by analysing published data from experimen-
tes where SD and ¹³C discrimination in leaf dry matter (δ) were measured concomitantly. We searched for data on δ and SD from controlled, mostly mono-factorial experiments with dicotyledonous plants. Results from 17 studies summarized in

<table>
<thead>
<tr>
<th>Age (DAW)</th>
<th>Cotyledons</th>
<th>First leaves</th>
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<tr>
<td></td>
<td>SD (mm⁻²)</td>
<td>PCD (mm⁻²)</td>
</tr>
<tr>
<td>7</td>
<td>439 (25)</td>
<td>1308 (70)</td>
</tr>
<tr>
<td>14</td>
<td>416 (11)</td>
<td>535 (30)</td>
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<td></td>
<td>(0.009)</td>
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Density of stomata (SD) and pavement cells (PCD) per mm² of projected leaf area (sum of adaxial and abaxial values) and stomatal index (SI) in cotyledons and first leaves of garden cress and responses of epidermal cell density to variations in the internal CO₂ concentration of the leaf (Cᵢ) were measured simultaneously under eight irradiances and equal Cᵢ. The results confirmed that cotyledons are less sensitive to Cᵢ than the first true leaves or are insensitive (Fig. 4A, B). The SD and PCD on the true leaves increased progressively at reduced Cᵢ. The SI in cotyledons did not respond to Cᵢ. However, in contrast to the previous experiment, SI significantly (P < 0.001) decreased with increasing Cᵢ in true leaves (Fig. 4C). The same data as in

Fig. 4 but plotted in the form of differences from the ‘control’ irradiance (310 μmol m⁻² s⁻¹) and shown separately for abaxial and adaxial leaf sides are presented in Supplementary Data Fig. S4. CO₂ response of SI was steeper for the abaxial (lower) than the adaxial (upper) leaf side.

DISCUSSION

In this study we demonstrate for the first time that the internal CO₂ concentration of the leaf, Cᵢ, correlates strongly with the development of stomatal and pavement cells expressed in terms of their densities. We applied several independent environmental factors to manipulate Cᵢ. Therefore, the experimental results suggest, although they do not prove, that Cᵢ or a Cᵢ-related cue integrates the effects of those environmental parameters and conveys their signal into the machinery controlling SD. Our tests with cotyledons, the first assimilatory organs in a plant’s life, support the previous evidence that adult leaves are essential in generating the signal (Lake et al., 2001, 2002 Miyazawa et al., 2006). The results show that photosynthesis- and stomata-controlled Cᵢ correlates with the number of stomatal and epidermal cells per unit of leaf surface: both insufficient stomatal conductance gₛ and/or an enhanced photosynthetic rate reduce Cᵢ, linking these processes directly or indirectly to the development of a new, acclimated leaf having more smaller stomata and pavement cells per mm² of the leaf surface. Conversely, elevated Cᵢ, caused for example by shading or elevated ambient CO₂ correlates with fewer stomata and pavement cells per mm² of the leaf surface and, consequently, larger epidermal cells develop (for examples of negative relationships between size and density of stomata, see Franks and Beerling, 2009; Franks et al., 2012). As this mechanism also operates at invariant Cᵢ, our experiments suggest that the internal rather than ambient CO₂ relates to the changes in SD and putatively exerts its feedback control over both the photosynthetic rate and stomatal conductance by modulating the SD and stomatal size, in analogy to the controls on stomatal aperture that were recognized long ago (Mott, 1988; Morison, 1998).

Lines of evidence for the involvement of a Cᵢ-related factor in stomatal density signalling

We present several pieces of indirect evidence indicating the involvement of Cᵢ or a Cᵢ-linked stimulus in adjustment of SD. First, coefficients of determination indicating the apparent role of Cᵢ in driving SD were fairly high (R² = 0.95) in our experiments with four different species and several environmental factors; 0.55 and 0.70 for stomata and pavement cells, respectively, in garden cress treated with sub- and super-ambient Cᵢ and a number of environmental factors; 0.73 and 0.61 in garden cress grown at eight different PPFD (see Figs 1, 2 and 4). Secondly, when shifting the axes in such a way that the values of SD and Cᵢ in controls are set to zero as was done in Figs 1 and 2, the points representing the different treatments fall near to each other on a line through the origin. This near [0,0] intercept, shown in Figs 1A, 2A and 5A, suggests to us a dominant role for the Cᵢ-related factor and only relatively minor effects of other factors.

In our estimates, sun-exposed beech leaves showed a 13.7 % increase in SD per 1 % of ¹³C enrichment, when compared with shaded leaves. Sunflower plants grown under high-light or low-light conditions yielded values of 13·2 (control), 16·3 (PEG-stressed) and 18·9 (ABA-fed) % (SD) %‰⁻¹ (δ). A 1 % difference in δ is equivalent to a change in Cᵢ of about 17 and 15 μmol mol⁻¹ in air and helox, respectively. Similar values of SD sensitivity to Cᵢ (derived from δ) have been observed (though not analysed) by other authors for a wide spectrum of plants and growth conditions [e.g. leaves of Vigna sinensis (Sekiya and Yano, 2008); fossil cuticles of the Cretaceous conifer Frenelopsis (Aucour et al., 2008); and the complex environmental factors acting along altitudinal gradients (Körner et al., 1988)]. Another line of evidence linking Cᵢ with frequency of stomata was obtained by analysing published data from experiments where SD and ¹³C discrimination in leaf dry matter (δ) were measured concomitantly. We searched for data on δ and SD from controlled, mostly mono-factorial experiments with dicotyledonous plants. Results from 17 studies summarized in
Supplementary Data Table S1 show that the values of SD sensitivity to $C_i$ range between $+10$ and $+20\%$ (SD) $\mu\text{mol} \text{mol}^{-1}$ (8). In terms of $C_i$ and at the present ambient CO2 concentration, SD typically increases by 1 % when $C_i$ drops by 1 $\mu\text{mol} \text{mol}^{-1}$, and vice versa. The differences in $\delta$ between treated and control plants, converted to differences in $C_i$ and plotted against the respective differences in SD (Fig. 5), show a similar pattern to those presented in Fig. 1.

$SD$ response to factors other than $C_i$

Signalling in stomatal differentiation requires membrane-bound receptors, regulatory peptide ligands, a mitogen-activated protein (MAP) kinase module and transcription factors with the
respective genes expressed mostly in the epidermis, but also in the mesophyll (Casson and Gray, 2008; Shimada et al., 2011; Pillitteri and Torii, 2012). Co-ordinated and light-dependent development of stomata and chloroplasts is also mediated by brassi-nosteroids and other phytohormones (Wang et al., 2012). The complex signalling pathway leads to division of protodermal cells, with an increasing proportion of stomata among all epidermal cells (SI), and a rising number of stomata per unit of leaf area (SD) in the early phase of epidermal development. Later on in the phase of intensive leaf area enlargement, presumably all epidermal cells extend in size proportionally, keeping SI stable and reducing SD (Asl et al., 2011). Therefore, the final density and size of stomata on mature leaves is the result of interactions between genomic and environmental factors controlling the entry of cells into the stomatal lineage and their expansion. The idea that such a complex signalling pathway would respond exclusively to only one environmentally modulated factor such as \( C_i \) has to be treated with caution. Indeed, a direct response of stomatal development to the red light-activated form of phytochrome B and to blue light signals has recently been observed in arabidopsis (Boccalandro et al., 2009; Casson et al., 2009; Kang et al., 2009). These wavelength-specific light effects on SD often do not conform to the negative SD~\( C_i \) relationship shown here. Thus, it seems that there could be two pathways in the light control of stomatal development: a photomorphogenesis-linked wavelength-specific mechanism and the \( C_i \)-mediated, probably \( \text{CO}_2 \) assimilation-based, control. Due to their specific response to \( C_i \), both pathways probably converge downstream of the putative \( C_i \) signalling point. Obviously, the SI sensitivity to \( C_i \), shown in Fig. 4C for true leaves of garden cress, contradicts this scheme. The reasons are not clear and remain to be revealed. Perhaps true leaves of plantlets grown at low irradiances were not fully developed and had a higher proportion of pavement cells and lower SI than at high irradiance. Alternatively, another unknown factor apart from those controlled here affected the proportion of stomata especially on the abaxial leaf side (Supplementary Data Fig. S4C).

**Is \( \text{CO}_2 \) the underlying factor?**

In this study, we show that SD correlates with \( C_i \) in true leaves. It does not necessarily mean that there is a causal relationship between abundance or activity of \( \text{CO}_2 \) molecules in the leaf interior and stomatal development. One aspect which must be considered is that the \( C_i \) values were inferred from discrimination against \(^{13}\text{CO}_2\), which takes place in the photosynthetically active leaf in light and is recorded in the leaf bulk dry mass (Farquhar et al., 1989). In addition, \( C_i \) estimated from \(^{13}\text{C}\) is not a simple time average but the photosynthesis-weighted value of \( C_i \) averaged over the photoperiod (Farquhar, 1989). Thus, supposing that the \( C_i \)-related link to SD exists, we can expect that the putative factor modulating stomatal density and/or size is either photosynthesis-weighted \( C_i \) (\( C_p \)) or some \( C_p \)-linked intermediate averaged over the photoperiod. However, the daylight specificity of \( C_i \) as a signal in stomatal size and density remains to be confirmed. Data on the diurnal course of \( C_i \) during leaf development and SD in the developed leaf are rare. Recently, Rogiers and Clarke (2013) showed that elevated root-zone temperature increased mid-day and reduced nocturnal \( C_i \) (determined via gas exchange) in grapevine. Leaves that emerged during root-zone warming had a lower SD. These results support our finding that higher \( C_i \) leads to reduced frequency of stomata and that the daytime, not nocturnal, \( C_i \) is the controlling factor. Photospiration is another powerful internal source of \( \text{CO}_2 \) in light-exposed leaves which could also be used for testing the \( C_i \) effect on SD. Ramonell et al. (2001) showed that downregulation of the photosynthetic source of \( \text{CO}_2 \) by the \( \text{O}_2 \) content in ambient atmosphere being reduced to 2.5 %, and presumably a reduced \( C_i \), increased both SD and starch content in newly developed leaves of arabidopsis. This indicates that \( \text{CO}_2 \) or a \( \text{CO}_2 \)-derived cue rather than non-structural assimilates could affect SD and stomatal size.

**Systemic vs. local signalling**

Whole plants (or whole branches in the case of beech) were subject to fairly homogeneous environmental conditions in our experiments. Therefore, it is not possible to judge whether the

![Graphical representation of the effect of various environmental factors on concomitant changes in the internal \( \text{CO}_2 \) concentration (\( C_i \)) and stomatal density (SD) of leaves. The SD values were calculated from carbon isotope discrimination data extracted together with SD values from 17 publications presenting factorial experiments. The differences between treatment and control plants in SD values (A) and in SD normalized to SD of control (B) are shown together with the best fits and 95% confidence intervals. The compiled data are shown in Supplementary Data Table S1, and come from the following studies: Bradford et al. (1983), Van de Water et al. (1994), Beering (1997), Sun et al. (2003), Gitz et al. (2005), Takahashi and Mikami (2006), Aucour et al. (2008), He et al. (2008), Sekiya and Yano (2008), Lake et al. (2009), Yan et al. (2009), Craven et al. (2010), Gorsch et al. (2010), He et al. (2012), Sun et al. (2012), Yan et al. (2012) and Rogiers and Clarke (2013).}
CO₂-derived signal was produced directly in the developing leaf or in a mature, lower insertion leaf and transmitted as a systemic signal (Lake et al., 2001, 2002; Coupe et al., 2006, and others). Our experiments in which we compared cotyledons and the first leaves of garden cress support this concept of systemic signalling. Despite the stomatal emergence extending through the period of advanced autotrophy, SD on mature cotyledons remained largely insensitive to Cᵢ, Cᵢₐ and light (Figs 2 and 4; Supplementary Data Fig. S4). Thus we suggest that, without a pre-existing carbon assimilation organ, the information on availability of CO₂ or a CO₂-related factor is not generated, cannot be conveyed to the developing cotyledons and cannot modulate the genetic programme of development of the stomata and epidermis. Nevertheless, cotyledons are probably competent in production of the systemic signal and transport to the true leaves at 7–9 DAW when they appear.

Conclusions

The SD and PCD of mature leaves co-vary with ¹³C discrimination caused by altering various environmental factors while keeping ambient CO₂ stable. This translates into an inverse association between SD, PCD and the daytime-integrated internal CO₂ concentration of the leaf. There is only a small (if any) change in the relative proportion of stomata to other epidermal cells (SI), with one exception, in a large number of experimental comparisons. Therefore, the results demonstrate overwhelmingly that the reduction in Cᵢ hinders the expansion of leaf area, increases SD and reduces the size of stomata. In contrast, elevated Cᵢ stimulates the expansion, increases the size of stomata and reduces SD. We suggest that the apparent Cᵢ-dependent modulation of SD and PCD could translate the mesophyll demand for CO₂ into a pattern of more numerous and smaller stomata on newly developed leaves. The putative Cᵢ-sensing mechanism could integrate several environmental factors and relies on a signal transported from older, photosynthetically competent organs, including cotyledons in the case of the first true leaves. In contrast, the absence of older photosynthetic organs probably prevented the adjustment of the SD of cotyledons in response to Cᵢ and environmental perturbations.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1: time course of ¹³C discrimination in garden cress plantlets grown from seed for up to 21 d after watering in air or helox atmosphere at high or low humidity, under total pressure reduced to one-half of normal pressure, and at three different atmospheric CO₂ mixing ratios. Fig. S2: time course of stomatal density in garden cress plantlets grown from seed for up to 21 d after watering in air or helox atmosphere at high or low humidity, under total pressure reduced to one-half of normal pressure, and at three different atmospheric CO₂ mixing ratios. Fig. S3: kinetics of seed-derived carbon in cotyledons of garden cress plants grown at three different ambient CO₂ concentrations from seeds for 14–21 d after the seed watering in an artificially mixed atmosphere. Fig. S4: details of the stomatal density, pavement cell density and stomatal index response of garden cress true leaves and cotyledons to leaf internal CO₂ concentration. Table S1: carbon isotope discrimination and stomatal density data compiled from published controlled factorial experiments with dicotyledonous plants.

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LITERATURE CITED


APPENDIX

Stomatal density correlates with leaf internal CO2

\[ \delta^{13} \text{C}- \text{based determination of the internal CO2 concentration in mixotrophic tissues of leaves} \]

Stomata develop at an early stage of leaf or cotyledon ontogeny. In \textit{L. sativum}, it was possible to observe the first differentiated stomata on folded cotyledons as early as 48 h after seed soaking (data not shown). Cotyledons are almost entirely heterotrophic and built from seed carbon at that stage of development, which prevents the determination of internal CO2 concentration in the cotyledon from \(^{13}\text{C}\) abundance in its dry mass. A fraction \(f\) of the carbon forming the cotyledon is supplied from seed storage and keeps (with presumably minimum change) its \(^{13}\text{C}\) abundance \((\delta_s)\). The rest of the cotyledon’s carbon, \(1 - f\), is assimilated by photosynthetic CO2 fixation and obeys the isotopic fractionation caused mainly by CO2 diffusion and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) carboxylation, resulting in \(^{13}\text{C}\) depletion of newly formed triose phosphates expressed as \(\delta_t\). Only the latter part provides isotopic information on actual growth conditions and can be used for the calculation of \(C_i\) in developing cotyledons, provided \(f\) is available.

The two-source carbon mixing model can be used to express the isotopic composition of the germinating plantlets \(\delta_p\):

\[ \delta_p = \delta_s f + \delta_t (1 - f) \quad (A1) \]

\(^{13}\text{C}\) depletion of the newly formed triose phosphates against ambient air, \(\Delta_i\), depends on the ratio of the internal CO2 of the cotyledons and the external CO2 concentrations, \(C_i/C_a\) (Farquhar et al., 1989), as:

\[ \Delta_i \approx \delta_a - \delta_t = a + [(b - a)C_i/C_a] \quad (A2) \]

where \(a\) and \(b\) denote fractionation factors due to diffusion in gas phase and carboxylation by Rubisco. Substitution for \(\delta_t\) from eqn (A2) into eqn (A1) yields:

\[ \delta_p = \delta_a + [(f - 1)(a + (b - a)C_i/C_a)] \quad (A3) \]

under the condition that the seeds and atmosphere have the same \(\delta\) values, \(\delta_s \approx \delta_a\), which is not unusual when compressed CO2 of fossil origin is used as a source for CO2 in the mixed atmosphere where the seeds germinate. In our case \(\delta_s\) and \(\delta_a\) were \(-28.19\) and \(-28.13\) ‰, respectively.

To calculate \(C_i\) from this equation, we have to know the fraction of seed carbon \(f\). We derived this value using the time course of isotopic composition \(\delta_p\) during the plantlets’ early development from germination \((f = 1\) at DAW = 0\) to the fully autotrophic stage \((f = 0\) at DAW = 14\) in our conditions). The typical time course of \(\delta_p\) had a sigmoid shape, approaching the most negative values between 14 and 21 DAW (Supplementary Data Fig. S3). The \(\delta_p\) asymptote, \(\delta_{pl}\), estimated from sigmoid regression of the \(\delta_p\) kinetics for true leaves, indicated the fully autotrophic stage. We re-scaled the \(\delta_s - \delta_{pl}\) values into the 1–0 range of \(f\) and calculated the fraction \(f\) in cotyledons of any particular age. The \(\delta_p\) and \(f\) kinetics were specific for various \(C_a\), indicating faster development at higher \(C_a\) concentrations (Supplementary Data Fig. S3). The kinetics also varied between plants grown in helox or air, with slightly higher slopes and faster development in helox. With \(f\) available, it was possible to calculate \(C_i\) in cotyledons and young true leaves as

\[ C_i = \left( \frac{\delta_p - \delta_a}{f - 1 - a} \right) \frac{C_a}{b - a} \quad (A4) \]