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Stomatal and pavement cell density linked to leaf internal CO_2 concentration

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• **Background and Aims** Stomatal density (SD) generally decreases with rising atmospheric CO_2 concentration, C_a. However, SD is also affected by light, air humidity and drought, all under systemic signalling from older leaves. This makes our understanding of how C_a controls SD incomplete. This study tested the hypotheses that SD is affected by the internal CO_2 concentration of the leaf, C_i, rather than C_a, and that cotyledons, as the first plant assimilation organs, lack the systemic signal.
• **Methods** Sunflower (*Helianthus annuus*), beech (*Fagus sylvatica*), arabidopsis (*Arabidopsis thaliana*) and garden cress (*Lepidium sativum*) were grown under contrasting environmental conditions that affected C_i while C_a was kept constant. The SD, pavement cell density (PCD) and stomatal index (SI) responses to C_i in cotyledons and the first leaves of garden cress were compared. ^{13}C abundance ($\delta^{13}C$) in leaf dry matter was used to estimate the effective C_i during leaf development. The SD was estimated from leaf imprints.
• **Key Results** SD correlated negatively with C_i in leaves of all four species and under three different treatments (irradiance, abscisic acid and osmotic stress). PCD in arabidopsis and garden cress responded similarly, so that SI was largely unaffected. However, SD and PCD of cotyledons were insensitive to C_i, indicating an essential role for systemic signalling.
• **Conclusions** It is proposed that C_i or a C_i-linked factor plays an important role in modulating SD and PCD during epidermis development and leaf expansion. The absence of a C_i–SD relationship in the cotyledons of garden cress indicates the key role of lower-insertion CO_2 assimilation organs in signal perception and its long-distance transport.

Key words: Stomatal density, stomata development, pavement cells, cotyledons, leaf internal CO_2, ^{13}C discrimination, *Lepidium sativum*, *Helianthus annuus*, *Fagus sylvatica*, *Arabidopsis thaliana*.

INTRODUCTION

Stomata are pores, which can vary in aperture, on plant surfaces exposed to the air: they control water and CO_2 exchange between plants and the atmosphere. Globally, about 17 % of atmospheric CO_2 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 CO_2 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 CO_2 concentration (C_i) of the leaf rather than to the external CO_2 concentration: pores open when C_i falls and close when it goes up (Mott, 1988; Willmer, 1988; Mott, 2009). It is tempting to speculate that similar sensing of C_i 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 CO_2 demand than by globally modulated availability of CO_2 in the free atmosphere. However, to our knowledge, there are no experiments on the relationship of SD to internal CO_2.

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 CO_2 (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 CO_2 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 CO_2 than ambient air. Sensing of the free atmospheric environment is thus unlikely. In addition to atmospheric CO_2, 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 ^{13}C content ($\delta^{13}C$) in assimilates (Sekiya and Yano, 2008). (For the sake of simplicity, we omit the '^{13}C' after δ from here on since carbon is the only element whose stable isotopes are considered here. Also, internal CO_2 refers to the CO_2 within the organ.) The degree of carbon isotope discrimination is considered a 'fingerprint' of the ratio of internal CO_2 concentration over ambient CO_2, C_i/C_a, 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 C_i/C_a 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 CO_2 concentration. We manipulated C_i by changing various environmental factors affecting photosynthetic rate and/or stomatal aperture (light quantity, ABA and osmotic stress) while keeping ambient CO_2 constant during the plant's growth. Four species were used for testing this question. In two species, we also investigated the C_i response of pavement cells and calculated the SI. Data from the literature for 16 plant species cultivated under different growth conditions were analysed to complement these experiments. Further, we wanted to know (2) whether SD in cotyledons is controlled by CO_2 in a way similar to that in true leaves. To manipulate C_i, we grew the plants at various C_a in mixed artificial atmospheres of air or helox [a mixture of gases similar to air but with nitrogen substituted by helium; CO_2 diffuses 2·3 times faster in helox than in air which can increase C_i (Parkhurst and Mott, 1990)] and at reduced total pressure of ambient atmosphere. C_i was estimated from the carbon isotope composition (δ) of leaf dry mass.

MATERIALS AND METHODS

Plants and growth conditions

Experiment 1: mature leaves at invariant C_a. 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 C_i. 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 C_i via stomatal conductance. All plants were exposed to a free atmospheric CO_2 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}. From the third day after germination, half of the plants were watered daily with 10 mL of water (controls) while the second half was given 10 mL of 10^{-4} M ABA solution per day. 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 C_a. 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 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 $\mu mol\ m^{-2}\ s^{-1}$, with a 16 h photoperiod and day and night air temperatures of 23–25 °C. A mixture of He and O_2 at a v/v ratio of 79/21 (helox), or artificial air mixed from N_2 and O_2 at the same v/v ratio (air), with the addition of CO_2 at one of three concentrations (180, 400 and 800 $\mu mol\ mol^{-1}$) 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 N_2 (both with a purity of 4·6), oxygen (3·5) and CO_2 (20 % of CO_2 in N_2; all Messer, Czech Republic). The δ of the source CO_2 was −28·2 ‰. CO_2 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 O_2 in the outlet atmosphere were measured with an He/O_2 analyser (Divesoft, Prague, Czech Republic) twice a day. Altogether, 18 treatments were applied: two different inert components of the gas mixture (He or N_2), each with three different CO_2 concentrations, and each in dry, humid and hypobaric variants (see Table 1). Different C_a, humidity, He instead of N_2, and reduced pressure in gas mixtures were used to manipulate C_i, the growth-integrated value of which was estimated by ^{13}C discrimination. Seven- and 14-day-old plants were harvested. For plants grown at 180 $\mu mol\ mol^{-1}$ of ambient CO_2, we also included harvest at 21 DAW.

Experiment 3: cotyledons and true leaves at different PPFDs and invariant C_a. In the third type of experiment, garden cress was cultivated at eight different PPFDs in glass cuvettes (volume 100 mL) flushed with air pumped from outside of the building (Multi-Cultivator MC 1000, PSI, Brno, Czech Republic). Single plantlets grew in fine perlite in temperature-controlled cuvettes irradiated individually with white light-emitting diodes (LEDs). Plants were grown for 21 d at PPFDs of 100, 170, 240, 310, 380, 450, 520 and 590 μmol (photons) $m^{-2}\ s^{-1}$, with a 16 h photoperiod and day/night air temperatures of 24/19 °C. The experiment was repeated six times.

Experiment 4: rosette leaves of arabidopsis at two different PPFDs and invariant C_a. *Arabidopsis thaliana* seeds, ecotypes Columbia Col-0 and C24, were incubated in the dark at 4 °C for 3 d. The plantlets were grown in soil in a growth chamber with a 10 h photoperiod, day and night temperatures of 18 and 15 °C, respectively, and air humidity of 50–70 %. Half of the plants were located on the upper shelf, closer to the fluorescence tubes, and exposed to high light (250 $\mu mol\ m^{-2}\ s^{-1}$; HL). The other half were kept at the bottom of the same growth chamber and exposed to low light (25 $\mu mol\ m^{-2}\ s^{-1}$; LL). Two independent repetitions with two different growth chambers (Sanyo, UK and Snijders Scientific, The Netherlands) were carried out. The carbon isotope composition of CO_2 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 ^{13}C 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 mm^2 each, randomly distributed across the leaf (apex, middle part and base). The results were expressed as counts of stomata or pavement cells per mm^2 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 ^{13}C over ^{12}C (δ) were measured in leaf dry matter and, in expt 2 (garden cress in desiccators), also in seeds with testa removed (δ_s) and in the source CO_2 (δ_a) used for mixing the artificial atmospheres. δ_a and δ_s were −28·19 ‰ and −28·13 ‰, respectively. δ_a 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 CO_2 separation, the $^{13}C/^{12}C$ 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: $\delta = (R_{sample}/R_{standard} - 1) \times 1000$. 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 CO_2 *concentration,* C_i

We used the δ of leaf dry mass to evaluate the intercellular CO_2 concentration integrated over the leaf's life time, C_i. The ^{13}C discrimination during photosynthetic CO_2 fixation, Δ, is related to C_i as (Farquhar *et al.*, 1989):

$$\Delta = a + [(b - a)C_i/C_a] \quad (1)$$

TABLE 1. *Overview of the design of the four experiments presented*

Experiment no.	Species (ecotype)	Treatment (PPFD μmol m^{-2} s^{-1})	Sub-treatment	C_a (μmol mol^{-1})	Leaf age at harvest (d)
1	*Helianthus annuus*	HL (700) [↓]	Control	390	56
			+PEG [↓]		
			+ABA [↓]		
		LL (70) [↑]	Control		
			+PEG [↓]		
			+ABA [↓]		
	Arabidopsis thaliana (Columbia, C24)	HL (200) [↓]		390	18
		LL (80) [↑]			
	Lepidium sativum	(500)	Control	390	14
			+ABA [↓]		
	Fagus sylvatica	HL (1301) [↓]	Season, leaf age	390	30–180 (18 harvests)
		LL (33) [↑]			
2	*Lepidium sativum*	Artificial air [↓]	LH [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			HH [↑]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			RP [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
		Helox [↑]	LH [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			HH [↑]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
			RP [↓]	180 [↓]	7, 14, 21
				400	7, 14
				800 [↑]	7, 14
3	*Lepidium sativum*	(100) [↑]		390	21
		(170) [↑]			
		(240) [↑]			
		(310) [↑]			
		(380) control			
		(450) [↓]			
		(520) [↓]			
		(590) [↓]			
4	*Arabidopsis thaliana* (Columbia, C24)	HL (250) [↓]		400	42
		LL (25) [↑]			

HL, high light; LL, low light; HH, high humidity; LH, low humidity; RP, reduced pressure; PPFD, photosynthetic photon flux density during growth.
Expected effect of the treatment and sub-treatment on C_i: increase [↑] or decrease [↓] with respect to the counterpart treatment or control.

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 Δ were considered to be small and were neglected. Δ is related to ^{13}C abundance in the plant, δ_p, and in air, δ_a, as $\Delta = (\delta_a - \delta_p)/[(\delta_p/1000) + 1]$ where both δ and Δ 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 $\Delta \cong (\delta_a - \delta_p)$ and rearrangement of eqn (1) for C_i yields:

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

In calculations of C_i in leaves grown in the free atmosphere and in artificial mixed atmospheres, we used δ_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 δ_a provided that the control and treated plants grew in the same mixed atmosphere, such as in our growth chamber experiments:

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

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 δ significantly different from free air. Our seeds, which were produced in free air conditions at δ_a

close to −8 ‰ and had $\delta_s = -28{\cdot}13$ ‰, grew in glass desiccators supplied with artificial air or helox with $\delta_a = -28{\cdot}19$ ‰. With an increasing proportion of autotrophy and f decreasing below 1, δ_p decreased to values more negative than δ_s, 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 their δ_p value [see Supplementary Data Fig. S3]. The sigmoid regressions of the δ_p time course were converted to the f course, ranging from 1 to 0, and used to determine f of 7- and 14-day-old cotyledons (see Appendix for more details). C_i of cotyledons grown in air or helox was then calculated with eqn (4) (derived in Appendix) as:

$$C_i = \left(\frac{\delta_p - \delta_a}{f-1} - a\right)\frac{C_a}{b-a} \quad (4)$$

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:

$$SEM_{SI} = \sqrt{\left[\frac{\partial SI}{\partial SD} SEM_{SD}\right]^2 + \left[\frac{\partial SI}{\partial PCD} SEM_{PCD}\right]^2} \quad (5)$$

After substituting the derivatives this becomes

$$SEM_{SI} = \sqrt{\left[\frac{\overline{PCD}\times 100}{(\overline{SD}+\overline{PCD})^2} SEM_{SD}\right]^2 + \left[\frac{-\overline{SD}\times 100}{(\overline{SD}+\overline{PCD})^2} SEM_{PCD}\right]^2} \quad (6)$$

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 δ 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} μmol^{-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

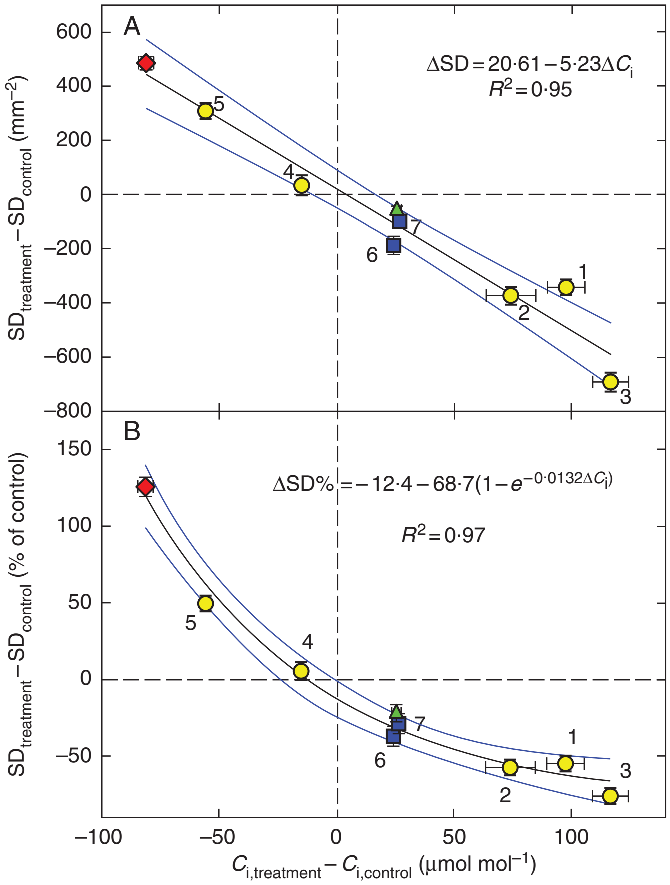

FIG. 1. Effects of shading and water stress treatments on internal CO_2 concentration of the leaf, C_i, as inferred from the $\delta^{13}C$ in leaf biomass, and on stomatal density SD. (A) Circles indicate increased (1, 2, 3) and decreased (4, 5) C_i, and concomitant changes in SD, under low light in sunflower grown in nutrient solution (1), with ABA (2) or PEG (3) added, and under high light in sunflower fed with ABA (4) or PEG (5). Values are differences between treatments and control, i.e. plants grown under high light in plain nutrient solution; $n_{ci} = n_{SD} = 6$–8. The triangle shows the effect of shading on beech leaves (single tree, sampled during the 2007 and 2009 seasons, $n_{ci} = 18$, $n_{SD} = 20$). Squares depict the shading effect in *Arabidopsis thaliana* rosette leaves grown for 18 d at irradiances of 200 or 80 μmol m^{-2} s^{-1}, respectively (ecotypes Columbia and C24, $n_{ci} = 6$, $n_{SD} = 18$). Diamonds indicate the ABA effect in cress plants ($n_{ci} = 5$, $n_{SD} = 48$). All plants experienced an atmospheric CO_2 concentration close to 390 μmol mol^{-1}. (B) The changes in SD from (A) expressed as a percentage of control. The points sharing the same C_i co-ordinate in (A) and (B) represent identical treatments. Standard errors of the mean (bars), regression lines and 95 % confidence intervals are shown.

of −81·1 % for C_i approaching a theoretical limit 10^6 (pure CO_2) and setting the limit of phenotypic plasticity in lowering SD to about 20 % of the original value.

Comparison of the C_i response in cotyledons and true leaves of garden cress

The results shown above were obtained at stable C_a. In controlled-atmosphere experiments with garden cress, we used ambient (400 μmol mol^{-1} as a control), sub-ambient (180 μmol mol^{-1}) or super-ambient (800 μmol mol^{-1}) CO_2 concentrations to grow cress plants for 14 d (or 21 d for sub-ambient CO_2) in air or in helox atmosphere, each with two different gas humidities and also under reduced total pressure. Variations in C_i were induced primarily by changing C_a, using helox instead of air [CO_2 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_2 plants had fewer stomata and pavement cells than the control plants. Conversely, in sub-ambient CO_2 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_i 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_i. 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_i.

The fraction of stomata among all epidermal cells (the SI) was almost invariant over the whole investigated range of C_i 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_i (slope = 0·0069 % mol μmol^{-1}, $R^2 = 0·32$; see Table 2). However, standard errors of SI means determined from SD and PCD data were high and the SI changes with C_i non-significant (Fig. 2C). We obtained qualitatively similar C_i responses of SD and PCD in the experiment with arabidopsis true leaves grown at two different irradiances and equal C_a (expt 4, Fig. 3A, B). The SD and PCD increased with C_i reduction in strongly irradiated leaves. The response was similar for both leaf sides and both ecotypes. The SI did not respond to C_i in any systematic way and showed a slightly negative average slope (Fig. 3C).

In order to verify the cotyledons' insensitivity to C_i in a way unbiased by estimation of the seed carbon fractions (see Appendix), we grew garden cress for a longer period (3 weeks)

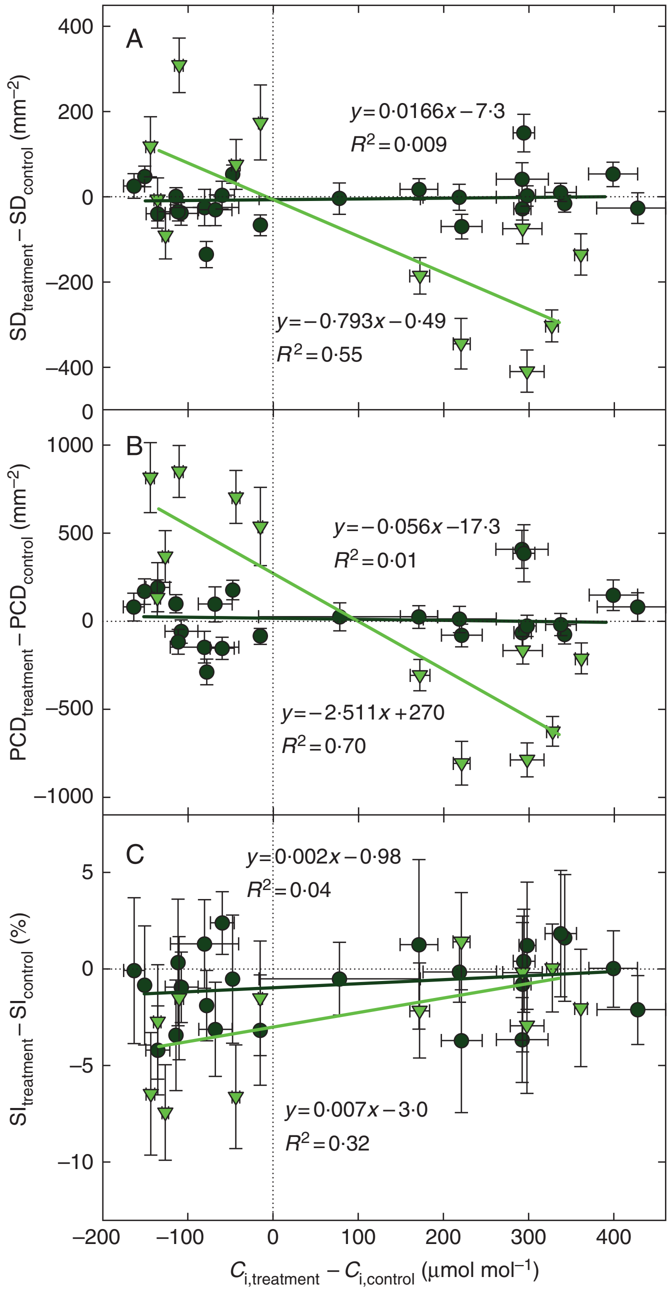

FIG. 2. Changes in internal CO_2 concentration (C_i) of the leaf in *Lepidium sativum* plants grown under sub-ambient or super-ambient CO_2 concentrations, as related to changes in stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C). The negative values of the C_i difference show by how much C_i was lowered when C_a was reduced from 400 to 180 μmol mol^{-1}; the positive values indicate the increase of C_i in plants grown at 800 compared with controls kept at 400 μmol mol^{-1}. Stomatal and pavement cell density in the first leaves (light green triangles and light green regression line) was much more sensitive to CO_2 concentration than in cotyledons (dark green circles and dark green regression line). Each point represents a difference between the C_a treatment (180 or 800) and C_a control (400) for one of three sub-treatments (two air humidities and hypobaric plants), each grown either in air or in helox gas mixture. Data for 14-day-old leaves and 7- and 14-day-old cotyledons are shown. The SD and C_i values used in calculation of the differences were means from three independent measurements (three plants per treatment). The C_i values were calculated from $\delta^{13}C$ in plant dry matter (see Appendix). Bars show the standard error of the mean, for SI calculated from the primary SD and PCD data [see eqn (6) in 'Statistical evaluation, meta-analysis'].

TABLE 2. *Comparison of density (number per unit area of leaf) and relative abundance of epidermal cells in cotyledons and the first leaves of garden cress and response of epidermal cell density to variations in the internal* CO_2 *concentration of the leaf* (C_i)

Age (DAW)	Cotyledons			First leaves		
	SD (mm^{-2})	PCD (mm^{-2})	SI (%)	SD (mm^{-2})	PCD (mm^{-2})	SI (%)
7	439 (25)	1308 (70)	25·2 (1·4)			
14	186 (11)	535 (30)	25·8 (2·4)	535 (84)	1304 (223)	29·4 (2·0)
Slope of C_i response (mol µmol^{-1} mm^{-2} or % mol µmol^{-1} where marked *), (R^2)	0·017 (0·009)	−0·056 (0·011)	0·0019* 0·0407)	−0·793 (0·549)	−2·511 (0·703)	0·0069* (0·317)

Density of stomata (SD) and pavement cells (PCD) per mm^2 of projected leaf area (sum of adaxial and abaxial values) and stomatal index (SI) in cotyledons 7 and 14 d after seed watering (DAW) and in the first leaves 14 DAW are given.

Means and standard error of the means (in parentheses) are shown of sets obtained by pooling data from plants grown in three growth CO_2 concentrations (180, 400 and 800 µmol mol^{-1}), two air humidities (60 and 90 %), two different inert host gases (N_2 and He) or reduced atmospheric pressure ($n = 18$, each n is a mean of three measurements on individual plants). Standard errors of SI means were calculated as means over all 18 treatments each obtained by the formula shown in the Materials and Methods.

simultaneously under eight irradiances and equal C_a. The results confirmed that cotyledons are less sensitive to C_i than the first true leaves or are insensitive (Fig. 4A, B). The SD and PCD on the true leaves increased progressively at reduced C_i. The SI in cotyledons did not respond to C_i. However, in contrast to the previous experiment, SI significantly ($P < 0·001$) decreased with increasing C_i 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. CO_2 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 CO_2 concentration of the leaf, C_i, correlates strongly with the development of stomatal and pavement cells expressed in terms of their densities. We applied several independent environmental factors to manipulate C_i. Therefore, the experimental results suggest, although they do not prove, that C_i or a C_i-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 C_i correlates with the number of stomatal and epidermal cells per unit of leaf surface: both insufficient stomatal conductance g_s and/or an enhanced photosynthetic rate reduce C_i, linking these processes directly or indirectly to the development of a new, acclimated leaf having more smaller stomata and pavement cells per mm^2 of the leaf surface. Conversely, elevated C_i, caused for example by shading or elevated ambient CO_2, correlates with fewer stomata and pavement cells per mm^2 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 C_a, our experiments suggest that the internal rather than ambient CO_2 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 C_i*-related factor in stomatal density signalling*

We present several pieces of indirect evidence indicating the involvement of C_i or a C_i-linked stimulus in adjustment of SD. First, coefficients of determination indicating the apparent role of C_i in driving SD were fairly high ($R^2 = 0·95$ 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 C_a 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 C_i 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 C_i-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 ^{13}C 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 C_i of about 17 and 15 µmol mol^{-1} in air and helox, respectively. Similar values of SD sensitivity to C_i (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 C_i with frequency of stomata was obtained by analysing published data from experiments where SD and ^{13}C 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

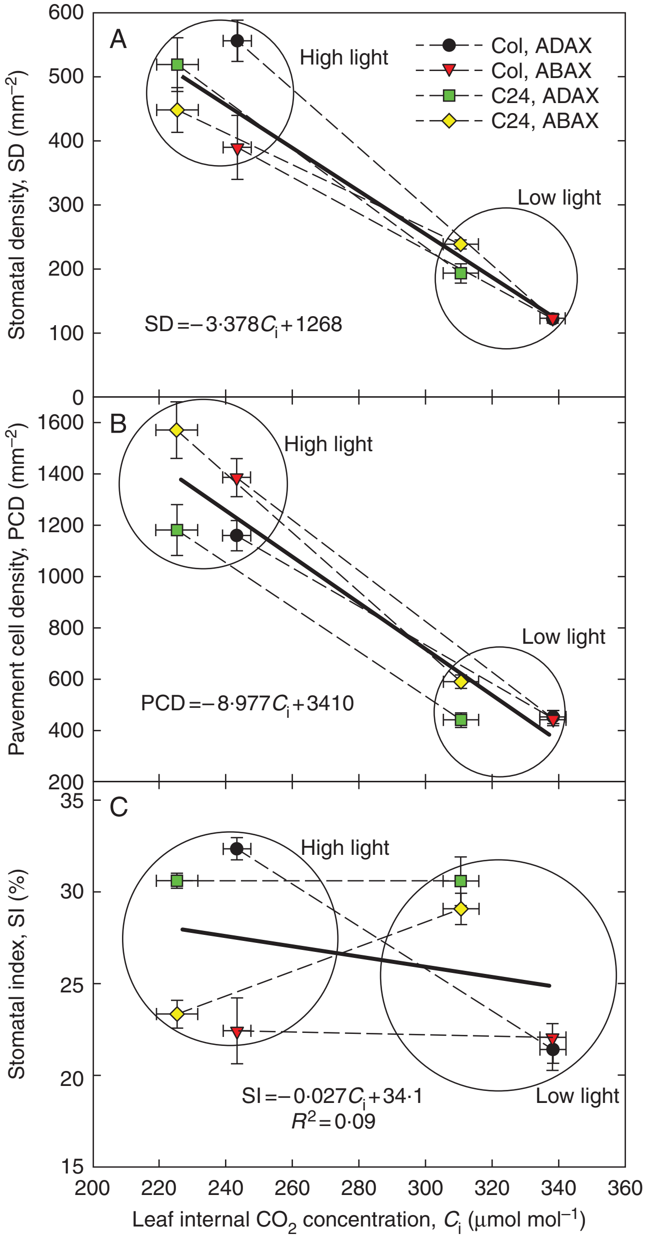

FIG. 3. Response to internal CO_2 (C_i) of stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C) of *Arabidopsis thaliana* leaves grown at two irradiances. Two wild ecotypes, Columbia (Col) and C24, were grown under low light (25 μmol m^{-2} s^{-1}) or high light (250 μmol m^{-2} s^{-1}) conditions in growth chambers. Mean values of SD, PCD and SI (symbols) for the adaxial and abaxial sides of mature first rosette leaves are shown as well as the standard error of the mean (bars, $n_{Ci} = 6$ and $n_{SD,PCD,SI} = 16$) and regression lines (thick solid line and equation) for information about leaf-averaged sensitivity (slope) of the relationship. The circles group the low and high light data.

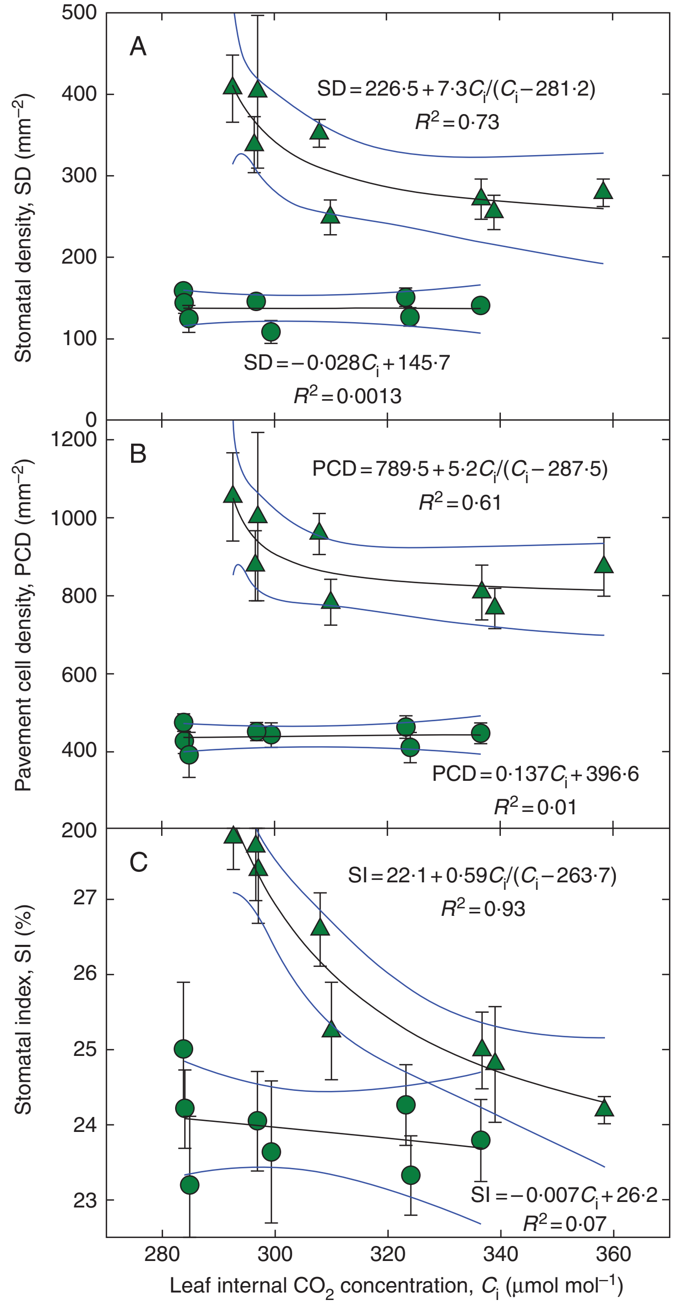

FIG. 4. Response of stomatal density (SD; A), pavement cell density (PCD; B) and stomatal index (SI; C) of *Lepidium sativum* leaves and cotyledons to the internal CO_2 (C_i) of leaves grown at various irradiances. The light green triangles and light green regression lines represent the first leaves; the dark green circles and dark green regression lines show the cotyledons' response. The plants were grown for 21 d under 7–8 photosynthetic photon flux densities (PPFDs), ranging from 100 to 590 μmol m^{-2} s^{-1}, in 100 mL glass cuvettes ventilated with ambient air. The points represent averages from six independent runs of the experiment. Mean values and the standard error of the mean are indicated. Linear or hyperbolic fits with 95 % confidence intervals are shown.

Supplementary Data Table S1 show that the values of SD sensitivity to C_i range between +10 and +20 % (SD) ‰$^{-1}$ (δ). In terms of C_i and at the present ambient CO_2 concentration, SD typically increases by 1 % when C_i drops by 1 μmol mol^{-1}, and vice versa. The differences in δ 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

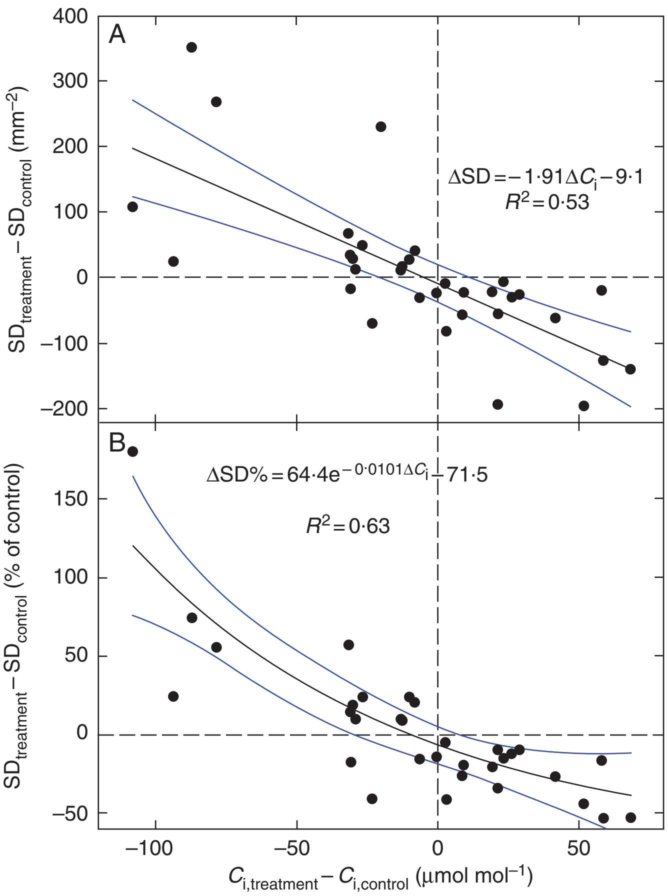

FIG. 5. The effect of various environmental factors on concomitant changes in the internal CO_2 concentration (C_i) and stomatal density (SD) of leaves. The C_i values were calculated from carbon isotope discrimination data extracted together with SD values from 17 publications presenting factorial experiments. 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), Beerling (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), Gorsuch *et al.* (2010), He *et al.* (2012), Sun *et al.* (2012), Yan *et al.* (2012) and Rogiers and Clarke (2013).

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 brassinosteroids 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 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 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 casual relationship between abundance or activity of 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 $^{13}CO_2$, 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}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_{iP}) or some C_{iP}-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. Photorespiration is another powerful internal source of 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 photorespiratory source of CO_2 by the 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 CO_2 or a 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

CO_2-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 C_i, C_a 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 CO_2 or a CO_2-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 ^{13}C discrimination caused by altering various environmental factors while keeping ambient CO_2 stable. This translates into an inverse association between SD, PCD and the daytime-integrated internal CO_2 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 C_i hinders the expansion of leaf area, increases SD and reduces the size of stomata. In contrast, elevated C_i stimulates the expansion, increases the size of stomata and reduces SD. We suggest that the apparent C_i-dependent modulation of SD and PCD could translate the mesophyll demand for CO_2 into a pattern of more numerous and smaller stomata on newly developed leaves. The putative C_i-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 C_i 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 ^{13}C 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 CO_2 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 CO_2 mixing ratios. Fig. S3: kinetics of seed-derived carbon in cotyledons of garden cress plants grown at three different ambient CO_2 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 CO_2 concentration. Table S1: carbon isotope discrimination and stomatal density data compiled from published controlled factorial experiments with dicotyledonous plants.

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APPENDIX

δ ^{13}C-based determination of the internal CO_2 concentration in mixotrophic tissues of leaves

Stomata develop at an early stage of leaf or cotyledon ontogeny. In *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 CO_2 concentration in the cotyledon from ^{13}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}C abundance (δ_s). The rest of the cotyledon's carbon, $1 - f$, is assimilated by photosynthetic CO_2 fixation and obeys the isotopic fractionation caused mainly by CO_2 diffusion and Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) carboxylation, resulting in ^{13}C depletion of newly formed triose phosphates expressed as δ_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 δ_p:

$$\delta_p = \delta_s f + \delta_t(1 - f) \quad \text{(A1)}$$

^{13}C depletion of the newly formed triose phosphates against ambient air, Δ_t, depends on the ratio of the internal CO_2 of the cotyledons and the external CO_2 concentrations, C_i/C_a (Farquhar *et al.*, 1989), as:

$$\Delta_t \approx \delta_a - \delta_t = a + [(b - a)C_i/C_a] \quad \text{(A2)}$$

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

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

under the condition that the seeds and atmosphere have the same δ values, $\delta_s \cong \delta_a$, which is not unusual when compressed CO_2 of fossil origin is used as a source for CO_2 in the mixed atmosphere where the seeds germinate. In our case δ_a and δ_s 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 δ_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 δ_p had a sigmoid shape, approaching the most negative values between 14 and 21 DAW (Supplementary Data Fig. S3). The δ_p asymptote, δ_{pl}, estimated from sigmoid regression of the δ_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 δ_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

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