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Effects of elevated carbon dioxide on stomatal characteristics and carbon isotope ratio of *Arabidopsis thaliana* ecotypes originating from an altitudinal gradient

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Stomatal functioning regulates the fluxes of CO_2 and water vapour between vegetation and atmosphere and thereby influences plant adaptation to their habitats. Stomatal traits are controlled by external environmental and internal cellular signalling. The objective of this work was to quantify the effects of CO_2 enrichment (CE) on stomatal density-related properties, guard cell length (GCL) and carbon isotope ratio ($\delta^{13}C$) of a range of *Arabidopsis thaliana* ecotypes originating from a wide altitudinal range (50–1260 m above sea level (asl)), and grown at 400 and 800 ppm [CO_2], and thereby elucidate the possible adaptation and acclimation responses controlling stomatal traits and water use efficiency (WUE). There was highly-significant variation among ecotypes in the magnitude and direction of response of stomatal traits namely, stomatal density (SD) and index (SI) and guard cell length (GCL), and $\delta^{13}C$ to CE, which represented a short-term acclimation response. A majority of ecotypes showed increased SD and SI with CE with the response not depending on the altitude of origin. Significant ecotypic variation was shown in all stomatal traits and $\delta^{13}C$ at each [CO_2]. At 400 ppm, means of SD, SI and GCL for broad altitudinal ranges, i.e. low (<100 m), mid (100–400 m) and high (>400 m), increased with increasing altitude, which represented an adaptation response to decreased availability of CO_2 with altitude. $\delta^{13}C$ was negatively correlated to SD and SI at 800 ppm but not at 400 ppm. Our results highlight the diversity in the response of key stomatal characters to CE and altitude within the germplasm of *A. thaliana* and the need to consider this diversity when using *A. thaliana* as a model plant.

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Abbreviations – [CO_2], CO_2 concentration; C_a, Atmospheric [CO_2]; pCO_2, Partial pressure of CO_2 in atmosphere; ACO_2, Ambient [CO_2]; ECO_2, Elevated [CO_2]; CE, CO_2 enrichment; ALT, Altitude of origin; SD, Stomatal density; SI, Stomatal index; ED, Epidermal cell density; GCL, Guard cell length; S, Stomatal size; PCI, Potential conductance index; g_{CO2}, Stomatal conductance to CO_2; g_{max}, Maximum stomatal conductance; $\delta^{13}C$, Carbon isotope ratio; WUE, Water use efficiency.

Introduction

Stomata play a crucial role in linking the atmosphere and terrestrial biosphere by regulating the fluxes of CO2 and water vapour. These fluxes may be optimized by short-term variations in stomatal aperture and longer-term changes in stomatal density (SD) and size (S) (Franks and Beerling 2009, Casson and Hetherington 2010, Franks et al. 2012, Haworth et al. 2015). An inverse relationship between SD and partial pressure of CO2 in the atmosphere (pCO2) has been shown from data spanning several decades (Wagner et al. 1996, Beerling and Kelly 1997, McElwain 2004, Wagner et al. 2005) to centuries (Woodward 1987, Penuelas and Matamala 1990, Woodward and Kelly 1995, Lammertsma et al. 2011) and fossil records (Beerling and Chaloner 1993, Beerling et al. 1993, Royer 2001, Beerling and Royer 2002, McElwain 2004). Increased maximum stomatal conductance (gmax) brought about by increased SD has enabled angiosperms to maintain higher maximum photosynthetic rates (Amax) during periods of decreasing atmospheric CO2 concentration (Ca) and thereby maintain a competitive advantage over gymnosperms during the Cretaceous Period (McElwain et al. 2016). In contrast to observations over longer time scales, short-term CO2 enrichment (CE) experiments have shown a range of responses for SD across a large number of plant species (Ferris and Taylor 1994, Woodward and Kelly 1995, Lauber and Körner 1997, Haworth et al. 2010, Haworth et al. 2015). Although Woodward et al. (2002) showed a mean reduction of 29% across 110 species with [CO2] doubling, individual species showed decreases, increases or no change.

Changes in stomatal traits have been an important component of plant adaptation to increasing altitude (Woodward 1986, Li et al. 2006, Qiuhong et al. 2013, Wang et al. 2014, Shi et al. 2015), which requires adaptation to decreasing availability of CO_2, air temperature and vapour pressure deficit coupled with increasing wind speed, irradiance and UV radiation (Körner 2007, Barry 2013). While many studies have shown increased SD and SI with increasing altitude (Körner and Cochrane 1985, Körner et al. 1986, McElwain 2004, Kouwenberg et al. 2007), some have reported a range of responses. For example, Shi et al. (2015) report contrasting stomatal and photosynthetic responses to increasing altitude. A deciduous shrub and a herbaceous annual showed increases in stomatal conductance (g_{CO2}) and photosynthetic capacity with altitude while an evergreen shrub showed decreases. Furthermore, SD and SI have been shown to be positively related to pCO_2 across an altitude gradient in the extant species *Quercus* *guyavifolia* (Hu et al. 2015) and in the herbarium sheets of *Typha orientalis* collected over period of $p\mathrm{CO}_2$ increase from 1931 to 2009 (Bai et al. 2015). Further illustrating the diversity of this relationship, SD of *Quercus aquifolioides* (Li et al. 2006), *Picea asperata* (Luo et al. 2006) and *Picea crassifolia* (Qiang et al. 2003) increased with altitude up to 2800–3000 m, beyond which the trend was reversed. Accordingly, it is possible that stomatal traits and their related ecological and physiological traits have a high degree of plasticity in their responses and in the consequent plant adaptation to habitat variation associated with altitude. Furthermore, the projected future increases in C_a (Stocker et al. 2013) are likely to impose differential impacts on plants adapted to the respective $p\mathrm{CO}_2$ at different altitudes.

The diversity of stomatal responses to increasing altitude and the associated reduction of $p\mathrm{CO}_2$ could be caused by the interacting effects of co-varying environmental factors (e.g. temperature, irradiance, vapour pressure deficit, wind speed etc.) and internal plant factors such as growth habit and leaf economic strategy (Shi et al. 2015). Woodward et al. (2002) concluded that variation of $p\mathrm{CO}_2$ with altitude induced significant changes in SD whereas changes in temperature, irradiance and wind speed did not influence the variation of SD significantly. In a survey of 100 species, Woodward and Kelly (1995) did not observe an influence of woodiness, growth form (trees vs. shrubs), habitat (cool vs. warm) and stomatal distribution on the leaf (amphistomatous vs. hypostomatous) on the response of SD to $p\mathrm{CO}_2$, which showed a 14.3% reduction in SD with CO_2 enrichment (CE). In a review consisting of leaf samples from wide altitudinal transects including sun and shade leaves of *Q. kelloggi* and *Nothofagus solandri*, supplemented with carefully-controlled growth chamber experiments simulating light and temperature gradients across altitude, Kouwenberg et al. (2007) concluded that clear increases in SD and SI with altitude are primarily a response to decreasing $p\mathrm{CO}_2$ and photosynthetic potential, with the effects of irradiance and temperature being insignificant.

Stomatal conductance is co-determined by SD and S. While the response of SD to C_a is well characterized, response of S has received less attention (Lomax et al. 2009). Franks and Beerling (2009) have shown that S, computed as the guard cell length (GCL) x closed stomatal width, has co-varied with SD in an inverse relationship when plants responded to variations in C_a over the last 400 million years. During periods of low C_a, g_{CO2} has been maximized by a combination of high SD and low S. Doheny-Adams et al. (2012) also have observed a strong negative correlation between SD and S in a collection of *Arabidopsis thaliana* mutants grown under a range of C_a and reduced soil water availability. In particular, they observed that the response of $g_{\max}$ of *A. thaliana* mutants to decreasing soil water occurred through reductions in S rather than SD.

Stomata exert a controlling influence on water use efficiency (WUE), the ratio between simultaneous rates of net photosynthesis and transpiration (Franks and Farquhar 2007). The functional basis of stomatal movements in response to environmental stimuli is to maximize carbon gain while incurring minimum water loss (Farquhar and Sharkey 1982). Accordingly changes in g_{CO2} induced by CE are likely to cause variations in WUE. As WUE is a key determinant of plant performance in water-limited environments, CO_2-induced changes in SD and S could have important implications on the fitness of different plant ecotypes in a given environment. The carbon isotope ratio of plant tissue ($\delta^{13}C_p$) is often used as an indication of WUE as a positive correlation exists between $\delta^{13}C_p$ and WUE (Farquhar and Richards 1984, Marshall et al. 2008).

The present work investigates the response of stomatal anatomical traits and $\delta^{13}C_p$ of 18 ecotypes of *Arabidopsis thaliana* originating from a diverse range of habitats across an altitudinal gradient to short-term CE in a growth chamber. The specific research questions that we aim to answer are: (1) Does *A. thaliana* show within-species variation in its stomatal anatomical traits and $\delta^{13}C_p$ that reflects adaptation to the respective altitudes of origin (ALT) of different ecotypes?; (2) Does the response of the above parameters to short-term CE differ among the different ecotypes and if so, is it related to ALT? ; (3) How do SD and S interact when different ecotypes adapt to their respective ALTs and when they respond to CE? ; (4) What are the inter-relationships among $\delta^{13}C_p$ and stomatal traits during the adaptation of ecotypes to their respective ALTs and during their response to CE?.

Materials and methods

Plant material and growth conditions

Eighteen accessions of *A. thaliana* (Table 1) originating from a wide altitudinal range (50–1260 m above sea level), were grown from seed to flowering in identical controlled environment chambers (Sanyo Gallenkamp, UK) (0.72 m² floor area) under ambient (400 ppm, ACO_2) and elevated (800 ppm, ECO_2) carbon dioxide concentrations. The ECO_2 was achieved using an external CO_2 cylinder (BOC gases, UK). CO_2 was monitored by infra-red gas analysers (ADC 2000, UK). A 16 h (hour) photoperiod at 201 ± 7.0 μmol (PAR) m^{-2} s^{-1}, with 21°C/18°C day-night temperatures and constant RH at 55% were maintained. All measurements and analyses were carried out on mature rosette leaves prior to flowering.

Determination of stomatal characteristics

Three leaves per plant were collected from five plants growing under each treatment. Stomatal impressions were taken using the method of Lake and Woodward (2008). These were taken from a point mid-way between the mid rib and leaf margin and halfway between the apex and the base of the leaf. The number of stomata and epidermal cells were recorded using light microscopy (Leitz Laborlux S, Leitz, Germany). Five fields of view per leaf sample were randomly selected for counting. The following characters were determined: SD (number of stomata per mm^{-2}); ED (number of epidermal cells per mm^{-2}) and SI (SD/[SD + ED] x 100). Guard cell length (μm) was measured on both leaf surfaces using a

Quantimet 500 image analysis system (Leica, Milton Keynes, Middlesex, UK) coupled to a light microscope (Leitz Laborlux S, Leitz, Germany). Five fields of view per leaf sample were randomly selected for measurements. Potential conductance index (PCI) was computed as PCI = $[\mathrm{GCL}]^2 \times \mathrm{SD} \times 10^{-4}$ (Holland and Richardson 2009), where GCL is given in μm and SD in stomata per mm^2, assuming that the stomatal aperture area was proportional to the GCL^2.

Leaf carbon isotope composition and discrimination using isotope ratio mass spectrometry

A sub-sample of six ecotypes representing four altitudes of origin (i.e. 50, 150, 550 and 790 m, Table 1) was used for isotope analysis. Leaf samples were dried to a constant weight at 50°C and finely ground. 1.0–1.5 mg was analysed on a Mass Spectrometer (ANCA GSL 20-20, PDZ Europa Ltd., Cheshire, UK) for isotope ratio determination. Composition of the source gas was taken from a previous ECO$_2$ experiment conducted in the same growth chambers. The isotope composition was determined relative to the standard CO$_2$ derived from fossil belemnite of the Pee Dee Formation (PDB), USA.

Absolute isotope ratios (R) were measured for sample and standard, and then the relative measure delta (δ^{13}C) was calculated as,

$$\delta^{13}\mathrm{C} = \left\{\frac{\mathrm{R(sample)} - \mathrm{R\ (standard)}}{\mathrm{R(standard)}}\right\} \times 1000‰ \qquad \text{(Eqn. 1)}$$

where, R (standard) is the molar abundance ratio of ^{13}C:^{12}C of the standard (PDB=0.01124) (Farquhar et al. 1989).

Statistical analysis

Significance of the effects of ECO$_2$ and individual ecotype on SD, ED, SI, GCL and PCI was determined by analysis of variance (ANOVA). As some ecotypes were not available at both CO$_2$ treatments, data were subjected to unbalanced ANOVA, using the GLM (General Linear Model) procedure of the Statistical Analysis System (SAS) version 9.0 (SAS Institute., Cary, N.C.). A separate ANOVA was carried out to determine significance of variation of the same stomatal characteristics with ECO$_2$ and altitude of origin (ALT) of ecotypes. As the respective numbers of ecotypes representing different ALTs were not equal (Table 1) the GLM procedure was used. Multiple comparisons among means was carried out by the Duncan's Multiple Range Test at $P = 0.05$ level.

Results

All measured stomatal characteristics (i.e. SD, ED, SI, GCL and PCI) differed significantly ($P<0.0001$) between the two leaf surfaces. Hence, they are presented separately.

Ecotypic and altitudinal variation of stomatal characteristics grown at ACO$_2$ and ECO$_2$

All stomatal characteristics of both leaf surfaces showed highly-significant ($P<0.0001$) ecotypic variation at the two respective [CO_2] (Figs 1–5). Of the 18 accessions, two accessions, Db-1 and Cvi-0, did not grow under ECO_2. Therefore, for these two, results are available only under ACO_2. All variables showed a broad range of variation, with substantial ecotypic variation within the ALTs of 50 and 150 m (Figs 1–5). With the exception of adaxial GCL (Table S2), all stomatal characteristics showed significant ($P<0.05$) variation among different ALTs (Tables S1–S3). In spite of reductions at certain ALTs, all stomatal characteristics showed an overall increasing trend across ALTs from 50 m to 1260 m.

Response of stomatal characteristics to CO_2 enrichment (CE)

Stomatal density-related characteristics of abaxial and adaxial surfaces

The direction of change of SD, ED and SI in response to ECO_2 and its magnitude differed for different ecotypes (Figs 1–3). Ba-1 showed reductions in all tested stomatal characteristics of both leaf surfaces while Col-0 showed increases. In contrast, ecotypes Su-0 and Mc-0 did not show significant variation. As a result, the ecotype x [CO_2] interaction effects on all three density-related stomatal characteristics of both leaf surfaces were highly significant ($P<0.0001$).

Stomatal density, ED and SI of different ALTs also showed differential responses to CE (Table S1). The ALTs 450 m and 1200 m, which were represented by Db-1 and Cvi-0 respectively, were excluded from this ANOVA as both ecotypes did not grow at ECO_2. Overall, at most ALTs, stomatal characteristics showed increases of varying magnitudes in response to ECO_2. The only exception was the 550 m altitude, where SD, ED and SI showed decreases with ECO_2, resulting in highly significant ($P<0.0001$) treatment x ALT interaction effects.

The responses to CE of abaxial and adaxial ED of individual ecotypes (Fig. 2) were broadly similar to the corresponding responses of their abaxial and adaxial SD (Fig. 1). In contrast, the response of SI to CE by a majority of ecotypes (Fig. 3) differed from the corresponding response of their SD. Only four ecotypes showed similar responses of SI and SD in both leaf surfaces. These were Col-0 and Mt-0 in which SI and SD of both surfaces increased with CE, Ba-1 in which both decreased and Mc-0 where abaxial SI and SD increased and adaxial SI and SD did not vary significantly. When the responses of the two leaf surfaces were considered separately, only 8 out of the 16 ecotypes showed similar directions of response to CE for both abaxial SD and SI. For the adaxial SD and SI, only 7 ecotypes showed similar directions of response to CE.

In Bla-1, Ts-1 and Rsch-4, abaxial SI did not respond significantly to CE despite their abaxial SD showing significant CE responses. In these three ecotypes, the abaxial ED responded significantly to CE, with the direction of response being similar to that of SD. Similarly, in Ksk-1, Lc-0, Pla-0, Lan-0 and Can-0 adaxial SI was not responsive to CE despite the corresponding adaxial SD being responsive. With the sole exception of Lc-0, in all the above ecotypes also, adaxial ED showed significant CE response in the same direction as adaxial SD. Conversely, in several ecotypes abaxial and adaxial SI showed significant responses to CE despite their respective SDs not showing significant CE responses. This was observed in Lc-0 and Ll-0 with respect to abaxial SI and SD and in Bla-1, Su-0, Ts-1 and Edi-0 with respect to adaxial SI and SD. In Lc-0, Ll-0 and Su-0, the respective ED also did not show a significant CE response. In contrast, in Bla-1, Ts-1 and Edi-0, the respective ED increased significantly while the SI decreased significantly. It was only rarely that the respective SD and SI showed opposite responses to CE. This was observed in Lan-0 and Wil-2 with respect to abaxial SD and SI, where SD increased while SI decreased. In both these ecotypes, the above changes in abaxial SD and SI were accompanied by significant increases in abaxial ED. In contrast, none of the ecotypes showed opposite responses to CE in their adaxial SD and SI.

Guard cell length (GCL) of the abaxial and adaxial surfaces

The ecotype x [CO_2] interaction was highly significant (P<0.0001) for GCL of both leaf surfaces. On each leaf surface, 8 of the 16 ecotypes showed significant variation in GCL in response to ECO_2 (Fig. 4). On both surfaces, the majority of ecotypes which showed significant variation of GCL with ECO_2 showed decreases relative to ACO_2. Four ecotypes (Bla-1, Lan-0, Rsch-4 and Can-0) showed significant reductions in GCL (i.e. smaller stomata) on both surfaces. In contrast, there were four ecotypes (Ksk-1, Su-0, Ts-1 and Mc-0) in which neither the abaxial nor adaxial GCL varied significantly in response to CE. In the few genotypes which showed reductions in GCL with ECO_2, they were often accompanied by increased SD (e.g. Lan-0 and Rsch-4). When ecotypes were grouped into different ALTs, ECO_2 decreased GCL at 150 and 1260 m and increased at 550 m while no significant variation was shown at 50 and 790 m (Table S2).

Potential Conductance Index (PCI) of the abaxial and adaxial surfaces

Five ecotypes increased their PCI on both leaf surfaces in response to ECO_2 (Fig. 5) and these were; Col-0, Pla-0, Wil-2, Rsch-4 and Mt-0. Lan-0 increased its PCI on the adaxial surface. Ba-1, Ts-1 and Ksk-1 decreased their PCI on both surfaces while Can-0 decreased its PCI on the abaxial surface. Consequently, the ecotype x [CO_2] interaction effects on PCI of both surfaces were highly significant (P<0.0001). All ecotypes which showed increased PCI in response to CE originated from 50 or 150 m altitudes. However, the ecotypes which showed decreased PCI originated from both high (Ba-1 and Can-0, 550 m and 1260 m) and low (Ksk-1, 50 m) ALTs (Fig. 5). At each ALT, the abaxial PCI was higher than the adaxial PCI under both [CO_2] (Table S3).

Correlations among stomatal characteristics within and between CO_2 treatments

Abaxial SD showed highly significant (P<0.001) positive correlations with abaxial ED and SI within each

[CO_2] (Fig. 6A). Similarly, adaxial SD showed significant ($P<0.05$) positive correlations with the respective adaxial characters (Fig. 6B). No significant correlations were observed between SI and ED on any leaf surface under any [CO_2] (*data not shown*). GCL was not significantly correlated to any of the other characters in either of the leaf surfaces. For all five stomatal characters measured, when the respective values of the two surfaces were averaged, there were significant ($P<0.05$) positive correlations between their mean values at ACO_2 and ECO_2 (Fig. 7).

Carbon isotope ratio of leaf tissue ($\delta^{13}C_p$)

Carbon isotope ratio ($\delta^{13}C_p$) of leaf tissue showed significant ($P<0.01$) variation among different ecotypes under both [CO_2] (Table 2), with Ba-1 and Ts-1 having greater $\delta^{13}C_p$ than the rest. When the ecotypes were categorized based on their ALT, $\delta^{13}C_p$ showed significant variation among different ALTs under both [CO_2] (Table S4). However, the altitudinal variation of $\delta^{13}C_p$ did not show a consistent increasing or decreasing pattern with ALT. Under ACO_2, significantly greater $\delta^{13}C_p$ was shown at 50 and 550 m in comparison to $\delta^{13}C_p$ at 150 and 790 m. Under ECO_2, $\delta^{13}C_p$ at 550 m was significantly greater while there was no significant variation among $\delta^{13}C_p$ of the rest of the ALTs. Mean $\delta^{13}C_p$ of the respective ecotypes growing in ACO_2 did not show significant correlations with any of the stomatal characteristics (Table 3). However, ecotypic mean $\delta^{13}C_p$ at ECO_2 showed strong negative correlations with the respective ecotypic means of SD, ED, SI and PCI at elevated CO_2. The low number of data points in the correlations (i.e. six) was responsible for these correlations not attaining statistical significance at $P=0.05$.

Discussion

Ecotypic variation of stomatal traits with altitude and associated CO_2 availability

Our results show substantial within-species variation in the response of stomatal traits of *A. thaliana* to varying CO_2. The ecotypic variation in SD and SI shown at ACO_2 (400 ppm) reflects long-term evolutionary adaptation to the environmental variation to which the plants had been exposed at different ALTs. Possible exchange of genetic material between *A. thaliana* plants at different altitudes (Beerling and Royer 2002) can be ruled out because of the geographical distances across the sites from which the genotypes had been collected (Table 1). Response to variation of CO_2 availability across altitudes probably had a determining influence on the ecotypic variation of stomatal traits as the observed increases of SD and SI with ALT (Table S1) agreed with the predominant trend reported previously (Körner and Cochrane 1985, Körner et al. 1986, Woodward and Bazzaz 1988, Hovenden and Brodribb 2000, McElwain 2004, Li et al. 2006, Kouwenberg et al. 2007). This also supports the suggestion that historical [CO_2] acts as a selective agent in determining stomatal traits (Beerling et al. 1993, Ward et al. 2000, Leakey and Lau 2012). Furthermore, the increase of PCI, a proxy for g_{CO2}, of both leaf surfaces with increasing ALT at both [CO_2]s observed in the present work is consistent with the observed responses of g_{CO2} to short-term variation of C_a across sub-ambient (180 ppm) to elevated (1000 ppm) [CO_2]s by Franks et al. (2012). The observed variation pattern of PCI with ALT also agrees with that computed by Franks and Beerling (2009) for a range of plant species in 400 Ma of fossil record.

However, the observed variations in SD and SI among ecotypes within each ALT at ACO_2 (Figs 1–3) meant that apart from pCO_2, many genetic and environmental factors also had an interacting influence. The possible genetic factors include the ecotypic variation in the number of amplifying divisions regenerating the meristemoid and sister cells during stomatal development of *A. thaliana* (Casson and Gray 2008). Differences in atmospheric demand for water as determined by variations in temperature and irradiance could also have influenced stomatal development. Notably, among the low-altitude (50 m) ecotypes, those from warmer environments (e.g. Pla-0, Ts-1, Bla-1 and Ll-0, Table 1) had lower SD and PCI under ACO_2 than those from cooler environments (e.g. Col-0 and Ksk-1). This was probably an adaptation to conserve water in warmer environments of high atmospheric demand. In addition, environmental variations tied to altitudinal change (Körner 2007) could have contributed to the ecotypic variation of stomatal traits at ACO_2. Increased irradiance under cloudless conditions at higher altitudes could cause SD to be higher (Furukawa 1997, Kouwenberg et al. 2007, Shi et al. 2015). For example, in the present work, the higher SD and PCI at ACO_2 of the high-altitude (1260 m) ecotype Can-0 could have been caused by the combination of lower pCO_2 and higher irradiance. On the other hand, Lake and Woodward (2008) speculate that increased precipitation and decreased irradiance could cause SD to decrease with increasing altitude because of decreased transpiration resulting from lowered vapour pressure deficits. Woodward (1986), Johnson et al. (2004) and Kouwenberg et al. (2007) argue that the altitudinal response of SD is not universal and could be controlled by environmental factors other than pCO_2. Our results support this argument.

Stomatal densities of ecotypes originating from higher altitudes have previously shown proportionately greater reductions than those originating from lower altitudes (Woodward and Bazzaz 1988). However, in the present study only one high altitude (i.e. 550 m ALT) ecotype (Ba-1) produced a similar result with a 37% decrease in SD on both leaf surfaces (Fig. 1 and Table S1) while the other high altitude ecotypes Mc-0 (790 m) and Can-0 (1260 m) were less responsive than low (50 m) and mid (150 m) altitude ecotypes. Therefore, the majority of ecotypes of the present work have not shown the greater sensitivity of SD to ECO_2 at higher ALTs.

Ecotypic variation of the response of stomatal traits to short-term CO_2 enrichment

The observed response of stomatal traits of different ecotypes to CE represented a short-term response over a single generation. In contrast to the typical mean response of a wide range of plant species

(Woodward 1987, Woodward and Bazzaz 1988, Ceulemans et al. 1995, Clifford et al. 1995, Woodward and Kelly 1995, Beerling et al. 1998, Tognetti et al. 2001, Woodward et al. 2002, Teng et al. 2006), the majority of ecotypes in the present work increased SD in response to CE, with only Ksk-1 and Ba-1 showing a decrease of SD in both leaf surfaces (Fig. 1). It is notable that in a study involving 63 accessions of *A. thaliana* (Woodward et al. 2002, Hetherington and Woodward 2003), the species exhibited both increases and decreases of SD in response to a doubling of [CO_2], with different ecotypes of *A. thaliana* showing a similar range of responses to CE as different plant species (Woodward et al. 2002, Royer 2001, Hetherington and Woodward 2003, Lake and Woodward 2008, Wang et al. 2014). Therefore, the diversity in the response of SD to CE in the present work is in accordance with the broader range of responses shown by *A. thaliana* and a range of other species (Royer 2001, Haworth et al. 2010, Haworth et al. 2016).

Despite the diverse responses to CE, mean SD, SI and PCI under ECO_2 increased with ALT (Tables S2 and S4) thus showing similar trends as those under ACO_2. This indicates that the genetic adaptation to ALT, and therefore to variation in CO_2 availability, is preserved under short-term CE as well (Beerling and Royer 2002). This conclusion is also supported by the significant positive correlations between the respective values at ACO_2 and ECO_2 for stomatal traits of the present study (Fig. 7). It is also in agreement with the observation of Lake and Woodward (2008) for SD of a range of *A. thaliana* ecotypes and mutants varying in transpiration. Whether the same holds true with long-term CE has to be investigated. Haworth et al. (2016) provide evidence from a temperate grassland that longer-term exposure (14 years) to moderate CE (480 ppm from 400 ppm) did not change the comparative order of SD among different species at ambient and elevated [CO_2], despite showing both decreases and increases in response to CE. In agreement with the above, Beerling and Chaloner (1993) and Franks et al. (2012) provide evidence that the evolutionary response of stomatal traits to long-term variation in atmospheric [CO_2] is similar to their phenotypic response to short-term CE. Furthermore, Royer (2001) observed that the expected reduction of SD with increasing [CO_2] (Woodward 1987, Franks and Beerling 2009) was less frequent in short-term experiments than in longer-term reconstructions from herbarium material or fossil leaves. Royer (2001) attributed this to the fact that short-term experiments show variable plastic responses while long-term measurements demonstrate genetic changes over several generations, thus supporting the diversity of SD responses to CE observed in the present study.

Possibility of physiological control of stomatal aperture

The absence of a response of SD to CE could have occurred due to changes in stomatal aperture (Reid et al. 2003, Young et al. 2006), which could still alter g_{CO2} without any change in SD. Reductions in stomatal aperture could occur due to reduced stomatal opening and/or smaller GCL. Elevated CO_2 has been shown to increase efflux of K^+ and anions (Brearley et al. 1997, Raschke et al. 2003) and trigger the release of Cl^- from guard cells (Hanstein and Felle 2002), thus leading to loss of guard cell turgor and reduced stomatal opening. Haworth et al. (2016) provide evidence of reduced stomatal size (S) being responsible for lower stomatal conductance at ECO_2 when SD either increased or remained unchanged. Furthermore, % reductions of SD and S in response to CE as reported by Haworth et al. (2016) for some grassland species were substantially lower than the observed reductions of g_{CO2}, thus indicating that appreciable reductions in stomatal opening must have occurred at ECO_2. Haworth et al. (2013, 2015) showed evidence that different species respond to CE either through active stomatal control (i.e. changes in stomatal opening over shorter time scales) or passive control (i.e. changes in SD). Therefore, it is highly likely that all the above regulatory mechanisms operate at different levels in the different ecotypes of *A. thaliana* in the present work, which could explain the observed range of responses to CE.

While growth parameters were not measured in the present study many other studies have confirmed an increase in growth of *A. thaliana* ecotypes in response to ECO_2 (Ward and Strain 1997, 1999, Woodward et al. 2002, Teng et al. 2006, Li et al. 2008). This positive growth response is brought about by increased carboxylation of RuBP and its reduced oxygenation (Gifford 2004, Long et al. 2004, Bernachchi et al. 2005, Ainsworth and Rogers 2007) and possibly reduced mitochondrial respiration (Ayub et al. 2015, Haworth et al. 2016). Although the above changes in photosynthetic and respiratory physiology increases efficiency of conversion of absorbed CO_2 to biomass, increased biomass at the whole plant level under ECO_2 would increase the demand for CO_2. It is plausible that the increase in SD seen under ECO_2 in some of the ecotypes of the present study (e.g. Col-0, Pla-0, Lan-0, Mt-0, Rsch-4 and Wil-2, Fig. 1) is associated with increased growth responses and that it occurs in order to fulfil a rapid increase in the demand for CO_2. In contrast, in the ecotypes which showed reductions (i.e. Ksk-1 and Ba-1) or no significant changes in SD (e.g. Ll-0 and Su-0), the increased CO_2 demand at ECO_2 could have been fulfilled by the increased $[CO_2]$ gradient at ECO_2 and by increasing stomatal aperture without having to increase SD. Therefore, changes of stomatal aperture could compensate for the absence of changes of SD to ensure an adequate supply of CO_2, thus causing the diversity of SD responses to ECO_2 observed in the present work. Similar conclusions have been made by Morison (1998), Franks and Beerling (2009) and Franks et al. (2012).

Possible contributions of changes in stomatal differentiation and epidermal cell expansion in observed responses of SD and SI to CE

The simultaneous responses of SD, SI and ED enable identification of whether the observed responses of SD to CE had occurred because of the effects of ECO_2 on stomatal differentiation or on epidermal cell expansion and/or division (Morison 1998, Poole et al. 2000, Casson and Gray 2008). The present work shows that all these mechanisms operate within the same species in different ecotypes (Table 4). In the ecotypes, in which significant CE responses were observed in SD but not in SI (e.g. Bla-1 and Ksk-1 for abaxial and adaxial surfaces respectively), the variation of SD was caused due to the effects of ECO_2 on epidermal cell expansion. Conversely, in the ecotypes, which showed significant SI responses to CE without significant responses in SD (e.g. Lc-0 and Bla-1 for abaxial and adaxial surfaces), the influence of ECO_2 could be different depending on the simultaneous response of ED as determined by the effects on epidermal cell division and/or expansion. This effect of ECO_2 on epidermal cell division or expansion could have occurred in addition to its effect on stomatal differentiation or as the sole mechanism causing the reduction of SI without a change in SD. Similar observations have been reported by Poole et al. (2000) and are supported by Taylor et al. (1994) who showed that ECO_2 influenced both cell division and expansion in leaves. In the ecotypes, which showed similar directional responses in SD and SI (e.g. both surfaces of Col-0), ECO_2 influenced stomatal differentiation, with varying effects on epidermal cell expansion and division depending on the direction of change of ED. The concurrent responses to CE of SD and SI were similar for the abaxial and adaxial surfaces in only four out of the 16 ecotypes. This showed that mechanisms through which ECO_2 influences stomatal differentiation and epidermal cell expansion and division are operating independently in the two leaf surfaces. This is in agreement with the observations and conclusions of Morison (1998), Lake et al. (2002) and Haworth et al. (2016).

Ecotypic variation of guard cell length with CE and ALT and its relationship with SD

Similar to stomatal density-related traits, stomatal size (S), as measured by GCL in the present work, also showed wide variation among different ecotypes in its response to CE (Fig. 4 and Table S2). Our results are only in limited agreement with the observed increases of GCL with CE on both geologic (Franks and Beerling 2009) and short experimental (Lomax et al. 2009) time scales as only two (Col-0 and Ba-1) and three (Lc-0, Ll-0 and Edi-0) ecotypes respectively showed increased abaxial and adaxial GCL in response to CE. However, Franks and Beerling (2009) noted that magnitude of change in S in short-term ECO_2 experiments was much lower than that observed in Phanerozoic reconstructions over geologic time scales as the short-term changes represent only phenotypic plasticity over a single generation. In agreement with our observations, Lake and Wade (2009) also observed differential variation of GCL of the two leaf surfaces in response to ECO_2.

The increasing trend of GCL with increasing ALT (Table S2) indicates that ecotypes originating at lower altitudes have smaller stomata. Having smaller stomata brings several advantages such as greater g_{CO2} for the same total pore area due to decreased length of flow path for CO_2 transfer (Franks and Farquhar 2007) and reduced proportion of leaf surface area for water loss (Franks and Beerling 2009). Furthermore, smaller stomata would entail a lower cost in terms of reduced epidermal surface area for

CO_2 absorption (de Boer et al. 2016). These properties would be advantageous at lower altitudes, where the higher pCO_2 is combined with higher temperatures, thus increasing the potential for both photosynthesis and transpiration. In such environments, smaller stomata can maximize photosynthetic carbon gain while minimizing transpiration and thereby maximize water use efficiency (WUE) (Franks and Beerling 2009, Haworth et al. 2016).

The absence of significant correlations between GCL and SD or SI showed that GCL is determined independently from stomatal density-related parameters among the ecotypes of *A. thaliana* used in the present study. While being in agreement with observations of Holland and Richardson (2009), this again contradicts the observed negative relationships between SD and GCL (Hetherington and Woodward 2003, Miller-Rushing et al. 2009, Lammertsma et al. 2011, Wang et al. 2014) or S (Franks and Beerling 2009, Franks et al. 2012) across a wide range of species covering all plant functional types. However, in agreement with our observations, for the response of *A. thaliana* to ECO_2, Hetherington and Woodward (2003) and Haworth et al. (2016) also show the absence of a negative correlation between SD and GCL or S.

De Boer et al. (2016) have shown that plants, across a wide range of phylogenetic groups, follow a strategy of optimally allocating epidermal surface area for stomata during responses aimed at maximizing g_{max} through increased SD. This optimal allocation is achieved by a concurrent reduction in stomatal size (S) which ensures that the epidermal surface area allocated for stomata does not increase. Interestingly, de Boer et al. (2016) show that the requirement for a concurrent reduction in S when SD increases is less stringent for amphistomatous dicots (e.g. *A. thaliana*) as both leaf surfaces are available for stomatal development. Therefore, when SD increases, the need to develop smaller stomata and thereby limit the cost of allocating a higher proportion of epidermal surface area to stomata is lessened. This could be a reason for the absence of a negative relationship between SD and GCL or S in our study.

Ecotypic variation of $\delta^{13}C_p$ with CE and ALT and its relationship with SD

In agreement with our observations, previous studies have reported genetic variation in $\delta^{13}C_p$ amongst *A. thaliana* ecotypes (Nienhuis et al. 1994, McKay et al. 2003). The range of $\delta^{13}C_p$ signatures obtained with six ecotypes at ACO_2, i.e. from –31.2 (Edi-0) to –29.8‰ (Ts-1) was similar, though narrower to that obtained by McKay et al. (2003), i.e. from –31.2 to –28.2‰, with 39 natural accessions of *A. thaliana* collected from a wide range of geographic locations across a wider altitudinal range (1–3400 m *asl*). In the study by Nienhuis et al. (1994), 31 *A. thaliana* ecotypes were analysed for $\delta^{13}C$ under ambient conditions with two, Col-0 and Edi-0, common to the present study. Col-0 was ranked as having a high WUE and Edi-0 the lowest. These results are consistent with those of the present study at ACO_2 (Table 2).

However, despite showing significant variation among ecotypes and ALTs, $\delta^{13}C_p$ in the present work did not show a consistent variation pattern with ALT at either ACO_2 or ECO_2. This is in contrast to the increase of $\delta^{13}C_p$ with altitude observed in several previous studies (Körner et al. 1988, Körner et al. 1991, Morecroft et al. 1992, Marshall and Zhang 1994, Morecroft and Woodward 1996, Cordell et al. 1998, Cordell et al. 1999, Hultine and Marshall 2000, Qiuhong et al. 2013, Shi et al. 2015). However, in agreement with our results, there are also reports of $\delta^{13}C_p$ increasing and decreasing with altitude in different years (Friend et al. 1989), decreasing with altitude (Zhang and Marshall 1995) and showing no consistent pattern of variation with altitude (Morecroft and Woodward 1990). While decreasing ratio of intercellular to atmospheric $p\mathrm{CO}_2$ (p_i/p_a) with altitude is regarded as the principal factor responsible for increasing $\delta^{13}C_p$ (Cordell et al. 1999), the interacting effects of other environmental factors linked to altitude such as temperature, irradiance and vapour pressure deficit and plant factors such as leaf nitrogen content, specific leaf area, photosynthetic carboxylation and electron transport capacities (i.e. Vc_{max} and J_{max}) and mesophyll conductance to CO_2 (g_m) have been shown to determine the variation of $\delta^{13}C_p$ (Zhang and Marshall 1995, Morecroft and Woodward 1996, Hultine and Marshall 2000, Boccalandro et al. 2009, Easlon et al. 2014). Hence, the observed variation of $\delta^{13}C_p$ in the our study could have occurred as a result of different *A. thaliana* ecotypes adapting to the environmental variations in their respective ALTs as phenotypic or genotypic adaptations (Morecroft and Woodward 1990, Körner et al. 1991, Morecroft et al. 1992, Cordell et al. 1998, Easlon et al. 2014).

The observed change in the order of ecotypes in terms of magnitude of $\delta^{13}C_p$ at ECO_2 (Table 2) demonstrates significant ecotypic variation in the response of $\delta^{13}C_p$ to CE. This variation indicates that shifts in the composition of *A. thaliana* populations are likely to occur with time due to increasing [CO_2] (Beerling and Chaloner 1993, Ward and Kelly 2004) because of the changes of WUE resulting from changes in $\delta^{13}C_p$ (Farquhar and Richards 1984, Marshall et al. 2008).

Co-variation of SD and $\delta^{13}C_p$ among *A. thaliana* ecotypes and during their response to CE showed broad agreement with the expected patterns of variation. An increase in SD increases g_{CO2} and transpiration, thereby decreasing $\delta^{13}C_p$ and WUE (Zhang and Marshall 1995, Hultine and Marshall 2000, Boccalandro et al. 2009, Qiuhong et al. 2013). In the present study, Col-0 showed the lowest $\delta^{13}C_p$ (i.e. lower potential WUE) under ECO_2 (Table 2). Among the ecotypes used for carbon isotope analysis, Col-0 showed the highest values for SD, SI, GCL and PCI for both leaf surfaces under ECO_2 (Figs. 1, 3–5). The higher stomatal numbers and dimensions would have led to the higher g_{CO2} and lower WUE observed under ECO_2. Furthermore, SD, SI and PCI showed negative correlations with $\delta^{13}C_p$ under ECO_2 (Table 3). Ts-1 which showed the highest $\delta^{13}C_p$ (i.e. higher WUE) under both treatments had the lowest values out of the six ecotypes for SD and PCI on both leaf surfaces under ambient and elevated CO_2. It also had the shortest abaxial and adaxial GCL under ECO_2 and the shortest abaxial GCL under ACO_2. Thus, the lower number of smaller stomata would have caused the lower g_{CO2} and higher WUE in Ts-1. Furthermore, out of the six ecotypes used in carbon isotope analysis, Ts-1 was the only ecotype of Mediterranean origin and would have evolved in a warmer and drier environment with a greater frequency of drought occurrence (Table 1). Such environments would select for higher $\delta^{13}C_p$ and WUE (Ehleringer and Cooper 1988, Ehleringer 1993, Heschel et al. 2002, Chen et al. 2005). In further agreement with the expected evolutionary adaptation patterns, Ba-1, a high altitude ecotype, showed the second highest values for $\delta^{13}C_p$ (the second highest WUE) under both ambient and elevated CO_2. Interestingly, Ba-1 was one of the few ecotypes in the present study that showed a significant decrease in SD and SI in response to CE (Figs. 1–3), thus contributing to its higher $\delta^{13}C_p$ and WUE under ECO_2.

Conclusion

The present work highlighted the substantial intraspecific diversity in the response of the stomatal traits of *A. thaliana* to long-term exposure to pCO_2 and other environmental parameters associated with altitudinal gradients and short-term CO_2 enrichment. This diversity of response indicates the involvement of multiple mechanisms in causing changes in stomatal traits. Hence, caution should be exercised when environmental responses of stomatal traits are defined based on work using a single ecotype.

Author contributions

H.I.U.C., F.I.W. and J.A.L. planned and designed the research. H.I.U.C. performed the experiment. H.IU.C. and W.A.J.M. De C. performed the statistical analyses, interpretation of experimental results and drafted the manuscript. W.A.J.M. De C. revised the manuscript based on the advice of J.A.L., F.I.W. and S.M.W.R..

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Figure legends

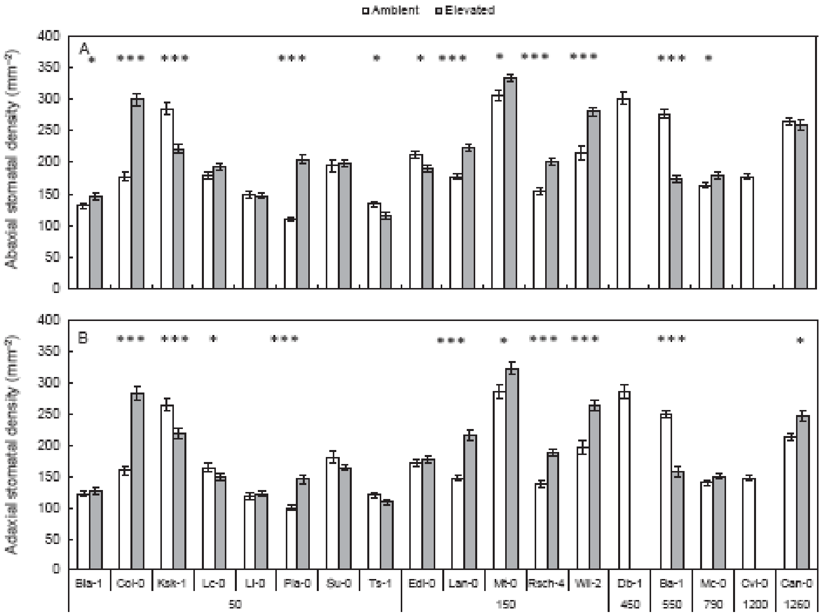

Fig. 1. Variation of stomatal density (SD) of *A. thaliana* ecotypes grown under ambient (400 ppm) and elevated CO_2 (800 ppm) treatment: (A) abaxial SD; (B) adaxial SD. Error bars indicate standard errors of mean (n= 75). Difference between elevated and ambient CO_2 significant at * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$. Numbers below ecotypes indicate their altitudes of origin (m above mean sea level).

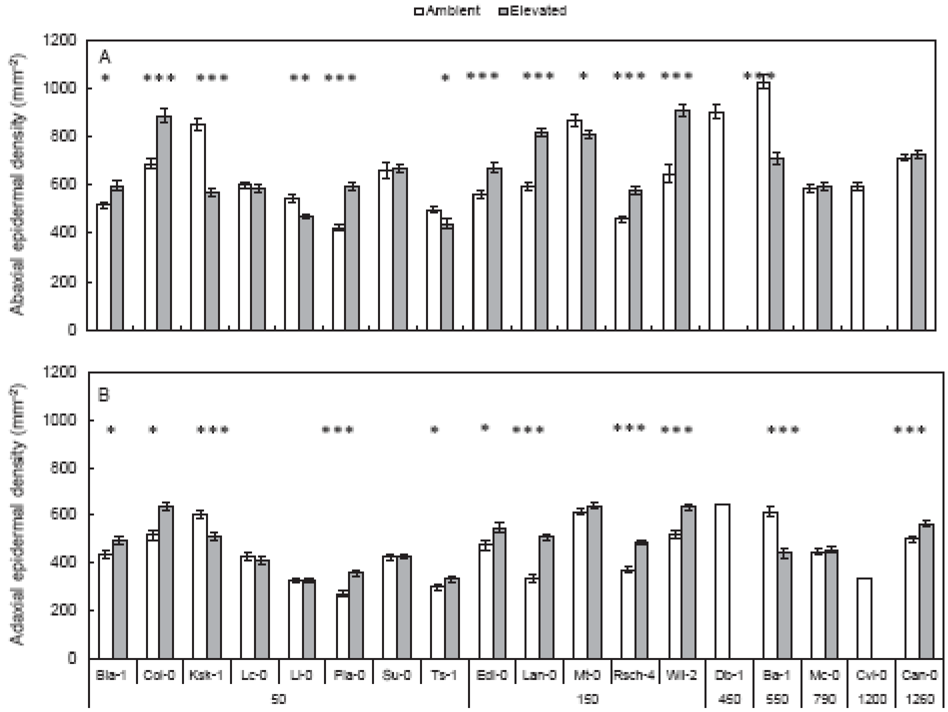

Fig. 2. Variation of epidermal density (ED) of *A. thaliana* ecotypes grown under ambient (400 ppm) and elevated CO_2 (800 ppm) treatment: (A) abaxial ED; (B) adaxial ED. See caption of Fig. 1 for details.

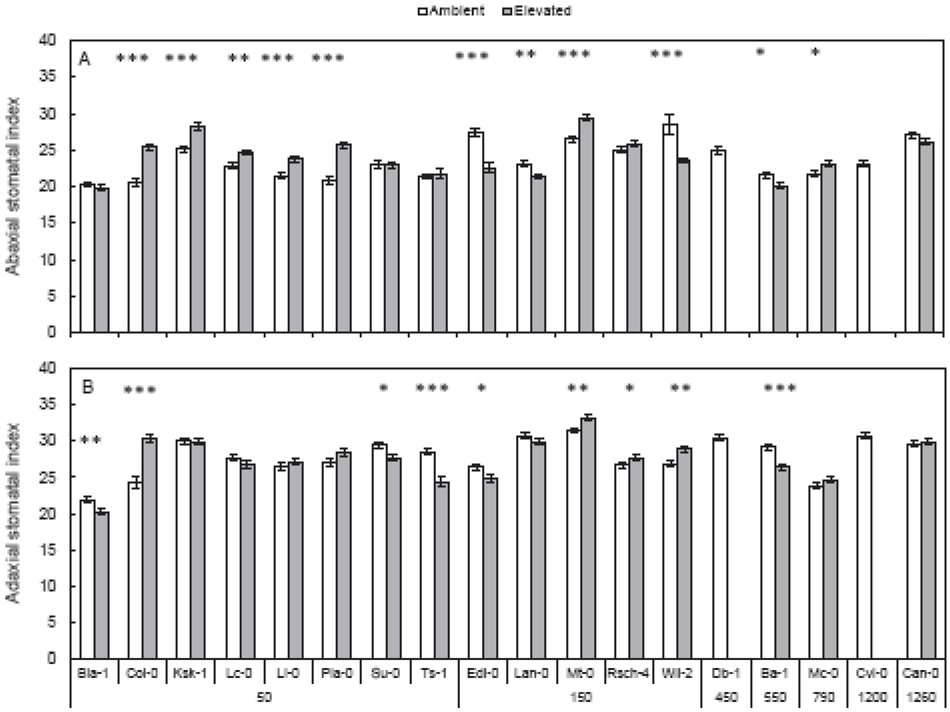

Fig. 3. Variation of stomatal index (SI) of *A. thaliana* ecotypes grown under ambient (400 ppm) and elevated CO_2 (800 ppm) treatment: (A) abaxial SI; (B) adaxial SI. See caption of Fig. 1 for details.

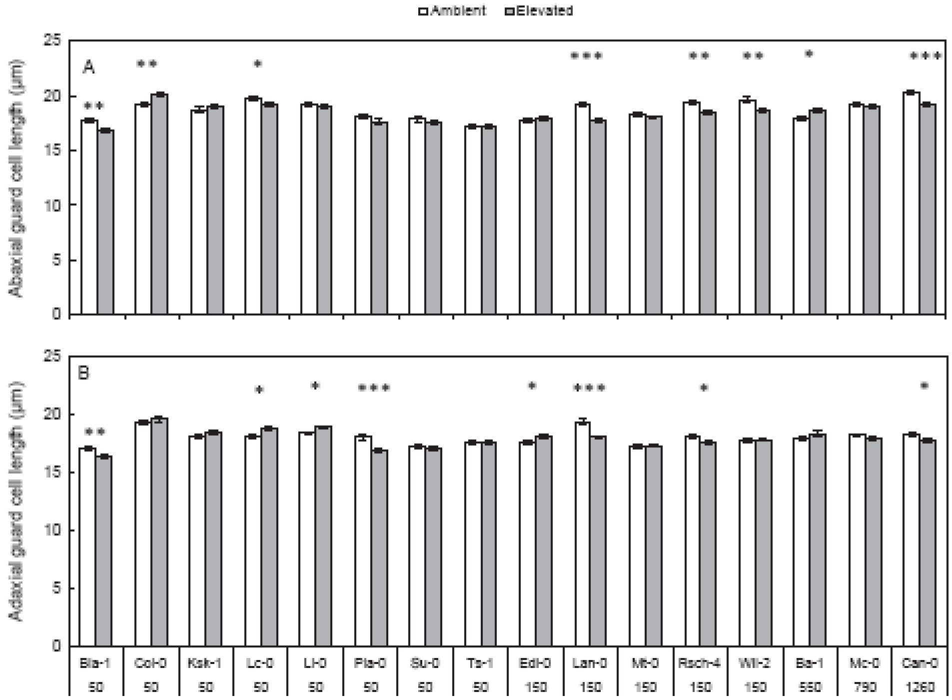

Fig. 4. Variation of guard cell length (GCL) of *A. thaliana* ecotypes grown under ambient (400 ppm) and elevated CO_2 (800 ppm) treatments: (A) abaxial GCL; (B) adaxial GCL. See caption of Fig. 1 for details.

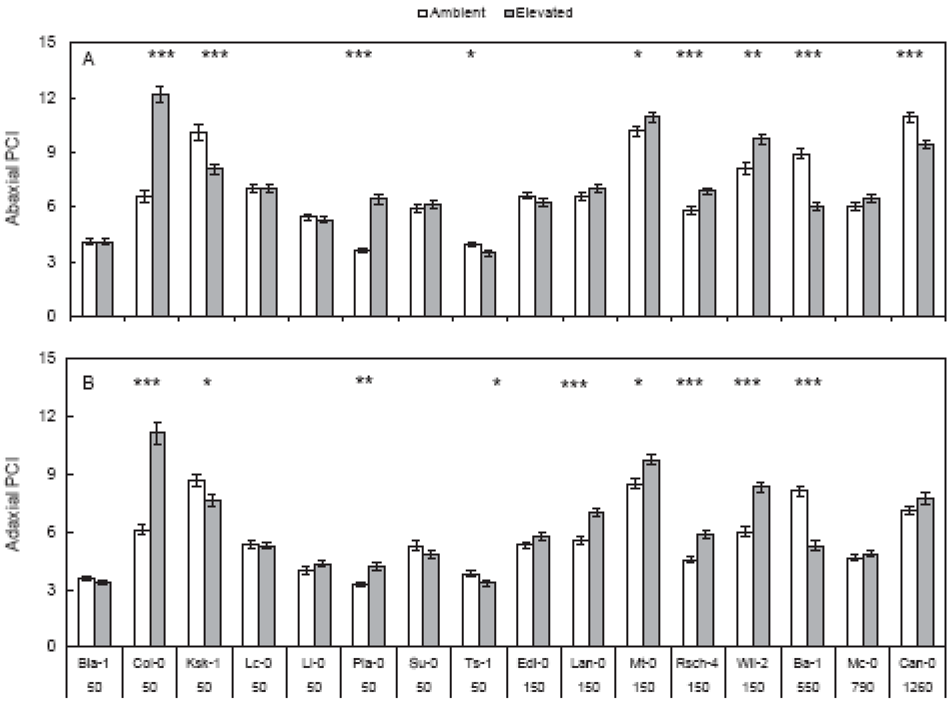

Fig. 5. Variation of Potential Conductance Index (PCI) of *A. thaliana* ecotypes grown under ambient (400 ppm) and elevated CO_2 (800 ppm) treatments: (A) abaxial PCI; (B) adaxial PCI. See caption of Fig. 1 for

details.

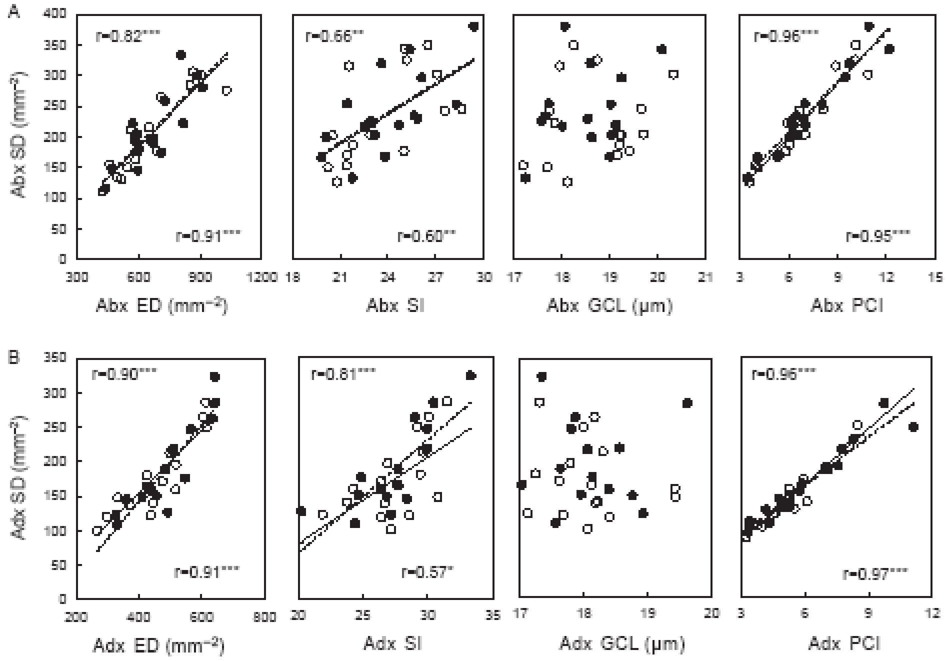

Fig. 6. Linear correlations among different stomatal characters of the abaxial (A) and adaxial (B) leaf

surfaces of *A. thaliana* ecotypes grown under ambient (solid line) and elevated CO_2 (dotted line). Correlations are between ecotypic means within a given CO_2 concentration. Each mean was from 75 replicate measurements. Correlations involving SD, ED and SI under ambient CO_2 are from 18 data points (i.e. 18 ecotypic means). The rest are from 16 ecotypic means. Correlation coefficient (r) above is for elevated CO_2 and that below for ambient CO_2. r is significant at * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$.

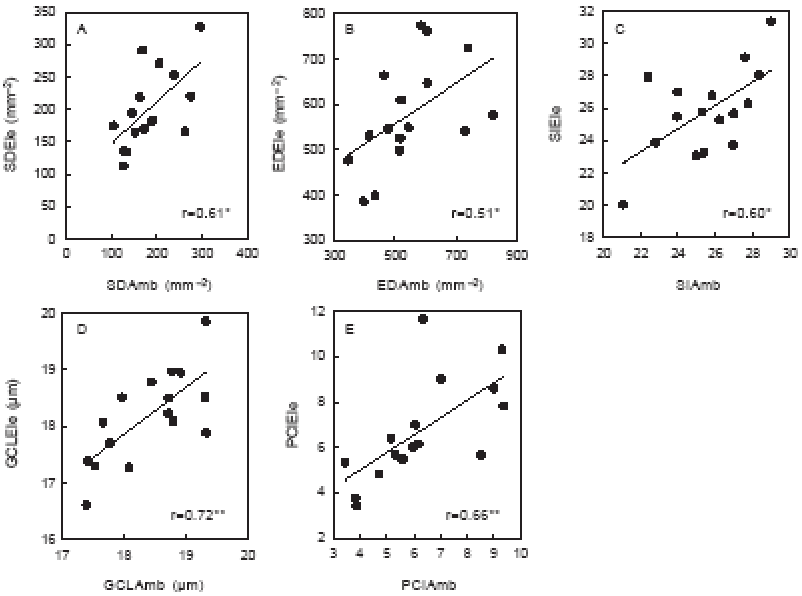

Fig. 7. Linear correlations between average (between abaxial and adaxial) ambient (Amb) and elevated (Ele) SD (A), ED (B), SI (C), GCL (D) and PCI (E) of *A. thaliana* ecotypes grown under ambient (400

ppm) and elevated (800 ppm) CO_2 concentrations. Correlations are between ecotypic means under ambient and elevated CO_2. Each mean was from 150 replicate measurements. Correlations involving SD, ED and SI under ambient CO_2 are from 18 data points (i.e. 18 ecotypic means). The rest of the correlations are from 16 ecotypic means. Correlation coefficient (r) is significant at * $P < 0.05$; ** $P < 0.001$.

Table 1. Description of *Arabidopsis thaliana* ecotypes used in the study (http://nasc.nott.ac.uk/;http://dbsgap.versailles.inra.fr/vnat/). T_{min} – Mean minimum monthly temperature; T_{max} – Mean maximum monthly temperature; P_{min} – Mean minimum monthly precipitation; P_{max} – Mean maximum monthly precipitation; NA – not available.

	Ecotype	**NASC ID**	**Location of origin**	**Altitude of origin (m)**	**Altitude class**	**Climate data**			
						T_{min} °C	**T_{max} °C**	**P_{min} (mm)**	**P_{max} (mm)**
1	Ba–1	N952	Blackmount, UK	550	High	4.0	14.7	81.0	164.0
2	Bla-1	N970	Blanes, Spain	50	Low	11.3	27.7	29.0	71
3	Can-0	N1064	Las Palmas, Canary Islands, Spain	1260	High	18.3	25.6	1.0	14.0
4	Col-0	N1092	Columbia, USA	50	Low	-1.2	18.2	31.0	76.0
5	Cvi-0	N1096	Cape Verde Islands	1200	High	22.0	27.0	1.0	80.0
6	Db-1	N1102	Tenne, Germany	450	High	-0.3	17.6	38.0	68.0
7	Edi-0	N1122	Edinburgh, UK	150	Mid	4.8	15.8	49.0	112.0
8	Ksk-1	N1634	Keswick, UK	50	Low	2.9	14.3	83.0	161.0
9	Lan-0	N1304	Lanark, UK	150	Mid	2.2	13.8	71.0	127.0
1	Lc-0	N1306	Loch Ness, UK	50	Low	0.3	12.1	83.0	184.0
1	Ll-0	N1338	Llagostera, Spain	50	Low	11.3	27.7	29.0	71.0

1	Mc-0	N1362	Mickles Fell, UK	790	High	1.1	13.5	72.0	129.0
1	Mt-0	N1380	Martuba, Libya	150	Mid	12.7	25.3	1.0	65.0
1	Pla-0	N1458	Playa de Aro, Spain	50	Low	NA	NA	NA	NA
1	Rsch-4	N1494	Rschew/Starize, Russia	150	Mid	-9.6	17.0	41	88
1	Su-0	N1540	Southport, UK	50	Low	2.0	13.6	74.0	130.0
1	Ts-1	N1552	Tossa de Mar, Spain	50	Low	11.0	18.0	NA	NA
1	Wil-2	N1596	Wilna, Lithuania	150	Mid	0.0	10.0	NA	NA

Table 2. Variation of carbon isotope ratio ($\delta^{13}C_p$) in leaf tissue of selected ecotypes of *A. thaliana* grown at two CO_2 concentrations. $\delta^{13}C_p$ - $^{13}C/^{12}C$ ratio of leaf tissue relative to $^{13}C/^{12}C$ ratio of the PDB standard. Within each column, means with the same letter are not significantly different at *P* =0.05. Each mean is from five replicate measurements.

		$\delta^{13}C_p$ (± Standard error of mean) (‰)	
Ecotype	Altitude (m)	400 ppm	800 ppm
Ba-1	550	–30.20 (±0.61) a	–44.07 (±0.56) a
Col-0	50	–30.48 (±0.10) ab	–46.77 (±0.12) b
Edi-0	150	–31.22 (±0.17) b	–46.58 (±0.19) b
Lan-0	150	–31.18 (±0.19) b	–46.23 (±0.18) b
Mc-0	790	–31.10 (±0.13) b	–45.88 (±0.14) b
Ts-1	50	–29.77 (±0.16) a	–43.13 (±0.55) a
P		0.009	<0.0001
CV (%)		–2.33	–1.59

Table 3. Pearson's correlation coefficients between the respective carbon isotope ratios ($\delta^{13}C_p$) and stomatal characteristics at ambient (400 ppm) and elevated (800 ppm) CO_2. SDA, EDA, SIA, GCLA and PCIA are respectively SD, ED, SI, GCL and PCI (means of abaxial and adaxial values) at 400 ppm CO_2. SDE, EDE, SIE, GCLE and PCIE are respectively SD, ED, SI, GCL and PCI (means of abaxial and adaxial values) at 800 ppm CO_2.

Stomatal character	$\delta^{13}C_p$ in 400 ppm	$\delta^{13}C_p$ in 800 ppm
SDA	0.031^{ns}	0.059^{ns}
EDA	0.170^{ns}	0.059^{ns}
SIA	$–0.244^{ns}$	0.076^{ns}
GCLA	$–0.445^{ns}$	–0.660 (*P*=0.154)
PCIA	$–0.084^{ns}$	$–0.142^{ns}$
SDE	$–0.340^{ns}$	–0.776 (*P*=0.069)
EDE	$–0.460^{ns}$	–0.811 (*P*=0.050)
SIE	$–0.209^{ns}$	–0.669 (*P*=0.146)
GCLE	$–0.074^{ns}$	$–0.544^{ns}$
PCIE	$–0.223^{ns}$	–0.713 (*P*=0.112)

Table 4. Summarized directions of significant responses stomatal characteristics to elevated [CO_2] of different ecotypes of *A. thaliana*. †Derived from Figures 1–4. + significant (*P*<0.05) increase; - significant (*P*<0.05) decrease; ns - response non-significant at *P*=0.05.

Ecotype	Direction of significant response to elevated [CO_2]†							
	Abaxial surface				Adaxial surface			
	SD	ED	SI	GCL	SD	ED	SI	GCL
Bla-1	+	+	ns	-	ns	+	-	-
Col-0	+	+	+	+	+	+	+	ns
Ksk-1	-	-	+	ns	-	-	ns	ns
Lc-0	ns	ns	+	-	-	ns	ns	+
Ll-0	ns	ns	+	ns	ns	ns	ns	+
Pla-0	+	+	+	ns	+	+	ns	-
Su-0	ns	ns	ns	ns	ns	ns	-	ns
Ts-1	-	-	ns	ns	ns	+	-	ns
Edi-0	-	+	-	ns	ns	+	-	+
Lan-0	+	+	-	-	+	+	ns	-
Mt-0	+	+	+	ns	+	ns	+	ns
Rsch-4	+	+	ns	-	+	+	+	-
Wil-2	+	+	-	-	+	+	+	ns
Ba-1	-	-	-	+	-	-	-	ns
Mc-0	+	ns	+	ns	ns	ns	ns	ns
Can-0	ns	ns	ns	-	+	+	ns	-