Global Change Biology

Global Change Biology (2017) **23**, 3781–3793, doi: 10.1111/gcb.13654

Contrasting responses of leaf stomatal characteristics to climate change: a considerable challenge to predict carbon and water cycles

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Abstract

Stomata control the cycling of water and carbon between plants and the atmosphere; however, no consistent conclusions have been drawn regarding the response of stomatal frequency to climate change. Here, we conducted a meta-analysis of 1854 globally obtained data series to determine the response of stomatal frequency to climate change, which including four plant life forms (over 900 species), at altitudes ranging from 0 to 4500 m and over a time span of more than one hundred thousand years. Stomatal frequency decreased with increasing CO_2 concentration and increased with elevated temperature and drought stress; it was also dependent on the species and experimental conditions. The response of stomatal frequency to climate change showed a trade-off between stomatal control strategies and environmental factors, such as the CO_2 concentration, temperature, and soil water availability. Moreover, threshold effects of elevated CO_2 and temperature on stomatal frequency were detected, indicating that the response of stomatal density to increasing CO_2 concentration will decrease over the next few years. The results also suggested that the stomatal index may be more reliable than stomatal density for determination of the historic CO_2 concentration. Our findings indicate that the contrasting responses of stomata to climate change bring a considerable challenge in predicting future water and carbon cycles.

Keywords: carbon and water cycles, climate change, drought stress, elevated CO_2 concentration, elevated temperature, life forms, stomatal frequency

Received 12 September 2016 and accepted 20 January 2017

Introduction

Stomata are small pores on the surfaces of leaves and stems that are essentially valves bounded by a pair of guard cells. They first appeared on vascular plants over 400 million years ago in the now extinct genus *Cooksonia* (Edwards *et al*., 1998; Haworth *et al*., 2011b) and played major roles in controlling global carbon (C) and water cycles (Hetherington & Woodward, 2003; Franks & Beerling, 2009). Although the total stomatal pore area may account for only 5% of the leaf surface (Willmer & Fricker, 1996), the loss of water vapor through stomata may reach as much as 70% of the total water loss of plants (Hetherington & Woodward, 2003), and terrestrial plants fix one-seventh of the total C in the atmosphere through their stomata (Ciais *et al*., 1997). Stomata are one of the most important participants in global water and C cycles, and they also promote speciation and evolution caused by changes in global environmental conditions (Janis, 1993; Woodward, 1998; Hetherington & Woodward, 2003). These facts have attracted the attention of scientists for at least three centuries (Meidner *et al*., 1987), and a great deal of knowledge related to the structure, development, and physiology of stomata has been acquired (Buckley, 2005; Bergmann & Sack, 2007; Berry *et al*., 2010). Studies of the changes in stomatal pore structure and function that occur in response to environmental changes are critically important to the prediction of climate change because stomata are the entry points for the exchange of gases (Edwards *et al*., 1998; Franks & Beerling, 2009; Berry *et al*., 2010). Thus, understanding stomatal changes in response to environmental changes will help us determine the plant responses and feedback mechanisms associated with future climate change (Hetherington & Woodward, 2003; Buckley, 2005; Berry *et al*., 2010).

In general, plants reduce stomatal frequency (stomatal density and index) in response to long-term increases in the atmospheric CO_2 concentration (Haworth *et al*., 2010). However, the effects of experimental single-step CO_2 enrichment on stomatal frequency may not be

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representative of plants' long-term responses to incremental increases of 1–2 ppm CO_2 per year. Therefore, the response of stomatal frequency to the global increase in the CO_2 concentration may be best studied using a historic leaf herbarium under natural conditions (Kürschner *et al*., 1996; Wagner *et al*., 1996; Kouwenberg *et al*., 2003). For example, Woodward (1987) reported an inverse relationship between stomatal frequency and CO_2 concentration using the leaf herbarium, indicated that trees significantly reduced their stomatal frequency in response to increasing CO_2 levels. This type of inverse relationship has been repeatedly demonstrated across a wide range of plant taxa in disparate geological and ecological settings, from the Paleozoic Era to the present, and it has been used to estimate paleo-CO_2 levels (Retallack, 2001; Bai *et al*., 2015; Steinthorsdottir & Vajda, 2015; Steinthorsdottir *et al*., 2016). Elevated CO_2 can stimulate cell division and expansion, resulting in thicker leaves with more numerous cells or cell layers and/or larger cells (Ferris *et al*., 2001; Luomala *et al*., 2005). In addition, different responses of stomatal density (SD, the number of stomatal pores per unit leaf area) to changes in the CO_2 concentration have also been reported (Haworth *et al*., 2011a; Bai *et al*., 2015). Experimental results have shown decreases, no effect or even increases in SD as the CO_2 concentration increases (Ferris & Taylor, 1994; Amthor, 1995; Dixon *et al*., 1995; Reddy *et al*., 1998; Ferris *et al*., 2002; Marchi *et al*., 2004; Luomala *et al*., 2005; Xu & Zhou, 2005). These differences might be attributed to variable responses of SD to CO_2 enrichment depending on the duration of the experiment, the plant species and/or genotype, the experimental facility, and environmental factors (Haworth *et al*., 2013; Xu *et al*., 2016). In addition, no consistent conclusions have been drawn on the response of SD or the stomatal index (SI; the ratio of stomata to the total number of epidermal cells) to seasonally or experimentally elevated temperature; no change, increases, and decreases have been reported (Ferris *et al*., 1996; Luomala *et al*., 2005; Xu & Zhou, 2005; Fraser *et al*., 2009). However, studies of the effects of drought stress on SD have shown that water deficit increases SD (Retuerto *et al*., 2000; Sekiya & Yano, 2008; Xu & Zhou, 2008) and decreases stomatal size (Xu & Zhou, 2008), altered gas exchange and increased water use efficiency, which is very important in water-limited environments (Poulos *et al*., 2007; Xu & Zhou, 2008; Fraser *et al*., 2009). The discrepancies in the results of these studies may be due to differences in the life forms and environmental conditions (Marchi *et al*., 2004; Sekiya & Yano, 2008; Soares *et al*., 2008). Haworth *et al*. (2015) reported that morphological stomatal control strategies differ among species; some species exhibit changes in SD, whereas others show little or no change in stomata in response to environmental conditions. However, the differences in the magnitudes of the responses of SD to environmental changes among different life forms remain unclear, and it is unknown whether these responses involve a threshold effect.

Compared with other stomatal traits, SD is relatively plastic (Richardson *et al*., 2001). Studies have reported that it is affected by environmental changes such as soil water availability (Sekiya & Yano, 2008; Xu & Zhou, 2008), light (Thomas *et al*., 2004), CO_2 (Woodward *et al*., 2002; Sekiya & Yano, 2008), temperature (Luomala *et al*., 2005; Xu & Zhou, 2005), and UV-B (Gitz *et al*., 2005). Moreover, highly variable responses of SD have been observed among life forms (Lin *et al*., 2001; Marchi *et al*., 2004) due to differences in morphological stomatal control strategies among species (Haworth *et al*., 2015). Thus, considerable caution is required when using SD as an indicator of a plant's response to elevated atmospheric CO_2 (Haworth *et al*., 2015; Xu *et al*., 2016). However, with the use of SI, the effects of other environmental factors, such as temperature, water stress, and humidity (Bai *et al*., 2015), can be eliminated because SI is a direct measure of the proportion of epidermal cells that have differentiated into stomata, whereas SD is also influenced by epidermal cell size, which can be modified by other factors (Ferris *et al*., 2001; Luomala *et al*., 2005). Thus, SI has been shown to be a reliable CO_2 proxy as it responds positively to CO_2 changes, and has been used to estimate the effects of increased atmospheric CO_2 (Haworth *et al*., 2011a; Rivera *et al*., 2014; Bai *et al*., 2015; Hu *et al*., 2015).

It is known that environmental changes can influence stomatal development (Fraser *et al*., 2009; Hill *et al*., 2015). Thus, an elevation gradient also provides a powerful setting in which to test the responses of stomatal frequency to changing environmental conditions because large changes in environmental factors can occur over short distances (Qiang *et al*., 2003; Wang *et al*., 2014). Studies of the effects of altitude on SD have shown a range of responses along elevation gradients, including decreases (Schoettle & Rochelle, 2000), increases (Kouwenberg *et al*., 2007), and no change (Wang *et al*., 2014). Some researchers observed an initial increase in SD followed by a decrease with increasing elevation (Qiang *et al*., 2003; Luo *et al*., 2006). Various theories have been proposed to explain the relationships between stomatal characteristics and elevation, including the reduced CO_2 availability theory (Kouwenberg *et al*., 2007), the drought stress theory (Luo *et al*., 2006), and the solar radiation theory (Körner *et al*., 1986), as plants may increase stomatal frequency to counteract the limited photosynthetic potential caused by decreased CO_2 partial pressure, increased UV radiation, and soil water availability with elevation

change. However, whether there is a trade-off among environmental factors (CO_2 concentration, temperature, and soil water availability) that influences the response of stomatal frequency to altitude remains unknown.

Due to the importance and the contradictory results obtained regarding stomatal responses to environmental changes, we conducted a meta-analysis of published data that included four life forms (over 900 species) across a time span of over one hundred thousand years to examine the responses of stomatal traits to environmental changes. The objectives of this study were as follows: (1) to investigate the specific responses of stomata to historic climate change among different species and life forms and to determine whether SI a better predictor of historic CO_2 concentrations than SD; (2) to compare the responses of stomata to experimentally induced environmental changes, including elevated CO_2 concentration, elevated temperature, and drought; and (3) to determine whether there is a trade-off among environmental factors (CO_2 concentration, temperature, and soil water availability) and/or a threshold effect associated with stomatal responses to environmental changes.

Materials and methods

Data preparation

Peer-reviewed journal articles were searched in the Web of Science and in the online databases of the Chinese Academy of Sciences (prior to June 2016) using the following keywords and phrases: stomatal density, stomatal index. To avoid bias in the selection of publications, articles were chosen based on the following criteria: (1) the study included at least two datasets (control and treatment) in experiments performed to test the effects of CO_2 enrichment, elevated temperature, drought stress, and nitrogen (N) addition on leaf traits; (2) the mean, standard deviation/error, and number of replicates in the control and treatment groups could be directly extracted from the text, tables, or digitized graphs (if standard errors were reported, standard deviations were calculated using the following equation: standard deviation = standard error $\times \sqrt{n}$, where n is the number of replicates); (3) reporting of standard deviation/error was not considered necessary in studies that reported the SD and SI for fossils or specimens in a natural environment; and (4) in studies of the effects of altitude on SD and SI, we used the data from the lowest altitude as control data. In total, 111 published papers were selected based on these criteria. The study material included four life forms (over 900 species), altitudes ranging from 0 to 4500 m and a time span of over one hundred thousand years (Data S1 and Fig. S1).

Data acquisition

For each selected study, the experimental location, plant species, life forms (fern, herb, shrub, or tree), experimental conditions, CO_2 concentration, temperature, and relative soil water (RSW) or drought intensity (mild, moderate, or severe) were obtained directly from the published material. In cases in which the drought intensity was not directly provided, the drought intensity was classified into one of the following three categories according to the RSW: mild drought (55% < RSW < 70%), moderate drought (40% < RSW < 55%), or severe drought (RSW < 40%). If possible, we also recorded the leaf area (LA), the specific leaf area (SLA), the leaf thickness, the adaxial SD and SI, the abaxial stomatal length (SL) and width (SW), the stomatal aperture and size, the guard cell length and width, the epidermal cell density, length, area and number, the palisade parenchyma thickness, the spongy parenchyma thickness, and the lower cuticle thickness. These values were obtained because close relationships between stomatal frequency and other traits, including precipitation, leaf traits, and stomatal area and length (Fig. S2), have often been observed, as reported by Loranger & Shipley (2010) and Wang *et al.* (2011). For studies in which the data were presented graphically, GET–DATA GRAPH DIGITIZER (ver. 2.20, Russian Federation) was used to digitize and extract the numerical data.

Data analysis

The response ratios (RRs, natural logs of the ratios of the mean values of the parameters in the treatment group to those in the control group) for the stomatal traits were evaluated using the following equation (Hedges *et al.*, 1999):

$$\mathrm{RR} = \ln(X_e/X_c) = \ln X_e - \ln X_c, \quad (1)$$

where X_e and X_c are the response values of each individual observation in the experimental and control treatments, respectively. The corresponding sample variance for each RR was calculated as follows:

$$\mathrm{vi} = (S_e/X_e)^2/n_e \times +(S_c/X_c)^2/n_c, \quad (2)$$

where n_e, n_c, S_e, S_c, X_e, and X_c are the sample sizes, standard deviations, and mean response values in the experimental and control groups, respectively. The reciprocal of the variance ($w = 1/\mathrm{vi}$) was considered as the weight of each RR. The mean weighted response ratio (RR_{++}) was calculated from the RR for individual pairwise comparisons between the treatment and control groups as follows:

$$\mathrm{RR}_{++} = \sum_{i=1}^{m}\sum_{j=1}^{k} w_{ij}\,\mathrm{RR}_{ij} / \sum_{i=1}^{m}\sum_{j=1}^{k} w_{ij}, \quad (3)$$

where m is the number of groups and k is the number of comparisons in the corresponding group. In addition, the standard error of RR_{++} was estimated as follows:

$$\text{standard error}\,(\mathrm{RR}_{++}) = \sqrt{\frac{1}{\sum_{i=1}^{m}\sum_{j=1}^{k} w_{ij}}}. \quad (4)$$

Meta-analysis was performed using the R software package (version 3.1.1; R Core Team, 2014). The natural logs of the RRs for the individual and combined treatments were determined by specifying the studies as random factors in the model with the 'METAFOR' package. The effect of environmental changes on a

leaf trait was considered significant if the 95% confidence interval (CI) of the RR did not overlap with zero. The 'MAPS' package was used to generate a map of the global site distribution (Fig. S1; Becker & Wilks, 2005). In addition, regression analysis was conducted to evaluate the following parameters: the relationship between the RRs of the SD and the SI and the altitude, temperature and RSW; the relationship between the SD and the SI and other leaf traits (Fig. S2) in a natural environment; and the frequency distributions of SD and SI at elevated CO_2 and under conditions of elevated temperature and drought stress (Fig. S3). The general linear model was used to compare differences between the regression equations for different species (Fig. 1a). Further, the 'MGCV' package was used to fit the curve between the CO_2 concentration and SD and SI; knots of 5 and 4 in the SD and SI curves, respectively, were chosen due to the lower Akaike information criterion (AIC) values and generalized cross-validation (GCV) scores and higher R^2 values (Table S1, Figs S4 and S5; Keele, 2008; Zuur *et al*., 2009). In addition, publication bias analysis was performed, and a funnel plot (Fig. S6) was generated to examine the influence of publication bias on the data ($P > 0.05$; Quintana, 2015).

Results

Changes in stomatal frequency with historic CO_2 concentration

SD and SI were significantly correlated with historic CO_2 concentrations, as shown in Fig. 1. In addition, the SD of some tree species decreased linearly with increasing CO_2 concentration, as shown in Fig. 1a and c. Differences in the slope of the curve describing the relationship between SD or SI and CO_2 concentration were observed among different tree species, with significant differences detected between *Quercus petraea* with both *Tsuga heterophylla* ($P = 0.002$) and *Betula pendula* ($P = 0.003$); however, no significant difference in slope was detected between *T. heterophylla* and *B. pendula* ($P = 0.141$). In addition, we observed variations in the SDs of some species (Fig. 1b), for example, a cubic function for *Quercus guyavifolia* (tree) and a quadratic function for *Typha orientalis* (herb) in response to historic CO_2 partial pressure. However, the SI showed a linear correlation with the historic CO_2 partial pressure (Fig. 1d). A comparison of the SD values obtained in 1995 with those obtained in 1927 indicated that the SD values decreased significantly in 1995, with significant decreases observed in herb SD compared with shrub and tree SDs (Fig. S7).

Changes in stomatal frequency with altitude

Large changes in environmental factors, including the CO_2 concentration, the temperature, and the soil water availability, can occur over short distances along altitude gradients. We found that altitude significantly

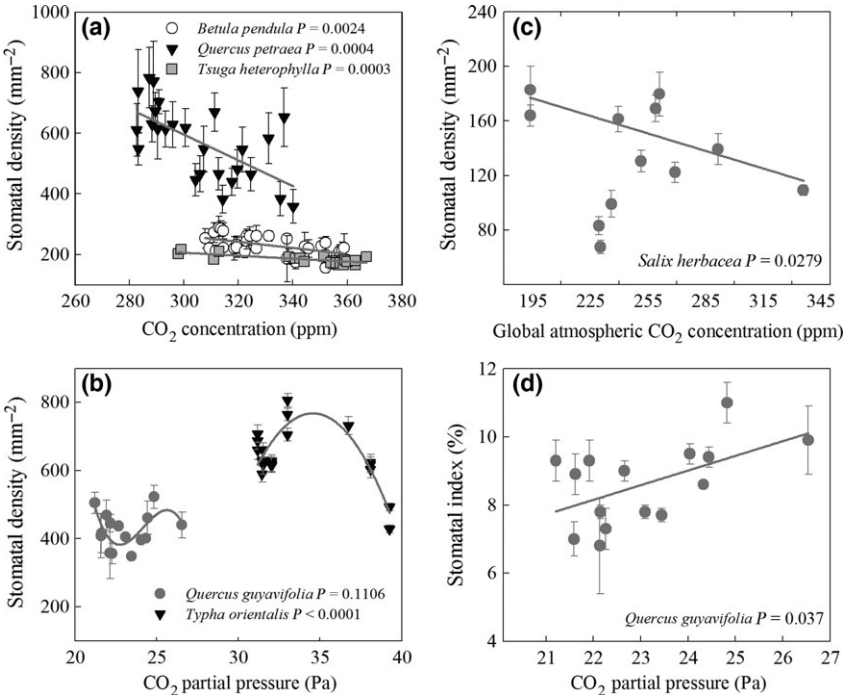

Fig. 1 Relationship between the stomatal density (SD) and the stomatal index (SI) of different species and the CO_2 concentration. The data presented in the figures are based on Wagner *et al.* (1996), Kürschner *et al.* (1996), and Kouwenberg *et al.* (2003) (a); on Beerling *et al.* (1993) (b); on Bai *et al.* (2015) and Hu *et al.* (2015) (c); and on Hu *et al.* (2015) (d).

affected the SI in contrast to the SD or SL. Specifically, in herbs, altitude negatively affected the SI but positively affected the SD, whereas it had no effect on shrub or tree SD (Fig. 2a). Notably, the RRs of SD and SI increased with increasing altitude at low altitudes but decreased as altitude continued to increase; the maximum values were observed at approximately 1950 m (Fig. 2c, d).

Effects of elevated CO_2 on leaf traits

Elevated CO_2 resulted in a significant increase in the LA, but it did not affect the SLA or the adaxial SD or SI (Fig. 3a). However, the abaxial SD and SI decreased significantly in response to increasing CO_2 concentration (Fig. 3a). Interestingly, the RR of the abaxial SD and SI showed variable changes in response to the CO_2 concentration, indicating a threshold effect. The RR of the SD decreased with increasing CO_2 concentration until the threshold of 409 ppm was reached, after which it increased as the CO_2 concentration increased to 600 ppm and subsequently decreased above that concentration (Fig. 4a). However, the RR of the SI increased to 1000 ppm and then decreased as the CO_2 concentration increased (Fig. 4b). Moreover, by comparing the different life forms, we found that elevated CO_2 had no effect on the adaxial SD or SI of herbs or trees (Fig. S8). However, differing responses of abaxial SD and SI to elevated CO_2 were observed among the different life forms, with significant decreases in ferns and trees but no changes in herbs (Fig. 3b). Elevated CO_2 resulted in significantly decreased SI of both deciduous and evergreen trees, but it had no effect on the SD of deciduous trees. In addition, the SI of broad- and needle-leaved trees decreased at elevated CO_2 concentrations, whereas the SD of broad-leaved trees did not change (Fig. 3b). Furthermore, different responses were observed under different experimental conditions, for example, decreased abaxial SI was detected in both chamber and greenhouse experiments, whereas no changes in SI were observed in free-air CO_2 enrichment (FACE) or open-top chamber (OTC) experiments, and decreased SD was only observed in FACE experiments (Fig. S9).

Effects of elevated temperature on leaf traits

Elevated temperature did not influence adaxial SD or SI, but it increased abaxial SD and SI significantly and decreased abaxial SL (Fig. 5a). Considering plant life forms, we found that elevated temperature increased the abaxial SD of herbs and shrubs as well as the

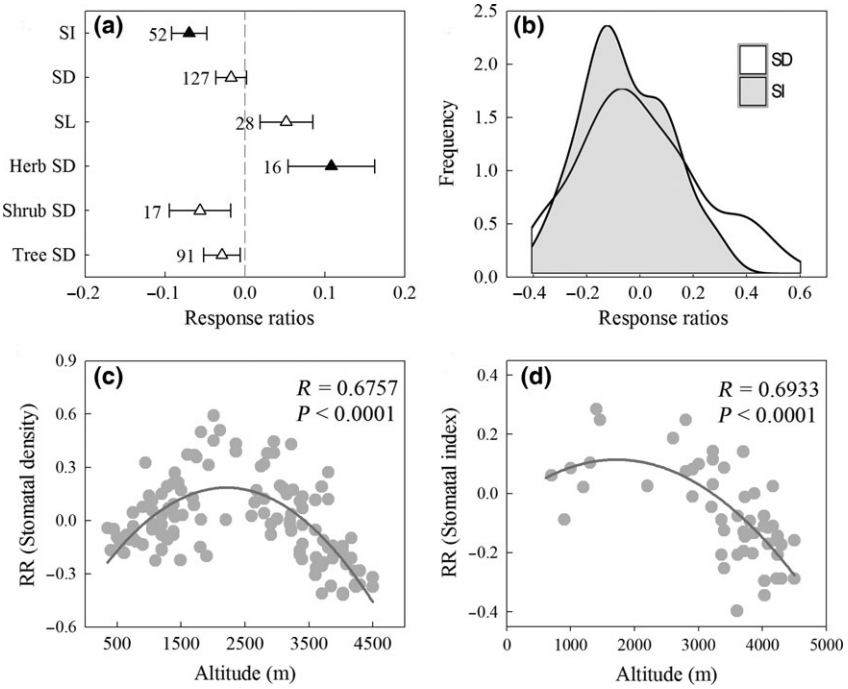

Fig. 2 Effects of altitude on stomatal index (SI), stomatal density (SD), and stomatal length (SL) (a). Frequency distributions of the response ratios (RR) of SI and SD (b), and relationships between the RR of SD (c) and SI (d) and altitude. The white and black triangles pointing upward represent insignificant ($P > 0.05$) and significant ($P \leq 0.05$) differences, respectively, between the response ratios and zero. The vertical dotted line represents a mean effect size of 0. The sample size of each variable is shown next to the CI (a).

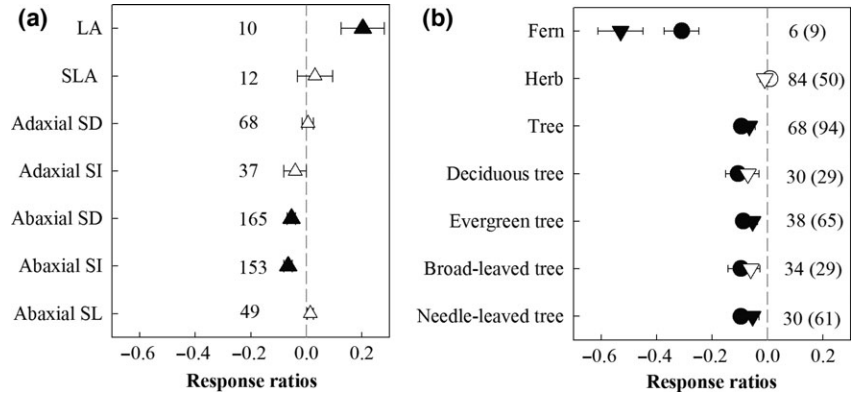

Fig. 3 (a) Effects of elevated CO_2 on leaf area (LA), specific leaf area (SLA), stomatal density (SD), stomatal index (SI), and stomatal length (SL) across all studies. (b) Effects of elevated CO_2 on abaxial SD (triangles pointed downward) and SI (circles); the black symbols indicate significant differences ($P \leq 0.05$) between the response ratios and zero. The vertical dotted line represents a mean effect size of 0. The sample size for each variable is shown next to the CI and represents the SD and SI from left to right.

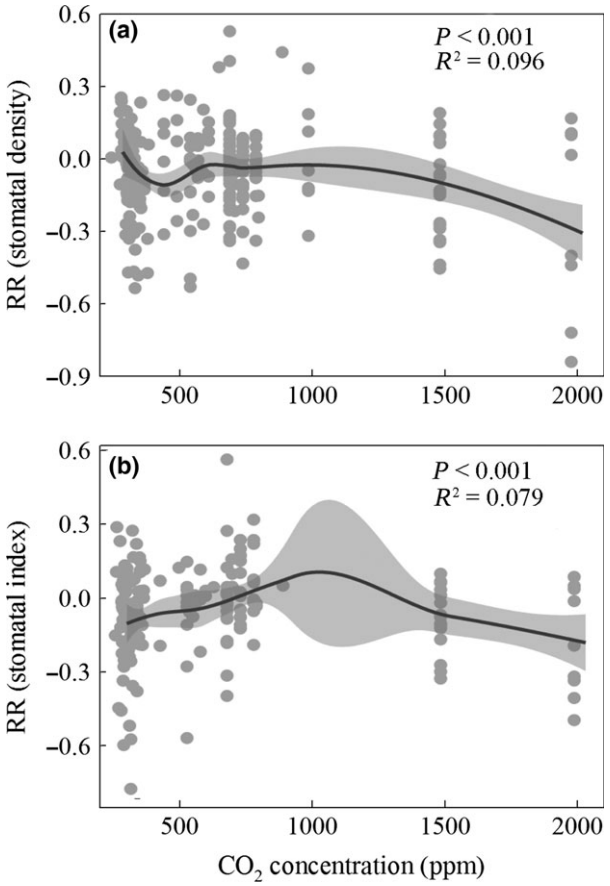

Fig. 4 Relationship of the response ratios of the stomatal density (SD) (a) and the stomatal index (SI) (b) to the CO_2 concentration. The 'MGCV' package in R was used to fit the curve between the CO_2 concentration and SD and SI, and knots of 5 and 4 were selected, respectively.

abaxial SI of shrubs, but that it decreased the SD of trees (Fig. 5b). Interestingly, we found a threshold effect of temperature on SD, with an increase in the RR of the SD and the SI with increasing temperature at temperatures lower than the threshold temperature, followed by a decrease with continuously increasing temperature (Fig. 6a and b).

Effects of drought stress on leaf traits

Drought stress had no effect on LA, SLA, adaxial SD or SI, or abaxial SW, but it significantly increased abaxial SD and SI and decreased abaxial SL (Fig. 7a). The abaxial SD of all species increased with drought intensity (Fig. 7b). In addition, the RR of the SD was significantly correlated with RSW, increasing linearly with a decrease in RSW, whereas no significant correlation was observed for the RR of SI (Fig. 6c and d). However, abaxial SD, SI, and SL showed differing responses to drought stress among the different life forms. Specifically, drought increased the SD and decreased the SL of herbs and trees, and it increased the SI of herbs and the SL of shrubs (Fig. 7b). In addition, it increased the SD of deciduous trees but had no effect on the SD of evergreen trees (Fig. 7b).

Discussion

Species-specific responses of stomatal frequency to environmental changes

A decrease in SD is considered a general response to elevated CO_2. However, several studies have observed differing magnitudes of SD responses among different tree species (Fig. 1a); for example, *Q. petraea* was reported to show a rapid decrease in SD with rising CO_2 concentration, which may be due to its high initial SD (Beerling & Kelly, 1997), whereas a smaller decrease was observed in *T. heterophylla* (coniferous species). These differences between species may be

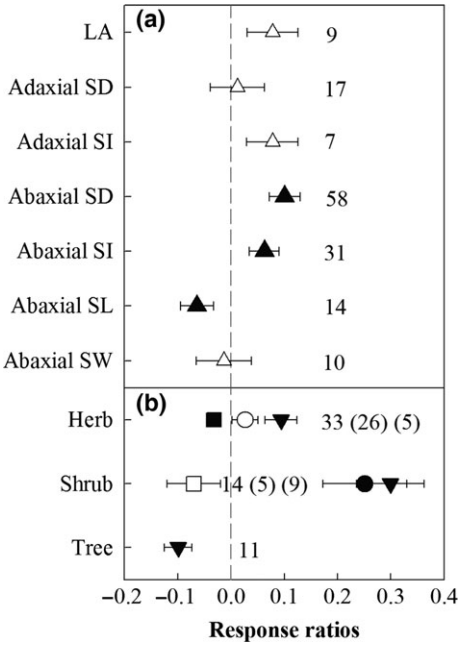

Fig. 5 (a) Effects of elevated temperature on leaf area (LA), stomatal density (SD), stomatal index (SI), stomatal length (SL), and stomatal width (SW) across all studies. (b) Effects of drought stress on SD (triangles pointed downward), SI (circles), and abaxial SL (squares); the black symbols represent significant differences ($P \leq 0.05$) between the response ratios and zero. The vertical dotted line represents a mean effect size of 0. The sample size for each variable is shown next to the CI and represents the SD, SI, and SL from left to right.

due to differences in stomatal formation (Kouwenberg *et al.*, 2003). Specifically, in broad-leaved trees, stomata are relatively randomly distributed across the entire leaf surface, whereas in conifers, their formation is initiated at the base of the needle, and the stomata develop longitudinally with needle growth (Croxdale, 2000). In addition, genetic factors might be responsible for the observed differences in stomatal responses to historic climate change (Casson & Hetherington, 2010). The stomatal characteristics of some species show high heritability (less sensitivity to environmental changes), whereas those of other species are more sensitive to environmental factors (Schoch *et al.*, 1980; Zhang *et al.*, 2012; Tanaka *et al.*, 2013). We found that the SD responses to climate change differed among different life forms (Fig. S7); there was a significant decrease in the SD of herbs, in contrast to the changes in SD observed in short-term experiments with elevated CO_2 (Fig. 3), which had no significant effect on the SD of herbs. Both the rising CO_2 concentration and other environmental factors are probably responsible for the decrease in the SD of herbs that occurred between 1927 and 1995 (Beerling & Kelly, 1997). In addition, the transition from a variable plastic response in short-term experiments to a genetic response on longer time scales can also explain the more frequent reduction in SD in longer-term measurements of stomatal responses (in herbarium material and fossil leaves) than in short-term

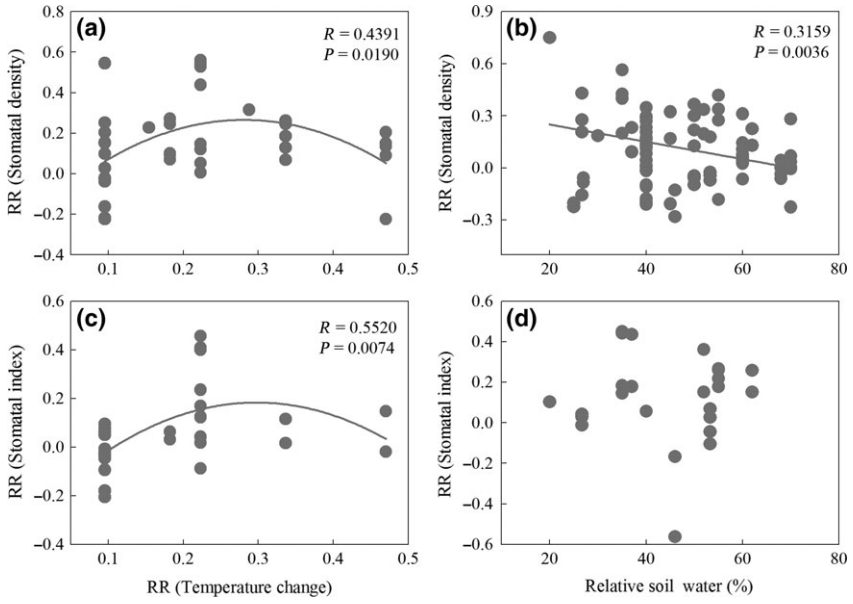

Fig. 6 Relationship of the response ratios of stomatal density (SD) and stomatal index (SI) to temperature change (a and b) and to relative soil water (RSW) content (c and d).

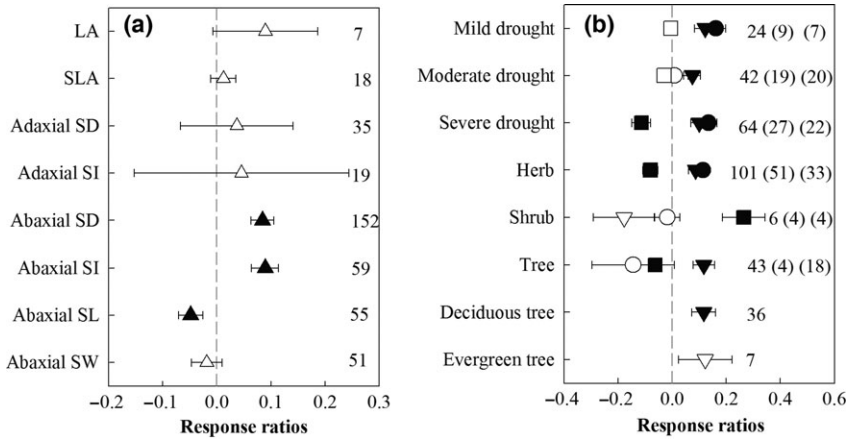

Fig. 7 (a) Effects of drought stress on leaf area (LA), specific leaf area (SLA), stomatal density (SD), stomatal index (SI), stomatal length (SL), and stomatal width (SW) across all studies. (b) Effects of drought stress on abaxial SD (triangles pointed downward), SI (circles), and abaxial SL (squares); the black symbols represent significant differences ($P \leq 0.05$) between the response ratios and zero. The vertical dotted line represents a mean effect size of 0. The sample size for each variable is shown next to the CI and represents the SD, SI, and SL from left to right.

field or growth chamber seasonal experiments (Hetherington & Woodward, 2003).

The different responses of SD to historic climate change are not only controlled by the CO_2 concentration but also by the temperature, soil water availability, and irradiance (Thomas *et al*., 2004; Luomala *et al*., 2005; Xu & Zhou, 2005, 2008; Sekiya & Yano, 2008). In this study, we observed a nonlinear relationship between SD and CO_2 partial pressure (Fig. 1b), indicating that the response of plants to the CO_2 concentration may involve other environmental factors. To study the response of stomatal frequency to complex environmental conditions, we analyzed the relationship of stomatal frequency to elevation, which involves changes in CO_2 concentration, temperature, and soil water availability over short distances. The results showed no significant changes in the RR of SD with increasing elevation with the exception of a significant increase in the RR of the herb SD (Fig. 2a). These findings suggest that the response of stomatal frequency differs among different plant life forms (Wang *et al*., 2014). Regression analysis revealed that the stomatal frequency initially increased and then decreased with increasing elevation (Fig. 2c and d), consistent with previous studies (Qiang *et al*., 2003; Luo *et al*., 2006), indicating that there is a trade-off among stomatal control strategies in plants' responses to changes in CO_2 concentration, temperature, and soil water availability because stomatal plasticity has been shown to vary with elevation (Wang *et al*., 2014). This trade-off can be explained by the reduced CO_2 availability theory (Kouwenberg *et al*., 2007), the drought stress theory (Luo *et al*., 2006), and the solar radiation theory (Körner *et al*., 1986). The results suggest that the response of stomatal frequency to climate change differs in different environments, which poses a great challenge to the prediction of the effects of climate change on C and water fluxes through plant stomata.

Contrasting stomatal responses to experimentally induced environmental changes

Although atmospheric CO_2, soil water availability, and temperature are known to affect stomatal frequency (Woodward *et al*., 2002; Thomas *et al*., 2004; Luomala *et al*., 2005; Xu & Zhou, 2005; Sekiya & Yano, 2008), the responses of stomatal frequency to environmental changes and the relationship between stomatal frequency and environmental conditions remain unresolved (Ferris & Taylor, 1994; Amthor, 1995; Dixon *et al*., 1995; Luomala *et al*., 2005; Xu & Zhou, 2005, 2008; Sekiya & Yano, 2008). Our meta-analysis, which was based on a considerable amount of data from around the world, revealed that abaxial stomatal frequency decreased significantly under global elevated CO_2, whereas adaxial stomatal frequency did not change (Fig. 3), consistent with the findings of many other studies (Ferris & Taylor, 1994; Amthor, 1995; Dixon *et al*., 1995). Considering the different genetic characteristics of life forms, we found that stomatal frequency did not change under elevated CO_2 condition in herbs. The SD of angiosperms often shows little or no response to an increase in the CO_2 concentration to above 400 ppm due to the evolutionary origin of angiosperms and their diversification during periods of low atmospheric CO_2 (i.e., <400 ppm; Kürschner *et al*.,

2008; Haworth *et al.*, 2015). It is likely that the SI was more sensitive than the SD of trees subjected to experimentally elevated CO_2 conditions, indicating that the elevated CO_2 resulted in increased epidermal cell number, as shown by our results (Fig. S10). Interestingly, no changes in the SD of deciduous or broad-leaved trees were observed, indicating that the decreased SI of these trees was mainly caused by an increased number of epidermal cells. In addition, the contradictory conclusions pertaining to the response of stomatal frequency to CO_2 enrichment among previous studies might be attributed to differences in the experimental conditions; for example, as shown in Fig. S9, the SI was more sensitive than the SD in chamber and greenhouse studies but was less sensitive in FACE studies, indicating that the experimental conditions affect the stomatal frequency. These findings suggest that the experimental conditions should be considered when simulating the response of stomatal frequency to elevated CO_2 (Marchi *et al.*, 2004; Sekiya & Yano, 2008) because the SD response may vary depending on the duration of the experiment, the plant species/genotypes, the experimental facility, and other environmental variables (Haworth *et al.*, 2013; Xu *et al.*, 2016). In addition, many studies have observed a 'ceiling response' to CO_2 concentrations of 350–400 ppm, above which SD and SI are no longer affected (Royer *et al.*, 2001; Kürschner *et al.*, 2008; Haworth *et al.*, 2013). Interestingly, when we combined the herbarium and experimental data, we found two inflection points for the RR of SD in the response to CO_2 concentration (Fig. 4) at 409 and 600 ppm, indicating that the response of SD increased with increasing CO_2 up to 409 ppm and then decreased up to 600 ppm; in addition, no differences in SD were observed at CO_2 concentrations below current ambient concentrations (herbarium studies) and those above 400 ppm (chamber and FACE studies) due to the near-symmetry of the response below and above 409 ppm. The threshold CO_2 concentration for effects on SD and SI is based on all plant life forms considered in this study; accurate assessment of the individual threshold of each plant life form will require additional data. In contrast, the RR of the SI showed a negative response to increasing CO_2 up to approximately 700 ppm; thus, SI may be a better and more reliable CO_2 proxy than SD because it responds in a sensitive way to CO_2 changes, as reported in previous studies (Haworth *et al.*, 2011a; Rivera *et al.*, 2014; Bai *et al.*, 2015; Hu *et al.*, 2015). In addition, SD is sensitive to factors that affect the initiation of stomata and cell expansion, and cell expansion is affected by many variables (e.g., light, temperature, and water status); thus, changes in these variables can mask the effects of signals that cause stomatal initiation. However, SI is sensitive only to factors affecting cell initiation, such as CO_2 concentration, which plays a stronger role in stomatal initiation than in epidermal cell expansion; thus, SI measurement allows normalization of the effects of epidermal cell expansion. SI should therefore yield more accurate CO_2 estimates than SD (Royer, 2001). Studies have also indicated that the SD response might decrease in the near future due to the rapid increase in the global CO_2 concentration, reporting that the magnitude of the SD response size will be only −0.026 by the end of the 21st century (900 ppm, Stocker *et al.* (2013)), although the response is much larger now (−0.108) for all plants, and that the response ratios for angiosperms and gymnosperms will be −0.025 and −0.088 at 900 ppm, respectively, as determined based on a fitted curve between CO_2 and SD ($P < 0.05$), consistent with the findings of Haworth *et al.* (2015), who reported that angiosperms frequently show little or no SD response to elevated CO_2 above 400 ppm. The above results provide a theoretical basis for assessing changes in gas exchange in response to climate change (Haworth *et al.*, 2013) and suggest that the benefits of CO_2 fertilization of the ecosystem will decrease (Peñuelas *et al.*, 2016).

Cell division is tightly regulated by temperature; thus, elevated temperature can alter stomatal traits (Luomala *et al.*, 2005). Our analysis indicated that elevated temperature increased abaxial SD and SI and decreased abaxial SL but had no effect on adaxial SD or SI. These results differ from those of several previous studies (Loveys *et al.*, 2002) that demonstrated decreased SD at elevated temperatures; however, they are consistent with the results of several other studies (Reddy *et al.*, 1998; Luomala *et al.*, 2005). In addition, SD and SI showed similar patterns at elevated temperature (Fig. 6a and b), and elevated temperature had no effect on epidermal cell number (Fig. S10), suggesting that changes in SI are mainly influenced by SD at elevated temperature (Reddy *et al.*, 1998). We also observed a threshold effect of temperature on stomatal frequency (Fig. 6a and b); notably, an increase in SD in response to increased temperature has been shown to affect the rate of photosynthesis (Tanaka *et al.*, 2013), indicating that the future impact of warming on the ecosystem may be greater than its impact at present (Peñuelas *et al.*, 2016).

The effects of drought on stomata have been studied extensively, and it is now widely accepted that plants exhibit increased stomatal frequency under drought stress (Retuerto *et al.*, 2000; Sekiya & Yano, 2008; Xu & Zhou, 2008). We observed an increase in abaxial stomatal frequency and a decrease in SL under drought, and SI showed a more sensitive response than SD, whereas no changes in adaxial stomatal frequency were observed (Figs 7 and 8). These findings are consistent

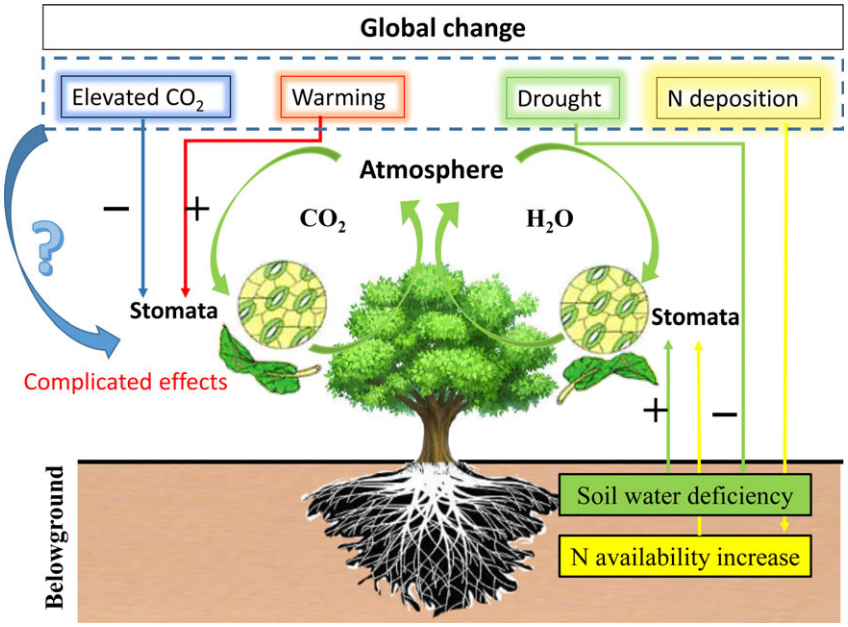

Fig. 8 Conceptual diagram of the influence of global climate change on processes controlling stomatal density. '+' and '−' represent positive and negative effects, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]

with those of previous studies (Retuerto *et al.*, 2000; Sekiya & Yano, 2008; Xu & Zhou, 2008) showing that both SD and SI may be affected by cell expansion, which is sensitive to the soil water status (Royer, 2001; Xu & Zhou, 2008). Increased SD in response to a gradual increase in drought stress is positively correlated with water use efficiency and might also indicate high acclimation capacity, which is very important in water-limited environments (Poulos *et al.*, 2007; Xu & Zhou, 2008; Fraser *et al.*, 2009). The stomatal traits of different life forms responded differently to drought; for example, the SD of herbs and trees increased significantly, whereas the SL of herbs and trees decreased and that of shrubs increased (Fig. 7b). In addition, we observed decreases in stomatal size and aperture. All of these changes could reduce transpiration and may represent adaptation strategies of plants in response to drought (Fig. S10; Reddy *et al.*, 1998).

We also found that different life forms (i.e., herbs, shrubs, and trees) responded differently to environmental changes, with a significantly higher SD and lower SL for trees compared with herbs and shrubs (Fig. S11). In addition, trees displayed the greatest stomatal size, whereas herbs showed the greatest guard cell length and epidermal cell area (Table S2). Moreover, the experimental environment affected the relationship between SD and SI (Fig. S12). The values of these variables exhibited a parabolic relationship in the natural environment, whereas a linear relationship between the RR of SD and SI was observed under elevated CO_2, elevated temperature, and drought stress conditions. These findings increase our current understanding of the stomatal response to climate change by showing that changes in SI in various experimental environments are affected by SD. We also observed decreased SD and increased SL in response to N addition (Fig. S13). The observed differences in stomatal responses among life forms and the observed trade-off among environmental factors in stomatal responses will pose great challenges to scientists attempting to predict stomatal responses to future climate change (Fig. 8).

Overall, the findings of our global study indicate that the effects of climate change on stomatal frequency are complex and extensive. Our results suggest that there is a trade-off among stomatal control strategies depending on specific environmental factors such as CO_2 concentration, temperature, and soil water availability. We demonstrated that elevated CO_2 concentrations result in decreased SD and that elevated temperature and drought lead to increased SD. Considering elevated CO_2 together with the effects of temperature, soil water availability, plant species, N deposition, and the experimental conditions on SD, it is difficult to predict stomatal responses to future climate change. This poses a considerable challenge to the prediction of global water and C cycles. In addition, the results of our study suggest that there is a threshold effect of elevated CO_2 and temperature on stomatal frequency and that the SD response may decrease in response to increasing CO_2 concentration, whereas it might increase with global warming over the next few years. This indicates that

the future impact of warming may be greater than the benefits of CO_2 fertilization of the ecosystem. The results also suggest that SI is more reliable than SD for predicting historic CO_2 concentrations.

Acknowledgements

This work was financially supported by the National Key Research and Development Program of China (2016YFC0501605) and the National Natural Science Foundation of China (41390463, 31370425).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Part 1. Supplementary tables and figures for the meta-analysis.
Table S1. Smoothing curves obtained by cubic regression splines between the response ratios of stomatal density (SD) and stomatal index (SI) and the CO_2 concentration.
Table S2. Leaf traits of plants of different life forms.
Figure S1. Global distribution of the study sites included in the meta-analysis.
Figure S2. Relationship of stomatal density (SD) and stomatal index (SI) to leaf traits and environmental factors in a natural environment.
Figure S3. Frequency distributions of the response ratios (RR) of stomatal density (SD) and stomatal index (SI) under elevated CO_2, elevated temperature and drought stress conditions.
Figure S4. Validation tools for the GAM model include one smoother for the response of stomatal density (SD) and CO_2 concentration.
Figure S5. Validation tools for the GAM model include one smoother for the response of stomatal index (SI) and CO_2 concentration.
Figure S6. Funnel plots illustrating the possible effect of publication bias on the stomatal density (SD) and stomatal index (SI) data under elevated CO_2 (a, d), elevated temperature (b, e) and drought stress (c, f) conditions.
Figure S7. Stomatal densities (SDs) of different life forms of plants from 1927 to 1995.
Figure S8. Effects of elevated CO_2 on the adaxial stomatal density (SD) and stomatal index (SI) of herb and tree species across all studies.
Figure S9. Effects of elevated CO_2 concentration on stomatal density (SD) (triangles pointed downward) and stomatal index (SI) (circles) across all studies.
Figure S10. Effects of elevated CO_2, elevated temperature and drought stress on leaf parameters across all studies.
Figure S11. Specific leaf area (SLA), stomatal density (SD), stomatal index (SI) and stomatal length (SL) of different life forms of plants.
Figure S12. Relationship between the log of stomatal density (SD) and the log of stomatal index (SI) in a natural environment (a). Relationship between the response ratios of SD and SI under elevated CO_2 (b), elevated temperature (c) and drought stress (d) conditions.
Figure S13. Effects of N addition on leaf parameters across all studies.
Part 2: The 111 papers from which data were extracted for this meta-analysis.
Data S1. Data used in this meta-analysis.