Effects of experimental warming on stomatal traits in leaves of maize (Zea may L.)

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Abstract
We examined the warming effects on the stomatal frequency, stomatal aperture size and shape, and their spatial distribution pattern of maize (Zea may L.) leaves using a light microscope, an electron scanning microscope, and geostatistical techniques. A field manipulative experiment was conducted to elevate canopy temperature by 2.08°C, on average. We found that experimental warming had little effect on stomatal density, but significantly increased stomatal index due to the reduction in the number of epidermal cells under the warming treatment. Warming also significantly decreased stomatal aperture length and increased stomatal aperture width. As a result, warming significantly increased the average stomatal aperture area and stomatal aperture circumference. In addition, warming dramatically changed the stomatal spatial distribution pattern with a substantial increase in the average nearest neighbor distance between stomata on both adaxial and abaxial surfaces. The spatial distribution pattern of stomata was scale dependent with regular patterns at small scales and random patterns at larger scales on both leaf surfaces. Warming caused the stomatal distribution to become more regular on both leaf surfaces with smaller L(t) values (Ripley’s K-function, L(t) is an expectation of zero for any value of t) in the warming plots than the control plots.

Introduction
Stomata are the pores on a leaf surface controlling gas exchanges, mainly CO2 and water vapor, between the atmosphere and plants (Woodward 1987; Hetherington and Woodward 2003), and thus regulate carbon and water cycles in various ecosystems (Franks and Beerling 2009; Haworth et al. 2010; Taylor et al. 2012). Globally, the gas exchanges between leaf surface and the atmosphere are massive at c. 440 × 1015 g CO2 per year through photosynthesis and 32 × 1018 g H2O per year through leaf transpiration (Ciais et al. 1997; Hetherington and Woodward 2003; Lake and Woodward 2008). Plant leaves usually optimize their gas exchange by altering stomatal pore openness, stomatal aperture size, stomatal frequency (stomatal density and stomatal index), and stomatal distribution pattern, which are regulated by both environmental factors (Lake et al. 2002; Hetherington and Woodward 2003; Schlüter et al. 2003; Casson and Gray 2008; Lake and Woodward 2008; Franks and Beerling 2009) and genetic signals (Bergmann 2004; Liang et al. 2005).
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2005; Shpak et al. 2005; Harra et al. 2007; Lampard et al. 2008; Hunt and Gray 2009; Hunt et al. 2010; Kondo et al. 2010; Sugano et al. 2010). In connection with the studies of photosynthetic acclimation under climate warming, stomatal response is of special interest, because stomatal traits set the limit for maximum stomatal conductance for gas exchange and thus has a potential to affect carbon gain and water use efficiency (Beerling 1997; Luomala et al. 2005).

Leaf maximum stomatal conductance has been widely used to quantify gas exchange efficiency, which is dependent on stomatal aperture size and shape, frequency, and distribution pattern (Buckley et al. 1997; Hetherington and Woodward 2003; Franks and Beerling 2009; Franks et al. 2009). Usually plants respond quickly to short-term environmental changes by changing the openness of the stomatal pore, a response also known as stomatal movement (Sharkey and Raschke 1981; Kwak et al. 2001; Guo et al. 2003; Young et al. 2006; Shimazaki et al. 2007; Shang et al. 2009). Many studies have shown that stomatal movement is controlled by light (Humble and Hsiao 1970; Sharkey and Raschke 1981; Kwak et al. 2001; Take-miya et al. 2006), CO₂ concentration (Ogawa 1979; Young et al. 2006; Lammertsma et al. 2011), temperature (Honour et al. 1995; Feller 2006; Reynolds-Henon et al. 2010), drought stress (Guo et al. 2003; Klein et al. 2004), air humidity (Lange et al. 1971; Schulze et al. 1974), and ultraviolet light (Hercik 1964; Eisinger et al. 2000). In addition to responses to short-term environmental changes through stomatal movement, long-term (decadal) environmental changes such as climate warming may also affect individual stomatal aperture size, stomatal frequency, and stomatal distribution pattern (Anderson and Brisk 1990; Lammertsma et al. 2011).

So far, no consistent conclusions have been drawn on the effect of warming on stomatal traits in the literature. Most studies found that warming had little effect on stomatal density and stomatal index (Apple et al. 2000; Hovenden 2001; Kouwenberg et al. 2007; Fraser et al. 2009), while other studies found that warming could decrease stomatal density (Beerling and Chaloner 1993) and index (Ferris et al. 1996) or increase stomatal density (Reddy et al. 1998; Xu et al. 2009) and stomatal index (Xu and Zhou 2005). In addition, warming could also change individual stomatal aperture size and shape (Ferris et al. 1996; Zuo et al. 2005; Zhang et al. 2010). For example, Ferris et al. (1996) found that experimental warming substantially increased the stomatal aperture length of a perennial ryegrass (Lolium perenne). By contrast, a more recent study reported that warming significantly decreased stomatal aperture length of four alpine meadow species including Thalictrum alpinum, Kobresia humilis, Gentiana straminea, Elymus nutans in the Qinghai-Tibetan plateau, China (Zhang et al. 2010).

In addition to the number, size and shape of stomata on the leaf surface, warming may also alter the spatial distribution pattern of stomata through cell division and cell differentiation (Croxdale 1998, 2000; Berger and Altmann 2000; Shpak et al. 2005) which are regulated by genetic signals (Nadeau and Sack 2002; Bergermann et al. 2004; Juarez et al. 2004; Shpak et al. 2005; Wang et al. 2007; Hunt et al. 2010) and environmental factors (Wang et al. 2007; Casson and Gray 2008) during stomatal development stages. The pattern of stomatal distribution is highly variable among species and recent advances in genetic studies have found that a number of genes, such as SDD1, EPF1, the putative receptors TMM, and the ERECTA-gene family, are involved in the determination of stomatal spacing (Nadeau and Sack 2002; Hunt et al. 2010). The spatial variation of stomata can be characterized at multiple scales, such as the adaxial versus abaxial surface, variations among different leaf sections, and the association/aggregation of individual stomata on a single leaf surface. Earlier studies have reported that stomatal density significantly differed between the adaxial and abaxial surfaces (Ciba and Brun 1975; Green et al. 1990; Ferris et al. 1996, 2002; Croxdale 1998, 2000; Reddy et al. 1998). Meanwhile, the distribution of stomata between leaf surfaces is associated with acclimation and adaptation to environmental factors such as temperature, water stress, light exposure, and CO₂ concentration (Parkhurst 1978; Mott et al. 1982; Ceulemans et al. 1995; Smith et al. 1998; Ferris et al. 2002; Driscoll et al. 2006; Soares et al. 2008). Moreover, the changes in the adaxial/abaxial ratio of stomata may also alter leaf function such as photosynthesis, because the stomata in the adaxial and abaxial leaf surfaces feature specific responses to environmental stresses such as CO₂ and temperature, thus result in the changes in leaf photosynthesis. Previous studies have found that growth at high CO₂ altered the regulation of photosynthesis on the adaxial and abaxial leaf surfaces of maize (Zea mays) (Driscoll et al. 2006) and Paspalum dilatatum (Soares et al. 2008) due to the changes in adaxial/abaxial ratio of stomata between leaf surfaces. In addition, the spatial variation of stomatal distribution was also seen among different leaf sections, such as the leaf tip, middle, and base section (Salisbury 1927; Sharma and Dunn 1969; Titcha 1982; Smith et al. 1989; Ferris et al. 1996; Zacchini et al. 1997; Stancato et al. 1999; Xu et al. 2009). However, several previous studies investigated stomatal features only collecting samples at the middle section of the abaxial or abaxial leaf surface (Beerling and Chaloner 1993; Hovenden 2001; Xu and Zhou 2005; Kouwenberg et al. 2007).

There are three photosynthetic pathways in terrestrial plants including C₃, C₄, and crassulacean acid metabolism
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(CAM). Globally, most plant species use the C₃ photosynthetic pathway, which is characterized by a low photosynthetic efficiency, because the process is compromised by photorespiration (Osborne and Freckleton 2009). However, C₄ pathway represents evolutionary advancements over the ancestral C₃ pathway (Ehleringer et al. 1997) due to high rates of photosynthesis and efficient use of water and nitrogen (Wang et al. 2009). It is noted that the performance of each pathway is significantly influenced by environmental conditions such as temperature (Ehleringer et al. 1997). Given the morphological and biochemical innovation, C₄ plants are proposed to better adapt to warming conditions than their C₃ counterparts (Dwyer et al. 2007; Sage and Kubien 2007). Maize (Zea mays L.) is an economically important food crop, which also uses C₄ photosynthetic pathway. So far, most studies mainly focused on the responses of leaf photosynthesis of maize plants to nitrogen (Muchow and Sinclair 1994; Correia et al. 2005), drought stress (Dwyer et al. 1992; Earl and Davis 2003), CO₂ concentration (Driscoll et al. 2006; Leahey et al. 2006), and salt stress (Khodary 2004; Sheng et al. 2008). To our knowledge, however, few studies have been reported investigating warming effects on the adaxial/abaxial ratio, the variation of stomata on different leaf sections, and the stomatal distribution pattern on single leaf surfaces of maize plants. The objectives of the current study are to examine warming effects on: (1) stomatal frequency; (2) stomatal aperture size; and (3) stomatal distribution pattern in maize leaves through a field warming experiment in northern China.

Materials and Methods

Site description

This study was conducted in the Yucheng Comprehensive Experiment Station (36°40′–37°12′ N, 116°22′–116°45′ E; an elevation of 28 m) which is located in the lower reach of the Yellow River in the North China Plain. The study area features a typical monsoon climate with average annual precipitation of 610 mm and annual mean temperature of 13.1°C. Approximately 70% of the annual precipitation is received between June and September (Hou et al. 2012). The soil at the station is classified as Calcaric fluvisols in the FAO-Uneson system with 66% silt; 22% clay; and 12% sand. The soils are chemically characterized with an average pH value of 8.5, organic matter content of 1.47 g/kg, and total N, P, and K concentration of 0.9 g/kg, 0.2% and 2.26%, respectively. A double cropping system with winter wheat (Triticum aestivum L.) and summer maize (Zea mays L.) has been practiced in this area for at least 50 years (Zhang and Ren 2012).

Materials and Methods

Warming experiment

The warming experiment, initiated in September 2009, consists of six 3 × 4 m plots with three of the plots as warming plots and the other three plots as control (three replicates). A 5 m buffer was established between the plots to reduce disturbances. The warming plots have been heated continuously since November 18th, 2009 using infrared radiators with a dimension of 165 × 1.5 cm in length and width (Kalglo Electronics Inc, Bethlehem, PA). Each infrared heater was suspended 2.25 m above the ground. A reflector associated with the heater can be adjusted so as to generate an evenly distributed radiant input to the plant canopy (Kimball 2005). In the control plot, one “dummy” heater with the same shape and size as the infrared radiator was suspended at the same height to eliminate shading effects of the infrared radiator.

Air temperature at 2.4 m above the ground and soil temperature at 5 cm depth were continuously monitored with thermocouple sensors and the averages were recorded hourly with PT 100 thermocouples (Unism Technologies Incorporated, Beijing, China). The foliar surface temperature was measured using a portable infrared thermometer (FLUKE 574; Fluke Inc., Carlsbad, CA). The warming, on average, has increased air, soil, and canopy temperature by 1.42 ± 0.18/1.77 ± 0.24 (day/night), 1.68 ± 0.9/2.04 ± 0.16 (day/night), 2.08 ± 0.72 (day), respectively, in comparison with the control during the maize growth period from June 24th to October 7th of 2011. Soil moisture (0–10 cm average) was also monitored in the middle of each plot with a FDS100 soil moisture sensor (Unism Technologies Incorporated, Beijing, China). All the plots were irrigated with normal management schedules to ensure that the soil moisture was not a limiting factor to plant growth. During the maize growth period the soil moisture (% by volume) in the top 10 cm averaged 26.02 ± 0.86% in the control plots and 25.04 ± 0.52% in the warming plots. Moreover, no significant difference was detected in the relative air humidity between the ambient and warming plots (data not shown).

In order to promote uniform germination, the seeds (cv. Zhengdan 958) of maize (Zea mays L.) were treated at 4°C in dark and wet environments for 2 days before they were sowed. Afterwards, the seeds were sown in the control and warming plots on June 24th, 2011. Given that the ear leaf of each plant is the most important for determining the maize yield, five fully expanded ear leaves were randomly collected, namely five plants in each treatment for field measuring and sampling on August 24th, 2011, 60 days after sowing.
Field gas exchange measurements and sampling

We randomly selected five maize plants grown in the three control plots or the three warmed plots for gas exchange measurements. Specifically, we selected maize plants from plot 1 (two plants), plot 2 (two plants), and plot 3 (one plant) among the three ambient plots. We also selected five maize plants from the three warmed plots with the same number of maize plants as the ambient plots; namely, two plants (plot 1), two plants (plot 2), and one plant (plot 3) were selected among the three warmed pots, respectively. Each fully expanded ear leaf of the five selected plants was used for the measurements of stomatal conductance (Gs) and transpiration rates (Tr) using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (P < 0.05).

To characterize the maximum stomatal pore size, we collected the stomatal samples at optimal conditions with a temperature of c. 30°C at a sunny day (August 24th, 2011) during 10:30–11:00 am. We sampled separately from the tip, middle, and base sections of the adaxial and abaxial surfaces using a colorless nail polish. The adaxial and abaxial epidermis of the leaves were cleaned first by a degreased cotton ball and then carefully smeared with nail varnish from the mid-area between the central vein and the leaf edge for about half an hour. The thin film (approximate 5 by 15 mm) was peeled off from the leaf surface and mounted on a glass slide. Then the thin film was immediately covered with a cover slip and pressured lightly with a fine-point tweezers. We used the same sampling method as in previous studies on the topic (Radoglou and Jarvis 1990; Ferris et al. 1996; Reddy et al., 2001; Xu and Zhou 2005; Xu et al. 2009; Zhang et al. 2010).

Laboratory measurements

The imprints were observed and photographed in the laboratory with a microscope (DM2500; Leica Corp., Biberach, Germany) equipped with a digital camera (DFC 300-FX; Leica Corp.). We took 15 images from five microscopic fields at each section (tip, middle, and base section) on the adaxial and abaxial surfaces of the five leaves sampled from the three replicated control or warmed plots. Then we randomly selected five images (subsamples) from each leaf section of the adaxial or abaxial surface per leaf (five subsamples* three sections* five leaves = 75 samples). Because the three ambient plots or the three warmed plots are the three replicates, all the data of the stomatal traits from the five sampled leaves (75 samples) were averaged within each plot, namely 30 samples (five subsamples* three sections* two leaves = 30 samples) from plot 1 or plot 2 and 15 samples (five subsamples* three sections* one leaf = 15 samples) from plot 3 in the three ambient plots or the three warmed plots. Moreover, we combined the subsamples of the adaxial and abaxial surfaces for estimating the stomatal characteristics for the whole leaf in the control or warmed plot (5 subsamples* 3 sections* 2 surfaces* 5 leaves = 150 samples). Stomata and epidermal cells were counted on the images. Stomatal density (SD), epidermal cell density (ECD), and stomatal index (SI) were calculated according to the methods outlined by Ceulemans et al. (1995) and Teng et al. (2006). Specifically, stomatal density (SD) and ECD were expressed as the number of stomata and epidermal cells per unit leaf area. The stomatal index (SD) was estimated as the percentage of stomata/(epidermal cells + stomata) × 100% (Xu and Zhou 2005; Xu et al. 2009).

To characterize the features of length, width, and area of stomatal pores and epidermal cells, we randomly selected six stomata and six epidermal cells from the above selected images (75 subsamples* six stomata/epidermal cells = 450 samples per leaf surface for stomatal pores or epidermal cells) for measuring stomatal apertures length (SAL), stomatal apertures width (SAW), stomatal apertures area (SAA), stomatal apertures circumference (SAC) and epidermal cell length (ECL), epidermal cell width (ECW), epidermal cell area (ECA), and epidermal cell circumference (ECC) with the Image J quantification software (NIH, Bethesda, MD). In addition, we also calculated stomatal aperture area index (SAAI) and stomatal aperture shape index (SASI). The SAAI is defined as the total stomatal aperture area per unit leaf area calculating as stomatal average density × stomatal aperture area per stoma × 100%. The SASI is calculated by the function that shape index = √A/P × 100%, where A is the stomatal aperture area and P is the stomatal aperture circumference. Both the stomatal traits (SD, SI, SAL, SAW, SAA, SAC, SAAI, and SASI) and epidermal features (ECD, ECL, ECW, ECA, and ECC) were statistically analyzed with one-way analysis of variation (ANOVA) followed by Duncan’s multiple range test (P < 0.05).

To measure the spatial distribution patterns of stomata on both the adaxial and abaxial surfaces, we randomly sampled two pieces (2 × 2 mm) in the middle section of each ear leaf from three maize plants grown under ambient or elevated temperature. These samples were treated with a fixative solution consisting of 2.5% (v/v) glutaraldehyde (in 0.1 M phosphate buffer, pH 7.0). Samples were

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stored at 4°C and transported to the laboratory immediately. Then, the samples were washed six times with the same buffer and postfixed in 1% (v/v) osmium tetroxide for 3 h at room temperature. After being washed with the same buffer, leaf tissues were passed through an ethanol dehydration series. Then the samples were critical point-dried, mounted on stubs, and coated with gold in a high-vacuum evaporation unit. The samples were examined and photographed at 10 KV under a Quanta 200 scanning electron microscope (FEI Corp, Hillsboro, OR).

Spatial pattern analysis
We randomly selected three scanning electron micrographs (a magnification of 100) from three maize leaves on each leaf surface to determine the spatial distribution pattern of stomata. For this analysis, we treated each stoma as a single point on the leaf surface by using the center of each aperture as the focal point. The selected micrographs were first digitized with a GIS software (ArcGIS 10.0; ESRI Inc., Redlands, CA). Then, the point pattern analysis was conducted with the Ripley’s K-function, a cumulative density function using the second moment of all point-to-point distances to evaluate two-dimensional distribution patterns at different scales (Ripley 1976). The results were plotted in the $L(t)$ values as shown below:

$$L(t) = \sqrt{K(t)/\pi - t} \quad (1)$$

where $L(t)$ is an expectation of zero for any value of $t$ when the pattern is Poisson random (Skarpe 1991; Haase et al. 1997). In order to estimate the boundaries of the 95% confidence level, we used the Monte Carlo simulation by running a random distribution for 1000 times. If the stomata are randomly distributed on the leaf surface at a given scale of $t$, then the calculated $L(t)$ value should be located within the 95% boundaries. If the $L(t)$ value is greater than the upper 95% boundary, then the stomata will follow a cluster distribution at that scale. Otherwise, the stomata would follow a regular distribution at the scale if the $L(t)$ value is smaller than the lower 95% boundary. Details of the Ripley’s K analysis can be found in Diggle (1983). The differences of the minimum $L(t)$ values of both leaf surfaces between the ambient plots and the warmed plots were statistically compared by one-way analysis of variation (ANOVA) followed by Duncan’s multiple range test ($P < 0.05$).

Statistical analysis
The warming effects on the features of stomata (SD, SI, SAL, SAW, SAA, SAC, SAAI, and SASI) and epidermal cells (ECD, ECL, ECW, ECA, and ECC) were tested using one-way analysis of variation (ANOVA) followed by Duncan’s multiple range test ($P < 0.05$). To estimate the interaction effects of temperature, leaf surface, and leaf section on the stomatal traits (SD, SI, SAL, SAW, SAA, SAC, SAAI, and SASI), we used three-way ANOVA followed by Duncan’s multiple range test ($P < 0.05$). All statistical analyses were performed using the SPSS 13.0 software (Chicago, IL).

Results
Stomatal frequency
Experimental warming had little effect on stomatal density (SD) on both leaf surfaces and sections except for the base section on the adaxial surface and the tip section on the abaxial surface (Tables 1 and 2). We found that the SD was significantly different between the adaxial and abaxial surfaces with an average stomatal density of 56(5) stomata/mm² on the adaxial surface and 77(3) stomata/mm² on the abaxial surface of maize leaves grown under ambient temperature. While warming barely increased the SD from 56(5) to 58(9) stomata/mm² on the adaxial surface and 77(3) to 81(1) stomata/mm² on the abaxial surface. Moreover, we found that experimental warming significantly increased the SD by 19.6% ($P = 0.023, F = 8.363, df = 149$) from 56(3) stomata/mm² to 67(9) stomata/mm² at the base section on the adaxial surface and by 11.0% ($P < 0.001, F = 14.901, df = 149$) from 73(1) stomata/mm² to 81(1) stomata/mm² at the tip section on the abaxial surface, while no significant difference was detected at other sections on the adaxial or abaxial surfaces (all $P > 0.05$). Our results also showed that the warming effect on the stomatal index (SI) was divergent on the adaxial and abaxial surfaces, where warming significantly increased the SI by 19.6% on the adaxial surface ($P < 0.001, F = 10.024, df = 299$) but only 6.2% on the abaxial surface ($P = 0.098, F = 6.954, df = 299$; Table 1). Similarly, experimental warming also had different effect on the SI among various leaf sections (Table 2). We found that warming significantly increased the SI by 23.6% at the middle section ($P = 0.014, F = 8.829, df = 149$) and by 37.9% at the base section ($P = 0.003, F = 14.921, df = 149$) of the adaxial surface and by 10.2% ($P = 0.025, F = 3.220, df = 149$) at the middle section of the abaxial surface (Table 2). However, experimental warming had little effect on the SI at the tip section on the adaxial surface and at the tip and base sections on the abaxial surface (all $P > 0.05$; Table 2).

In addition to the stomatal frequency of leaf surfaces and sections, experimental warming also had different effects on the adaxial/abaxial ratio of stomatal density and
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Table 1. Effects of experimental warming on stomatal features of maize leaves.

| Parameters | Ambient temperature | Elevated temperature | Increase (%) | P-value |
|------------|---------------------|-----------------------|--------------|---------|
|            | Adaxial | Abaxial | Adaxial | Abaxial |                  |              |
| SD (stomata/mm²) | 56 (5) | 77 (3) | 58 (9) | 81 (1) | – | – |
| SI (%) | 13.8 (0.1)c | 19.4 (0.5)ab | 16.5 (0.3)b | 20.6 (0.8)a | – | – |
| SAL (µm)* | 36.2 (2.9)ab | 35.5 (1.5)a | 31.0 (2.7)b | 28.5 (3.0)b | – | – |
| SAW (µm)* | 3.6 (0.4)bc | 3.2 (0.7)c | 4.6 (0.7)ab | 4.5 (0.9)a | – | – |
| SAA (µm²) | 119 (10)bc | 100 (19)c | 150 (25)a | 135 (19)ab | – | – |
| SAC (µm) | 75 (9)bc | 70 (4)c | 87 (14)a | 77 (7)b | – | – |
| SAAI (%) | 0.66 (0.24)c | 0.77 (0.12)bc | 0.87 (0.26)b | 1.10 (0.25)b | – | – |
| SASI (%) | 14.7 (2.1)a | 14.3 (1.7)a | 14.5 (2.4)a | 15.1 (2.2)a | – | – |

Values given are means ± standard deviation for SD, SI, SAAI, and SASI (75 subsamples and three replicates), and for SAL, SAW, SAA, and SAC (450 subsamples and three replicates). Mean values were compared by the one-way analysis of variance (ANOVA) at P < 0.05. Different letters indicate P < 0.05 and the same letters indicate P > 0.05. SD, stomatal density; SI, stomatal index; SAL, stomatal aperture length; SAA, stomatal aperture area; SAC, stomatal aperture circumference; SAAI, stomatal aperture area index; SASI, stomatal aperture shape index. *Stomatal aperture length is the longest dimension, and the stomatal aperture width is the widest dimension.

stomatal index (Table 3). We found that warming significantly increased SI from 0.71 to 0.80 (P = 0.028, F = 4.738, df = 299), but had little effect on the ratio of SD between the adaxial and abaxial surfaces (P = 0.803, F = 0.033, df = 299). The adaxial/abaxial ratio of SD was almost a constant, with a value of 0.73 in ambient temperature and 0.72 in elevated temperature (Table 3). However, we found that warming significantly increased the adaxial/abaxial ratio of SD and SI by 18.1% and 30.6% at the base section (all P < 0.05), while had little effect on those at the tip and middle sections of the maize leaves (all P > 0.05; Table 3).

Stomatal aperture size and shape

Experimental warming not only affected stomatal frequency but also changed individual stomatal size. Our results showed that warming significantly decreased stomatal aperture length, but increased stomatal aperture width (Table 1). We found that warming decreased stomatal aperture length from 35.9 µm to 29.8 µm, about 17.0% (P < 0.001, F = 7.010, df = 149; Table 1). Specifically, warming significantly decreased stomatal aperture length at the tip, middle, and base sections by 15.7% (P = 0.032, F = 6.158, df = 149), 16.0% (P < 0.001, F = 135.652, df = 149), and 9.7% (P = 0.005, F = 13.228, df = 149) on the adaxial surface and by 21.4% (P < 0.001, F = 2.767, df = 149), 14.9% (P < 0.001, F = 78.053, df = 149), and 23.0% (P < 0.001, F = 121.950, df = 149) on the abaxial surface (Table 2). By contrast, experimental warming significantly increased the stomatal aperture width from 3.4 µm to 4.6 µm, about 33.8% (P < 0.001, F = 31.660, df = 449; Table 1). Meanwhile, experimental warming also had different effects on stomatal aperture width among leaf sections (Table 2). Compared with the ambient temperature, the stomatal aperture width was significantly increased 32.4% (P = 0.010, F = 10.114, df = 149) at the middle section of the adaxial surface and 48.4% (P = 0.013, F = 9.152, df = 149) and 51.4% (P < 0.001, F = 4.910, df = 149) at the middle and base sections of the abaxial surface under elevated temperature (Table 2). These results were also confirmed by our directly scanning electron microscopic observation where we compared the microscopic images of leaves grown under elevated and ambient temperatures. We also observed shorter and wider stomata on both adaxial and abaxial surfaces of leaves grown at elevated temperatures than those leaves grown at ambient temperature (Fig. 1).

As a result, warming significantly increased the stomatal aperture area (SAA) by 30.1% (P < 0.001, F = 38.531, df = 449) and stomatal aperture area index (SAAI), defined as the stomatal aperture area per unit leaf area, by 39.9% (P < 0.001, F = 35.940, df = 449; Table 1). We found that warming significantly increased the SAA and the SAAI at various leaf sections on both leaf surfaces (all P < 0.05) except for the tip section on the adaxial surface (Table 2). Meanwhile, warming also significantly
Table 2. Warming effects on stomatal and epidermal cell characteristics at different leaf sections of maize.

| Features | Adaxial surface | Abaxial surface |
|----------|-----------------|-----------------|
|          | Ambient temperature | Elevated temperature | Ambient temperature | Elevated temperature |
|          | Tip | Middle | Base | Tip | Middle | Base | Tip | Middle | Base | Tip | Middle | Base |
| SD | 51 (5) | 56 (3) | 50 (2) | 67 (9) | 73 (1) | 58 (4) | 52 (4) | 66 (9) | 78 (10) | 64 (5) | 67 (9) | 61 (3) |
| ECD | 330 (27) | 350 (35) | 320 (40) | 343 (21) | 309 (28) | 324 (19) | 343 (21) | 350 (35) | 36 (15) | 338 (21) | 350 (35) |
| SI | 35 (2.3) | 31 (2.2) | 27 (1.6) | 31 (2.2) | 33 (2.2) | 31 (2.2) | 31 (2.2) | 35 (2.3) | 33 (2.2) | 31 (2.2) | 33 (2.2) |
| SAL | 34 (2.3) | 38 (2.3) | 35 (2.3) | 42 (2.3) | 35 (2.3) | 40 (2.3) | 35 (2.3) | 42 (2.3) | 40 (2.3) | 35 (2.3) | 40 (2.3) |
| SAW | 112 (4.6) | 126 (9) | 132 (9) | 140 (9) | 146 (9) | 152 (9) | 140 (9) | 146 (9) | 152 (9) | 140 (9) | 146 (9) |
| SAC | 74 (7) | 73 (7) | 71 (7) | 81 (9) | 83 (9) | 81 (9) | 71 (7) | 81 (9) | 83 (9) | 71 (7) | 81 (9) |
| SAA | 14.4 (1.7) | 15.5 (2.1) | 10.7 (1.4) | 16.2 (2.0) | 15.7 (1.4) | 11.7 (1.4) | 16.2 (2.0) | 15.7 (1.4) | 11.7 (1.4) | 16.2 (2.0) | 15.7 (1.4) |

Values given are means ± standard deviation for SD, ECD, SI (25 subsamples and three replicates) and for SAL, SAW, and SAA (150 subsamples and three replicates). Mean values were compared by the ANOVA followed by Duncan’s multiple range test, and the different letters represent statistical differences at \( P < 0.05 \). SD: Stomatal density (number per mm\(^2\)); ECD: Epidermal cell density (number per mm\(^2\)); SI: Stomatal index (%); SAL: Stomatal aperture length (\( \mu \)m); SAW: Stomatal aperture width (\( \mu \)m); SAA: Stomatal aperture area (\( \mu \)m\(^2\)); SAAI: Stomatal aperture area index (%).
A regular pattern occurred at a scale of 4.80 for the abaxial surface (leaves and only increased by 181% (from 3.1 to 5.6 mmol/m²/s) under warming significantly decreased the average minimum value of the warmed leaves was significantly lower than that of the leaves in the ambient temperature. Specifically, warming significantly increased stomatal conductance from 2.43 to 4.43 for the adaxial surface and –3.3 for the abaxial surface for leaves grown in the ambient temperature (Fig. 2A–C). We also found that warming made the stomata more regularly distributed on both leaf surfaces because the average minimum L(t) value of the warmed leaves was significantly lower than that of the leaves in the ambient temperature. Specifically, warming significantly decreased the average minimum L(t) value from –1.81 to –4.43 for the adaxial surface (P < 0.001, F = 241.9, df = 5; Fig. 2) and from –3.25 to –4.80 for the abaxial surface (P = 0.003, F = 41.399, df = 5; Fig. 3). Moreover, warming also increased the scale range of the regular distribution with the most regular pattern occurred at a scale of c. 60 μm for the warmed leaves and only c. 30 μm for the control leaves (Figs 2 and 3). This warming effect on stomatal distribution pattern, in general, was greater on the adaxial surface than the abaxial surface and the warming effect was consistent among all the replicate leaves (Figs 2 and 3).

### Stomatal conductance and transpiration rate

Experimental warming not only changed stomatal traits but also increased stomatal conductance (Gs) and transpiration rate (Tr) of maize leaves (Fig. 4). We found that warming significantly increased stomatal conductance from 174 to 457 mmol/m²/s by 163% (P = 0.001, F = 16.970; df = 9; Fig. 4). Similarly, transpiration rate was also increased by 181% (from 3.1 to 5.6 mmol/m²/s) under warming conditions (P = 0.017, F = 8.483; df = 9; Fig. 4).

### Discussion

#### Warming effects on stomatal density and stomatal index

The responses of stomatal density (SD, the number of stomata per unit area) and stomatal index (SI, the proportion of stomata in relation to total number of epidermal plus stomatal cells) to global warming are important in determining the potential efficiency of leaf-level gas exchange, and thus ecosystem carbon cycles under future global warming (Woodward 1987; Ferris et al. 1996; Apple et al. 2000). In this study, we found that warming had little effect on SD but significantly increased SI of the leaves of maize plants (Zea may L.) because warming significantly decreased the number of epidermal cells and meanwhile had no effect on the number of guard cells. Previous studies have reported that increasing temperature enhanced epidermal cell expansion on maize leaves but had little effect on the epidermal cell division (Erwin et al. 1991, 1994; Tardieu et al. 2000). This suggests that the decrease of the number of epidermal cells per unit leaf area in the warming plots in the current study was mainly due to the greater expansion of the individual epidermal cells, rather than greater differentiation to guard cells under the warming treatment. Our results also confirmed that the average size of the individual epidermal cells was significantly larger in the warming plots than in the control plots (Fig. 1 and Table S1), resulting in the asymmetric warming effect on guard cells and epidermal cells on the maize leaves. Similarly, several studies have also found that plant leaves respond to elevated CO2 concentration with adjusting their stomatal densities through changes in epidermal cell numbers rather than stomatal numbers (Ferris et al. 2002; Driscoll et al. 2006; Soares et al. 2008).

In addition to temperature, stomatal frequency is also affected by other environmental factors, such as drought, irradiance, light intensity, and relative air humidity (Apple et al. 2000; Fraser et al. 2009; Xu et al. 2009). The inconsistent conclusions in the literatures concerning warming effects on stomatal frequency may result from different warming methods, different warming intensity and possibly different species. Different warming methods, such as using open-top chamber (Fraser et al. 2009), greenhouse (Ferris et al. 1996; Reddy et al. 1998; Apple et al. 2000; Luomala et al. 2005; Xu and Zhou...
2005), and growth chamber (Hovenden 2001; Xu et al. 2009; Jin et al. 2011), may alter other environmental factors, such as soil water content, light intensity, and relative humidity, in addition to temperature. For example, Niu et al. (2007) compared the disadvantages of different warming facilities for simulating climate warming and concluded that open-top chamber and greenhouse altered the microclimates including light, humidity, and rainfall inside them except for temperature. Luomala et al. (2005) pointed out that higher VPD inside the elevated temperature growth chambers during growth season might affect stomatal density. Moreover, several studies have compared stomatal density of plant leaves growing in different geographical locations (Beerling and Chaloner 1993), and altitudinal gradients (Kouwenberg et al. 2007) with different air temperatures for simulating

Figure 1. Scanning electron micrographs (SEM) showed the characteristics of stomata and epidermal cells at the middle section of maize leaves grown in ambient (A–C) and elevated temperature (D–F). Note that shorter and wider stomatal pores were observed on both the adaxial surface (B) and abaxial surface (C) of maize leaves grown at elevated temperature than those of their counterparts (E and F) grown at ambient temperature. In addition, elevated temperature also increased the width of epidermal cells. Bars, 10 μm (A and D) and 40 μm (B, C, E, and F).
climate warming, which apparently changed climate conditions such as precipitation, soil moisture, and light intensity and period. As a result, the different growth conditions may contribute to the inconsistent results of warming effects among species and ecosystems. The current study examined the warming effects on the stomatal frequency of maize leaves with an infrared radiator in field conditions, which had little disturbance on the

| Parameters                     | SD    | SI    | SAL   | SAW   | SAA   | SAC   | SAAI  | SASI  |
|--------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Temperature                    | 0.038 | <0.001| <0.001| <0.001| <0.001| <0.001| <0.001| 0.756 |
| Leaf surface                   | <0.001| <0.001| 0.061 | 0.982 | <0.001| <0.001| <0.001| 0.861 |
| Leaf section                   | <0.001| <0.001| <0.001| <0.001| <0.001| 0.583 | <0.001| <0.001|
| Temperature × leaf surface      | 0.608 | 0.042 | 0.628 | 0.873 | 0.409 | 0.512 | 0.006 | 0.281 |
| Temperature × leaf section      | 0.307 | 0.002 | <0.001| 0.357 | 0.076 | 0.658 | 0.008 | 0.811 |
| Leaf surface × leaf section     | 0.019 | 0.013 | 0.359 | 0.018 | 0.421 | 0.253 | 0.428 | 0.225 |
| Temperature × leaf surface × leaf section | 0.025 | 0.083 | 0.722 | 0.774 | 0.090 | 0.002 | 0.010 | 0.006 |

Values given are means ± standard deviation for SD, SI (25 subsamples and three replicates) and for SAL, SAW, and SAA (150 subsamples and three replicates). Mean values were compared by the ANOVA followed by Duncan’s multiple range test, and the different letters represent statistical differences at P < 0.05. The bold value indicates P < 0.05. SD: Stomatal density (number per mm²); ECD: Epidermal cell density (number per mm²); SI: Stomatal index (%); SAL: Stomatal aperture length (μm); SAW: Stomatal aperture width (μm); SAA: Stomatal aperture area (μm²); SAAI: Stomatal aperture area index (%) which is the total stomatal aperture area per unit leaf area calculating as stomatal average density × stomatal aperture area per stoma.

Figure 2. Point pattern analyses of stomata on the adaxial surface in leaf 1, leaf 2, and leaf 3 of maize plants grown at ambient temperature (A–C) and in leaf 1, leaf 2, and leaf 3 of maize plants grown at elevated temperature (D–F), respectively. The dotted lines give a 95% confidence envelope for complete spatial randomness. The data were given for three leaves from three ambient or warmed plots.
microclimates and few difference in relative air humidity between the control and warmed plots (Niu et al. 2007; Zhang et al. 2010).

**Warming effects on stomatal aperture size and shape**

Plants in response to climate warming not only alter stomatal frequency (Apple et al. 2000; Luomala et al. 2005; Xu and Zhou 2005), but also change stomatal aperture size anatomically (Hetherington and Woodward 2003; Franks and Beerling 2009; Casson and Hetherington 2010). It is noted that the stomatal aperture size here refers to the stomatal aperture length which is the linear distance between the junctions of the guard cells at each end of the stomata. The stomatal aperture size is different from stomatal pore openness which responds simultaneously to environmental variables such as light (Humble and Hsiao 1970; Sharkey and Raschke 1981; Kwak et al. 2001; Takemiya et al. 2006), temperature (Honour et al. 1995; Feller 2006; Reynolds-Henne et al. 2010), humidity (Lange et al. 1971; Schulze et al. 1974), and CO₂ concentration (Ogawa 1979; Young et al. 2006; Lammertsma et al. 2011). Anatomically, the length of guard cells mainly determines stomatal aperture length, because when stomata open or close the short axis (ventral and dorsal lengths) of the guard cells can increase or decrease but the long axis remains the same (Willmer and Fricker 1996; Beaulieu et al. 2008). Several studies have found that stomatal aperture size was regulated and modified by environment factors such as CO₂ concentration (Hetherington and Woodward 2003; Franks and Beerling 2009; Casson and Hetherington 2010). However, recent studies found a strong positive relationship between angiosperm genome size (nuclear DNA amount) and stomatal guard cell length and this predictive relationship was independent of environmental conditions (Beaulieu et al. 2008; Lomax et al. 2009). For example, Lomax et al. (2009) examined the effects of environmental variables (CO₂ concentration, drought, relative humidity, irradiance, ultraviolet radiation, and pathogen attack) on the guard cell length of *Arabidopsis thaliana* and found that guard cell length responded to all these variables, but the predictive relationship between genome size and guard cell...
length was not changed by these environmental variables. Unfortunately, the study did not examine the temperature effect on the guard cell length. Interestingly, in the current study, we found that warming significantly reduced the guard cell length (Table S1), thus resulting in the reduction in stomatal aperture length (Table 1 and Fig. 1). This finding supports a recent study that experimental warming significantly decreased the stomatal aperture length of four alpine grass species (Zhang et al. 2010). It is noted that the decrease in stomatal length with warming is accompanied with the increase in the optimal stomatal aperture width (measured at optimal conditions). As a result, we found that warming significantly increased the stomatal aperture area (SAA) and the SAA index ($P < 0.01$; Table 3), suggesting that the adaxial/abaxial ratio of SD in maize leaves is genetically controlled and independent of the changes in environmental condition such as warming. Moreover, we also found that the adaxial/abaxial ratio of SI was significantly increased by experimental warming ($P = 0.037$; Table 3), which was mainly due to the decrease of the ECD ratio between the adaxial and abaxial surfaces ($P < 0.001$; Table 3). These results suggest that maize plants in response to global warming may alter the dorsoventral distribution of stomata by changing the adaxial/abaxial ratio of ECD.

In addition to the stomatal frequency between the adaxial and abaxial leaf surfaces, experimental warming also resulted in uneven effects on stomatal features along different sections such as tip, middle, and base within a leaf surface (Table 2). These results suggested that the warming effects feature high within-surface and within-leaf variations in stomatal dimensions, distribution, and characteristics of maize leaves. Similar results were also found in two perennial grasses, *Lolium perenne* (Ferris et al. 1996) and *Leymus chinensis* (Xu et al. 2009). However, many previous studies examined warming effects on stomatal features only at the middle section on the abaxial leaf surface (Beerling and Chaloner 1993; Hovenden 2001; Xu and Zhou 2005; Kouwenberg et al. 2007). Therefore, it is noted that the sampling method of stomata should be developed for evaluating the stomatal characters across the whole leaf.

Experimental warming not only changes stomatal distribution features at a leaf scale but also affects spatial distribution pattern of stomata at a smaller scale in leaf sections. Previous studies mainly focused on the one-dimensional pattern of stomatal distribution (Juarez et al. 2004; Driscoll et al. 2006). Our results showed that warming had little effect on the adaxial/abaxial ratio in SD ($P = 0.856$; Table 3), suggesting that the adaxial/abaxial polarity of the SD in maize leaves is genetically controlled and independent of the changes in environmental condition such as warming. Similar results have been reported in maize plants under CO$_2$ enrichment conditions by Driscoll et al. (2006), who found that the dorsoventral pattern (adaxial/abaxial) of stomata in maize plants is independent of and not affected by CO$_2$ concentrations.

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**Figures 4.** Stomatal conductance (A) and transpiration rate (B) of maize leaves grown under ambient temperature or elevated temperature. The data given are means ± standard deviation for five leaves from three ambient or warmed plots. Note that experimental warming significantly increased stomatal conductance ($G_s$) and transpiration rate ($T_r$) 163% and 81%, respectively.
2004; Shpak et al. 2005; Wang et al. 2007) or the behavior of a single stoma (Shimazaki et al. 2007; Shang et al. 2009), because it is difficult to characterize the spatial distribution pattern of stomata (Croxdale 2000; Martins et al. 2012). In this study we examined the warming effects on the spatial distribution pattern of stomata using the geostatistical method (Ripley’s K-function), which is considered as an accurately mathematical technique for analyzing spatial distribution patterns (Skarpe 1991; Haase et al. 1997). We observed a more regular spatial distribution pattern of stomata in elevated temperature than that in ambient temperature. This suggested that experimental warming may enhance leaf gas exchange efficiency of maize plants, because the most regular distribution pattern of stomata features the shortest CO₂ diffusion distance to other stomata. In the current study, we also found that warming increased stomatal conductance and transpiration rate (Fig. 4) which was partly attributed to the more regular spatial distribution pattern of the stomata. This warming effect on stomatal spatial distribution pattern may also be a strategy for plants to adapt to global warming because the increased transpiration with the increase of stomatal conductance can cool the leaves, especially in the hot summer with daytime temperature around 40°C in northern China.

**Warming, stomata, and C₄ photosynthetic pathway evolution**

Many of the most productive crops such as maize and sugarcane use the C₄ photosynthetic pathway, which offers C₄ plants the potential to achieve higher rates of leaf photosynthesis and more efficient use of water and nitrogen than C₃ plants (Osborne and Freckleton 2009; Taylor et al. 2012). In comparison with C₃ plants, the higher photosynthetic capacity of C₄ plants is mainly due to their unique mode of CO₂ assimilation, featuring strict compartmentation of photosynthetic enzymes into two distinct cell types, mesophyll and bundle sheath (Wang et al. 2009). In this study, our results showed that experimental warming not only increased individual size of stoma, but also resulted in more regular spatial distribution pattern of stomata in maize leaves, which may reduce the CO₂ diffusion distance from each stoma to photosynthetic site and thus increased the photosynthesis rates of maize plants. These results suggested that global warming may improve the evolution of C₄ photosynthesis through the changes in stomatal traits including stomatal frequency, stomatal size, and spatial distribution pattern of stomata. Moreover, our findings also help to better understand the role of stomatal changes in the long-term evolution of wild C₄ crop progenitors in a subambient CO₂ condition from the origin of agriculture (Sage 1995; Cunniff et al. 2008; Aliscioni et al. 2012). In addition, the enhancement in C₄ photosynthesis efficiency may increase the aboveground biomass accumulation of C₄ plants (Luo et al. 2009; Hou et al. 2012). For example, Luo et al. (2009) showed that experimental warming stimulates aboveground biomass accumulation through enhancing C₄ dominance in a North America tallgrass prairie. Therefore, our results suggested that future global warming may affect the contribution of agroecosystems to CO₂ sequestration in a warmer world.

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**Conflict of Interest**

None declared.

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**Supporting Information**

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

**Table S1.** The characteristics of epidermal cells and guard cells in maize leaves grown at ambient temperature or elevated temperature.