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Effects of artificial warming on the structural, physiological, and biochemical changes of maize (*Zea mays* L.) leaves in northern China

Yunpu Zheng · Ming Xu · Ruichang Shen · Shuai Qiu

Abstract We examined the effects of artificial warming on physiological, biochemical, and structural changes in leaves of maize plants (*Zea mays* L.) with a field warming experiment in the North China Plain. Stomatal characters, leaf anatomy and ultrastructure, gas exchange, and carbohydrate and mineral nutrition concentrations were examined using light microscopy, electron microscopy, portable photosynthesis system (Licor-6400), and inductively coupled plasma atomic emission spectroscopy. We found that artificial warming (about 2 °C) increased both the stomatal index and stomatal size, and thus increased net photosynthesis rate (A), stomatal conductance (g_s), and transpiration rate (E). Artificial warming also significantly increased the profile area of chloroplast and mitochondria, but decreased leaf width and thickness, mesophyll thickness, and mesophyll cell size (mainly palisade cell size). In addition, artificial warming also significantly increased the foliar C:N ratio and soluble sugar contents (glucose, fructose, and sucrose), but not the mineral nutrients and starch contents. Our findings suggest that future global warming may affect the maize growth and production in northern China due to the direct warming effects on the structures (anatomy and ultrastructure), biochemical properties and gas exchanges of the maize leaves.

Keywords Elevated temperature · Gas exchange · Image analysis · Leaf anatomy · Mineral nutrition · Carbohydrates · Stomatal characteristics

Introduction

It is widely evidenced that the concentrations of greenhouse gases (GHG), such as CH_4, CO_2 and N_2O, in the atmosphere have dramatically increased since the beginning of the industrial revolution mainly due to fossil fuel combustion and land use change (IPCC 2007). Meanwhile, the increased GHG concentration has led to a rapid rise in global temperature over the past decades (Gunderson et al. 2000; Wigley and Raper 2001). Moreover, it is estimated that the mean global surface temperature will continue to increase by 1.1–6.4 °C over this century depending on GHG emissions (IPCC 2007). This projected global warming is expected to cause shifts in community species composition and distribution (Epstein et al. 2004; Klein et al. 2004; Walker et al. 2006; Parolo and Rossi 2008), changes in ecosystem structure and function (Peñuelas et al. 2004; Biasi et al. 2008a, b; Jägerbrand et al. 2009), and even local/global extinctions of plant species in terrestrial ecosystems (Parmesan and Yohe 2003; Franco et al. 2006; Malcolm et al. 2006). Global warming may also generate profound alterations in plant structure and

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function (Kudo and Suzuki 2003; Llorens et al. 2004a; Yin et al. 2008; Niu et al. 2008; Zhao and Liu 2009), and thus alter biomass accumulation and allocation (Prieto et al. 2009a; Lin et al. 2010; Wang et al. 2012), and/or net primary production of terrestrial plants (Alward et al. 1999; Peñuelas et al. 2007; Lin et al. 2012; Hou et al. 2012).

In addition to the elevated mean global surface temperature, global precipitation may decline and become unevenly distributed both temporally and spatially (IPCC 2007; Chun et al. 2011). Drought stress caused by a decline in precipitation is also a major factor limiting plants growth and yield. The two abiotic stresses drought and warming are becoming increasing concerns because drought and warming often occur simultaneously in the summer in many areas all over the world (Jiang and Huang 2000, 2001). Previous studies reported that drought and warming alone or in combination affect leaf structure and function such as photosynthesis and respiration (Rachmilevitch et al. 2006), and thus the growth and yield of plants under future climate change. Moreover, most plant species have an optimal growth temperature and only grow in a certain temperature range (Jin et al. 2011). Thus, some species are likely to adapt to warmer temperatures by changing their growth and development or by shifting their ranges, provided that the optimum temperatures are not exceeded. In contrast, some species may fail to adapt to this global change and may even become extinct if the air temperature is too high (Thomas et al. 2004; Colwell et al. 2008; van Mantgem et al. 2009). Meanwhile, many recent studies have found that global warming may come with more frequent extreme climate events such as heat waves resulted in more days with extreme high temperatures (Meehl and Tebaldi 2004; Schär et al. 2004; Tebaldi et al. 2006; Fischer and Schär 2010). It should be noted that there is usually a growth temperature threshold for each development stage of plants/crops, and thus plants/crops grow in a temperature exceeding their growth temperature threshold that may result in more damages than exposure to longer warmer period.

Global warming usually affects plant functions through direct effects on metabolic rates, such as photosynthesis and respiration (Tjoelker et al. 1999; Zha et al. 2001; Llorens et al. 2004b; Niu et al. 2008; Han et al. 2009; Prieto et al. 2009b; Albert et al. 2011), and indirect effects on soil nitrogen mineralization and availability (Rustad et al. 2001; Peñuelas et al. 2004; Sardans et al. 2008a, b), soil moisture (Wan et al. 2005; Holsten et al. 2009), and growing season length (Menzel and Fabian 1999; Walther et al. 2002; Cleland et al. 2006). Most of the impacts of global warming on plant functions are resulted from the changes in plant structure, especially in leaves (Niinemets et al. 2007; Jin et al. 2011; Xu et al. 2012; Smith et al. 2012). Many studies found that leaf thickness and mesophyll volume are strongly correlated with leaf area-based

carbon assimilation (Higuchi et al. 1999; Niinemets et al. 2007; Xu et al. 2012) and leaf mass per area (LMA) (Gorsuch et al. 2010). Previous studies found that plants grown at higher temperatures had thinner leaves, which was mainly caused by reductions in the thickness of epidermal, palisade, and spongy layers, and an associated decrease in the size of mesophyll cells (Hartikainen et al. 2009; Gorsuch et al. 2010; Jin et al. 2011). Earlier studies also found that the number and size of chloroplasts are highly correlated with leaf photosynthetic rates (Jin et al. 2011; Xu et al. 2012). For example, in *Arabidopsis thaliana*, it was observed that the number of chloroplast per cell remained unchanged under 2.5 °C warming, but decreased by 22 % under 5 °C warming (relative to a control day/night temperature of 23/18 °C); Meanwhile the size of chloroplasts also decreased with warmer growth temperature (Jin et al. 2011).

The concentration of TNC reflects the balance between net photosynthetic carbon uptake and irreversible investments for structures (plant growth) or loss of carbon. Most studies have found that global warming generally decreases the TNC concentration in plant leaves (Zha et al. 2001; Tingey et al. 2003; Jin et al. 2011; Wang et al. 2012; Smith et al. 2012). In contrast, several studies found that global warming may increase (Djanaguiraman et al. 2011) or have little effect on the TNC concentration in plant leaves (Tjoelker et al. 1999; Xu et al. 2012). For example, Tjoelker et al. (1999) reported that warming had no effect on the carbohydrate concentration in four boreal tree species including *Betula papyrifera*, *Larix laricina*, *Pinus Banksiana*, and *Picea mariana*, but increased the carbohydrate concentration in *Populus tremuloides*. Previous studies have found that global warming increases leaf C:N ratio (Tolvanen and Henry 2001; Olszyk et al. 2003; Biasi et al. 2008a, b; Yang et al. 2011).

Maize (*Zea may* L.), a widely distributed C_4 plant species, is one of the most important crops in many regions and thus the maize production determines the safety of future food supply all over the world. It is important to note that the global warming will alter evapotranspiration and water use efficiency (WUE) of crop (Thomas 2008; Mo et al. 2009) and thus affect crop physiological process and growth, which lead to alteration of crop yield (Mo et al. 2009; Liu et al. 2010; Guo et al. 2010). Previous studies have found that global warming decreases the maize yield in many areas of the world (Lobell and Asner 2003). The North China Plain (NCP) is one of the most important regions for crop production in China, accounting for approximately 69 % of wheat (*Triticum aestivum* L.) and 35 % of maize (*Zea mays* L.) grain yields of the whole country (Liu et al. 2010). Recent modeling results have shown that global warming may reduce the maize production by 22.8 % for a 5 °C increase and 13.2 % for a

2 °C increase in the NCP (Liu et al. 2010). However, the underlying mechanisms of climate change on the maize production are far from understood, especially the leaf structure, physiology and biochemistry. The objectives of this study are to examine warming effects on: (1) leaf anatomy and ultrastructure, (2) leaf gas exchange, and (3) carbohydrate and nutrient concentration of maize leaves through a field warming experiment with infrared heaters in northern China.

Materials and methods

Site description

We conducted this study in the Yucheng Artificial Station operated by the Chinese Academy of Sciences (36°40′–37°12′N, 116°22′–116°45′E; an elevation of 28 m). The station is located in the lower reach of the Yellow River in the NCP. The study area features a semi-arid climate with average annual precipitation of 610 mm and temperature of 13.1 °C. Approximately 70 % of annual precipitation occurs between June and September. The soil could be classified as calcaric fluvisols in the FAO-Uneson system with 66 % silt; 22 % clay; and 12 % sand. The chemical properties of the soil are as follows: pH 8.5, organic matter 1.47 g/kg, total N 0.9 g/kg, total P (P_2O_5) 0.2 % and total K (K_2O) 2.26 %. Winter wheat (*T. aestivum* L.) and summer maize (*Z. mays* L.) double cropping is predominant in the NCP.

Warming experiment

The warming experiment features six 3 × 4 m plots with three of the plots serving as treatments and the others as control plots. The warmed plots have been heated continuously since 4 February 2010 using infrared radiators (165 × 1.5 cm, MSR-2420, Kalglo Electronics Inc., Bethlehem, PA) suspended 2.25 m above the ground. In the control plot, one "dummy" heater with the same shape and size as the infrared radiator was also suspended 2.25 m above the ground to simulate the shading effects of the heater. The distance between the control and the warmed plot was approximately 5 m to avoid heating the control plot by the infrared radiator. Air temperature and soil temperature were hourly monitored with PT 100 thermocouples (Unism Technologies Incorporated, Beijing, China) at 2.4 m above and at the depth of 5 cm in the soil. 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 24 June to 7 October 2011. Soil moisture in the top 0–10 cm soil layer was also recorded with a FDS100 soil moisture sensor (Unism Technologies Incorporated, Beijing, China). During the maize growing season, the mean soil moisture (%volume) in the warmed plots was slightly lower than that in the control plots, 25.04 ± 0.52 vs. 26.02 ± 0.86 %, none of which were likely to limit the maize growth.

Field sampling

Seeds of maize (*Zea may* L.) were exposed to a dark, cold, and wet treatment at 4 °C for 2 days before planting to promote uniform germination. Thereafter, they were sown in the field soil in the control and warmed plots in 24 June 2011 and both the maize seedlings in the control and warmed plots grew above the soil surface in 1 July 2011. Maize plants were irrigated with below-ground water to avoid drought stress during the growing period from 24 June 2011 to 7 October 2011. Given that the ear leaf is the most important leaf for determining the crop yield, we sampled the fully expanded ear leaves of maize at the heading stage in 24 August 2011, 60 days after sowing, for the following investigations.

Gas exchange and stomatal characteristics

Three fully expanded leaves were selected from each of five plants grown in ambient temperature and elevated temperature to measure the net CO_2 assimilation rate (A), stomatal conductance (g_s) transpiration rate (E), intercellular CO_2 concentration (C_i), and leaf dark respiration (R_d) using an Li-6400 Portable Photosynthesis System (LI-COR Inc. Lincoln, Nebraska, USA). The WUE was determined by the value of A and E. The measurements were conducted with leaf temperature at 30 °C, PAR at 1,500 μmol m^{-2} s^{-1}, CO2 concentration at 380 μmol mol^{-1}, and cuvette vapor pressure deficit (VPD) at 2.0 kPa.

We randomly selected five fully expanded ear leaves at the heading stage in each of the warming and control plots on 24 August 2011. To determine the stomatal and epidermal cell characteristics, we sampled impressions from the tip, middle, and base sections of the adaxial and abaxial leaf surfaces using 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 (approximately 5 × 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. The imprints were observed and photographed in the laboratory with a microscope (DM2500, Leica Corp, Germany) equipped with a digital camera (DFC 300-FX, Leica Corp,

Germany). Thirty separate fields of 0.16 mm were analyzed in each section of the sampled leaves. Stomata and epidermal cells at the tip, middle, and base sections on each surface were counted and combined for calculating stomatal density (SD), epidermal cell density, and stomatal index (SI) (the ratio of stomata to epidermal cells) on the adaxial surface and abaxial surface, respectively. Moreover, we also randomly selected six digital photographs from each leaf section of the adaxial and abaxial surfaces to measure the stomatal aperture length, width, and area using Image J quantification software (NIH, USA). Then the data from the tip, middle, and base sections of both leaf surfaces were combined and averaged for estimating the warming effects on stomatal aperture size.

For visualizing and comparing the differences of stomata between the warming and control plots under scanning electron microscopy (SEM), we snapped three pieces (2 × 2 mm) from the middle section of each leaf with a fixative solution consisting of 2.5 % (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.0). Samples were stored at 4 °C and transported to the laboratory as soon as possible. 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. Samples were examined and photographed at 10 kV under a Quanta 200 scanning electron microscope (FEI Corp, USA).

Anatomical measurements and ultrastructure observation

We used the same ear leaves for further anatomical observations and measurements. The internal anatomy of the selected leaves was assessed on leaf sections harvested from the middle of the leaf and prepared for light microscope observation as described by Sage and Williams (1995). Images of leaf cross-sections were used to estimate leaf anatomical features using Image J software (NIH, USA). Leaf mesophyll thickness was measured between epidermal layers at five points in each cross-section and the interveinal distance was measured between two adjacent vascular bundles (Pengelly et al. 2010). The percentage of the leaf cross-section covered by M or BS tissue was determined by laying a grid of 200 random points over cross-sections of images and calculating the proportion of points falling on M or BS cells (Parkhurst 1982; McKown and Dengler 2007; Riyadh et al. 2007).

For examining the ultrastructure of cellular organelles, we took the advantage of the transmission electron microscopes. Five fully expanded ear leaves of maize plants were dissected and immediately fixed in 2.5 % (v/v) glutaraldehyde (0.1 M phosphate buffer, pH 7.0) for 2 h at 4 °C. Then the samples were washed four times with the same buffer and postfixed in 1 % osmium tetroxide for 3 h. After being washed with the same buffer, leaf tissues were passed through an ethanol dehydration series, and then infiltrated and embedded in Spurr's resin. Sections were cut using an LKB-V ultramicrotome (LKB, Bromma, Sweden). Thin sections were stained with uranyl acetate and lead citrate, then observed and photographed under a transmission electron microscope (JEOL Ltd, Tokyo, Japan).

Biochemical composition analysis

The sampled leaves were oven-dried to constant weight at 75 °C, and ground to fine powder with a ball mill (MM2, Fa. Retsch, Haan, Germany). Samples were assayed for non-structural carbohydrates according to Hendrix (1993) and Way and Sage (2008). Glucose, fructose, sucrose, and starch concentrations were determined spectrophometrically (UV-1750, Shimadzu Corp., Tokyo, Japan), using a glucose kit (GAHK-20, Sigma, St Louis, MO, USA). Phosphoglucose isomerase (P5381-1 KU, Sigma) was used to convert fructose to glucose, and invertase (I-4504, Sigma) was used to convert sucrose to glucose. Total carbon (C) and nitrogen (N) contents in leaves were determined using an elemental analyzer (Vario Max CN; Elemnetar Corp., Germany). The contents of potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), sodium (Na), and Iron (Fe) were determined using an ICP-OES (Optima 5300DV, PerkinElmer Corp., USA) (Luomala et al. 2005). All the chemical analyses were repeated five times and expressed on a percentage dry matter basis.

Statistical analysis

The differences of the physiological, anatomical, and biochemical variables between the warmed and control plots were tested with the Student's t test ($P < 0.05$) using software SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Stomatal characters and gas exchange

We found no significant difference in stomatal density on adaxial and abaxial leaf surfaces between ambient temperature and elevated temperature, whereas stomatal index significantly increased c. 20 and 6 % on the adaxial and abaxial surfaces, respectively, (Table 1). Only a slight increase in SD was observed under elevated temperature

Table 1 Stomatal and physiological properties of maize leaves grown at ambient temperature (control) or elevated temperature (warming)

Parameter	Control	Warming	% Increase	P value
Stomatal data				
Stomatal density (adaxial number mm^{-2})	56 ± 6	58 ± 9	3.6	0.377
Stomatal density (abaxial number mm^{-2})	77 ± 7	81 ± 6	5.2	0.059
Stomatal index on the adaxial surface (%)	13.8 ± 0.1	16.5 ± 0.3	19.6	0.004
Stomatal index on the abaxial surface (%)	19.4 ± 0.5	20.6 ± 0.8	6.2	0.013
Stomatal aperture length (μm)	36.2 ± 2.1	29.7 ± 3.1	−18.0	<0.0001
Stomatal aperture width (μm)	3.5 ± 0.5	4.4 ± 0.8	25.7	<0.0001
Stomatal aperture area (μm^2)	110 ± 34	142 ± 38	29.1	0.003
Stomatal aperture area per unit leaf area (mm^2)[a]	0.7 ± 0.2	1.0 ± 0.3	39.4	<0.0001
Physiological data[b]				
A (μmol m^{-2} s^{-1})	12.8 ± 4.4	19.5 ± 4.8	52.3	0.019
g_s (mmol m^{-2} s^{-1})	174 ± 78	457 ± 164	163	0.001
E (mmol m^{-2} s^{-1})	3.1 ± 1.1	5.6 ± 2.0	80.6	0.017
C_i (μmol mol^{-1})	210 ± 75	262 ± 39	24.8	0.130
R_d (μmol m^{-2} s^{-1})	3.7 ± 0.5	2.8 ± 0.4	−24.3	0.006
WUE (mmol mol^{-1})	4.7 ± 2.5	4.0 ± 1.7	14.9	0.521

Values given are mean ± standard deviation for five fully expanded ear leaves sampled from five maize plants ($n = 5$ for physiological data; $n = 400$ for epidermal data). Mean values were compared by Student's t test at $P < 0.05$ level

A net CO_2 assimilation rate, g_s stomatal conductance, E transpiration rate, C_i intercellular CO_2 concentration, R_d dark respiration, WUE water use efficiency

[a] The stomatal aperture area per unit leaf area is calculated as mean stomatal density × stomatal aperture area per stoma

[b] The physiological data were determined at 1,500 μmol m^{-2}s^{-1} PAR, 2.0 kPa VPD, 380 μmol mol^{-1} CO_2, and 25 °C leaf temperature

compared with ambient temperature (Table 1). Compared with ambient temperature, the mean stomatal aperture length was decreased by 18 %, but the stomatal aperture width and area as well as the stomatal aperture area per unit leaf area at elevated temperature were significantly increased by c. 26, 29, and 39 %, respectively, (Table 1; Fig. 1).

Artificial warming not only changed leaf stomatal features but also affected leaf physiological characteristics of maize plants. Our results showed that warming significantly increased A, g_s and E ($P < 0.05$), but decreased R_d ($P < 0.05$; Table 1). In contrast, warming had little effect on WUE (Table 1).

Anatomy and ultrastructure

We found that artificial warming significantly decreased both the width and thickness of maize leaves ($P < 0.05$), but it barely affected leaf length (Table 2). Leaf mesophyll thickness, as defined in Fig. 2, was c. 10 % less in elevated temperature than ambient temperature. As no differences were observed in the average number of cell layers between the two groups of plants, the difference in leaf mesophyll thickness was partially due to changes in cell size rather than the number of cell layers (Table 2). Warming significantly reduced the distance between

vascular bundles (defined as the interveinal distance in Fig. 2) by c. 10 % and the area of vascular bundle by c. 30 %, resulting in more and smaller vascular bodies as evidenced in Fig. 2. Relative to ambient temperature, the cell size of bundle sheath (BS) was not markedly affected by artificial warming (Table 2; Fig. 2), whereas warming caused a decrease of 22 % in the cells size of mesophyll (M), which was mainly due to the significant decreases in cell area of palisade mesophyll (PM) since the cell area of spongy mesophyll (SM) was not affected by artificial warming (Table 2). However, we found no significant difference in the M-to-BS cell area ratio between the elevated temperature and ambient temperature (Table 2). Similarly, although warming significantly enhanced the % M by c. 13 %, both % BS and the M-to-BS tissue ratio were not significantly changed (Table 2). Moreover, no difference was observed in sub-stomatal cavity area between the ambient and elevated temperature (Table 2), but warming significantly reduced the cell wall thickness by c. 30 % (Table 2; Fig. 3c, e).

Relative to ambient temperature, elevated temperature significantly increased the average length and width of chloroplast by 46 and 50 %, respectively, resulting in a dramatically c. 140 % enhance in the chloroplast profile area (Table 2; Fig. 3b, e). However, warming had little effect on the number of chloroplasts per mesophyll (Fig. 3a, d) and the

Fig. 1 Scanning electron micrographs (SEM) showed the stomatal and epidermal cell characteristics of maize leaves grown at ambient temperature (**a**, **b**) and elevated temperature (**c**, **d**). Note that shorter and wider stomatal apertures were observed on the leaves of maize grown at elevated temperature than those of their counterparts grown at ambient temperature. *Bars* 10 μm (**a**, **c**) and 40 μm (**b**, **d**)
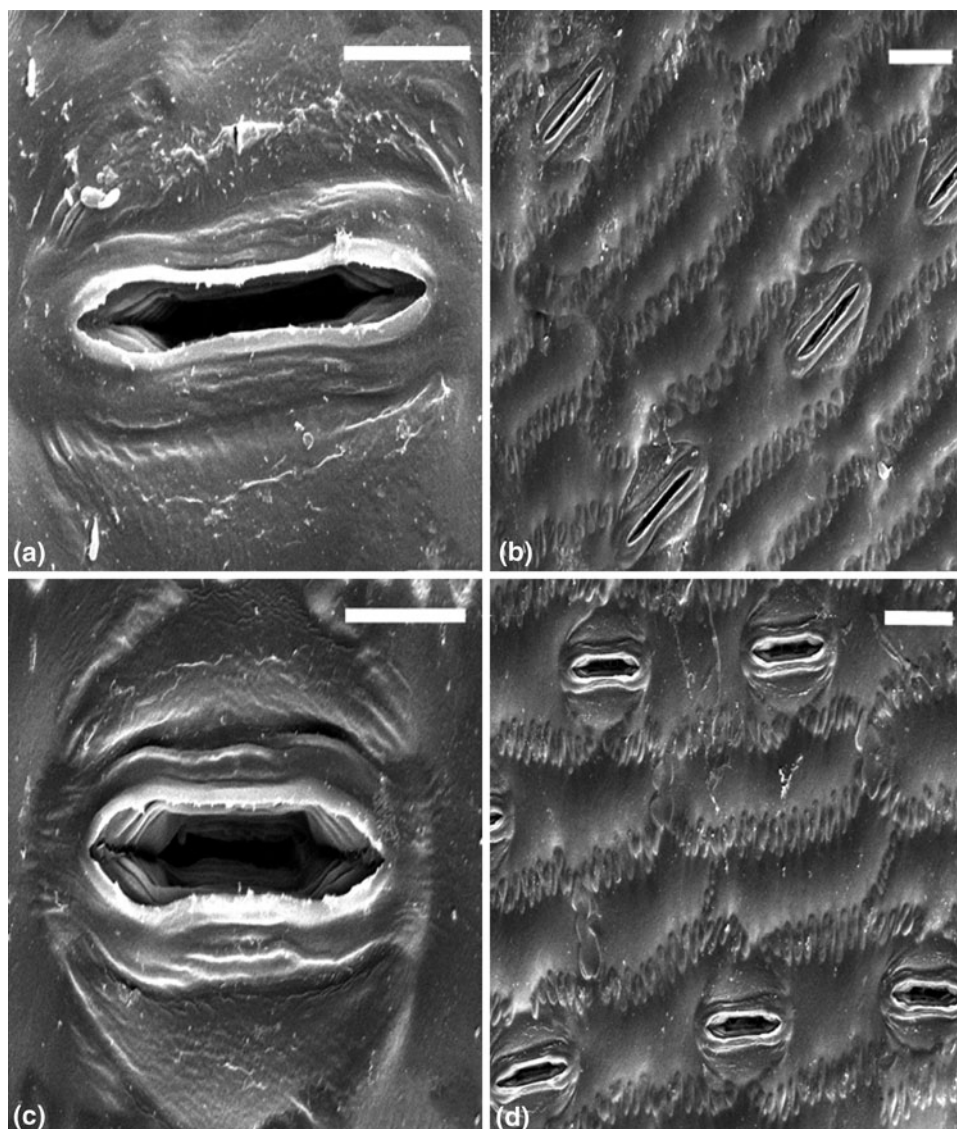
plastoglobuli per chloroplast (Fig. 3b, e; Table 2). In addition, warming also statistically enhanced the mitochondrial size by c. 53 % (Table 2; Fig. 3c, f).

Carbohydrate and mineral nutrient concentration

We found that artificial warming profoundly increased the concentration of TNC mainly due to the increase of the concentration of soluble sugar because warming had little effect on the concentration of starch (Table 3). Compared to ambient temperature, the concentration of soluble sugar at elevated temperature was significantly enhanced by c. 44 %, resulting from the dramatic increase in glucose of 25 %, fructose of 53 %, and sucrose of 83 %, respectively, ($P < 0.05$; Table 3). As a result, artificial warming significantly increased the TNC concentration by c. 30 %, although the concentration of starch not affected by warming (Table 3).

Our results showed no significant difference on the concentrations of all the foliar nutrients between the two temperatures on a dry weight (DW) basis, whereas artificial warming significantly increased the C:N ratio in maize leaves ($P < 0.05$). In addition, artificial warming had no effect on the average concentrations of N, P, K, Ca, and Mg (Table 4). Interestingly, we also found that artificial warming substantially enhanced the C:N ratio by c. 10 % although the concentrations of C and N were not significantly affected by elevated temperature (Table 4).

Discussion

The stomatal responses to artificial warming may be variable and species dependent (Ferris et al. 1996; Reddy et al. 1998; Kouwenberg et al. 2007). Many previous studies have found that warming affected stomatal density but did

Table 2 Morphological and anatomical features of maize leaves grown at ambient temperature (control) or elevated temperature (warming)

| Parameter	Control	Warming	% Increase	P value
Morphological data				
Leaf length (cm)	78.4 ± 3.1	79.9 ± 2.2	1.9	0.256
Leaf width (cm)	10.2 ± 0.7	9.8 ± 0.6	−3.9	0.017
Leaf thickness (μm)	167 ± 11	150 ± 25	−10.2	<0.0001
Leaf mass per unit area (g m^{-2})	14.7 ± 1.1	14.5 ± 1.2	−1.4	0.768
Anatomical data				
Mesophyll thickness (μm)	122 ± 11	110 ± 20	−9.8	0.001
Number of cell layer	8.1 ± 0.7	8.6 ± 0.8	6.2	0.068
Interveinal distance (μm)	122 ± 19	109 ± 29	−10.7	0.027
Vascular bundle area (μm^2)	4,946 ± 1,251	3,538 ± 1,568	−28.5	0.001
Sub-stomatal cavity area (μm^2)	742 ± 271	761 ± 344	2.6	0.851
Palisade mesophyll cell area (μm^2)	374 ± 81	246 ± 153	−52.0	<0.0001
Spongy mesophyll cell area (μm^2)	226 ± 88	216 ± 85	−4.4	0.525
Mesophyll cell size (μm^2)	297 ± 57	231 ± 91	−22.2	<0.0001
Bundle sheath cell size (μm^2)	487 ± 225	459 ± 170	−5.8	0.446
Mesophyll:bundle sheath (cell area ratio)	0.79 ± 0.42	0.64 ± 0.37	−20.0	0.093
%Mesophyll	51.9 ± 7.4	45.2 ± 7.7	−12.9	0.010
%Bundle sheath	28.1 ± 5.3	27.8 ± 5.8	−1.1	0.872
Mesophyll:bundle sheath (tissue ratio)	1.9 ± 0.5	1.7 ± 0.6	−10.5	0.270
Cell wall thickness (μm)	0. 6 ± 0.1	0.4 ± 0.1	−31.5	0.031
Chloroplast and mitochondria data				
Chloroplast length (μm)	6.1 ± 1.2	8.9 ± 0.9	45.9	<0.0001
Chloroplast width (μm)	1.4 ± 0.2	2.1 ± 0.2	50.0	<0.0001
Area of chloroplast profile (μm^2)	6.3 ± 1.1	15.2 ± 1.4	141.3	<0.0001
Number of chloroplasts per cell	8.8 ± 2.6	11.8 ± 2.4	22.7	0.240
Number of plastoglobuli per chloroplast	8.6 ± 1.1	9.9 ± 1.8	15.3	0.151
Mitochondrial area (μm^2)	0.17 ± 0.04	0.26 ± 0.06	52.9	0.01

Values given are mean ± standard deviation. Mean values were compared by Student's t test at $P < 0.05$ level. The leaf mesophyll thickness was measured between epidermal layers at eight points in each cross-section and the interveinal distance was measured between two adjacent vascular bundles. The length of chloroplast is the longest dimension, and the width of chloroplast is the widest dimension

not influence stomatal index (Beerling and Chaloner 1993; Reddy et al. 1998; Xu and Zhou 2005; Xu et al. 2009a, b). However, several studies showed that warming had no effect on stomatal density but affected stomatal index due to the changes in epidermal cell density (Beerling and Chaloner 1993; Ferris et al. 1996). Moreover, no changes in both stomatal density and stomatal index were observed under warming conditions in some other studies (Apple et al. 2000; Hovenden 2001; Kouwenberg et al. 2007; Fraser et al. 2009). In our study, the artificial warming significantly increased stomatal index, but had no effect on stomatal density on both the adaxial and abaxial leaf surfaces of maize plants, indicating that the increased stomatal index mainly resulted from the reduction in epidermal cell density, given that the stomatal density was not affected. These results suggested that artificial warming may have directly affected the differentiation and expansion of epidermal cells, but had little effect on the initiation of the number of stomata during ontogenesis. Moreover, we also found that stomatal aperture area was statistically increased by elevated temperature. These results suggested that the stomata of maize leaves in response to global warming may stimulate not only stomatal index but also stomatal aperture area. In addition, leaf stomatal conductance (g_s) and transpiration rate (E) are mostly dependent on stomatal aperture size and stomatal frequency including stomatal density and stomatal index (Buckley et al. 1997; Hetherington and Woodward 2003; Franks and Beerling 2009; Franks et al. 2009). Our results also showed that artificial warming substantially increased g_s and E by 163 and 81 % possibly due to the increases of stomatal index and stomatal aperture size of maize leaves.

Previous studies have not found consistent results on leaf photosynthetic rates in response to warming. Several studies reported no changes in leaf gas exchange rates (Wookey et al. 1994; Nijs et al. 1996; Loik et al. 2000;
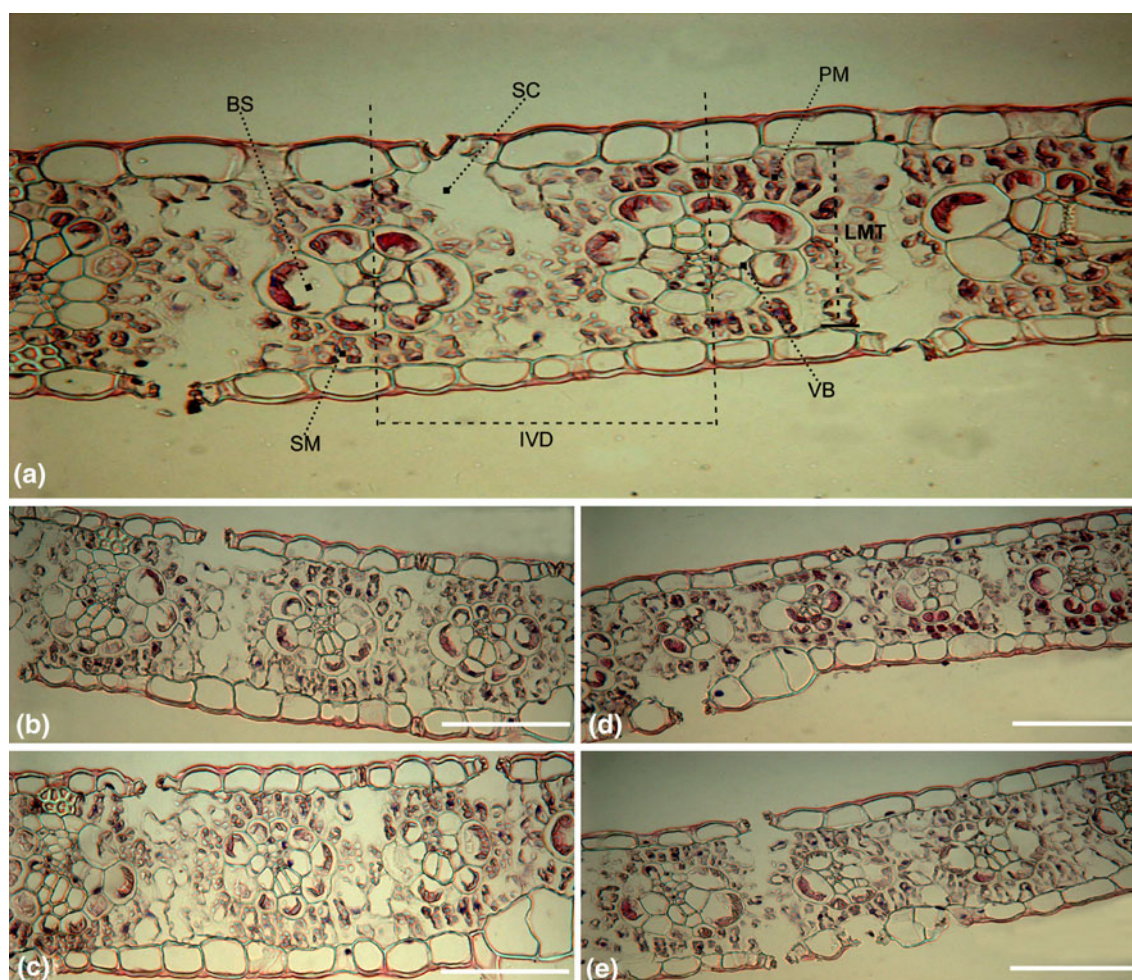
Fig. 2 Light micrographs of cross-section through leaves of maize grown at ambient temperature (**a**–**c**) and elevated temperature (**d**, **e**). Note that more and small bundle sheaths were observed in leaves of plants grown in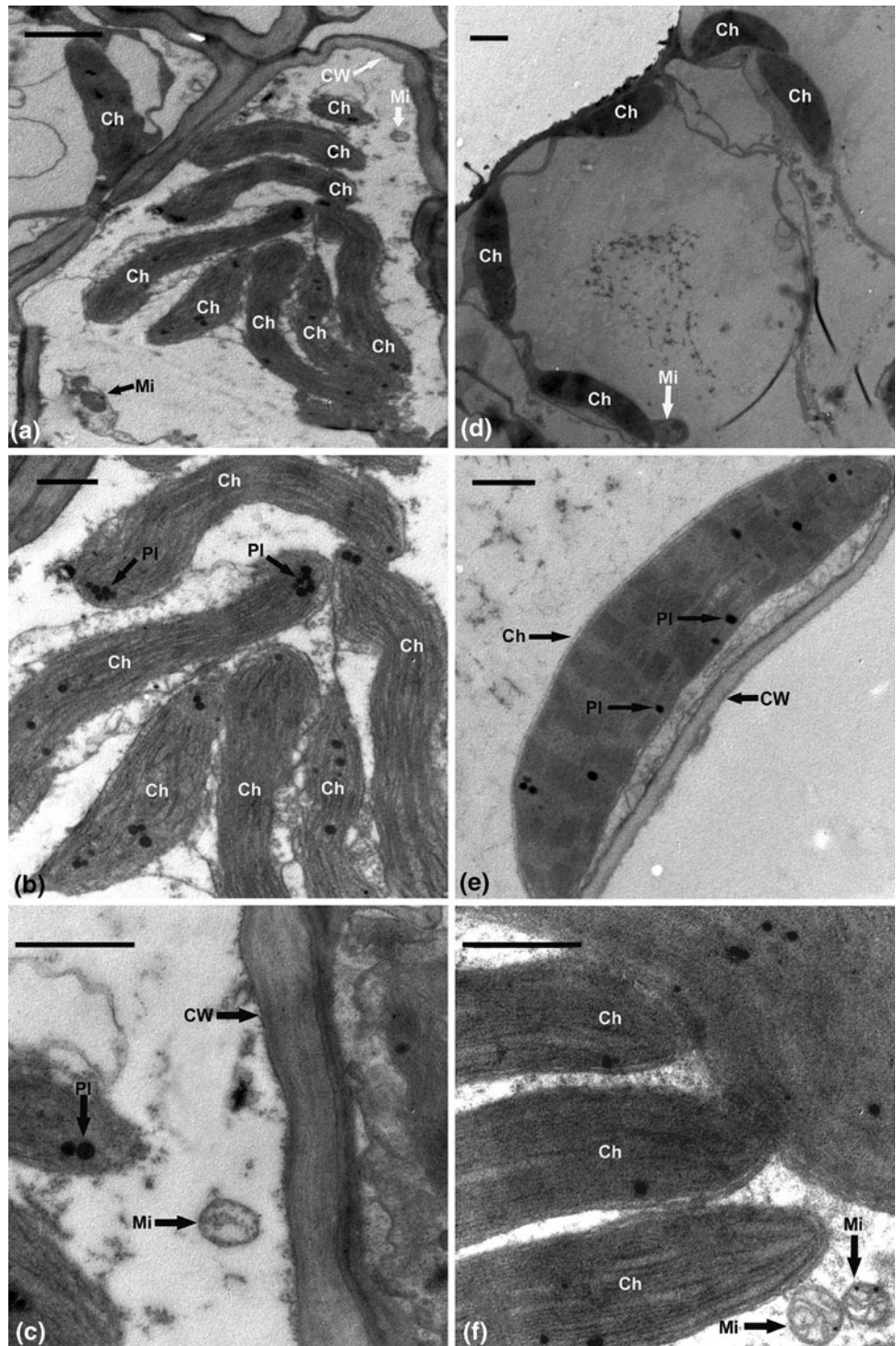 elevated temperature. Moreover, elevated temperature decreased leaf thickness. *BS* bundle sheath, *SC* substomatal cavity, *PM* palisade mesophyll, *SM* spongy mesophyll. *Bar* 100 μm (**e**)

Starr et al. 2000; Llorens et al. 2004a, b), whereas others found deceases (Callaway et al. 1994; Roden and Ball 1996; Djanaguiraman et al. 2011) or increases in leaf photosynthetic rates (Chapin and Shaver 1996; Apple et al. 2000; Yin et al. 2008; Han et al. 2009; Prieto et al. 2009a; Jin et al. 2011). However, comparisons among studies are difficult because of the different characteristics of warming treatments among the experiments and the different temperature sensitivities and optimal temperatures for photosynthesis among species and ecotypes (Shaw et al. 2000; Niu et al. 2008). Moreover, leaf photosynthetic rates in response to warming are often strongly affected by other interacting factors such as nutrient availability, the internal plant water status and the water vapor pressure difference between the leaf and the surrounding air (Chaves 1991; Llorens et al. 2004a). Our results that artificial warming stimulated the net photosynthesis rates of maize plants by 52 % support conclusions from previous reports that global warming may increase plant photosynthesis and growth (Huxman et al. 1998; Niu et al. 2008; Yang et al. 2011). Indeed, we found that the enhancement of leaf photosynthesis might be explained by the significant increases in the chloroplast length, chloroplast width, and chloroplast profile area though the number of chloroplasts per cell was not affected. This is also confirmed by our results that warming significantly increased leaf stomatal conductance (Jin et al. 2011). These results suggested that the increased maize photosynthetic rates in the warming plots were attributed to the increased chloroplast profile area and stomatal conductance. It should be noted that the maize plants in the current study were well watered and fertilized, thus they had the optimal growth conditions. However, it is possible that the photosynthetic response to increased temperature may be negative under the nitrogen or other nutrients deficit conditions. In addition, artificial warming resulted in the maize plants characterizing higher photosynthesis,

Fig. 3 Transmission electron micrographs showed chloroplast, mitochondria, and cell wall ultrastructure of maize leaves grown under ambient temperature (**a**–**c**) or elevated temperature (**d**–**f**). Note that larger chloroplasts and mitochondria were observed in leaves of plants grown under elevated temperature. In addition, elevated temperature decreased the thickness of cell wall. *Ch* chloroplast, *Mi* mitochondria, *CW* cell wall, *Pl* plastoglobuli. *Bar* 2 μm (**a**, **d**) or 1 μm (**b**, **c**, and **e**–**f**)

bigger stomatal aperture size and stomatal index, and higher leaf transpiration, which may be disadvantageous for maize plants when the periods of drought occurred simultaneously with elevated temperature.

Many of the most productive crops such as maize use the C_4 photosynthetic pathway, which offers C_4 plants the potential to achieve higher rates of leaf photosynthesis and more efficient use of water and nitrogen than C_3 plants (Osborne and Freckleton 2009; Taylor et al. 2012). In comparison with C_3 plants, the higher photosynthetic capacity of C_4 plants is mainly due to their unique mode of CO_2 assimilation, featuring strict compartmentation of photosynthetic enzymes into two distinct cell types, mesophyll and bundle sheath (Wang et al. 2009). In

Table 3 Content of carbohydrates in leaves of maize grown at ambient temperature (control) or elevated temperature (warming)

Carbohydrates (mg g^{-1} DW)	Control	Warming	% Increase	P value
Glucose	16.2 ± 2.7	20.2 ± 1.8	24.9	0.048
Fructose	11.4 ± 1.8	17.4 ± 0.9	52.8	0.003
Sucrose	5.1 ± 1.1	9.4 ± 1.9	83.0	0.009
Soluble sugars	32.7 ± 4.6	47.0 ± 1.4	43.7	0.001
Starch	37.5 ± 4.3	45.0 ± 3. 6	20.0	0.080
Total non-structural carbohydrates	69.9 ± 5.2	91.3 ± 4.5	30.8	0.006

Values given are mean ± standard deviation. The ear leaves of maize plants were sampled and mixed for the determination of carbohydrates. Mean values ($n = 5$ samples, with five plants per sample) were compared by Student's t test. Total non-structural carbohydrates = soluble sugars + starch

DW dry weight

Table 4 Nutrient concentration in leaves of maize grown at ambient temperature (control) or elevated temperature (warming)

Element (mg g^{-1} DW)	Control	Warming	% Increase	P value
C	436 ± 3	439 ± 3	0.8	0.179
N	28.5 ± 1.6	26.4 ± 0.7	−7.4	0.058
P	2.9 ± 0.4	2.5 ± 0.1	−13.8	0.110
K	12.7 ± 2.2	12.0 ± 1.3	−5.5	0.618
Ca	6.5 ± 0.8	5.4 ± 1.8	−17.0	0.249
Mg	6.9 ± 1.6	5.1 ± 1.6	−26.8	0.077
C:N	15.4 ± 0.7	16.6 ± 0.3	7.8	0.028

Values given are mean ± standard deviation. The ear leaves of maize plants were sampled and mixed for the determination of carbohydrates. Mean values ($n = 5$ samples, with five plants per sample) were compared by Student's t test

DW dry weight

addition, the higher photosynthetic capacity of C_4 plants can also be attributed to the higher CO_2 concentration and the limitation of photorespiration in the leaves of C_4 plants. In this study, we also found that warming significantly increased net CO_2 assimilation rates (52.3 %), which may result from the increases in the chloroplast size, stomatal index, and stomatal conductance. However, artificial warming increased the soluble sugar concentration in maize leaves, which may be a signal to decrease photosynthesis. Moreover, our results that artificial warming decreased the palisade mesophyll cell area by 52 % also suggested a reduction of photosynthesis of maize plants. Therefore, an opposite trends in photosynthesis and growth may be observed with warming experiments continued for several vegetative seasons. In addition, the enhancement in C_4 photosynthesis efficiency may increase the above-

ground biomass accumulation of C_4 plants (Luo et al. 2009). For example, Luo et al. (2009) showed that artificial warming stimulates above-ground biomass accumulation through enhancing C_4 dominance in a North America tallgrass prairie. However, a recent study from the same warming experiment as the current study found that artificial warming increased the above ground biomass but had little effect on the yield of winter wheat. Therefore, our results suggested that future global warming may affect the contribution of agroecosystems to CO_2 sequestration in a warmer world.

It is reported that warming decreased the thickness of plant leaves (Luomala et al. 2005; Jin et al. 2011; Yang et al. 2011). For example, Jin et al. (2011) found that elevated temperature decreased the leaf thickness by 8 % in *A. thaliana*. Similarly, Yang et al. (2011) observed that warming decreased leaf thickness by 10 % in one of the dominant alpine sedge (*Kobresia pygmaea*) in the Qinghai-Tibetan Plateau, China. In this study, our results also showed that artificial warming decreased the leaf thickness by c. 10 %, which was mainly due to the thinner mesophyll tissue in maize leaves under elevated temperature. This thinner mesophyll tissue was mainly attributable to the decreased mesophyll cell size since the number of cell layer of mesophyll tissue was not changed. Moreover, warming significantly decreased palisade mesophyll cell area by 52 %, but had little effect on the spongy mesophyll cell area. These results showed that warming may decrease leaf thickness mainly via reducing the palisade mesophyll cell area of maize leaves. In addition, our results showed that warming decreased interveinal distance and vascular bundle area by c. 10 and 30 %, respectively, (Table 2). We also observed that maize leaves in elevated temperature have more and smaller vascular bundles than those in ambient temperature (Fig. 2). Similar results have been reported recently by Yang et al. (2011), who found that warming significantly decreased the width and length of the vascular bundle by 18 and 22 %, respectively. Our results suggested that more and smaller vascular bundles per unit leaf area may be more efficient for CO_2 assimilation under warmer conditions. Therefore, the stimulated photosynthetic rate may be also attributed to the decreases in the number and size of the vascular bundles in maize leaves.

The concentration of non-structural carbohydrates generally decreases in plants grown under warm conditions due to the accelerated leaf respiration (Zha et al. 2001; Tingey et al. 2003; Jin et al. 2011; Wang et al. 2012). Interestingly, in this study, we found that artificial warming significantly increased the TNC concentration, especially the concentration of soluble sugars including glucose, fructose, and sucrose. Similarly, a recent study also showed that artificial warming increased the concentration of

soluble sugar in soybean leaves (Djanaguiraman et al. 2011). In the current study, we found that artificial warming increased photosynthesis but decreased R_d (Table 1), thus leading to the carbohydrate accumulation in maize leaves (Table 4). Our results also suggest that warming may alter the sink-source balance, thus changing the carbon allocation in maize plants (Eamus and Jarvis 1989; Körner et al. 1995).

Our result that warming significantly increased the leaf C:N ratio suggests that the maize plants may become N limited in the NCP under future global warming. Other mineral nutrients, such as K, Ca and Mg, also have similar trends though their leaf concentrations were not statistically different between the warming and the control plots. Our results were supported by previous studies that warming will increase foliar C:N ratio in trees (Tjoelker et al. 1999; Olszyk et al. 2003; Wang et al. 2012), shrubs (Tolvanen and Henry 2001; Biasi et al. 2008a, b; Sardans et al. 2008b), and grasses (Yang et al. 2012). In addition, we found that warming had little impact on foliar P concentration of maize plants (Table 4). This result was in accordance with Sardans et al. (2008b), who found that warming had little effect on foliar P concentration in a shrub species *Globularia alypum*. However, Peñuelas et al. (2004) found that warming decreased leaf P concentration by 11 % in *G. alypum*. By contrast, Sardans et al. (2008a, b) reported that warming increased P concentration by 42 % in *Erica multiflora* leaves. These results suggest that the warming effects on foliar P concentration are more complicated and further studies are needed to clarify the differences.

There are many warming methods for simulating climatic warming in field conditions including greenhouse, open-top chamber, soil heating pipes and cables, infrared reflector, and infrared radiator (Niu et al. 2007). In this study, we examined the warming effects on physiological, biochemical, and structural characteristics of maize leaves with infrared radiators in northern China. It is important to note that the warming effects from the suspended infrared radiators on soil temperature and canopy temperature may be changed during the experiment, because the warming effects may also dependent on the increase of maize growth and plant size within the artificial period. The growing maize plants are closer and closer to the radiators and simultaneously the soil surface was more and more shaded during the artificial period, which may result in artifacts of warming effects on the characteristics of maize leaves. Therefore, in terms of the increase of soil temperature, the soil was heated with the method of heating pipes and cables may be better for controlling the soil temperature.

Author contribution Y. Zheng and M. Xu designed the experiment and wrote the manuscript. Y. Zheng and R. Shen conducted the experiment. Y. Zheng and S. Qiu analyzed data.

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