Warming and elevated CO$_2$ alter the transcriptomic response of maize (Zea mays L.) at the silking stage

Yulan Huang$^{1,5}$, Rui Fang$^{2,5}$, Yansheng Li$^2$, Xiaobing Liu$^2$, Guanghua Wang$^2$, Kuide Yin$^{1,4}$, Jian Jin$^{2,3,5}$ & Stephen J. Herbert$^4$

Exploring the transcriptome of crops in response to warming and elevated CO$_2$ (eCO$_2$) is important to gaining insights of botanical adaption and feedback to climate change. This study deployed Illumina sequencing technology to characterize transcriptomic profile of maize plants at the silking stage, which were grown under warming (2 °C higher than ambient temperature) and eCO$_2$ (550 ppm) conditions. The treatment of ambient temperature and ambient CO$_2$ concentration was considered as control (CK). Warming, eCO$_2$ and warming plus eCO$_2$ resulted in 2732, 1966 and 271 genes expressing differently (DEGs) compared to the CK, respectively. Among the DEGs, 48, 47 and 36 gene ontology (GO) terms were enriched in response to warming, eCO$_2$ and warming plus eCO$_2$ compared to the CK, respectively. The majority of genes were assigned to the biological process category and the cellular component category. Elevated CO$_2$ significantly inhibited gene expressions in terms of photosynthesis and carbohydrate biosynthesis pathways. Warming not only negatively affected expressions of these genes, but also secondary pathways of nitrogen (N) metabolism, including key enzymes of GST30, GST7, GST26, GST15, GLUL and glnA. These results indicated the negative biochemical regulation and physiological functions in maize in response to warming and eCO$_2$, highlighting the necessity to improve the genetic adaptability of plant to future climate change.

The increase in atmospheric concentration of CO$_2$ is the main driver of global warming. According to the Intergovernmental Panel on Climate Change\textsuperscript{1}, atmospheric CO$_2$ concentration has exceeded 400 parts per million (ppm) with a consequence of global temperature increase by 0.85 °C since the industrial revolution. The atmospheric CO$_2$ concentration is predicted to reach 500–700 ppm by 2050 and 650–1200 ppm by 2100, which is likely to contribute to a global temperature increase of 2 to 4 °C by the end of this century\textsuperscript{2}.

Crop production is likely to be influenced by elevated CO$_2$ (eCO$_2$) and global warming. In general, eCO$_2$ stimulates photosynthesis and increases yield in C3 crops while the effect may become less pronounced in C4 crops such as maize\textsuperscript{3} due to greater CO$_2$ concentration inside the bundle sheath cells than atmospheric CO$_2$ saturating Rubisco\textsuperscript{4}. The yield response of maize to eCO$_2$ are not consistent among studies with positive\textsuperscript{5–7} or no effect\textsuperscript{8}. However, the growth of maize plants may be sensitive to temperature. Using models, Hatfield et al. (2016) predicted that every 2 °C increase may result in yield reduction of up to 8% in the Corn Belt region, U.S\textsuperscript{9}. In north-west India, Abebe et al. (2011) found that the 1.5 °C rise of temperature decreased maize yield by 15%\textsuperscript{7}.

A better understanding the mechanisms of eCO$_2$ and warming interactive effect on maize yield requires explorations at the genetic level, linking the gene-manipulated physiological functions to plant growth and productivity under climate change. High through-put transcriptome profiling has been widely deployed to identify genes in soybean or maize, which are differentially expressed in response to those environmental factors\textsuperscript{10–12}. There were total of 1390 genes in the maize leaf either that were up- or down-regulated during 14 days of exposure to eCO$_2$\textsuperscript{10}. However, to our knowledge, very few studies have been performed regarding the transcriptome analysis on maize in response to warming plus elevated CO$_2$.

With the RNA sequencing technology, we investigated the gene expression profile of maize in response to eCO$_2$ and warming at the silking stage which is critical to yield formation. We hypothesized that the expression

\textsuperscript{1}College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Daqing, 163319, China. \textsuperscript{2}Key Laboratory of Mollisols Agroecology, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin, 150081, China. \textsuperscript{3}Centre for AgriBioscience, La Trobe University, Melbourne Campus, Bundoora, Vic, 3086, Australia. \textsuperscript{4}Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA, 01003, USA. \textsuperscript{5}These authors contributed equally: Yulan Huang and Rui Fang. \textsuperscript{*email: yinkuide@163.com; jinjian29@hotmail.com}

\textsuperscript{1}The increase in atmospheric concentration of CO$_2$ is the main driver of global warming. According to the Intergovernmental Panel on Climate Change\textsuperscript{1}, atmospheric CO$_2$ concentration has exceeded 400 parts per million (ppm) with a consequence of global temperature increase by 0.85 °C since the industrial revolution. The atmospheric CO$_2$ concentration is predicted to reach 500–700 ppm by 2050 and 650–1200 ppm by 2100, which is likely to contribute to a global temperature increase of 2 to 4 °C by the end of this century\textsuperscript{2}.

\textsuperscript{2}Crop production is likely to be influenced by elevated CO$_2$ (eCO$_2$) and global warming. In general, eCO$_2$ stimulates photosynthesis and increases yield in C3 crops while the effect may become less pronounced in C4 crops such as maize\textsuperscript{3} due to greater CO$_2$ concentration inside the bundle sheath cells than atmospheric CO$_2$ saturating Rubisco\textsuperscript{4}. The yield response of maize to eCO$_2$ are not consistent among studies with positive\textsuperscript{5–7} or no effect\textsuperscript{8}. However, the growth of maize plants may be sensitive to temperature. Using models, Hatfield et al. (2016) predicted that every 2 °C increase may result in yield reduction of up to 8% in the Corn Belt region, U.S\textsuperscript{9}. In north-west India, Abebe et al. (2011) found that the 1.5 °C rise of temperature decreased maize yield by 15%\textsuperscript{7}.

\textsuperscript{3}A better understanding the mechanisms of eCO$_2$ and warming interactive effect on maize yield requires explorations at the genetic level, linking the gene-manipulated physiological functions to plant growth and productivity under climate change. High through-put transcriptome profiling has been widely deployed to identify genes in soybean or maize, which are differentially expressed in response to those environmental factors\textsuperscript{10–12}. There were total of 1390 genes in the maize leaf either that were up- or down-regulated during 14 days of exposure to eCO$_2$\textsuperscript{10}. However, to our knowledge, very few studies have been performed regarding the transcriptome analysis on maize in response to warming plus elevated CO$_2$.

\textsuperscript{4}With the RNA sequencing technology, we investigated the gene expression profile of maize in response to eCO$_2$ and warming at the silking stage which is critical to yield formation. We hypothesized that the expression
of genes regulating the photosynthetic pathway might be inhibited by warming, but eCO₂ might alleviate this negative trend. The results would provide novel data on the biochemical regulation and physiological functions in maize in response to the two environmental factors, which will help to develop better management strategies for crops adapting to future climate changes.

Materials and Method
Experimental design and plant growth. A pot experiment was conducted in open-top chambers (OTC) at the Northeast Institute of Geography and Agroecology (45°73′N, 126°61′E), Chinese Academy of Sciences, Harbin, China. The experimental design was a random block design with two atmospheric CO₂ concentrations (ambient CO₂ and eCO₂) and two temperature levels (ambient temperature and warming). Thus, there were four treatments in total, i.e. ambient CO₂ and ambient temperature as control (CK), warming only, eCO₂ only, and warming plus eCO₂. Each treatment had three replicates. Thus, there were 12 octagonal OTCs. Each OTC had a steel frame body with 2.0 m high and a 0.5 m high canopy, which formed a 45° angle with plane. The OTCs were covered with polyethylene film. The transparency of the film was tested with a portable quantum sensor (MQ-100, Apogee Instruments, Inc. USA) to determine photosynthetically active radiation (PAR), of which the wave length ranges from 409 to 659 nanometers. The photosynthetic photon flux measured inside OTC was more than 95% of the full photo flux outside, indicating that the film cover won’t affect plant photosynthesis in this study. A number of CO₂-associated studies used similar designs of OTC. A digital CO₂-regulating system (Beijing VK2010, China) was installed to monitor the CO₂ level inside OTCs and automatically regulated the supply of CO₂ gas (99.9%) through CO₂ cylinders to achieve CO₂ concentrations at 550±30 ppm for eCO₂ and at 400±30 ppm for ambient CO₂. An electronic pump was installed at the ground level in each OTC to circulate inner air through a temperature-regulating conditioner system to achieve the OTC-inner temperature as same as ambient temperature or 2±0.5°C higher than ambient temperature. The temperature records during plant growth were presented in Fig. S1.

Soil used in this study was collected at Puyang in Henan province (116°52′E, 35°20′N), China. The soil type is Fluvisol. The soil had 18.0 g kg⁻¹ of organic C, 1.1 g kg⁻¹ of total N, and a pH (1:5 in 0.01 M CaCl₂) 5.8. The soil was air-dried under a shade and then sieved (<4 mm). Thirteen kg of the soil was loaded into each pot (30 cm diameter, 35 cm height). Basal nutrients were added into soil at the following rates: 100 mg N kg⁻¹ as urea, 50 mg P kg⁻¹ and 63 mg K kg⁻¹ as KH₂PO₄ and 45 mg Ca kg⁻¹ as CaCl₂·2H₂O. The rates of other nutrients were as follows: 4.2 mg Mg kg⁻¹ as MgSO₄·7H₂O, 1.7 mg Fe kg⁻¹ as Fe-EDTA, 2.2 mg Mn kg⁻¹ as MnSO₄·H₂O, 2.4 mg Zn kg⁻¹ as ZnSO₄·2H₂O, 2.0 mg Cu kg⁻¹ as CuSO₄, 0.12 mg B kg⁻¹ as H₃BO₃, 0.03 mg Co kg⁻¹ as CoCl₂·6H₂O, and 0.08 mg Mo kg⁻¹ as Na₂MoO₄·2H₂O. All pots were allocated into respective OTCs before sowing. Three maize seeds (Zea mays L. cv. Xiangyu 998) were sown in each pot and then thinned to 1 plant 10 days after emergence. Soil water content was maintained daily at 80±5% of field capacity by weighing and watering.

Measurement. At the silking stage (55 days after emergence), approximately 1 g of the top fully extended leaf was sampled from each pot. In particular, the sampling positions were on both sides of the main leaf vein about 20 cm away from leaf tip. The sampling area was similar across plants with about 4×7 cm². Leaf samples were immediately frozen in liquid N and stored at −80°C for the RNA extraction. Before RNA extraction, leaf samples were homogeneously ground in liquid N. For chlorophyll measurement, 1 g of fresh leaf on the same position near of each leaf was sampled and ground in alcohol and acetone mixture (1:1). The leaf chlorophyll a and b concentrations in the supernatant of the solution was measured using a spectrophotometer at 663 and 645 nm, respectively. Shoots were also cut at this time at ground level. Then the root system was separated from soil, and washed with tap water to remove soil and sand particles adhering to the roots. Shoot and root samples were oven-dried at 105°C for 30 min, and then dried at 80°C for three days. The dry biomass of shoot and root were weighed and recorded. The dried plant samples were ground using a ball mill (Retsol MM2000, Retsch, Haan, Germany) and N concentration was determined by an ELEMENTAR III analyzer (Hanau, Germany).

RNA extraction, cDNA library construction and sequencing. The leaf RNA was extracted using a RNeasy® Plant Mini kit (Qiagen, Shanghai, China) according to the manufacturer’s instructions. A cDNA library was constructed using a TruSeq™ RNA Sample Preparation Kit (Illumina, Inc.) and sequenced using an Illumina HiSeq. 2000 platform at Shanghai Majorbio Biopharm Technology Co., Ltd (Shanghai, China). Quality control was performed to eliminate low-quality reads, adaptor polluted reads and ambiguous Ns reads.

Gene functional annotation and expression analysis. The quality-controlled sequencing data were aligned to the maize genome (NCBI BioProject Accession NO. PRJNA10769). The identification of differentially expressed genes (DEGs) was detected with the Bioconductor package “edgeR” between CK and any other treatments. The different expression was indicated with fold-change (FC) ≥ 1 and the screening criteria were used as the screening criteria. The gene expression among functional groups was analyzed by the Wilcoxon signed-rank test and the influence of CO₂ and temperature at the transcriptomic level was evaluated. The P value ≤ 0.05 was considered as statistical significance.

Differentially expressed genes were further processed with Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. GO and KEGG analyses were performed to identify which DEGs were significantly enriched in GO terms or metabolic pathways. Based on Wallenius non-central hyper-geometric distribution, GO enrichment analysis of the DEGs was conducted using GOSep R packages. GO terms with P value ≤ 0.05 were considered significantly enriched among the DEGs. The enriched DEGs in KEGG pathways were analyzed with the KOBAS (v2.0.12) software. A P value < 0.05 also was the threshold for DEGs in KEGG pathways. Plant MetGenMAP, a web-based system was used to assign DEGs to metabolic pathways. Sequences of selected unigenes were aligned within the Plant Transcription Factor Database v3.0.
Gene expression verification by quantitative real time PCR (qRT-PCR). According to RNA-Seq data, 8 DEGs were randomly selected to verify expressions in sequencing via the qRT-PCR. Selected genes and primers were shown in Table S1. Total RNA (0.3 μg) from each selected gene was treated with gDNA wiper mix and translated into the first strand cDNA, which was stored at −20°C for the subsequent analysis. All qRT-PCRs were performed in three technical replicates in following steps: pre-denaturation for 5 min at 95°C and 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 56°C and extension for 1 min at 72°C. Outliers were manually discarded. The housekeeping gene GAPDH was used as internal standard to estimate the relative expression. Then the gene expressions were standardized to transcript levels for PtACTIN calculated with the 2−ΔΔct method.

Statistical analysis. Data were analysed with Genstat 13 (VSN International, Hemel Hemsplead, UK) including plant biomass, N concentration, chlorophyll concentration, DEGs and KEGG enrichment. One-Way ANOVA tests were used to assess the differences between treatments at significant levels at P < 0.05 and P < 0.01.

Results

Plant biomass, N and chlorophyll concentrations. Neither warming nor eCO2 significantly influenced the shoot dry biomass. A similar result was found in root dry biomass (Table 1). The leaf N concentration was not affected by warming or eCO2, but warming plus eCO2 resulted in a significant increase in N concentration compared with the CK. Similar trend was found in stem N concentration. Warming did not affect the root N concentration. However, the concentrations of chlorophyll a and chlorophyll a + b were decreased by 20.3% and 18.1% in response to warming compared to the CK. Elevated CO2 did not affect chlorophyll concentrations (Table 1).

RNA-seq and transcriptomic profile. To comprehensively investigate the transcriptomic and gene expression profiles of maize under eCO2 and warming, three cDNA samples extracted from leaves were prepared and sequenced using Illumina NovaSeq. An overview of the RNA-Seq reads of libraries was presented in Table S2. After the low-quality reads were removed, 29 Gb clean reads were obtained with an average of 7.3 Gb reads for each sample, and the proportion of Q30 was greater than 93.33%, which indicates that the sequencing results were highly accurate.

With the Trinity program, the total of 190,928 transcripts were obtained from clean reads. Among them, the length of 99,410 transcripts was more than 1800 bp. The length distribution of transcripts was given in Table S2, suggesting that the sequencing assembly was ideal. The average correlation between the replicates in each treatment reached 0.901. The variation of transcription among replicates was marginally acceptable due to the sampling size of leaf and the influence of other environmental factors such as temperature change during the day.

Analysis of DEGs and GO annotation. Wilcoxon signed-rank test showed that the ratio of DEGs was not more than 1% of the total number of genes in maize. Warming, eCO2 and warming plus eCO2 caused 2732, 1966 and 271 DEGs compared to the CK, respectively (Fig. 1A). In particular, warming resulted in 2476 up-regulated and 274 down-regulated DEGs, and eCO2 led to 741 up-regulated and 1225 down-regulated DEGs. However, only 141 up-regulated and 130 down-regulated DEGs were observed for warming plus eCO2 (Fig. 1B). These results indicated that warming and eCO2 interactively reduced the expression of numerous genes in maize.

Among the DEGs, 48, 47 and 37 GO terms were enriched in warming, eCO2, eCO2 + warming compared with the CK, respectively, which were mainly in biological process, cellular component and the molecular function categories (Fig. 2). In the biological process category, warming and eCO2 resulted in 1008 and 749 enriched DEGs respectively, but only 102 for eCO2 plus warming. In the cellular component category, there were 691 and 499 of DEGs for warming and eCO2, respectively, while only 59 for eCO2 plus warming. Regarding molecular function, the numbers of DEGs reached 939 and 673 for warming and eCO2, respectively, but only 101 for warming plus eCO2.

KEGG enrichment analysis. To further assess the biological functions of these DEGs, pathway-based analysis was performed using KEGG. Compared to the CK, warming, eCO2, and warming plus eCO2 significantly enriched 14, 15, and 3 pathways (Fig. 3), respectively. The warming-induced enrichment in metabolic pathways not only included photosynthesis, carbon fixation in photosynthetic organisms, and glyoxylate and dicarboxylate metabolism, but also the secondary metabolisms, such as N metabolism (Fig. 3A). Elevated CO2 mainly affected photosynthesis and carbohydrate metabolism pathways (Fig. 3B). Interestingly, only three metabolism pathways were enriched in response to warming plus eCO2. They were plant hormone signal transduction, glycolysis, serine, threonine, starch and sucrose metabolisms (Fig. 3C).

| Treatment | Shoot dry biomass (g plant^−1) | Root dry biomass (g plant^−1) | Leaf N concentration (mg g^−1) | Stem N concentration (mg g^−1) | Root N concentration (mg g^−1) | Chlorophyll a (mg g^−1 FW) | Chlorophyll b (mg g^−1 FW) | Chlorophyll a + b (mg g^−1 FW) |
|-----------|-------------------------------|-------------------------------|-----------------------------|-----------------------------|-----------------------------|---------------------------|---------------------------|-------------------------------|
| CK        | 286 ± 1.11a                   | 53.6 ± 0.96a                  | 15.1 ± 1.01b                | 9.03 ± 0.52ab               | 6.35 ± 0.45ab               | 11.8 ± 0.18a               | 53.6 ± 0.97a               | 16.6 ± 0.87a     |
| Warming   | 293 ± 5.04a                   | 47.3 ± 0.19a                  | 16.1 ± 0.37b               | 9.41 ± 0.33a               | 6.84 ± 0.73ab               | 9.4 ± 1.67b               | 47.3 ± 0.19a               | 13.6 ± 2.35b     |
| eCO2      | 317 ± 16.8a                   | 57.0 ± 7.25a                  | 14.1 ± 0.47b               | 7.68 ± 0.18b               | 5.63 ± 0.08b               | 12.3 ± 0.66a              | 57.0 ± 7.25a              | 17.2 ± 0.27a     |
| Warming + eCO2 | 301 ± 8.51a             | 52.2 ± 1.01a                  | 18.7 ± 0.03a               | 11.5 ± 0.49a               | 8.16 ± 0.4a                | 9.4 ± 1.18b               | 52.2 ± 1.01a              | 13.1 ± 1.37b     |

Table 1. Effects of warming and elevate CO2 (eCO2) on plant dry biomass, N concentration and chlorophyll concentrations at the silking stage. The data are means of three replicates ± standard deviation. Different letters indicate significant difference between treatments (P > 0.05). FW represents fresh weight.
Differential expression of photosynthesis-related genes. Compared to the CK, 22 DEGs involved in the photosynthesis pathways, including Photosystem II core complex proteins (psbY), Photosystem I reaction center subunit (psaK) and Photosystem I reaction center subunit N (psaN). Oxygen-evolving enhancer protein 1 (psbO) and Oxygen-evolving enhancer protein 3–1 (psbQ1) were identified as significant down-regulated genes under warming ($P < 0.05$ & $|\log_{2}FC| \geq 1$). Similarly, eCO$_2$ significantly altered these DEGs as well. However, eCO$_2$ plus warming resulted in only 6 down-regulated genes compared to the CK (Table S3).

Figure 1. Summary of differentially expressed genes (DEGs) in response to warming, elevated CO$_2$ (eCO$_2$) and warming + eCO$_2$ in a Venn chart (A) and up- and down-regulated DEGs in a bar chart (B).

Figure 2. Differentially expressed genes (DEGs) annotated against Gene Ontology (GO) function in response to warming, elevated CO$_2$ (eCO$_2$) and warming + eCO$_2$. The horizontal axis shows the secondary annotation of three primary categories in GO. The left vertical axis indicates the percentage of DEGs to the total annotated genes, and the right vertical axis indicates the number DEGs in this secondary classification of GO.
Differential expression of carbohydrate biosynthesis-related genes. Warming and eCO\textsubscript{2} had similar effects on carbohydrate biosynthesis-related genes (Fig. 4). Seventeen DEGs in carbohydrate metabolism pathways were significantly down-regulated in response to warming, such as genes responsible for glutathione S-transferase (GST 30), glucose-1-phosphate adenylyltransferase (glgC), fructose-bisphosphate aldolase (ALDO), pyruvate phosphate dikinase (ppdK), gluconokinase (gntK) and glutathione S-transferase (GST). Only two genes were up-regulated,
which were associated with pyruvate dehydrogenase (PDHB) and dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase (DLAT). These genes expressed the similar trends in response to eCO2 except for the chitinase activity associated genes. Only 7 genes responded to warming plus eCO2 (Table S4).

**Differential expression of primary and secondary metabolism-related genes.** In response to warming, 14 DEGs were significantly down-regulated, while 8 were up-regulated in primary and secondary metabolism pathways. Warming resulted in the down-regulated genes including 1-aminocyclopropane-1-carboxylate synthase activity (ACS), terpene synthase activity (TPS11), 12-oxophytodienoate reductase (OPR), cytokinin dehydrogenase (CKX) and lipoxygenase (LOX2S). However, eCO2 did not affect these genes. For the up-regulated genes, warming and eCO2 had similar effects. There were 10 DEGs, of which 7 were up-regulated expressions and 3 were down-regulated under warming plus eCO2 (Table S5).

**qRT-PCR verification for RNA-Seq.** To confirm the accuracy of the Illumina RNA-Seq results, 8 of the transcripts related to photosynthesis, sugar and secondary metabolism were selected for qRT-PCR (Table S1). The expression levels of these DEGs with qRT-PCR were compared to those of DEGs with RNA-Seq. A significant correlation (R² = 0.819) was observed between the RNA-Seq and qRT-PCR (Fig S3). The qPCR results were consistent with their transcript-abundance in RNA-seq, which verified the accuracy of DEGs from RNA-seq analyses in this experiment.

Figure 4. Summary of gene expressions in photosynthesis, sugar, primary and secondary metabolisms in response to warming, elevated CO2 (eCO2) and warming + eCO2. Annotated Genes and enzymes in the metabolic pathway are shown in green letters. C, W and CW represent eCO2, warming and eCO2 + warming, respectively.
Discussion

Atmospheric CO2 concentration and temperature are expected to be the most important factors impacting the crop production in agriculture. Although maize is the most widely produced crop worldwide, few studies have evaluated the interactive effects of CO2 and warming on transcriptome in relevant to photosynthesis and growth. In this study, we examined the gene expression profiles in transcriptome. Although the RNA expression may not directly alter the metabolism and hormonal metabolism at the silking stage of maize,41,42. Therefore, the plant growth response and relevant gene expression under warming in the pot experiment may differ from field.

The eCO2-induced inhibition of sugar metabolism was likely to contribute to the down-regulation of photosynthesis pathways. In this study, significant down-regulations in transcripts pertaining to PsaD, PsaE, PsaG, PsaK, PsaO subunit in PSI, and PsbY, Psb27, Psb28, PetE and PetF in PSI1 were found under eCO2. Chen et al.30 and Ainsworth et al.31 reported long-term exposure to eCO2 can reduce photosynthetic capacity of plants due to accumulation of carbohydrates in the leaf. As C4 crop species have the CO2-concentrating mechanism (CCM) in photosynthesis,32 leaf photosynthesis is closely correlated with the Rubisco. Genes encoding sub-units rbcS that are related with Rubisco were inhibited in this experiment. The eCO2-induced down-regulations of the expression of genes encoding for sub-units rbcS have been observed in other species such as wheat and pea.32–34. However, the link of photosynthetic capacity with the expression of gene rbcS needs further experimental test under eCO2. Moreover, the down regulation of PPDK may affect phosphoenolpyruvate, xaloacetate, and alanine in the C4-dicarboxylic acid cycle (Fig. 3), which is consistent with the findings by Zhang et al.35.

Warming also negatively affects the expressions of photosynthesis-associated genes (Fig. 3, Table S3), contributing to significant down-regulation in transcripts pertaining to PsAD, PsAF, PsAG, PsAK, PsaO subunit in PSI and PsBY, Psb27, Psb28, PetE and PetF in PSI1 (data not shown). Warming not only affects enzymes in all biochemical reactions associated with photosynthesis, but also the fluidity and integrity of the chloroplast membrane.43 At the current CO2 concentration, Rubisco activity is the main limiting factor for photosynthesis. A study has shown that increasing leaf temperature can affect the expression of Rubisco carboxylase and reduce Rubisco activity, which in turn affects the enzyme activity involved in electron transfer,44 supporting the results of this study. Although leaf temperature was not measured in this study, a significant relationship between leaf temperature of maize and air temperature (r = 0.948, P < 0.001) was found in a preliminary study. When the leaf temperature is beyond the optimum temperature range for plant growth, the balance between chlorophyll biosynthesis and catabolism is likely broken, resulting in reduce in leaf chlorophyll content.45,46. Thus, the increase of leaf temperature due to warming may inhibit the photosynthetic activities.47 These impaired photosynthetic metabolisms may cause the decrease in chlorophyll concentration of maize in response to warming (Table 1). In addition, warming also fundamentally affects secondary metabolisms such as galactose metabolism, and N metabolism. In particular, the glutamine synthetase (GS) is a key enzyme involved in plant N metabolism, which can catalyze glutamate to synthesize glutamine (Gln). Some Glns are substrates for the production of amino acids in plant.48 In this study, the expression of GST 30, GST7, GST26, GST15, GLUL and glnA were significantly down regulated under warming (Tables S4, S5), which may inhibit the N metabolism and the synthesis of amino acids. The slow-down N metabolism under warming may potentially lower N accumulation during the reproductive stage of maize (Fig. 3, Table S5). Research showed that warming reduced leaf N concentration of maize, which may be associated with photosynthetic acclimation.49 Therefore, the change in N metabolism under warming in this study might be another contributor to the reduction of gene expression in relevant to photosynthetic pathways. It is worthy to note that plant performance in pots in this study may be slightly different from the field condition, as the effect of warming on root and soil microbial activity, and subsequent nutrient mineralization and uptake by plants is likely stronger than the field condition. The air warming normally influence the surface soil only in the field. Moreover, the leaf N concentration in this pot experiment (Table 1) was lower than the critical N level in plant.50 The N deficiency may influence maize metabolisms in response to warming as previous studies showed that low N decreased the production of numerous proteins associated with C and N metabolism and hormonal metabolism at the silking stage of maize.51,52 Therefore, the plant growth response and relevant gene expression under warming in the pot experiment may differ from field.

In recent years, the interactive effects of warming and eCO2 on plant biology have drawn increasing attention.43–45. However, the number of DEGs in response to warming and eCO2 was much less than either the warming or eCO2 effect. This may be due to the trade-off effect between warming and eCO2 on plant physiological characteristics,46,47, and consequent non-effect on plants biomass (p > 0.05) (Table 1). Ruiz-Vera et al.53 also reported that there was no significant effect of warming and eCO2 on plant biomass of soybean. However, the reason for the trade-off of environmental factors on maize warrants further investigation, which is important to predict how future climate change may affect plant functions.
Conclusions
This study not only provided transcriptomic datasets of maize at the silking stage under warming and eCO2 conditions, but also new biological insights into the expression of genes associated with photosynthesis, carbohydrate biosynthesis and secondary pathways. As a summary diagram showed (Fig. 4), eCO2 significantly inhibited gene expressions involved in photosynthesis and carbohydrate biosynthesis pathways. Warming not only negatively affected these genes expression, but also secondary pathways such as N metabolism. The inhibition of these genes in response to warming and eCO2 may limit the plant adaptability to future climatic changes.

Received: 6 February 2019; Accepted: 4 November 2019;
Published online: 29 November 2019

References
1. IPCC. Climate change 2014 impacts, adaptation, and vulnerability. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1150 (2014).
2. Lin, E. et al. Climate change impacts on crop yield and quality with CO2 fertilization in China. *Philos Trans R Soc Lond B Biol Sci.* **360**, 2149–2154 (2005).
3. Ghanbourn, O., Von caemmerer, S., Ziska, L. H. & Conroy, J. P. The growth response of C4 plants to rising atmosphere CO2 partial pressure: a reassessment. *Plant Cell Environ.* **23**, 931–942 (2000).
4. Kürats, O., Peter, J. L. & Vincent, R. Franceschi, & Gerald, E. E. Bundle sheath diffusive resistance to CO2 and effectiveness of C4 photosynthesis and refixation of photorespired CO2 in a C4 cycle mutant and wild-type Amaranthus edulis. *Plant Physiol.* **130**, 964–976 (2002).
5. Leaky, A. D. B., Ainsworth, E. A., Bernacchi, C. J., Rogers, A. & Long, S. P. Elevated CO2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. J. Exp. Bot. **60**, 2859–2876 (2009).
6. Vanaja, M. Variability in growth and yield response of maize genotypes at elevated CO2 concentration. *Adv. Plants Agric. Res.* **2**, 42 (2015).
7. Abebe, A. et al. Growth, yield and quality of maize with elevated atmospheric carbon dioxide and temperature in north–west India. *Agr. Eco. Environ.* **218**, 66–72 (2016).
8. Kim, S. H., Dennis, C. G., Richard, C. S., Jeffery, T. B. & Dennis, J. T. Temperature dependence of growth, development, and photosynthesis in maize under elevated CO2. *Environ. Exp. Bot.* **64**, 224–236 (2007).
9. Hatfield, J. L., Boote, K. J. & Kimball, B. A. Climate impacts on agriculture: implications for crop production. *Agron. J.* **103**, 351–370 (2011).
10. Ge, Y. et al. Transcriptome analysis identifies differentially expressed genes in maize leaf tissues in response to elevated atmospheric [CO2]. J. Plant Interact. **13**, 373–379 (2018).
11. Ainsworth, E. A., Rogers, A., Vodkin, L. O., Walter, A. & Schurr, A. The effects of elevated CO2 concentration on soybean gene expression. An analysis of growing and mature leaves. *Plant Physiol.* **142**, 135–147 (2006).
12. Prins, A. et al. Acclimation to high CO2 in maize is related to water status and dependent on leaf rank. *Plant Cell Environ.* **34**, 314–331 (2001).
13. Zhang, W. W., Wang, G. H., Lai, X. B. & Feng, Z. H. Effects of elevated O3 exposure on seed yield, N concentration and photosynthesis of nine soybean cultivars (*Glycine max* (L.) Merr.) in Northeast China. *Plant Sci.* **226**, 172–181 (2014).
14. Chaturvedi, A. K., Bahuquna, R. N., Shah, D., Pal, M. & Jagdish, S. K. High temperature stress during flowering and grain filling in wheat and rice. *J. Exp. Bot.* **67**, 2699–2714 (2016).
15. Hu, Z. H. et al. Effectiveness of elevated CO2 in mediating bacterial communities in the soybean rhizosphere depends on genotypes. *Agr. Ecosyst. Environ.* **231**, 229–232 (2016).
16. Wu, unesco. Soil Map of the World, 1:5 000 000 vol. X. Australia. UNESCO, Paris (1976).
17. Yu, Z. H., Liu, S. J., Liu, X. B., Li, Y. S. & Wang, G. H. Carbon flux in the plant-soil-microbe continuum of different growth stages of maize grown in a Mollisol. *Agron. Agric. Soil Sci.* **63**, 362–374 (2017).
18. Strain, H. H. & Svec, W. V. Extraction, separation, estimation and isolation of the chlorophylls. *Agron. J.* **57**, 289–300 (1995).
19. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B (Methodological).* **57**, 289–300 (1995).
20. Leng, N., Dawson, J., Thomson, J., Ruotti, B. & Rissman, A. EBSeq: An empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics.* **29**, 1035–1043 (2013).
21. Mao, X. Z., Cai, T., Olyarchuk, J. G. & Wei, L. P. Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. *Bioinformatics.* **21**, 3787–3793 (2005).
22. Poussin, N., Boudjikan, T., Brisseau, A. & Olyarchuk, J. G. Plant MetGenMAP: an integrative analysis system for plant systems biology. *Plant Physiol.* **151**, 1758–1768 (2009).
23. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **39**, e45 (2011).
24. Steel, R. G. & Torrie, J. H. Principles and procedures of statistics: A biometrical approach. 2nd ed. *McGraw-Hill, New York* (1980).
25. Grabherr, M. et al. Full length transcriptome assembly from RNA Seq data without a reference genome. *Nature Biotech.* **29**, 644–652 (2011).
26. Ursulam, R. V., Mathew, H. S., David, W. D., Donald, R. O. & Carl, J. B. Canopy warming caused photosynthetic acclimation and reduced seed yield in maize grown at ambient and elevated [CO2]. *Glob. Change Biol.* **21**, 4237–4249 (2015).
27. Dermedy, O., Long, S. P., McConnaughey, K. & DeLuca, H. E. How do elevated CO2 and O3 affect the interception and utilization of radiation by a soybean canopy? *Glob. Chang. Biol.* **14**, 556–564 (2008).
28. Long, S. P., Ainsworth, E. A., Rogers, A. & Ort, D. R. Rising atmospheric carbon dioxide: plants FACE the future. *Ann. Rev. Plant Biol.* **55**, 591–628 (2004).
29. Ainsworth, E. A. Rice production in a changing climate: a metaanalysis of responses to elevated carbon dioxide and elevated ozone concentration. *Glob. Chang. Biol.* **14**, 1642–1650 (2008).
30. Chen, G. Y. et al. Photosynthetic acclimation in rice leaves to free-air CO2 enrichment related to both ribulose-1, 5-bisphosphate carboxylation limitation and ribulose-1, 5-bisphosphate regeneration limitation. *Plant Cell Physiol.* **46**, 1036–1045 (2005).
31. Ainsworth, E. A. & Long, S. V. What have we learned from 15 years of free-air CO2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO2. *New Phytol.* **165**, 351–372 (2005).
32. Yin, X. Y., Peter, E. L., Putten, V. D., Steven, M. D. & Struik, C. Temperature response of bundle-sheath conductance in maize leaves. *J. Exp. Bot.* **67**, 2699–2714 (2016).
33. Takatani, N. et al. Effects of high CO2 on growth and metabolism of Arabidopsis seedlings during growth with a constantly limited supply of nitrogen. *Plant Cell Physiol.* **55**, 282–292 (2014).
34. Zhang, X. Y., et al. Characterization of maize leaf pyruvate orthophosphate dikinase using high throughput sequencing. *Journal of Integrative Plant Biol.* **60**, 670–690 (2018).
35. Way, D. A., Oren, R. & Kroner, Y. L. The space-time continuum: the effects of elevated CO2 and temperature on trees and the importance of scaling. *Plant Cell Environ.* **38**, 991–1007 (2015).
36. Crous, K. Y. et al. Photosynthesis of temperate Eucalyptus globulus trees outside their native range has limited adjustment to elevated CO₂ and climate warming. *Glob. Chang. Biol.* **19**, 3790–3807 (2013).
37. Tewari, A. K. & Tripathy, B. C. Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat. *Plant Physiol.* **117**, 851–858 (1998).
38. Zhou, H. et al. Exogenous spermidine delays chlorophyll metabolism in cucumber leaves (*Cucumis sativus* L.) under high temperature stress. *Acta Physiol. Plant* **38**, 224 (2016).
39. Fontaine, J. et al. Characterization of a NADH-Dependent Glutamate Dehydrogenase mutant of Arabidopsis demonstrates the key role of this enzyme in root carbon and nitrogen metabolism. *Plant Cell* **24**, 4044–4065 (2012).
40. Reuter, D. & Robinson, R. B. *Plant analysis: an interpretation manual.* CSIRO publishing (1997).
41. Liao, C. S. et al. Proteomic analysis revealed nitrogen-mediated metabolic, developmental, and hormonal regulation of maize (*Zea mays* L.) ear growth. *J. Exp. Bot.* **14**, 5275–5288 (2012).
42. Pan, X. et al. Asymmetric transcriptomic signatures between the cob and florets in the maize ear under optimal- and low-nitrogen conditions at silking, and functional characterization of amino acid transporters ZmAAP4 and ZmV AAT3. *J. Exp. Bot.* **66**, 6149–6166 (2015).
43. Chang, J. D. et al. Effects of elevated CO₂ and temperature on Gynostemma pentaphyllum physiology and bioactive compounds. *J Plant Physiol.* **196**, 41–52 (2016).
44. Duan, H. et al. Elevated [CO₂] does not ameliorate the negative effects of elevated temperature on drought-induced mortality in Eucalyptus radiata seedlings. *Plant Cell Environ.* **37**, 1598–1613 (2014).
45. Miao, S. J., Qiao, Y. F. & Zhang, F. T. Conversion of Cropland to Grassland and Forest Mitigates Global Warming Potential in Northeast China. *Pol. J. Environ. Stud.* **24**, 1195–1203 (2015).
46. Bishop, K. A., Andrew, D. B. L. & Elizabeth, A. A. How seasonal temperature or water inputs affect the relative response of C3 crops to elevated [CO₂]: a global analysis of open top chamber and free air CO₂ enrichment studies. *Food Energy Security* **3**, 33–45 (2014).
47. Ursula, M. R. et al. Global warming can negate the expected CO₂ stimulation in photosynthesis and productivity for soybean grown in the Midwestern United States. *Plant Physiol.* **162**, 410–423 (2013).
48. Ruiz-Vera, U. M. et al. Global warming can negate the expected CO₂ stimulation in photosynthesis and productivity for soybean grown in the Midwestern United States. *Plant Physiol.* **162**, 410–423 (2013).

**Acknowledgements**

The project was funded by the National Key Research and Development Program of China (2017YFD0300300), the Hundred Talents Program of Chinese Academy of Sciences, La Trobe University Research Focus Area project (RFA) (2000002982) and the China Studies Seed-funding Research Grant. We much appreciate that Professor Chunxia Xu at the Institute of Food Crops (IFC), Yunnan Academy of Agriculture Science (YAAS) and Professor Zhijun Su at Inner Mongolia University kindly provided soils for this experiment.

**Author contributions**

R.F. and Y.L. performed the experiment. Y.L. and Y.H. analysed the data. X.L., G.W., K.Y., and J.J. designed the experiment. Y.H., S.J.H., and J.J. discussed the results and wrote up the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** is available for this paper at [https://doi.org/10.1038/s41598-019-54325-5](https://doi.org/10.1038/s41598-019-54325-5).

**Correspondence** and requests for materials should be addressed to K.Y. or J.J.

**Reprints and permissions information** is available at [www.nature.com/reprints](http://www.nature.com/reprints).

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

---

© The Author(s) 2019