Plant Science 285 (2019) 239–247

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Photosynthesis and yield response to elevated CO_2, C_4 plant foxtail millet behaves similarly to C_3 species

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ARTICLE INFO

Keywords:
Elevated [CO_2]
Foxtail millet
Photosynthesis
Yield
Gene expression

ABSTRACT

Foxtail millet (*Setaria italica*) is a nutrient-rich food source traditionally grown in arid and semi-arid areas, as it is well adapted to drought climate. Yet there is limited information as how the crop responses to the changing climate. In order to investigate the response of foxtail millet to elevated [CO_2] and the underlying mechanism, the crop was grown at ambient [CO_2] (400 μmol mol^{-1}) and elevated [CO_2] (600 μmol mol^{-1}) in an open-top chamber (OTC) experimental facility in North China. The changes in leaf photosynthesis, chlorophyll fluorescence, biomass, yield and global gene expression in response to elevated [CO_2] were determined. Despite foxtail millet being a C_4 photosynthetic crop, photosynthetic rates (P_N) and intrinsic water-use efficiency (WUEi), were increased under elevated [CO_2]. Similarly, grain yield and above-ground biomass also significantly increased ($P < 0.05$) for the two years of experimentation under elevated [CO_2]. Increases in seeds and tiller number, spike and stem weight were the main contributors to the increased grain yield and biomass. Using transcriptomic analyses, this study further identified some genes which play a role in cell wall reinforcement, shoot initiation, stomatal conductance, carbon fixation, glycolysis / gluconeogenesis responsive to elevated [CO_2]. Changes in these genes reduced plant height, increased stem diameters, and promote CO_2 fixation. Higher photosynthetic rates at elevated [CO_2] demonstrated that foxtail millet was not photosynthetically saturated at elevated [CO_2] and its photosynthesis response to elevated [CO_2] were analogous to C_3 plants.

1. Introduction

The atmospheric CO_2 concentration [CO_2] is likely to increase from the current level of around 401 μmol mol^{-1} to 1000 μmol mol^{-1} by the end of the 21st century [1]. Increasing CO_2 can directly impact on the growth, development and yield of crops [2,3]. Generally, as yield increases in response to elevated [CO_2] [4] variation in response are observed in major crop. For example, in response to elevated [CO_2], the yield of C_3 crops (wheat, rice and barley) increases by about 19%, C_3 grain legumes (soybean, pea, peanut, common bean) increases by about 16%, whereas C_4 grain crops (sorghum and maize) show slightly decreased yield [3]. Although there are relatively small number of C_4 species, they contribute to more than 25% of the total terrestrial net productivity [5,6] and 30% of all global agricultural grain production [7]. C_4 crop foxtail millet, used to be the main crop for a long time in Chinese history, now as major supplement crop in China, has a high nutritional value and is tolerant of drought and barren soil [8–10]. Further, foxtail millet is an excellent C4 model plant as it has a small genome (490 Mbp), small plant size and quick generation time [9,11,12]. However, there is very limited understanding on how this species will respond to future climate, particularly, rising [CO_2] concentration.

The primary mechanism of C_3 plant response to elevated [CO_2] has been well demonstrated [4,13,14]. Increased photosynthesis, reduced stomatal conductance and decreasing photorespiration are recognized as primary factors [14–18]. Growth of C_4 plants also increases at elevated [CO_2] but this was not through the direct effect of photosynthesis [19] because C_4 photosynthesis is saturated at current atmospheric [CO_2] [20,21]. Further, this argument was supported by increased yields in C_4 crops without changes in the photosynthetic rates [22–25].

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https://doi.org/10.1016/j.plantsci.2019.05.006
Received 11 December 2018; Received in revised form 28 February 2019; Accepted 8 May 2019
Available online 25 May 2019

Some studies showed that elevated [CO_2] stimulated C_4-crops only under drought [23,25–27]. Thus, it becomes essential to understand how C_4 plants respond to elevated [CO_2], which could be used to improve C_4 crop productivity under future [CO_2] rich environment.

The molecular mechanisms that underlie the response of plants to elevated [CO_2] have been reported for a few species: poplar trees [28,29], *Arabidopsis* [30,31], soybean [32], sugar and rice [33]. Photosynthetic responses to elevated [CO_2] for an extended period was commonly known as down-regulation of photosynthesis [13,31,34–36]. Several genes involved in light harvesting were down-regulated by elevated CO_2 [31,35,37–39]. Down-regulation of photosynthesis was associated with the suppression of the synthesis of the RbcS (Rubisco small subunit) transcript at elevated [CO_2] compared to plants grown at ambient [CO_2] [33,38,39]. Under elevated [CO_2], transcripts abundance of many chloroplast-related functional genes were down-regulated [30], whereas functions associated with light fixation, development, defense and signaling were up-regulated [28–31]. The transcript level of several genes encoding chloroplast transporters and sugar transporters were up-regulated and the abundance of some transcripts encoding mitochondrial transport proteins was also increased in response to elevated [CO_2] [38,40]. Shi et al. [41] reported that the sequential production of NADPH oxidase-dependent H_2O_2 and NR-dependent NO act downstream of OST1, and were involved in the elevated CO_2-induced stomatal closure. Elevated [CO_2] up-regulated respiratory genes including the genes for glycolysis, mitochondrial electron transport chains, and the tricarboxylic acid cycle [42]. In the present study, foxtail millet was exposed to elevated [CO_2] to investigate the physiological and molecular mechanism of growth responses to elevated [CO_2]. The following questions were asked in our experiment: (1) Will the leaf photosynthetic physiology, chlorophyll fluorescence and yield of foxtail millet be altered under elevated [CO_2]? (2) Will the elevated [CO_2] alter gene expression in foxtail millet, and (3) Is there a correlation between photosynthetic physiology, yield and gene expression? The overall aim of our experiment was to help to understand to some extent the mechanisms of growth response in the C4 cereal to elevated [CO_2].

2. Materials and methods

2.1. Site description

The experiment was conducted at the open-top chamber (OTC) facility at Shanxi Agricultural University (37.42 °N, 112.55 °E), Taigu, Shanxi, China. Each chamber was 4 m in diameter and 2.5 m high. The ambient and elevated CO_2 concentrations were 385 ± 20 and 590 ± 40 μmol mol^{-1}, respectively. An automatic control system using CO_2 sensors (*Vaisala*, Finland) was used to adjust CO_2 to the target [CO_2] by regulating the influx rate of CO_2 or air. The ECO_2 treatment was conducted from the beginning of crop emergence stage to harvest time. The upper part of the OTCs was made a frustum of 0.5 m at 2.5 m height, which was kept open to maintain the near natural conditions of temperature and relative humidity. The temperature and relative humidity of the two chambers were measured during the growth period. They were 22.6 °C and 66.5% in CK OTC, and 22.1 °C and 71.2% in ECO_2 OTC in 2014. They were 23.3 °C and 64.2% in CK OTC, and 23.2 °C and 65.1% in ECO_2 OTC in 2015.

2.2. Foxtail millet cultivation, fertilization and irrigation

A landrace of foxtail millet, YPM, from Yuanping county, Shanxi province, was sown in 40 cm × 60 cm rectangular pots (28 cm deep) on 16 June 2014 and 17 June 2015, respectively. Ten plants were evenly grown in each pot and 10 replicates were included in each chamber. The [CO_2] treatments were unreplicated. The soil had a clay loam with a pH of 8.5 and contained 1.37% organic carbon (C) and 0.12% total N. Each pot was fertilized with 11.04 g N and 12.24 g P during the elongation stage. Irrigation equivalent to 10–20 mm of rainfall was applied every 3–5 days after seedling emergence both in two chambers. This level of water was maintained to guarantee that no water stress was imposed on the plants.

2.3. Gas exchange measurements

Measurements of P_N (net photosynthetic rate) were conducted just before heading (49 days after sowing in 2014), anthesis (65 days after sowing in 2014 and 64 days after sowing in 2015), and grain-filling (83 days after sowing in 2014 and 82 days after sowing in 2015). Gas exchange was measured on attached fully expanded flag leaves using a portable gas exchange system (LI-COR 6400; Lincoln, Neb, USA) between 09:00 am and 11:30 am local time. The [CO_2] in the leaf chamber was regulated by the LI-COR CO_2 injection system, which was set to 400 μmol mol^{-1} in current [CO_2] treatment and 600 μmol mol^{-1} in elevated [CO_2] treatment. An irradiance of 1400 μmol (photons) m^{-2} s^{-1} was achieved using a built-in LED lamp (red/blue). Temperature in the 2 × 3 × 2.5 cm^3 leaf chamber was maintained at approximately 28℃. The vapour pressure deficit (VPD) range on the leaf surface was between 1.9 and 2.1 kPa. P_N, transpiration rate (*Tr*), and stomatal conductance (g_s) were measured at the same irradiance, temperature and vapour pressure. Intrinsic water-use efficiency (WUEi, WUEi = P_N/g_s) were also calculated. A (CO_2 assimilation rate)/C_i (intercellular-CO_2 concentration) response curves were made at anthesis (67 days after sowing in 2015). The irradiance was maintained at 1600 μmol (photons) m^{-2} s^{-1} in the leaf chamber, using a built-in LED lamp (red/blue). The [CO_2] surrounding the leaf for all control and treatment leaves was regulated across the series of 400, 300, 200, 100, 0, 400, 600, 800, 1000, and 1200 μmol mol^{-1}, and data were recorded after values became stable but with a minimum waiting time of 3 min at each step within the sequence. Other environmental conditions of leaf chamber were same as for the P_N measurements. Measurements were conducted between 09:00 and 14:00 h local time. Four fully-expanded flag leaves were used in CK OTC and ECO_2 OTC, respectively. Each individual curve took approximately 40 min to complete.

2.4. Chlorophyll fluorescence

To examine the effects of high CO_2 on PSII and post-PSII electron transport, the chlorophyll fluorescence parameter maximal quantum yield of PSII photochemistry (Fv/Fm), effective quantum yield of PSII photochemistry (Φ_{PSII}), photochemical quenching coefficient (qP), intrinsic efficiency of PSII (Fv'/Fm'),and non-photochemical quenching (NPQ) were measured with a miniaturized pulse-amplitude modulated fluorescence analyzer (Mini-PAM, Walz, Effeltrich, Germany). The fluorescence parameters of minimal fluorescence yield of the light-adapted state (F_0') and maximal fluorescence yield of the light-adapted state (F_m') were determined at incident photosynthetic photon flux density (PPFD) between 08:30 and 12:00 h. One upper most fully-expanded leaf per pot was used after the gas exchange measurements on the same day. Ten replications were used per chamber. Maximal fluorescence yield of the dark-adapted state (F_m) and minimal fluorescence yield of the dark-adapted state (F_0) were determined between 23:00 and 01:00 h with the same leaves on the same day. To measure saturated fluorescence, the high light flash was made a PPFD of 4000 μmol (photon) $m^{-2}s^{-1}$and a duration of 800 ms. All chlorophyll fluorescence parameters were calculated according to the methods in Rascher et al. [43].

2.5. Differentially expressed genes by high-throughput sequencing

The flag leaves of foxtail millet at anthesis in four pots in CK OTC and ECO_2 OTC with the same leaf age were used for analysis of gene expression using high-throughput sequencing in 2014. All samples were taken and plunged in liquid nitrogen immediately. Flash-frozen samples

Table 1
Gene-specific primers used for real-time RT-PCR gene expression studies.

Accession Number	Description	Pathway		Primer Sequence
Si014344	Caffeoyl-CoA O-methyltransferase	Phenylpropanoid biosynthesis	-F	5' ACGTGGGGGCGTTCGAC 3'
			-R	5' TGAGGTCCCTGATGGCGG 3'
Si030198	Protein phosphatase	Plant hormone signal transduction	-F	5' TCCTCGGACCACAAGCCC 3'
			-R	5' GCCCTCCCAGAAGATGACG 3'
Si000717	Type I inositol polyphosphate 5-phosphatase, arath	Inositol phosphate metabolism	-F	5' TCGTGAGCAAGCAGATGGT 3'
			-R	5' GGTGGCAGCACACAAAGC 3'
Si035574	Glycosyl hydrolases family	Plant hormone signal transduction	-F	5' CGCCTACAACGACTACTACCA 3'
			-R	5' CGAAAACGCAAGACCCTGA 3'
Si036462	Peroxidase	Phenylpropanoid biosynthesis	-F	5' CTCACACGTTTGGCAGGGTA 3'
			-R	5' GCGATAGGAATGCTCGGTAA 3'
Si000774	NADP + -dependent malic enzyme	Carbon fixation in photosynthetic organisms	-F	5' TTGCTCAGCAGGTCTCAGAA 3'
			-R	5' CAGCGGTAGTTGCGGTAAA 3'
Si013194	Lipoxygenase	Linoleic acid metabolism	-F	5' AAGGAGATTGAGGGGATCAT 3'
			-R	5' GTCACGCCTTCCTGAGAGA 3'
Si034948	Phosphoglycerate mutase	Glycolysis / Gluconeogenesis	-F	5' TGAGCAAGTGGGTGGCATT 3'
			-R	5' TCCTTGTCACGGAGCGGT 3'
Si035904	Alpha-galactosidase/alpha-n-acetylgalactosaminidase	Galactose metabolism Glycosphingolipid biosynthesis Sphingolipid metabolism glycerolipid metabolism	-F	5' CTGCACAAGACGCTGGACA 3'
			-R	5' CCTGGACTTGAGCACGAACAT 3'
Si010446	Leucine-rich repeat-containing protein	Plant-pathogen interaction	-F	5' GGAACCCGCTGGTGTGTC 3'
			-R	5' GCCGTGAAGGTGCCGTA 3'

were stored at −80 °C until analysis. The experimental processes including sample preparation and transcriptome sequencing were carried out by BGI, Shenzhen, China. Two analytical replicates were performed for each experimental treatment. The main instruments used were Illumina HiSeq™2000, Agilent 2100 Bioanaylzer and ABI Step One Plus Real-Time PCR System.

The gene expression level was calculated using the *RPKM* method [44]. Differentially expressed genes (DEGs) were identified using the method by [45]. In gene functional-enrichment analysis, GO and KEGG were performed to identify DEGs compared with the whole-transcriptome background.

2.6. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

We identified the key genes by GO and Pathway analysis at corrected P-value ≤ 0.05 using the formula as described by Hao et al. [46] and Shi et al. [47]. The optimal primers (Table 1) were designed and synthesized by Sangon Biotech Corporation, China. Total RNA isolated from foxtail millet leaf tissues was first converted to cDNA using MMLV reverse transcriptase (Baosheng Corporation, China), according to the manufacturer's specifications, with random hexamer primers from Sangon Biotech Corporation, China. Reactions (20 μL) were conducted in triplicate in 96-well plates (Temp Plate Scientific, BIO-RAD, China). Each replicate sample was run three times as technical replicates with the iCycler Real-Time PCR Detection System (Bio-Rad Laboratories INC., USA) by employing the two-step amplification plus melting curve protocol. The expression patterns of selected genes by comparing the expression levels in samples between the elevated [CO_2] and the normal [CO_2] treatments were analyzed by using relative quantitative method delta-delta CT ($2^{-\triangle\triangle CT}$) [48].

2.7. Harvesting

At maturity, foxtail millet plants were hand-harvested on 8th October 2014 and 4th October 2015, respectively. After drying, random subsamples of 5 plants from each pot were assessed for height, panicle length, stem diameter, tiller number, panicle weight per m^2, leaf weight per m^2, stem weight per m^2 and number of seeds per plant. Then, all plants were separated into leaves, stems, panicles and seeds, air dried and weighed. Yield and above-ground biomass were also calculated.

2.8. Statistical analysis

The significance of differences between the means were subjected to the analysis of variance at 0.05 percent probability using SAS System 8.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. The response of yield component and above-ground biomass to elevated [CO_2]

Elevated [CO_2] did not affect the growth and development period of millet in both two years (Table 2). Different yield and above-ground biomass was observed under elevated [CO_2]. The total yield of foxtail millet increased by 32% and 11%, and the above ground biomass increases by 19 and 8%, in 2014 and 2015, respectively (Figs. 1and 2). Similarly, compared to plants grown under ambient [CO_2], panicle and leaf weight (g/m^2) increased by 18% and 19% in 2014, 20% and 4% in 2015, respectively ($P < 0.05$, Table 3). Furthermore, seed number per plant also increased by 25% in 2014 and 8% in 2015 under elevated [CO_2]; thousand seed weight was also increased in 2014 and 2015.

Table 2
Effects of elevated [CO_2] on growth stage of foxtail millet.

Year	Growth [CO_2]	Growth stage				
		Sowing date	Heading stage	Anthesis	Grain-filling stage	Mature stage
2014	CK	June 16th	August 8th	August 17th	August 27th	October 6th
	ECO_2	June 16th	August 8th	August 17th	August 27th	October 6th
2015	CK	June 17th	August 7th	August 16th	August 25th	October 1st
	ECO_2	June 17th	August 7th	August 16th	August 25th	October 1st

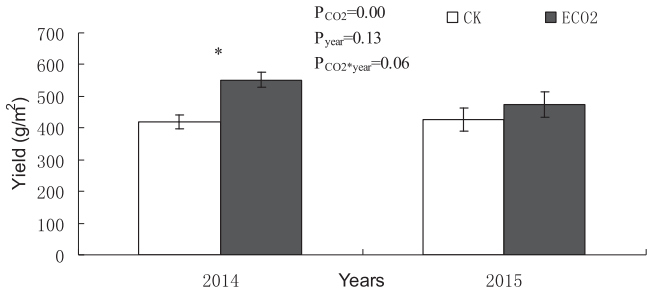

Fig. 1. Effects of elevated [CO_2] on yield in foxtail millet. Values are means ± standard error of variables across the three replicates, * – the significant differences at 0.05 levels.

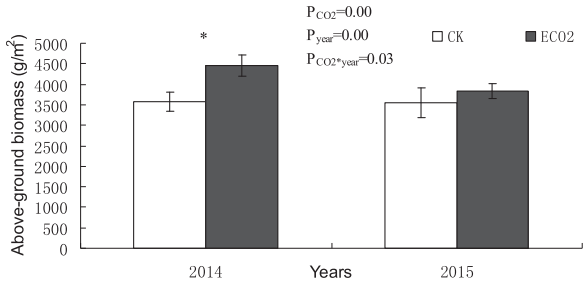

Fig. 2. Effects of elevated [CO_2] on above-ground biomass in foxtail millet. Values are means ± standard error of variables across the three replicates, * – the significant differences at 0.05 levels.

Stem diameter was increased by 16% in 2014 and 12% in 2015 under elevated [CO_2] ($P < 0.01$, Table 4). Tiller number was increased by 27% in 2014 and 14% in 2015 under elevated [CO_2] ($P = 0.01$, Table 4). Plant height was reduced with increased [CO_2] during both years. The changes in panicle length were not consistent between the two years (Table 3).

3.2. The response of P_N and gas exchange parameters to elevated [CO_2]

Elevated [CO_2] increased P_N at all stages of plant development ($P \leq 0.05$, Table 5). P_N was increased by 21%, 73% and 123% under elevated [CO_2] at heading, anthesis and at grain-filling in 2014, and by 7% and 19% at anthesis and at grain-filling in 2015, respectively. Stomatal conductance was significantly increased under elevated [CO_2] except at heading stage in 2014, whereas in 2015 it was decreased under elevated [CO_2] (Table 5). The change in Tr was similar to the change in g_s (Table 5). Consequently, elevated [CO_2] increased WUEi by 77%, 8% and 41% at heading, anthesis and grain-filling in 2014, and by 45% and 56% at anthesis and grain-filling stage in 2015, respectively (Table 5). An increase in the initial slope of the response curve illustrated that photosynthetic acclimation did not occur in foxtail millet grown under elevated [CO_2] (Fig. 3). [CO_2] was not saturated at 600 μmol mol^{-1} in foxtail millet.

3.3. The response of chlorophyll fluorescence to elevated [CO_2]

There was no effect of elevated [CO_2] on optimal photochemical efficiency of PSII (Fv/Fm) and non-photochemical quenching (NPQ) during the two years (Table 6). Effective quantum yield of PSII (Φ_{PSII}), photochemistry quenching (qP) and the highest photosynthetic electron transport (ETR) was increased under elevated [CO_2] at all the growth stages in 2014, whereas all of them were decreased in 2015 (Table 6).

3.4. Gene expression

Differentially expressed genes were identified by high-throughput sequence analysis; among them, 19 genes were up-regulated and 47 genes were down-regulated (Table 7). The pathway enrichment analysis of differentially expressed genes is listed in Table 7. Elevated [CO_2] showed a significant effect on plant hormone signal transduction, phenylpropanoid biosynthesis, galactose metabolism, glycosphingolipid biosynthesis - globo series, and cutin, suberine and wax biosynthesis (Table 7). The results obtained from high-throughput RNA sequencing were confirmed by RT-PCR (Fig. 4), suggesting the high reliability of high-throughput RNA sequencing data.

In the lignin biosynthesis pathway, the gene encoding peroxidase was up-regulated, whereas the expression of caffeoyl-CoA O-methyltransferase was down-regulated. Caffeoyl-CoA O-methyltransferase catalyses the synthesis of sinapoyl-coa, and peroxidase catalyses the formation of guaiacyl lignin from coniferyl alcohol. Protein phosphatase, involved in plant hormone signal transduction leading to stomatal closure, was down-regulated in foxtail millet grown under elevated [CO_2]. Leucine-rich repeat-containing protein, involved in cell wall reinforcement, was up-regulated under elevated [CO_2]. In inositol phosphate metabolism, the gene encoding type I inositol polyphosphate 5-phosphatase was down-regulated under elevated [CO_2]. Elevated CO_2 had negative effects on the glycosyl hydrolases family, which is involved in plant hormone signal transduction leading to shoot initiation. Elevated [CO_2] also decreased the expression of lipoxygenase which catalyses the synthesis of 13-Oxo-10(E)-dodecenoic Acid (13-OxODE). The expression of alpha-galactosidase/alpha-n-acetylgalactosaminidase was significantly down-regulated under elevated [CO_2]. Further, elevated [CO_2] significantly inhibited the gene expression of $NADP^+$-malic enzyme (NADP-ME). The expression of phosphoglycerate mutase, involved in glyceraldehyde-3 P and glyceraldehyde-2 P reciprocal transformation, was significantly down-regulated under elevated [CO_2].

4. Discussion

We demonstrated that foxtail millet responded positively in the terms of yield and biomass to the elevated [CO_2], the highest growth or yield response reported so far for a C_4 species. This is not in accordance with previous studies in other C_4 crops. It was previously assumed that C_4 plant species will not respond to elevated [CO_2] compared to C_3 species [27,49,50]. Positive stimulating effect by elevated [CO_2] are observed only under drought conditions in C_4 crops [23,25,50,51]. It

Table 3
Effects of elevated [CO_2] on weight in foxtail millet. Values are means ± standard error of variables across the ten replicates. Mean values with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

Year	Growth [CO_2]	Panicle weight per plant [g/m^2]	Leaf weight per plant [g/m^2]	Stem weight per plant [g/m^2]	Thousand seed weight [g]	Seeds number per plant
2014	CK	535.19 ± 26.27 c	181.13 ± 7.59 c	253.65 ± 10.34 c	2.59 ± 0.08a	3578.31 ± 229.98 b
	ECO_2	632.71 ± 33.48 ab	214.92 ± 20.77 bc	320.73 ± 19.03 b	2.75 ± 0.12 a	4456.53 ± 268.66 a
2015	CK	608.15 ± 39.52 bc	256.64 ± 4.42 ab	485.97 ± 56.95 a	2.65 ± 0.05 a	3559.94 ± 363.98 b
	ECO_2	731.03 ± 37.45 a	265.68 ± 11.49 a	401.61 ± 50.13 ab	2.71 ± 0.10 a	3831.52 ± 178.50 b
Pvalues	Year	0.22	0.00	0.00	0.85	0.11
	CO_2	0.00	0.04	0.83	0.13	0.01
	Year × CO_2	0.75	0.20	0.01	0.51	0.13

Table 4
Effects of elevated [CO_2] on growth in foxtail millet. Values are means ± standard error of variables across the ten replicates. Mean values with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

Year	Growth [CO_2]	Plant height[cm]	Panicle length[cm])	Stem diameter[cm])	Tiller number	Number of leaves
2014	CK	109.72 ± 2.63 a	17.67 ± 0.56 a	0.57 ± 0.02 a	1.42 ± 0.05 b	12.16 ± 0.22 a
	ECO_2	102.29 ± 2.15 a	16.84 ± 0.61 a	0.66 ± 0.04 a	1.80 ± 0.23 a	12.75 ± 0.31 a
2015	CK	92.93 ± 0.61 b	15.39 ± 0.09 b	0.49 ± 0.01 b	1.32 ± 0.13 b	10.88 ± 0.27 b
	ECO_2	90.95 ± 4.68 b	16.93 ± 0.48 a	0.55 ± 0.03 a	1.51 ± 0.11 ab	9.46 ± 0.29 c
Pvalues	Year	0.00	0.01	0.00	0.87	0.11
	CO_2	0.13	0.35	0.00	0.01	0.00
	Year × CO_2	0.20	0.00	0.42	0.71	0.09

could be due to the fact that foxtail millet is well adapted to drought condition. In our study, aboveground biomass production of foxtail millet was significantly increased under elevated [CO_2] in both two years, which was associated with the production of more panicle and leaf weight per unit ground area. Increased tiller and seed numbers were the main contributors to increases in grain yield in both years under elevated [CO_2]. Elevated [CO_2] significantly increased stem diameter and reduced the height of foxtail millet. Similar changes in plant morphology has been reported in elsewhere, for example elevated [CO_2] significantly increased the plant height of maize [52] and sugarcane [40]. While the growth stage of foxtail millet was not affected by elevated [CO_2]. Springer and ward [53] observed that all possible responses including delayed, accelerated, and no change in flowering time both among species as well as within species in response to elevated [CO_2]. This indicates the need for more studies addressing the effects of elevated [CO_2] drivers on developmental processes in plants. Being a C_4 panicoid crop, foxtail millet is efficient in photosynthesis and has higher WUE [54]. This species (C_4) has developed physiological and molecular strategies to improve WUE and WUEi, thus contributing to positive growth at elevated [CO_2] [55,56]. In our study, foxtail millet was very effective in water use both under ambient and elevated [CO_2] conditions (Table 5). It has been demonstrated that elevated [CO_2] increased C_4 photosynthesis under a combination of water stress conditions [22,55,57–59]. On the other hand, some studies reported that there was no stimulation of photosynthesis, *in vivo* or *in vitro* photosynthetic enzyme activities, biomass or yield under elevated [CO_2] [23]. In our study, photosynthesis was significantly increased in foxtail millet under elevated [CO_2] regardless of the ontogenetic stage of the leaf blades in both years of the study (Table 5); it was increased by 26% and 61% at anthesis and grain-filling stage in 2013 [60]. An increase in the initial slope of photosynthetic against intercellular-CO_2 concentration suggested that foxtail millet was different from other C_4 plants that possess a near-saturating photosynthetic capability at elevated [CO_2]

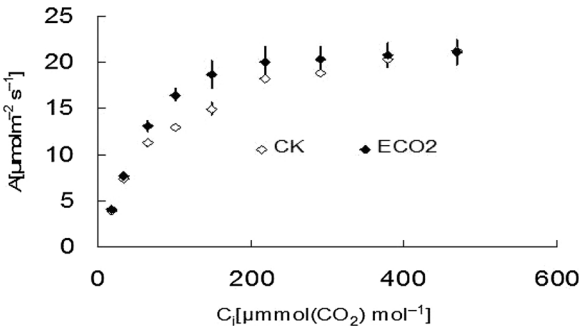

Fig. 3. [CO_2] response curves (intercellular [CO_2]: *ci*) of CO_2 assimilation rate (A) in foxtail millet.

(Fig. 3). The maintenance of high photosynthetic rate during leaf ontogeny is likely to play a key role in determining the large growth response to elevated [CO_2]. The C_4 photosynthetic response to water stress is as diverse as those reported for C_3 photosynthesis. Some studies reported an inhibition of C_4 photosynthesis under water stress conditions mainly due to stomatal closure, while others concluded that non-stomatal factors play a major role [61]. Foxtail millet showed a higher average stomatal conductance and transpiration rate under elevated [CO_2] in 2014. This strategy may help to lower leaf temperatures, which may decrease heat-related damage in foxtail millet plants compared with other C_4 species from the same habitat. In line with other findings [5,56], lower g_s coupled with higher photosynthetic capacity in the leaves of foxtail millet under elevated [CO_2] in 2015 resulted in higher water use efficiency in comparison to the leaves of C_3 plants.

Elevated [CO_2] significantly increased ETR, qP and Φ_{PSII} in foxtail millet in 2013 [60] and in 2014 (Table 6). Previously, it has been reported that ETR and Φ_{PSII} increased under elevated [CO_2] in another C_4 species *Z. mays* [56]. Therefore, it is suggested that increased ETR and

Table 5
Effects of elevated [CO_2] on gas exchange parameters in foxtail millet. Measurement was taken on their respective [CO_2]. Values are means ± standard error of variables across the ten replicates. Mean values with different letters are significantly different ($P <$ 0.05) according to Duncan's multiple range test.

	Growth stage	Growth [CO_2]	P_N[molm^{-2} s^{-1}]	g_s[mol(H_2O) m^{-2} s^{-1}]	Tr[mmol(H_2O) m^{-2} s^{-1}]	WUEi[mol (CO_2)mol(H_2O)$^{-1}$]
2014	Heading stage	CK	20.62 ± 0.94 b	0.17 ± 0.01 a	2.74 ± 0.14 a	132.40 ± 4.49 c
		ECO_2	24.91 ± 1.36 a	0.13 ± 0.01 b	2.32 ± 0.13 ab	209.65 ± 7.21 a
	Anthesis	CK	11.62 ± 0.58 c	0.10 ± 0.00 c	2.10 ± 0.09 b	116.75 ± 2.13 cd
		ECO_2	20.20 ± 0.71 b	0.16 ± 0.01 a	2.77 ± 0.12 a	125.89 ± 3.38 c
	Grain-filling stage	CK	10.44 ± 0.61 c	0.07 ± 0.00 d	1.46 ± 0.08 c	155.59 ± 2.19 b
		ECO_2	23.26 ± 0.35 a	0.11 ± 0.00 b	2.19 ± 0.07 b	219.77 ± 5.17 a
	Pvalues	Growth stage	0.00	0.00	0.00	0.00
		CO_2	0.00	0.00	0.00	0.00
		Growth stage × CO_2	0.00	0.00	0.00	0.00
2015	Anthesis	CK	16.55 ± 1.24 ab	0.12 ± 0.01 a	3.90 ± 0.37 a	138.17 ± 7.00 b
		ECO_2	17.76 ± 0.82 ab	0.09 ± 0.01 b	3.77 ± 0.27 a	200.19 ± 12.36 a
	Grain-filling stage	CK	15.86 ± 1.01 b	0.12 ± 0.01 a	2.37 ± 0.21 b	136.88 ± 10.74 b
		ECO_2	18.91 ± 1.07 a	0.09 ± 0.01 b	1.79 ± 0.17 c	213.95 ± 16.21 a
	Pvalues	Growth stage	0.82	0.89	0.00	0.61
		CO_2	0.05	0.01	0.19	0.00
		Growth stage × CO_2	0.39	0.87	0.41	0.54

Table 6
Effects of elevated [CO_2] on chlorophyll fluorescence parameters in foxtail millet. Values are means ± standard error of variables across the ten replicates; The statistical significance level for the effects of [CO_2] treatment was tested. Mean values with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

	Growth stage	Growth [CO_2]	Fv/Fm	ΦPSII	ETR	qP	NPQ
2014	Heading stage	CK	0.78 ± 0.00 a	0.31 ± 0.02 b	181.81 ± 11.19 b	0.68 ± 0.03 b	1.96 ± 0.13 a
		ECO_2	0.78 ± 0.20 a	0.37 ± 0.02 a	223.39 ± 9.73 a	0.79 ± 0.02 a	1.87 ± 0.18 a
	Anthesis	CK	0.75 ± 0.00 c	0.21 ± 0.02 d	122.92 ± 9.31 d	0.49 ± 0.04 cd	1.52 ± 0.26 ab
		ECO_2	0.75 ± 0.01 c	0.27 ± 0.01 c	158.74 ± 3.10 c	0.59 ± 0.08 bc	1.64 ± 0.20 ab
	Grain-filling stage	CK	0.76 ± 0.00 b	0.19 ± 0.04 d	112.22 ± 13.92 d	0.43 ± 0.05 cd	1.37 ± 0.08 bc
		ECO_2	0.75 ± 0.00 c	0.27 ± 0.01 c	160.93 ± 8.32 c	0.60 ± 0.03 bc	1.47 ± 0.14 ab
	Pvalues	Growth stage	0.00	0.00	0.00	0.00	0.03
		CO_2	0.81	0.00	0.00	0.02	0.74
		Growth stage × CO_2	0.11	0.46	0.46	0.60	0.56
2015	Anthesis	CK	0.75 ± 0.01 ab	0.23 ± 0.02 a	137.71 ± 9.64 a	0.64 ± 0.03 a	2.27 ± 0.14 a
		ECO_2	0.74 ± 0.00 bc	0.12 ± 0.01 c	76.71 ± 8.83 b	0.41 ± 0.04 c	2.41 ± 0.14 a
	Grain-filling stage	CK	0.72 ± 0.01 cd	0.23 ± 0.01 a	135.09 ± 8.47 a	0.56 ± 0.03 b	1.65 ± 0.11 b
		ECO_2	0.76 ± 0.01 a	0.19 ± 0.01 b	114.98 ± 11.54 a	0.45 ± 0.04 bc	1.63 ± 0.09 b
	Pvalues	Growth stage	0.28	0.08	0.08	0.56	0.00
		CO_2	0.17	0.00	0.00	0.00	0.62
		Growth stage × CO_2	0.03	0.05	0.05	0.07	0.47

Table 7
The pathway enrichment analysis of different expression genes.

#	Pathway	Pvalue	Differentially expressed genes
1	Plant hormone signal transduction	0.01	Si036015 m.g, Si013648 m.g, Si030198 m.g, Si010073 m.g, Si029090 m.g, Si035574 m.g
2	Phenylpropanoid biosynthesis	0.01	Si014344 m.g, Si036462 m.g, Si029879 m.g, Si001092 m.g
3	Galactose metabolism	0.01	Si029000 m.g, Si035904 m.g
4	Glycosphingolipid biosynthesis - globo series	0.03	Si035904 m.g
5	Cutin, suberine and wax biosynthesis	0.03	Si006073 m.g, Si019489 m.g
6	Linoleic acid metabolism	0.06	Si013194 m.g
7	Stilbenoid, diarylheptanoid and gingerol biosynthesis	0.07	Si014344 m.g, Si029879 m.g
8	Biosynthesis of secondary metabolites	0.07	Si006073 m.g, Si037596 m.g, Si014344 m.g, Si019489 m.g, Si036462 m.g, Si029879 m.g, Si001092 m.g, Si022485 m.g
9	Flavonoid biosynthesis	0.08	Si014344 m.g, Si029879 m.g
10	Phenylalanine metabolism	0.09	Si014344 m.g, Si036462 m.g
11	Sphingolipid metabolism	0.10	Si035904 m.g
12	Biosynthesis of unsaturated fatty acids	0.11	Si035778 m.g
13	Protein processing in endoplasmic reticulum	0.17	Si002731 m.g, Si003151 m.g
14	Glycerolipid metabolism	0.17	Si035904 m.g
15	Inositol phosphate metabolism	0.17	Si000717 m.g
16	Starch and sucrose metabolism	0.18	Si006474 m.g, Si001092 m.g
17	Cysteine and methionine metabolism	0.20	Si037596 m.g
18	alpha-Linolenic acid metabolism	0.21	Si013194 m.g
19	Phosphatidylinositol signaling system	0.21	Si000717 m.g
20	Cyanoamino acid metabolism	0.23	Si001092 m.g
21	Carotenoid biosynthesis	0.25	Si022485 m.g
22	Circadian rhythm - plant	0.28	Si017194 m.g
23	Metabolic pathways	0.32542	Si037596 m.g, Si021485 m.g, Si014344 m.g, Si006474 m.g, Si000717 m.g, Si013194 m.g, Si036462 m.g, Si001092 m.g, Si022485 m.g
24	Pyrimidine metabolism	0.622732	Si021485 m.g
25	Plant-pathogen interaction	0.920756	Si010446 m.g
26	Carbon fixation in photosynthetic organisms		Si000774 m.g
27	Glycolysis / Gluconeogenesis	0.00704	Si034948 m.g

Φ_{PSII} at elevated [CO_2] was related to greater photosynthesis. Increased PSII activity helped to generate enough NADPH and ATP to fix additional carbon through photosynthesis. Elevated [CO_2] decreased ETR, qP and Φ_{PSII} in foxtail millet in 2015. The different changes in ETR, qP and Φ_{PSII} during the two years of the experiment need to be clarified with further research.

Transcriptome profiling is a powerful and complementary tool to uncover the mechanism of C_4 response to elevated [CO_2]. In our study, we identified 66 differentially expressed genes in foxtail millet by transcriptome resequencing. The low number of differentially expressed genes may be related to possible strong adaptation of foxtail millet to the carbon rich atmosphere. Previously, it has been suggested that foxtail millet was well adapted to drought and poor soils, and was suggested as an ideal crop in an era of climate change [10]. This was supported by physiological and transcriptomic evidence. Changes in carbon metabolism seem to be more pronounced at elevated [CO_2], possibly as an attempt to adjust carbon partitioning between the organs at the organelle level. Particularly, lignin biosynthesis pathway of sinapoyl-coa was inhibited, while the pathway of coniferyl alcohol enzymes were over expressed (Fig. 4). Similar findings have been reported elsewhere. Körner et al. [62] demonstrated that lignin concentrations were significantly reduced under elevated [CO_2] and suggested that a shift in carbon partitioning from recalcitrant to more labile compounds occurs under elevated [CO_2].

Further, our results demonstrate that elevated [CO_2] inhibited the expression of glycosyl hydrolase family genes that promote shoot initiation. At the same time, elevated [CO_2] up-regulated leucine-rich repeat-containing protein which was beneficial to cell wall reinforcement. Some genes involved in cell wall composition were up-regulated by elevated CO_2 [31,32,40,63]. All these findings support our results for

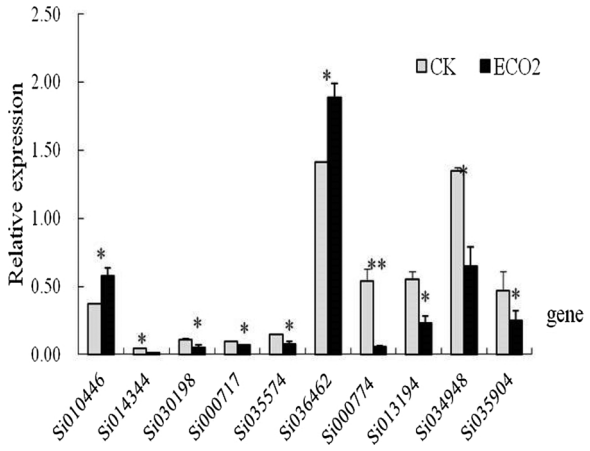

Fig. 4. Effects of elevated [CO_2] on gene expression in foxtail millet. Values are means ± standard error of variables across the three replicates, * – the significant differences at 0.05 levels. ** – the significant differences at 0.01 levels.

morphological adjustment, decreased plant height and increased stem diameters in foxtail millet is likely to be associated with changes in carbon metabolism. Reducing plant height and increasing the mechanical strength of the stem are more conducive to overcoming the lodging of millet at the later maturity stage and promoting the increase of biomass and yield in foxtail millet, in agreement with the study of Tian et al. [64].

The involvement of stomata in plant responses to elevated CO_2 has been well established; however, the underlying mechanism of how elevated CO_2-induces stomatal closure remains largely unknown. In our study, stomatal conductance largely remained unchanged at elevated [CO_2] at anthesis and grain-filling stage in 2014 (Table 5). These findings were further supported by down-regulation of the gene encoding protein phosphatase which induced stomatal closure at elevated [CO_2]. Elevated [CO_2] had a negative effect on the expression of alpha-galactosidase/alpha-n-acetylgalactosaminidase suggesting that galactose metabolism, glycosphingolipid biosynthesis, sphingolipid metabolism, and glycerolipid metabolism were inhibited at elevated [CO_2]. The inositol phosphate metabolism is a signaling pathway in plants that increases in response to many stimuli such as gravity, light and salt stress [65–67]. Increases in transcripts associated with enzymes in inositol phosphate biosynthesis was observed under elevated [CO_2] [32]. But our study showed that elevated [CO_2] suppressed one part of the inositol phosphate metabolism in foxtail millet. Khodakovskaya et al. [68] showed that reduced basal level of inositol-(1,4,5)-trisphosphate (InsP3) and increased flux through the inositol phosphatases causes an increase in cytosolic Pi concentration which could increase CO_2 fixation. We assumed it would be similar for foxtail millet under elevated [CO_2]. $NADP^+$-dependent malic enzyme (NADP-ME), a key enzyme in the C_4 pathway in the NADP-ME subtype of C_4 plants, is located in the chloroplasts of the bundle sheath cells where it is decarboxylated by NADP-ME enzyme, releasing CO_2 for photosynthesis [69]. Our result showed that elevated [CO_2] suppressed the gene expression of NADP-ME which may affect carbon fixation in foxtail millet. $NADP^+$-dependent malic enzyme catalyzed the conversion of malate to pyruvate in C_4-Dicarboxylic acid cycle malate. However, we did not observe a down-regulation of photosynthesis suggesting that there may be other routes of carbon supply to the C_3 photosynthetic cycle in foxtail millet. This phenomenon is currently under investigation in our laboratory.

Further, elevated [CO_2] suppressed the expression of phosphoglycerate mutase, suggesting that elevated [CO_2] inhibited glycolysis/gluconeogenesis, which may lead to a reduction of Glyceraldehyde-3 P and hence affect carbon fixation and foliar respiration in foxtail millet under high [CO_2] environment. Suppression of glycolysis has been reported with C_3 wheat [70]. On the other hand, elevated [CO_2] increased the transcript abundance of genes encoding enzymes of glycolysis in soybean, through the glycolytic pathway which was diverted into secondary metabolism, in particular, lignin, and fatty acid biosynthesis at elevated [CO_2] [32]. We found remarkable similarities in response to elevated [CO_2] between C_4 crops plants and foxtail millet. Such understandings are essential to adapt major C_4 crops to the inevitable climate change.

Some studies argued that the release of pyrimidine nitrogen via the catabolic pathway played a significant role in remobilization of nitrogen at the nitrogen metabolism [71,72]. Our study showed that Si021485 involved in pyrimidine metabolism was downregulated, which ultimately affects nitrogen metabolism.

Rubisco is the key enzyme in photosynthesis and photorespiration. Rubisco initial activity decreased under elevated [CO_2] in all the compiled studies, and the extent of this decrease partly determined the response of an increase in A_N [73]. The reduction of rubisco under elevated [CO_2] needs to be further verified in millet studies.

5. Conclusions

This study demonstrates that despite foxtail millet being a C_4 plant, it showed a consistent increase in both biomass and yield when grown under elevated [CO_2], mainly due to a higher photosynthetic rate. We identified some genes which play an important role in cell wall reinforcement, shoot initiation, stomatal conductance, plant hormone signal transduction, carbon fixation, glycolysis / gluconeogenesis being responsive to elevated [CO_2]. Changes in these genes reduced plant height, increased stem diameters, and promote CO_2 fixation. The results presented here suggest that under increasing CO_2 concentrations in the future, foxtail millet, a C_4 species will not forfeit its advantage to C_3 crops.

Funding

This work was supported by the National Natural Science Foundation of China [31601212, 31501276], Research on Science and Technology of Shanxi Province [20150311006-2], National Science and Technology Major Project of China [2017BAD11B02-5], Scientific and Technological Project in Shanxi Province [201703D221033-1].

Conflict of interest

No conflict of interest.

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