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The effects of elevated CO_2 (0.5%) on chloroplasts in the tetraploid black locust (*Robinia pseudoacacia* L.)

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Abstract

Some ploidy plants demonstrate environmental stress tolerance. Tetraploid (4×) black locust (*Robinia pseudoacacia* L.) exhibits less chlorosis in response to high CO_2 than do the corresponding diploid (2×) plants of this species. We investigated the plant growth, anatomy, photosynthetic ability, chlorophyll (chl) fluorescence, and antioxidase activities in 2× and 4× black locusts cultivated under high CO_2 (0.5%). Elevated CO_2 (0.5%) induced a global decrease in the contents of total chl, chl a, and chl b in 2× leaves, while few changes were found in the chl content of 4× leaves. Analyses of the chl fluorescence intensity, maximum quantum yield of photosystem II (PSII) photochemistry (*Fv/Fm*), K-step (V_k), and J-step (V_J) revealed that 0.5% CO_2 had a negative effect on the photosynthetic capacity and growth of the 2× plants, especially the performance of PSII. In contrast, there was no significant effect of high CO_2 on the growth of the 4× plants. These analyses indicate that the decreased inhibition of the growth of 4× plants by high CO_2 (0.5%) may be attributed to an improved photosynthetic capacity, pigment content, and ultrastructure of the chloroplast compared to 2× plants.

KEYWORDS

chloroplast, diploid, elevated CO_2, photosynthesis, tetraploid, tetraploid black locust (*Robinia pseudoacacia* L.)

1 | INTRODUCTION

Over the last two centuries, the global atmospheric carbon dioxide concentration [CO_2] has increased faster than predicted and will double within the next hundred years (Meehl et al., 2007; Peters et al., 2011). Elevated CO_2 (EC) is largely responsible for plant growth and yield. Generally, an increase in CO_2 concentration increases photosynthesis, stimulates growth, and improves yield (Reef et al., 2015; Stiling et al., 2013; Taub, 2008). Furthermore, EC influences the morphology, respiration, and proliferation of plants (Barnes, Ollerenshaw, & Whitfield, 1995; Benlloch-Gonzalez, Berger, Bramley, Rebetzke, & Palta, 2014; Gifford, 1995; Roden and Ball 1996; Wang et al., 2012). For plants, these positive effects of EC may be due to higher stomatal conductance and water-use efficiency (WUE) (Onoda, Hirose, & Hikosaka, 2009). Previous studies have mostly focused on the responses of crops such as wheat (Gifford, 1995), cotton (Hu et al., 2013), and soybean (Bunce, 2014) or non-fast-growing tree species, including oak (Stover, Day, Ke, & Hinkle, 2010) and pine (Phillips, Finzi, & Bernhardt, 2011), to EC conditions. Or some metabolic networks have been analyzed due to their biotechnological and basic science importance: the photosynthetic carbon metabolism in a general leaf, the *Rhodobacter spheroides bacterium*, and the *Chlamydomonas reinhardtii alga* (Carapezza et al., 2013). However, for polyploidy plants, the effects of elevated CO_2 on photosynthesis and growth are not clear.

Tetraploid (4×) black locust (*Robinia pseudoacacia* L.) (TBL), which is native to Korea, not only is a preferred tree species in timber forests

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because of its rapid growth and good wood texture but also can be used as a superior feed for domestic fowl and livestock because its fleshy leaves are rich in vitamins and minerals (Meng, Pang, Huang, Liu, & Wang, 2012; Wang, Wang, Liu, & Meng, 2013). Moreover, TBL has a wide range of adaptability to adverse abiotic and biotic environmental conditions (Li et al., 2009; Podda et al., 2013; Zhang & Forde, 2000). Therefore, TBL has higher economic and ecological value due to these properties.

At present, polyploidy induction has become an important method to gain insight into physiological mechanisms of plants in the response to environmental stress (Comai, 2005; Woode et al., 2009). In fact, many polyploidy plants have a superior tolerance to environmental stresses compared to their corresponding diploid (2×) plants (Huang et al. 2002; Zhang, Hu, & Yao, 2010). Chloroplasts, one of the primary organelles, are more sensitive to various environmental stresses than other organelles because photosynthesis and other biochemical and biophysical processes occur within these structures (Barry & John, 1981; Wang et al., 2013). However, the mechanisms by which EC affects chloroplasts are not completely understood. In particular, few studies have explored the special response mechanisms of chloroplasts from polyploidy woody plants to specific EC conditions. Indeed, changes in the response to EC of the chloroplasts from polyploidy plants could have a significant ecological impact.

The aim of this study was to determine the effects of a particular EC concentration (0.5%) on two black locust species (2× and 4×). Based on the response of TBL to other abiotic stresses (Meng et al., 2012; Wang et al., 2013), we proposed the following hypotheses: (1) TBL (fast-growing trees) may adapt better to EC conditions compared to its 2× counterpart, and (2) TBL may adapt to an EC environment via adjustments at the chloroplast level. Accordingly, to determine the physiological and biochemical responses of the 2× and 4× plants to a particular EC condition (0.5%), we measured and observed the morphology, anatomy, photosynthetic ability, antioxidase activities, and ultrastructure and other parameters of the chloroplasts.

2 | MATERIALS AND METHODS

2.1 | Plant growth

All of the materials were introduced into China directly from Korea by Beijing Forestry University. The 2× and 4× black locust (*Robinia pseudoacacia* L.) seedlings were from same germplasm and had the same genetic base (Wang et al., 2013). In June 2014, 30 plants (1 year old) from among the 2× and 4× species were selected for size uniformity and were grown in plastic pots (10 cm in diameter and 10 cm in depth) filled with a 2:1 (v/v) mixture of soil and sand. This experiment was carried out in a high-performance CO_2-controlled growth chamber (Huanghua Faithful Instrument Co., Ltd., Hebei, China) under 0.5% CO_2 concentrations as treated with a light/dark cycle of 16 hr/8 hr, at temperatures of 30/25°C, 50/60% humidity, and 250 μmol photons m^{-2} s^{-1} light. The seedlings were harvested and measured followed by treatment as control (day 0) and then harvested and measured again at 6 days after the treatment (day 6).

2.2 | Determination of leaf morphology, leaf nitrogen (N), and carbon isotope composition ($\delta^{13}C$)

On day harvested, the leaves morphology was photographed. For the determination of nitrate concentrations and carbon isotope composition ($\delta^{13}C$), the leaves were immediately weighed and dried at 80°C for 48 hr to a constant weight. Nitrate concentrations were determined according to the method of Yin et al. (2012). $\delta^{13}C$ was analyzed using a mass spectrometer (Finnegan MAT Delta-E, Germany) (Li et al., 2009).

2.3 | Determination of chlorophyll pigment

Total chlorophyll, chlorophyll *a*, and chlorophyll *b* contents were estimated according to the methods of Meng et al. (2012). 300 mg of fresh leaves was ground in 80% cold acetone and centrifuged at 12,000 *g* for 20 min. Then, the supernatant was collected and fixed in 10 ml of 80% acetone and detected at 470, 645, and 663 nm.

2.4 | Determination of photosynthesis and chlorophyll fluorescence

Net photosynthetic rates (Pn), stomata conductance (Gs), intercellular CO_2 (Ci) of leaves were measured from 09:00 to 11:30 in the morning using a LI-6400 photosynthesis system (Li-Cor, LI-COR Biosciences, Lincoln, NE, USA). The ambient CO_2 concentration, leaf temperature, humidity, and leaf-to-air vapor pressure deficit were 390 ± 10 μmol/L, 25°C, 50%, 1–1.3 kPa. Chlorophyll fluorescence parameters were recorded using a Handy PEA (Hansatech Instruments, Ltd., King's Lynn Norfolk, UK) (Meng et al., 2012). Measurements were repeated at least five times for each treatment.

2.5 | Isolation of chloroplasts

To isolate and purify chloroplasts from the leaves, we followed protocols described by Yin et al. (2011) with minor modifications. All of the steps were performed at 4°C. In total, 30 g of leaves was harvested and ground in 200 ml of isolation buffer I containing 50 mmol/L HEPES (hydroxyethyl piperazine ethanesulfonic acid)/KOH (pH 7.5), 5 mmol/L hexanoic acid, 0.3% bovine serum albumin (BSA) (w/v), 0.3 mol/L sucrose, 10 mmol/L β-mercaptoethanol, 20 mmol/L ethylenediaminetetraacetic acid (EDTA), 30 mmol/L Na-ascorbate, and 1% (w/v) polyvinylpyrrolidone (PVP). Then, the homogenate was filtered through six layers of mesh nylon cloth (40 × 40 μm) and centrifuged at 4,000 *g* for 10 min. The supernatant was centrifuged at 20,000 *g* for 10 min. The precipitate was carefully suspended in buffer II containing 20 mmol/L HEPES/KOH (pH 7.5), 330 mmol/L sorbitol, 10 mmol/L NaCl, 2 mmol/L EDTA, and 5 mmol/L Na-ascorbate and washed twice. Subsequently, the resuspended chloroplasts were loaded onto a Percoll gradient consisting of a 6:6:6:3 ratio, top to bottom, of 10%, 40%, 70% and 90% Percoll. The mixture was centrifuged for 0.5 hR at 40,000 *g*, and the chloroplasts were present between the 40% and 70% interface. Then, the intact chloroplasts were collected, washed, and centrifuged at 10,000 *g* for 15 min in buffer II medium.

2.6 | Measurement of the lipid peroxides and hydrogen peroxide of chloroplasts

Lipid peroxides were estimated using a modified method of Janik-Papis et al. (2009). In total, 0.5 ml of chloroplast supernatant was added to a test tube containing 2 ml of a mixture of 20% TCA (Trichloroacetic acid), 0.01% butylated hydroxytoluene, and 0.6% thiobarbituric acid. The mixture was heated in boiling water for 30 min and then quickly cooled on ice. After centrifugation at 12,000 *g* for 10 min, the absorbance of the supernatant was determined at 450, 532 and 600 nm. Hydrogen peroxide (H_2O_2) was detected spectrophotometrically according to the method of Sergiev et al. (1997). A volume of 0.5 ml of chloroplast supernatant was homogenized in 0.1% TCA in an ice bath. After centrifugation at 12,000 *g* for 10 min, 0.5 ml of the extracted solution was mixed with 0.5 ml of potassium phosphate buffer (pH 7.5) and 1 ml of potassium iodide (1 mol/L). The absorbance of the supernatant was measured at 390 nm, and the concentration of H_2O_2 was obtained using a standard curve.

2.7 | Measurement of the enzymatic activities of chloroplasts

Superoxide dismutase (SOD, EC1.15.1.1) activity was measured following the method of Beauchamp and Fridovich (1971). The reaction mixture contained 20 μl of enzyme extract, 50 mmol/L sodium phosphate buffer (pH 7.8), 100 μmol/L EDTA, and 10 mmol/L pyrogallol. Enzymatic activity was detected spectrophotometrically at 420 nm. Glutathione reductase (GR, EC1.6.4.2) activity was determined via nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm. The reaction mixture contained 10 μl of enzyme extract, 100 mmol/L potassium phosphate buffer (pH 7.8), 0.2 mmol/L NADPH, 2 mmol/L EDTA, and 0.5 mmol/L glutathione. The reaction was initiated by adding NADPH at 25°C (Carlberg & Mannervik, 1975). Peroxidase (POD, EC.1.11.1.7) activity was measured according to the method of Nickel and Ba (1969). Ascorbate peroxidase (APX, EC1.11.1.11) activity was assayed by the method of Nakano and Asada (1981). The reaction mixture contained 50 mmol/L sodium phosphate buffer (pH 7) including 0.2 mmol/L EDTA, 0.5 mmol/L ascorbic acid, and 50 mg of BSA. The reaction was started by adding H_2O_2 at a final concentration of 0.1 mmol/L.

3 | RESULTS

3.1 | Changes in morphology and MDA and H_2O_2 levels

High EC led to chlorosis in the leaves of both 2× and 4× seedlings compared with their corresponding controls (Figure 1a). Furthermore, the 2× leaves exhibited more obvious and severe chlorosis than the leaves from the 4× seedlings (Figure 1b). In addition, H_2O_2 and MDA (malondialdehyde) accumulated significantly in the 2× plants under 0.5% CO_2 conditions. Conversely, there were no differences in the H_2O_2 and MDA contents of the 4× plants between the control and treated (0.5%) samples (Figure 1c,d).

3.2 | Chlorophyll content, N content, and carbon isotope composition ($\delta^{13}C$)

The contents of total chlorophyll (chl), chl *a* and chl *b* of 2× and 4× plants are presented in Table 1. In the 2× species, statistically significantly lower contents of total chl (52.86%), chl *a* (51.11%), and chl *b* (57.14%) were detected after 0.5% CO_2 treatment, whereas no significant changes were found in total chl, chl *a*, and chl *b* of the 4× plants under 0.5% CO_2 conditions. In contrast, 0.5% CO_2 did not significantly affect the carbon isotope composition ($\delta^{13}C$) or N content of the 2× and 4× plants.

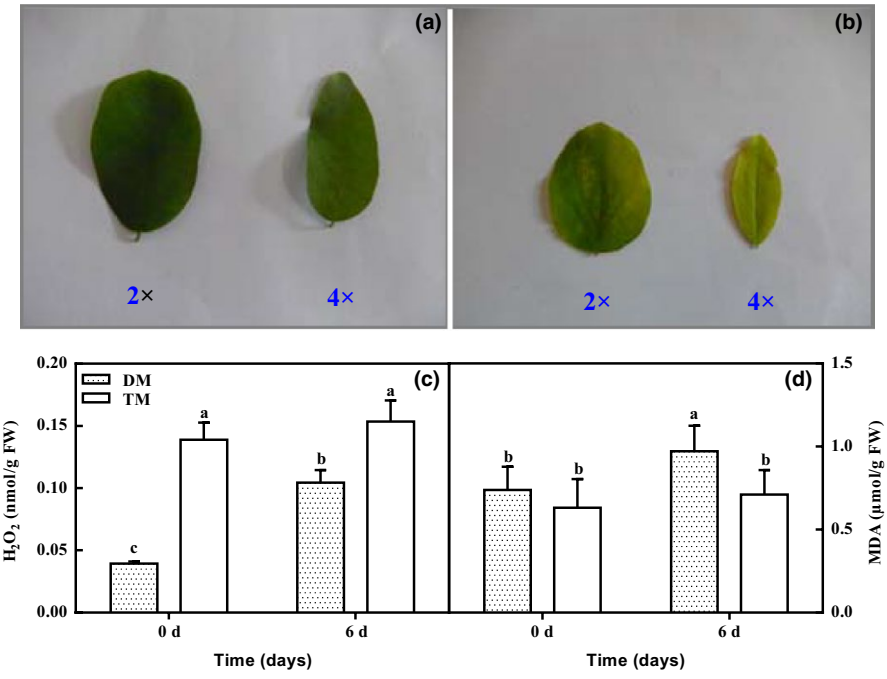

FIGURE 1 Morphology (a and b), H_2O_2 (c), and MDA (d) contents in chloroplasts of leaves in 2× and 4× at 0 and 6 days, respectively. (a) Morphology of leaves of 2× (diploid black locust) and 4× (tetraploid black locust) at 0 day; (b) morphology of leaves of 2× and 4× at 6 days; (c) H_2O_2 content of leaves of 2× and 4× at 0 and 6 days, respectively; (d) MDA content of leaves of 2× and 4× at 0 and 6 days, respectively; DM, 2×; TM, 4×

TABLE 1 Chlorophyll *a*, chlorophyll *b*, total chlorophyll contents, N content, and carbon isotope composition ($\delta^{13}C$) in leaves of 2× and 4× affected by high CO_2 condition

		Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total chlorophyll (mg/g)	$\delta^{13}C$ (‰)	N (g/kg)
2×	Control	0.765 ± 0.057[a]	0.336 ± 0.029[a]	1.101 ± 0.083[a]	−29.28 ± 0.17[b]	12.10 ± 0.28[a]
	Treatment	0.374 ± 0.058[b]	0.144 ± 0.031[b]	0.519 ± 0.088[b]	−29.05 ± 0.10[b]	12.80 ± 0.18[a]
4×	Control	0.807 ± 0.040[a]	0.337 ± 0.033[a]	1.144 ± 0.072[a]	−33.08 ± 0.06[a]	11.95 ± 0.23[a]
	Treatment	0.786 ± 0.113[a]	0.323 ± 0.053[a]	1.109 ± 0.166[a]	−32.37 ± 0.16[a]	12.08 ± 0.24[a]

Different small letters mean significant differences in the same parameters ($p < .05$).

3.3 | Chloroplast ultrastructure

The ultrastructures of the 2× and 4× species are depicted in Figure 2. The ultrastructure and arrangement of the chloroplasts of both the 2× and 4× seedlings were normal under control conditions, but injuries were apparent in the 2× plant under 0.5% CO_2 conditions (Figure 2A). Under control conditions, grana (Gr) of both the 2× and 4× seedlings were well developed and highly stacked with normal thylakoids (Figure 2). In the 2× plants, 0.5% CO_2 induced a disturbance of the chloroplast structures. Swollen, instead of smooth and spindly, chloroplasts and marked swelling of the Gr and an incompact structure of thylakoids in the chloroplasts were found. The effects of starch and osmiophilic globule accumulation were also observed relative to controls (Figure 2A). In contrast, the 4× plants maintained structural integrity of the chloroplasts compared with those of the 2× plants after 0.5% treatment (Figure 2B). An important difference between diploid and tetraploid chloroplasts is the amount of osmiophilic bodies- in the diploid. About 0.5% CO_2 induced increased numbers of osmiophilic globules in 2× plants (Figure 2A, e and f).

3.4 | Measurement of gas exchanges

The photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 (Ci) in the 2× and 4× leaves decreased after exposure to 0.5% CO_2 (Table 2). However, the decreases in the 2× leaves were much greater than those in the 4× leaves. Notably, the Pn and Ci in the 2× leaves decreased dramatically after high CO_2 treatment.

3.5 | Measurement of chl fluorescence

The 2× and 4× leaves exhibited a typical O-J-I-P transient chl fluorescence upon exposure to 0.5% CO_2 conditions and controls (Figure 3a). However, 0.5% CO_2 lowered the fluorescence intensity, especially in I to P step of the 2× plants; thus, the shape of the O-J-I-P transient was altered (Figure 3a). In addition, according to the *Vo*-p, the V_J (J-step) (which occurred at approximately 2 ms) was decreased, and there was no difference in the V_I between the 2× and 4× plants after 0.5% CO_2 treatment (Figure 3b). The values of V_k (K-step) and V_J in the O-J phase of the 4× samples were decreased after 0.5% CO_2 treatment (Figure 3c). However, the 2× samples displayed little change in the values of V_k and V_J in the O-J phase (Figure 3d). Furthermore, the maximum quantum yield of the PSII photochemistry (Fv/Fm) was reduced in the high-CO_2-treated leaves of 2× plants (Figure 3e). The photosynthetic performance index (PI_{ABS}) of both the 2× and 4× species increased after 0.5% CO_2 treatment, while that of the 4× was higher compared with that of the 2× species (Figure 3f).

3.6 | SOD, GR, POD, and APX activities

The application of 5% CO_2 decreased the activities of SOD, GR, and POD in the 2× plants by 27.49%, 52.21%, and 6.05%, respectively, while the APX activity exhibited a significant increase (38.38%) (Figure 4). However, in the 4× plants, the SOD and GR activities were marginally decreased, while the APX activity was greatly stimulated by 0.5% CO_2 (Figure 4). In addition, the SOD, GR, POD, and APX activities in the 4× plants were much higher than those in the 2× plants both in control and treatment.

4 | DISCUSSION

Chloroplasts are one of the vital organelles in plant cells because photosynthesis occurs in these structures. Many plant species display ultrastructural damage from environmental stress (Liu, Zhang, Qi, & Li, 2014). Accordingly, this damage to the chloroplast may lead to a significant decrease in photosynthesis (Zhang et al., 2010). This decrease in photosynthesis may result from rearrangements of the light-harvesting complex I and light-harvesting complex II (Garstka et al., 2005). In the current study, 0.5% CO_2 induced a disturbance in the chloroplast structure by increasing the number of starch and osmiophilic globules (Figure 2). In other words, high CO_2 conditions inhibit the growth of 2× plants due to a decrease in the Pn, reduction in the pigment content, destruction of the ultrastructure of the chloroplast. In contrast, there was no significant effect of high CO_2 on the growth of the 4× plants. These results are consistent with results of previous studies (Jiang, Yang, & Zhang, 2007; Velikova et al., 2009). For example, potato plants accumulated more starch under long-term CO_2 enrichment (Sun, Li, & Liu, 2011). Hao et al. (2013) also reported that the accumulated starch within chloroplasts can cause a decline in photosynthesis with CO_2 enrichment. This accumulation of starch grains may result in the arrangement of Gr thylakoids. Accordingly, such an alteration may lead to an inhibition of light energy absorption and a decrease in photosynthesis. Thus, 0.5% CO_2 can affect a vital function in chloroplast structure regulation. However, the physiological, molecular, and biochemical mechanisms of starch accumulation and subsequent photosynthesis inhibition under high CO_2 conditions remain unclear and require further investigation.

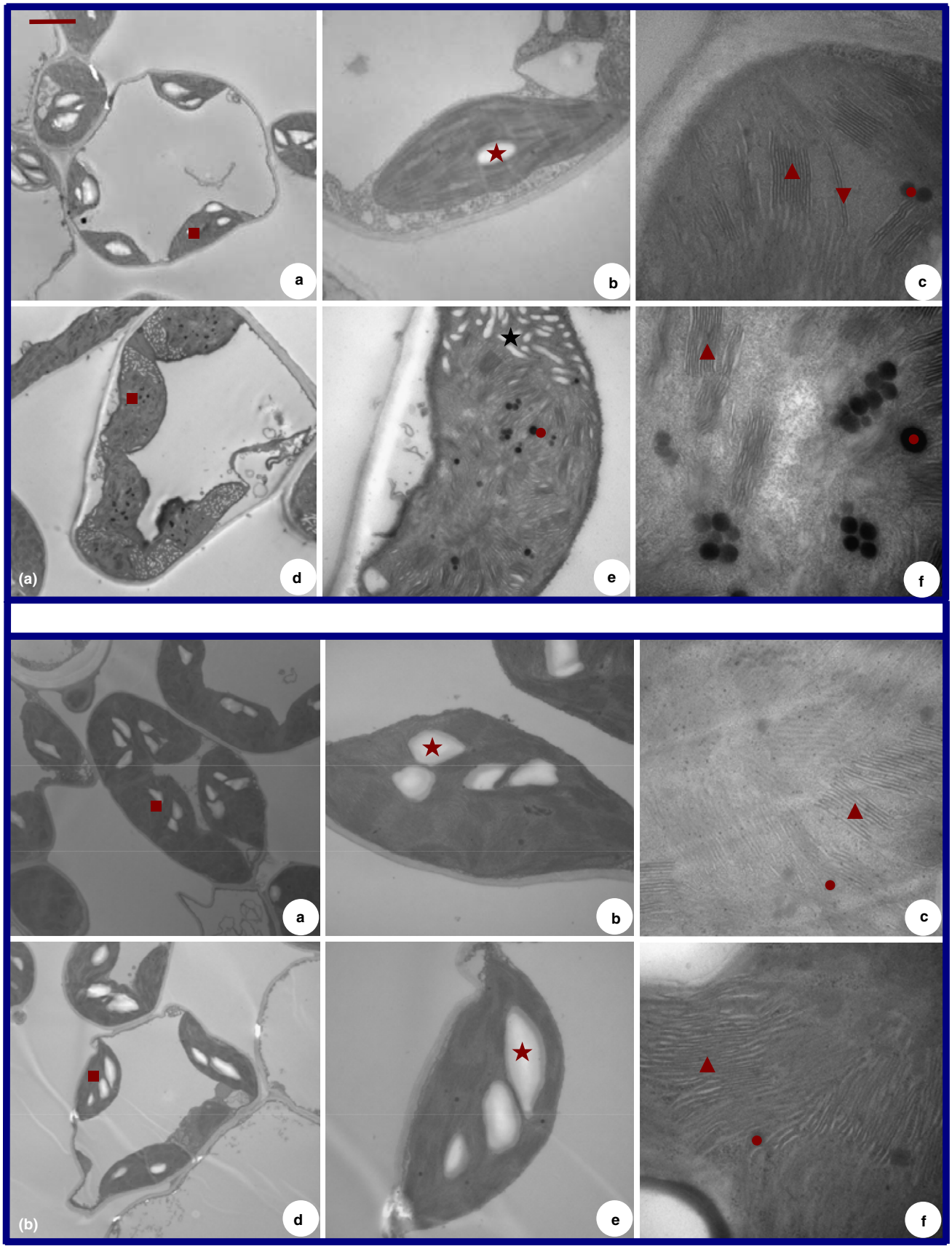

FIGURE 2 Transmission electron micrographs of chloroplasts in 2× (a) and 4× (b) at 0 (up) and 6 (down) days, respectively. 2×, 4×, a and d (bar = 0.25 μm), arrangement of chloroplast; b and e (bar = 0.5 μm), ultrastructure of chloroplast; c and f (bar = 100 nm), grana thylakoids of chloroplast. Five-pointed star (★), starch grains; square (■), chloroplast; up-triangle (▲), grana; down-triangle (▼), thylakoids; ball (●), liposomes

TABLE 2 Gas exchange and chlorophyll fluorescence in leaves of 2× and 4× as affected by 0.5% CO_2

Species	Treatment (days)	Pn ($\mu mol\ m^{-2}\ s^{-1}$)	Gs ($mmol\ m^{-2}\ s^{-1}$)	Ci ($\mu mol\ CO_2\ mol^{-1}$)	Tr ($mmol\ m^{-2}\ s^{-1}$)	NPQ
2×	0	7.21 ± 0.85^a	0.26 ± 0.06^a	236.81 ± 35.6^a	2.14 ± 0.70^a	0.72 ± 0.14^b
	6	2.20 ± 0.41^b	0.14 ± 0.01^b	73.52 ± 47.92^c	0.30 ± 0.06^b	1.26 ± 0.03^a
4×	0	9.88 ± 0.41^a	0.30 ± 0.07^a	247.36 ± 72.11^a	2.49 ± 0.06^a	1.35 ± 0.29^b
	6	4.95 ± 0.81^b	0.21 ± 0.05^b	150.55 ± 22.92^b	1.10 ± 0.21^b	2.02 ± 0.04^a

Pn, photosynthesis rate; Gs, stomatal conductance; Tr, transpiration rate; Fv/Fm, maximal efficiency of PS II; NPQ, nonphotochemical quenching. Data are presented as the mean ± *SD*. Different small letters mean significant differences in the same parameters ($p < .05$).

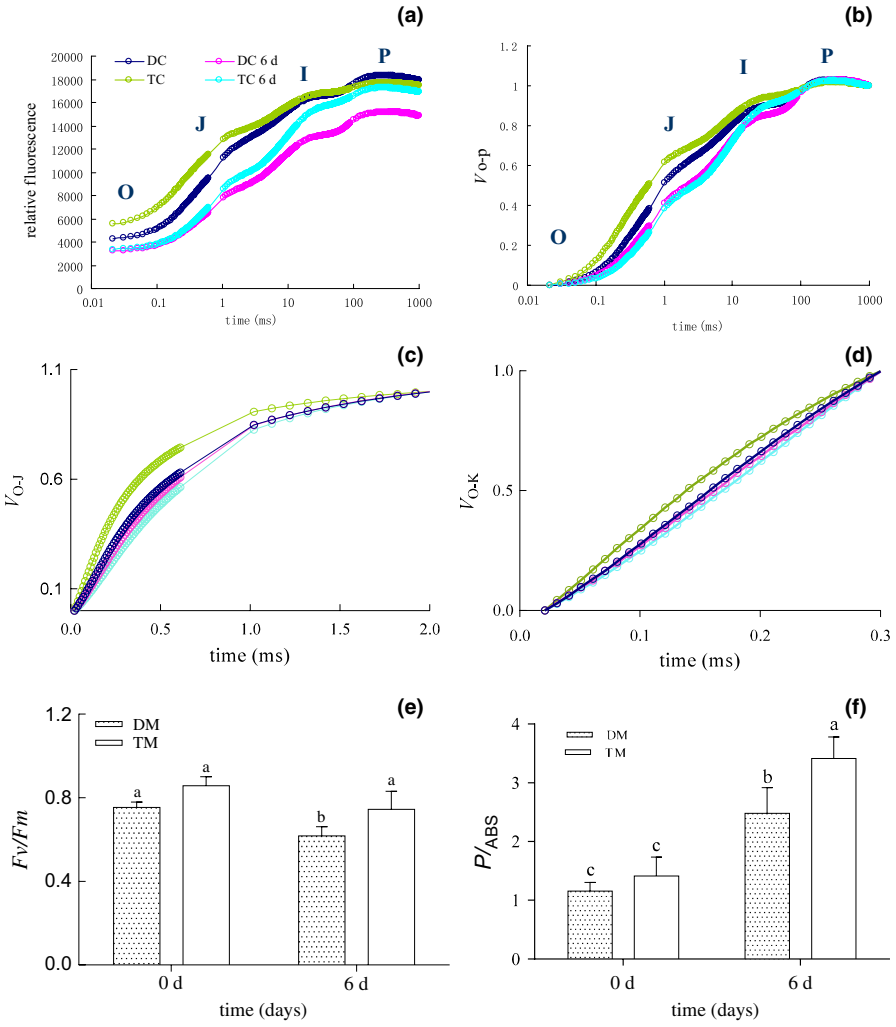

FIGURE 3 Chlorophyll fluorescence including relative fluorescence (a), $V_{o\text{-}P}$ (b), $V_{o\text{-}J}$ (c), $V_{o\text{-}k}$ (d), *Fv/Fm* (e), and PI_{ABS} (f) in leaves of 2× and 4× at 0 and 6 days, respectively. 2×, diploid black locust; 4×, tetraploid black locust; DC, 2× at 0 day; TC, 4× at 0 day; DC6d, 2× after 0.5% CO_2 treatment of 6 days; TC6d, 4× after 0.5% CO_2 treatment of 6 days; DM, 2×; TM, 4×

Both mineral nitrogen (N) and CO_2 are involved in plant growth (Peterson et al., 1998). Our present study suggested that 0.5% CO_2 increased the leaf N content of the 2× and 4× seedlings by 0.06% and 0.01%, respectively; however, this effect was not statistically significant (Table 1). This finding led us to conclude that elevated CO_2 does not have a significant effect on N content. In fact, N is also a part of chl (Yin et al., 2012). However, elevated CO_2 induced a decrease in chl *a*, chl *b*, and total chl of the 2× plants by approximately 50%, which could partly explain the decrease in the Pn (Table 2). Thus, the decrease in chl depends not on the effects of the N content but on other factors.

For plants, $\delta^{13}C$ is chiefly controlled by the photosynthetic pathway (Lomax, Knight, & Lake, 2012). Farquhar, O'Leary, and Berry (1982) reported that the Tr can be estimated by measuring the $\delta^{13}C$ in leaves during photosynthesis. Similar results have also been obtained in some legume crops, such as bean, cowpea, groundnut, and soybean (Kashiwagi et al. 2006). Therefore, there is a close relationship between the $\delta^{13}C$ and Tr. However, no significant correlation was observed between the $\delta^{13}C$ and Tr when the 2× and 4× plants were grown under 0.5% CO_2 conditions (Tables 1 and 2). A similar result has been reported in sunflower (Virgona, Hubick, Rawson, Farquhar, & Downes, 1990). This indicated that the $\delta^{13}C$ discrimination was limited in describing the Tr under elevated CO_2 conditions. Alternatively, our results on black locust may indicate that the differences in the Tr are attributed to changes in Gs rather than by changes in mesophyll

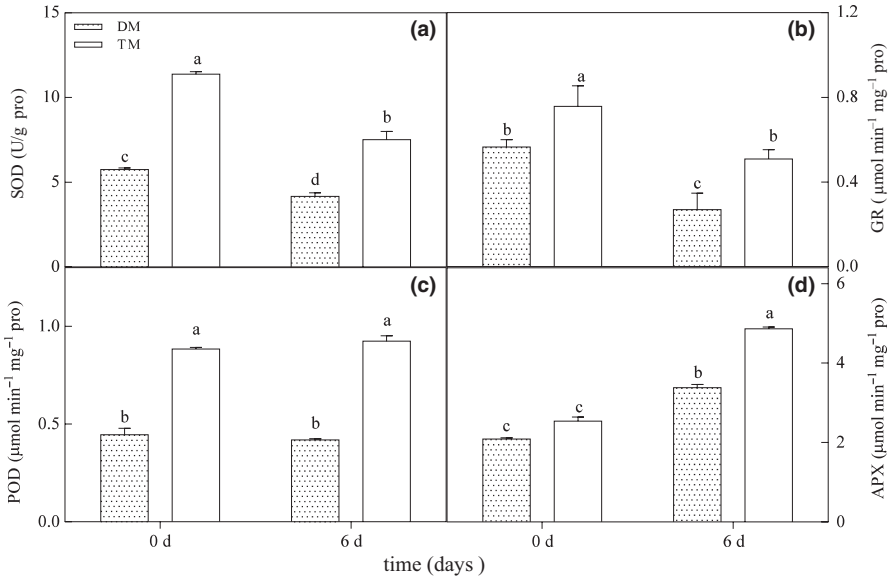

FIGURE 4 SOD (a), GR (b), POD (c), and APX (d) activities of chloroplasts in leaves of 2× and 4× at 0 and 6 days. 2×, diploid black locust; 4×, tetraploid black locust; DM, 2×; TM, 4×

efficiency. These findings were extended, indicating that elevated CO_2 negatively affects the Pn or Tr at N and $\delta^{13}C$ levels.

Generally, an OJIP curve reflects the photochemical state in PSII (Tóth, Schansker, Garab, & Strasser, 2007). It is accepted that the shape of the initial fluorescence rise is reflected by the energetic connectivity or probability of exciton exchange between the PSII units (Yordanov et al., 2008). In higher plants, the OJIP curve possesses a sigmoidal shape under optimal conditions and is sensitive to various abiotic stresses (Lu, Qiu, Wang, & Zhang, 2003; Pierangelini, Stojkovic, Orr, & Beardall, 2014; Zhang et al., 2011). In our experiment, a decreased rate of the J-step and I-step was found in the 2× and 4× species after 0.5% CO_2 treatment (Figure 3a,b). One may speculate that this effect could be related to the low absorption cross section of PSII and higher segregation of the photosystems, thereby reducing energetic connectivity between individual PSII units (Govindjee, 1995; Schansker, Tóth, & Strasser, 2005). The OJIP curves of the CO_2-treated 2× and 4× plants declined significantly (especially for the 2× sample), which indicated that an impairment of electron flow from PSII to the PQ pool occurred (Antal et al., 2006). In addition, a prominent decline in the P point was found in the 4× plant after 0.5% CO_2 treatment, which may be due to the influence of nonphotochemical quenching (Antal et al., 2009).

To further characterize the function of the photosynthetic apparatus in response to the strain under the special EC condition (0.5% CO_2), we also analyzed selected parameters derived from the OJIP curves. Generally, an increased rate of *V*k in the O-J phase is a convenient indicator of the degree of damage of the oxygen-evolving complex (OEC) activity (Pospíšil & Dau, 2000). The OEC in the membrane-bound protein complex PSII catalyzes the water oxidation reaction that takes place in oxygenic photosynthetic organisms (Carina et al., 2013). In this study, the Vk value in the O-J phase in both the 2× and 4× plants tended to decrease under elevated CO_2 (0.5%) compared to the 2× plants (Figure 3c). The magnitude of this drop was higher in the 4× than in the 2× plants, indicating that the special CO_2 may induce OEC activity to protect PSII. However, these changes were not confirmed by the structural changes of thylakoids in the 2× chloroplasts (Figure 2) because the inhibition of the OEC activity can lead to lipid peroxidation damage, an increase in membrane permeability, and the release of more liposomes (Takahiro, Mitsue, & Yasusi, 2004). Generally, the degradation of thylakoids can result in the formation of liposomes (Ma, Zhang, Wang, Song, & Zhang, 2013). Therefore, in the 2× plants, an incompact structure of thylakoids was found in the chloroplasts with more liposomes compared with the 4× chloroplasts, which suggests that the damage to the OEC in the 2× plants by 0.5% CO_2 treatment was not more significant than that in the 4× plants (Figure 2). We also noted that the *L* value (V_L, 0.15 ms) in the O-K phase of the 4× sample was decreased after 0.5% CO_2 treatment; however, this value only changed a little (Figure 3d). In general, the absorption, transmission, and transformation of light energy are carried out in the thylakoid membranes (Chow, Haehnel, & Anderson, 2006). The increase of the V_L value is an indicator of thylakoid dissociation. The structure of thylakoids is related to PSII function (Kirchhoff et al., 2007). Accordingly, the 4× samples had improved function of the thylakoids after 0.5% CO_2 treatment. In addition,

The equation ϕ_{po} = Fv/Fm is widely used to reflect the maximum potential efficiency of PSII or the intrinsic quantum efficiency of the PSII units (Kitajima & Butler, 1975). As a multiparametric expression of the three main functional steps of photosynthetic activity by a PSII reaction center complex, the overall photosynthetic performance index (PI_{ABS}) is also suitable to distinguish the photosynthetic performance of plants under various environmental conditions (Lepeduš et al., 2012). As shown in Figure 3, when exposed to 0.5% CO_2, the 2× and 4× seedlings all exhibited decreases in Fv/Fm, indicating that 0.5% CO_2 affected the behavior of PSII. At the same time, the different Fv/Fm ratio with 0.5% CO_2 suggested that the photochemical reaction in 4× seedlings was different from that of 2× seedlings. Furthermore, the results of this study clearly reveal that the 4× species maintained a higher level of PI_{ABS} than did the 2× species under 0.5% CO_2 conditions. Therefore, the photosynthetic system was damaged more severely in the 2× than in the 4× plants by 0.5% CO_2 treatment.

As a consequence of the inhibition of PSII, excess light energy will accordingly lead to photoinhibition and oxidative stress, which can

lead to a burst of reactive oxygen species (ROS, such as H_2O_2, ·OH, and $O_2^{\cdot-}$) or lipid peroxidation (Asada, 1999; Yin, Pang, & Chen, 2009). Furthermore, an increase in ROS will result in chl loss and a decrease in photosynthetic CO_2 assimilation (Ahmed et al., 2013). As evidence of ROS and lipid peroxidation generation, significantly increased H_2O_2 and MDA were observed in the 2× seedlings after 0.5% CO_2 treatment (Figure 1). Additionally, Pn and chl contents were decreased in the 2× plants by 0.5% CO_2 treatment (Figure 1, Tables 1 and 2). These observations confirmed that more serious oxidative stress occurred in the 2× than in the 4× plants after 0.5% CO_2 treatment; that is, the 4× species had less oxidative stress.

To alleviate the damage initiated by oxidative stress, plants can modulate antioxidative enzymes such as SOD, POD, GR, etc. (Smirnoff and Wheeler 2000). The SOD, APX, and GR activities in leaves increase when exposed to elevated CO_2 (Guo, Zhou, & Zhang, 2015). In our experiments, SOD, GR, and POD activities were decreased in the chloroplasts from the 2× leaves under the 0.5% CO_2 condition (Figure 4). In contrast, the activities of POD and APX of the 4× leaves were remarkably stimulated by 0.5% CO_2 (Figure 4). This result suggested that high CO_2 stimulated an increase in the antioxidant enzyme activities of the 4× leaves to alleviate oxidative damage and indicted that the 4× species has a highly efficient defense system under elevated CO_2 conditions to some extent. Although SOD, GR, and POD activities showed different changes between diploid and tetraploid black locust under specially elevated CO_2 (0.5%), some sensitive enzymes have not been identified in the photosynthetic carbon metabolism. For instance, Umeton et al. (2012) found that 11 sensitive enzymes are confirmed to play a key role in terms of maximal CO_2 uptake and minimal nitrogen consumption. Thus, in the future, these parameters or molecular methods should be used to gain a systematic and comprehensive understanding of polyploidy plants under elevated CO_2.

In some studies, increased numbers of osmiophilic globules in the cytoplasm and nucleolus-associated bodies as well as electron dense material in vacuoles were observed in cadmium tolerant cells (Gzyl, Przymusiński, & Gwóźdź, 2009). And Bréhélin also found that the tocopherols stored in osmiophilic globules would be delivered to thylakoid membranes to scavenge ROS under oxidative stress (Bréhélin, Kessler, & van Wijk, 2007). In *Tillandsia albida (Bromeliaceae)*, Papini found an increase in plastoglobules in plastids involved in autophagy (Papini, Mosti, & van Doorn, 2014). And Sallas et al. also reported that a non significant decrease in the number of plastoglobules in response to elevated CO_2 in Norway spruce while an increase in number of chloroplast plastoglobules in Scots pine (Sallas, Luomala, Utriainen, Kainulainen, & Holopainen, 2003). It indicated that the response of plastoglobules depends on the plant species.

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CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Fanjuan Meng and Yuan Cao conceived and designed the experiments; Yuan Cao and Mingquan Jiang performed the experiments; Shuo Liu, Mingquan Jiang, and Fuling Xu analyzed the data; Yuan Cao and Mingquan Jiang wrote the manuscript; Shuo Liu provided editorial advice.

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