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ORIGINAL ARTICLE

Nitric oxide enhances development of lateral roots in tomato (*Solanum lycopersicum* L.) under elevated carbon dioxide

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Abstract Elevated carbon dioxide (CO_2) has been shown to enhance the growth and development of plants, especially of roots. Amongst them, lateral roots play an important role in nutrient uptake, and thus alleviate the nutrient limitation to plant growth under elevated CO_2. This paper examined the mechanism underlying CO_2 elevation-induced lateral root formation in tomato. The endogenous nitric oxide (NO) in roots was detected by the specific probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA). We suggest that CO_2 elevation-induced NO accumulation was important for lateral root formation. Elevated CO_2 significantly increased the activity of nitric oxide synthase in roots, but not nitrate reductase activity. Moreover, the pharmacological evidence showed that nitric oxide synthase rather than nitrate reductase was responsible for CO_2 elevation-induced NO accumulation. Elevated CO_2 enhanced the activity of nitric oxide synthase and promoted production of NO, which was involved in lateral root formation in tomato under elevated CO_2.

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Keywords Elevated CO_2 · Lateral root · Nitric oxide · Nutrient acquisition · Root system

Abbreviations

cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
DAF-FM DA	4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate
L-NAME	N^G-Nitro-L-arginine methyl ester
LR	Lateral root
NO	Nitric oxide
NOS	Nitric oxide synthase
NR	Nitrate reductase
SNP	Sodium nitroprusside

Introduction

Current Intergovernmental Panel on Climate Change (IPCC) projections indicate that atmospheric carbon dioxide (CO_2) concentration will increase over this century, reaching 730–1,020 ppm by 2100 (Meehl et al. 2007). It has been confirmed that elevated CO_2 enhances the photosynthesis and inhibits the mitochondrial respiration metabolism, thus promoting sucrose accumulation and plant growth (Bunce 1994; De Souza et al. 2008). In order to acquire enough nutrients, the plants need to form a strong root system to enhance nutrient acquisition, among which the lateral root (LR) development is vitally important (BassiriRad et al. 2001). Lateral roots are formed in the pericycle cells, which differentiate and proliferate to form LR primordia, and then further differentiate and elongate causing the LR to emerge through the epidermis (Malamy and Benfey 1997). It has been shown that elevated CO_2

(800 μL L^{-1}) significantly increases LR number, total root length, root surface area, root diameter and root volume in tomato (Wang et al. 2009). Our recent study demonstrated an important effect of elevated CO_2 on development of root hairs in *Arabidopsis* through the auxin signaling pathway (Niu et al. 2011). It has been suggested that the changes in root morphology provide the plant an efficient strategy to alleviate the limitation of nutrients under elevated CO_2. However, detailed mechanisms underlying the enhancement of LR development by elevated CO_2 are not fully understood.

Several lines of evidence suggest that nitric oxide (NO) emerged as a freely diffusible signaling molecule, and plays an important role in diverse physiological processes, including seed germination (Bethke et al. 2004), plant growth and development (Guo and Crawford 2005; Neill et al. 2006), stomata movement (Neill et al. 2003), and resistance to biotic and abiotic stresses (Tian et al. 2007; Asai et al. 2008). In addition, NO is involved in the growth and development of lateral roots in tomato (Correa-Aragunde et al. 2004), which acts downstream of auxin in regulating Fe-deficiency-induced tomato root branching (Jin et al. 2011). Under normal growth conditions, auxin and other important components in the auxin signaling are critical for LR development (Himanen et al. 2002; Benková et al. 2003). Recent studies showed that elevated CO_2 increased auxin level and response (Li et al. 2002; Teng et al. 2006; Niu et al. 2011), and that production of NO was promoted in tomato roots under co-treatment with elevated CO_2 and iron deficiency (Jin et al. 2009a). Consequently, NO may be involved in the CO_2 elevation-induced LR development. However, the direct evidence supporting this hypothesis is still lacking.

Although many processes are controlled by NO in plants, the molecular mechanisms responsible for the biosynthesis of this radical remain controversial. In animals, NO is generated by nitric oxide synthase (NOS and NOS-like enzymes), which converts L-arginine to L-citrulline and NO (Mayer and Hemmens 1997). In plants, although the direct homologs of any animal enzyme and protein have not been identified, N^G-nitro-L-arginine methyl ester (L-NAME) as an inhibitor of mammal NOS, significantly inhibits the activity of NOS and decreases the level of NO (Tian et al. 2007). Constitutive NOS activity, which appeared to be regulated by plant part and developmental stage, was detected in the leaf, stem and root of pea seedlings (Corpas et al. 2006). Importantly, Flores et al. (2008) showed that NOS played a crucial role in the formation of lateral roots in *Arabidopsis*. Another enzymatic source of NO is nitrate reductase (NR) in plants. It has been suggested that NR uses nitrite as a substrate to generate NO in vitro and in vivo (Dean and Harper 1986; Neill et al. 2003). NR has also been demonstrated a potential physiological role in mediating auxin-induced LR formation (Kolbert and Erdei 2008). Until recently, there was no consistent evidence for an increase of NR activity in elevated CO_2. It was reported that elevated CO_2 leads to increased NR activity in *Plantago major* and tobacco as compared with ambient CO_2 (Fonseca et al. 1997; Geiger et al. 1998); on the contrary, an inhibitory effect was shown in wheat, maize and tobacco (Ferrario-Méry et al. 1997). This discrepancy may result from different plant species, tissues, development stage and growing conditions.

Therefore, the objective of this study was to determine whether NOS and/or NR is involved in LR response to elevated CO_2. Tomato was used as an experimental material. We hypothesized that elevated CO_2-induced accumulation of NO was critical for the formation of lateral roots through increasing activity of NOS.

Materials and methods

Seeding culture

Seeds of tomato (*Solanum lycopersicum* L. cv. Micro-Tom, kindly provided by Dr. Chongwei Jin, Zhejiang University, China) were germinated in a 0.5 mM $CaSO_4$ solution. Four days after sowing, seedlings were transferred to an aerated hydroponic system containing nutrient solution (pH 6.8) with the following composition (in μM): H_3BO_3, 10; $MnSO_4$, 0.5; $ZnSO_4$, 0.5; $CuSO_4$, 0.1; $(NH_4)_6Mo_7O_{24}$, 0.1; Fe-EDTA, 25; KH_2PO_4, 500; $MgSO_4$, 500; $CaCl_2$, 1000; KNO_3, 1500. All plants were grown at 60 % humidity under a daily cycle of 25 °C, 14-h day and 22 °C, 10-h night in Conviron E7/2 growth chambers (Controlled Environment Ltd., Winnipeg, Manitoba, Canada). The daytime light intensity was 200 μmol photons m^{-2} s^{-1}. The nutrient solution was renewed every 3 days and the solution pH was adjusted daily. After 15 days, CO_2 treatments were initiated by growing plants in chambers with a CO_2 concentration of either 350 (ambient CO_2) or 800 μL L^{-1} (elevated CO_2).

Experimental design

Uniform seedlings were grown in basal nutrient solution with or without the NO scavenger [0.5 mM cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide], the NO donor (0.2 mM SNP, sodium nitroprusside), the NOS inhibitor (0.2 mM L-NAME) or the NR inhibitor (0.1 mM tungstate). The nutrient solution was renewed every 2 days. After treatment for 2 days, the roots of seedling were harvested to determine the level of NO in both LR primordia and root tips, as well as the activities of NOS and NR. The number of LR (≥1 mm) in the range of

15 cm from the root tip was recorded under the treatment for 4 days.

Determination of NO level in roots

In vivo measurements of NO was conducted using the NO-specific fluorescent probe [4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA)] (Foresi et al. 2007). Segments of 4 cm from the root apex of plant seedlings were excised and incubated with 5 μM DAF-FM DA in Hepes–KOH buffer (pH 7.5) for 30 min, followed by washing three times with Hepes–KOH buffer to remove excess fluorescent probe. DAF-2T fluorescence was visualized using a microscope with excitation and emission wavelengths of 488 and 515 nm, respectively. The fluorescence intensity of fluorescent image acquired using a digital camera (Nikon), was determined by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The fluorescence intensity, named as relative fluorescence unit, was expressed in color level on a scale ranging from 0 to 255.

Protein extraction and quantification

The concentration of total protein in plants was extracted as described by Tian et al. (2007) with some modifications. Briefly, 0.2 g tomato roots were frozen in liquid nitrogen and ground to a fine powder; the powder was then homogenized in 2 mL extraction solution. The solution was composed of 100 mM Hepes–KOH buffer (pH 7.5), 1 mM EDTA, 10 % glycerol, 5 mM dithiothreitol, 0.1 % Triton X-100, 0.5 mM phenylmethylsulfonyl, 20 μM FAD, 25 μM leupeptin, 5 μM Na_2MoO_4 and 1 % polyvinylpyrrolidone. The solution was centrifuged at 13,000*g* for 20 min at 4 °C. The clear supernatant was used to determine the activity of NR and NOS. The concentration of protein was measured using the method of Bradford (1976), with BSA as a standard.

Determination of NOS activity

A NO synthase assay kit (Beyotime, Haimen, China) was used to determine NOS enzyme activity according to the manufacturer's instructions (Xiong et al. 2009; Ding and Zhang 2012). Briefly, 0.2 mL of clear supernatant was added to 0.1 mL assay mixture containing NADPH, L-arginine and DAF-FM DA, and then reacted at 37 °C in the dark for 1 h. The concentration of NO was detected with a laser confocal scanning microscope (Leica Microsystems, Mannheim, Germany), and the excitation and emission wavelengths were 488 and 515 nm, respectively. The pixel intensity of fluorescence was determined by ImageJ software. Values were corrected for the blank control. The fluorescence intensity of the control was defined as 100 %, and the relative NOS activity was expressed with relative fluorescence unit compared to the control value.

Determination of NR activity

The NR activity was determined as described by Tian et al. (2007) with some modifications. A total of 0.2 mL of clear supernatant was added into 0.4 mL of pre-warmed assay buffer containing 100 mM Hepes buffer, 5 mM KNO_3 and 0.25 mM NADH. The mixed solution was reacted at 30 °C for 1 h, and then Zn-acetate was added to the solution to stop the reaction. The amount of nitrite produced was measured colorimetrically at 540 nm after application of 1 mL of 1 % sulfanilamide in 3 M HCl and 1 mL of 0.2 % *N*-(1-naphthyl)-ethylenediamine.

Statistical analysis

The data were subjected to statistical analysis by analysis of variance using SPSS for Windows version 18.0 (CoHort Software, Berkeley, CA, USA). Least significant difference test was applied to compare the treatment means.

Results

Enhancement of LR development under elevated CO_2

Increasing evidence suggests that elevated CO_2 induces the accumulation of carbohydrates and thus promotes plant root growth and development (De Souza et al. 2008). In the present study, the effect of elevated CO_2 on the development of tomato LRs was observed after 4 days of treatment. The number and length of LRs are significantly higher under elevated CO_2 than under ambient CO_2 (Fig. 1a). The number of LRs increased by 75 % under elevated CO_2 compared with ambient CO_2 (Fig. 1b). This result is consistent with the observation of Wang et al. (2009).

Role of NO in the formation of LRs under elevated CO_2

The levels of NO in LR primordia and root tips were determined using the cell NO-specific fluorescent probe (DAF-FM DA). Because DAF-FM DA reacts with NO derived to yield a fluorometric molecule (DAF-2 T), NO levels in roots were measured according to the intensity of fluorescence (Foresi et al. 2007). As shown in Fig. 2b, c, the levels of NO in both LR primordia and root tips were 67 and 62 % higher, respectively, under elevated CO_2 than under ambient CO_2. This suggests that elevated CO_2 promoted the production of NO in LR primordia and root tips.

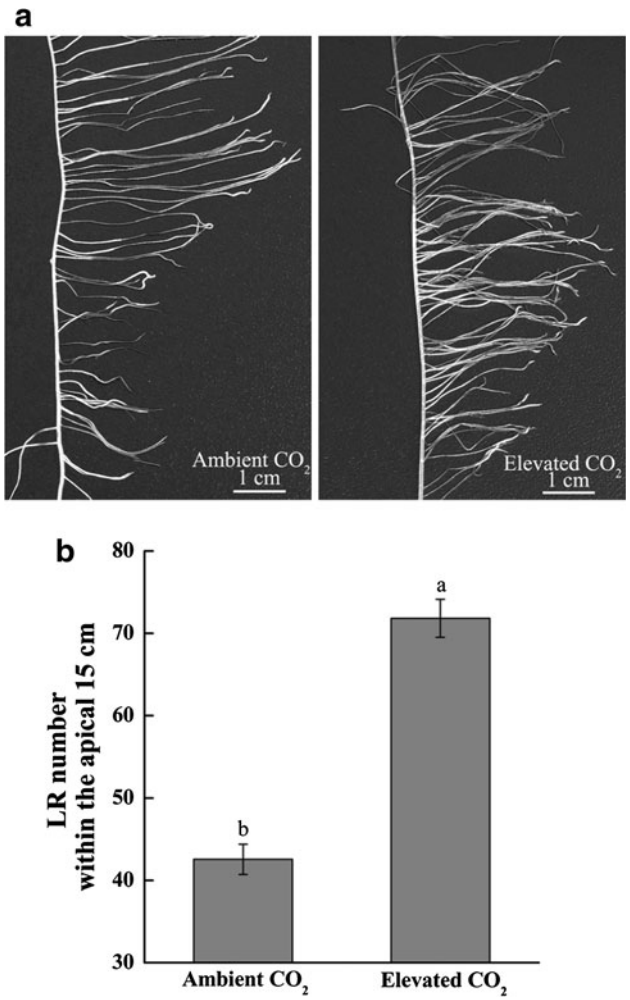

Fig. 1 Elevated CO_2 enhanced the formation of lateral roots (LRs) in tomato (*Solanum lycopersicum* L.). **a** The photos of LR region of a plant grown under ambient CO_2 or elevated CO_2 for 4 days, **b** the LR number of the root treated for 4 days with ambient CO_2 or elevated CO_2. *Error bars* represent the standard error of means ($n = 6$). *Different letters* indicate a significant difference ($P < 0.05$) between treatments

To verify the role of NO in mediating CO_2 elevation-induced LR development, the NO donor SNP (Bethke et al. 2004) and the NO scavenger cPTIO (Planchet et al. 2006) were used to shift the regulatory function of NO in tomato. When cPTIO was applied, the LR number decreased by 70 % under elevated CO_2 (Fig. 2a), and the levels of NO in LR primordia and root tips decreased by 40 and 44 %, respectively, similar to those under ambient CO_2 (Fig. 2b, c). Moreover, SNP increased the levels of NO in LR primordia and root tips by 300 and 100 %, respectively; and the formation of LRs by 100 % (Fig. 2). These data suggest that the LR number was positively related with the level of NO in the LR primordia, and confirmed that NO was involved in CO_2 elevation-induced LR formation in tomato.

NOS rather than NR is essential for CO_2 elevation-induced NO accumulation

To identify the enzymatic source of endogenous NO, we examined the role of NOS and NR in CO_2 elevation-induced NO accumulation. Under elevated CO_2, L-NAME decreased the number of LRs by 46 % (Fig. 3), and inhibited the accumulation of NO in LR primordia and root tips by 33 and 50 %, respectively (Fig. 4). The inhibitory effect was reversed when SNP was supplemented, and the level of NO and the number of LRs were similar to those under elevated CO_2 (Figs. 3, 4).

In a previous study, tungstate as an inhibitor of plant NR inhibited the activity of NR and decreased the production of NO (Xiong et al. 2009). According to the literature, treatment with tungstate for 2 days was not sufficient to induce a deficit in protein biosynthesis. Actually, in our experiment, we did not observe any sign of nitrogen deficiency. The present experiment showed that the LR number was unaffected by tungstate under elevated CO_2 (Fig. 3), while endogenous NO levels in both LR primordia and root tips did not decrease significantly (Fig. 4). The addition of SNP increased the level of NO by 33 % in LR primordia (Fig. 4a, c), but did not affect the number of LRs (Fig. 3) or the concentration of NO in the root tips (Fig. 4b, c). Therefore, the results indicate that it was NOS rather than NR responsible for the increased NO under elevated CO_2.

The effect of elevated CO_2 on the activities of NR and NOS

To further identify the roles of NR and NOS in CO_2 elevation-induced NO accumulation and LR development, we measured the activities of NR and NOS enzymes. The activity of NOS tripled under elevated CO_2 compared with ambient CO_2 using the fluorometric assay method (Fig. 5a). In comparison, elevated CO_2 had not significantly affected the activity of NR in the roots (Fig. 5b).

Discussion

It has been reported that tomato plants grown under elevated CO_2 have greater total root length, root surface area, root diameter, root volume and number of LRs than those under ambient CO_2, leading to a greater root system (Wang et al. 2009). As a result, elevated CO_2 significantly increased the uptake of N, P, K, Ca, Mg and micronutrients (Cu, Fe, Mn and Zn), which in turn promoted plant growth and development (Prior et al. 1998). In the present experiment, we observed that both number and length of LRs increased significantly by elevated CO_2 (Fig. 1). The results are consistent with the previous conclusion that

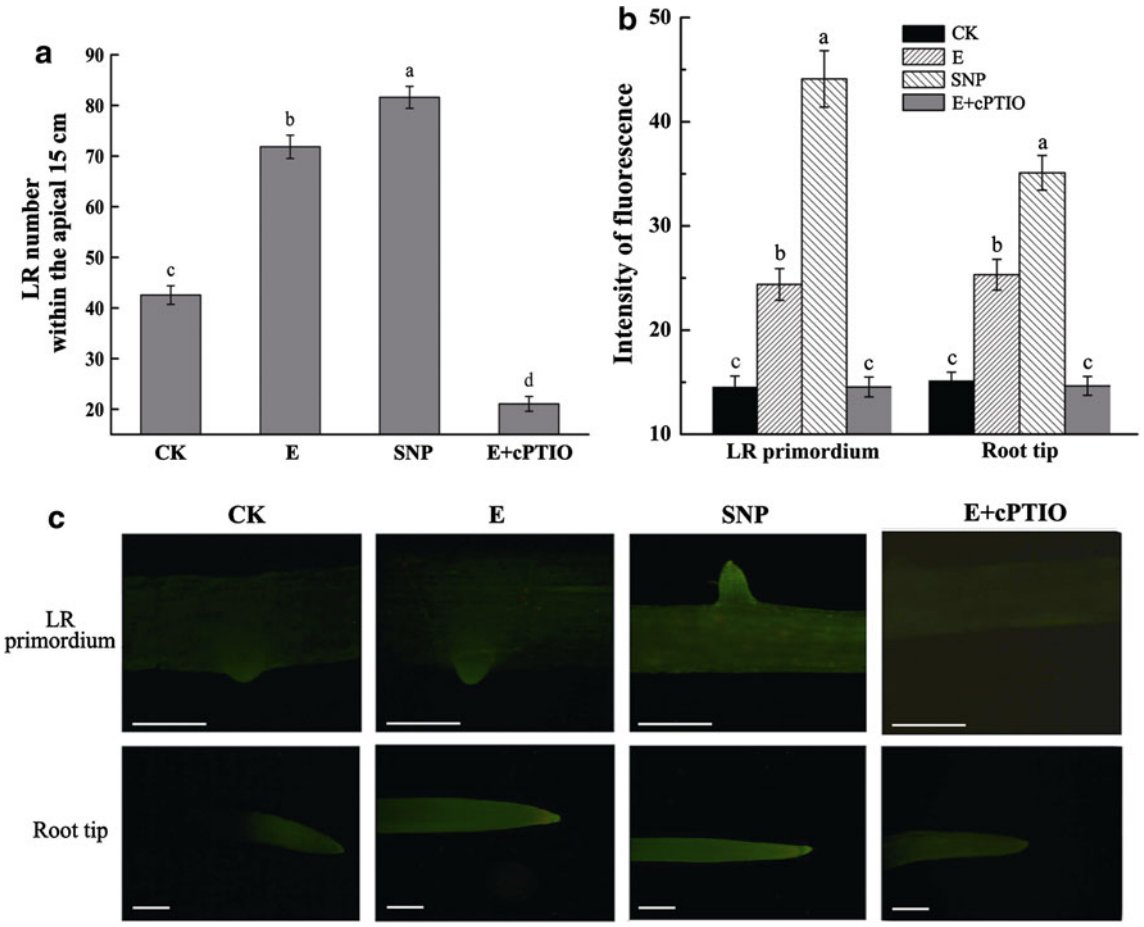

Fig. 2 Effect of NO on the formation of LRs in tomato under elevated CO_2. The plants were treated with ambient CO_2 or elevated CO_2 alone or with SNP (0.2 mM) and cPTIO (0.4 mM), respectively. **a** The LR number of roots treated for 4 days, **b** pixel intensity of fluorescence in LR primordia and tips of the root treated for 2 days. The data are mean ± SE ($n = 6$). *Different letters* indicate significant differences ($P < 0.05$) between treatments and **c** representative fluorescence images of DAF-FM DA-loaded roots treated for 2 days. *Bars* 1 mm. *CK*, ambient CO_2 alone; *E*, elevated CO_2 alone; *SNP*, supplement with SNP under ambient CO_2; *E + cPTIO*, supplement with cPTIO under elevated CO_2

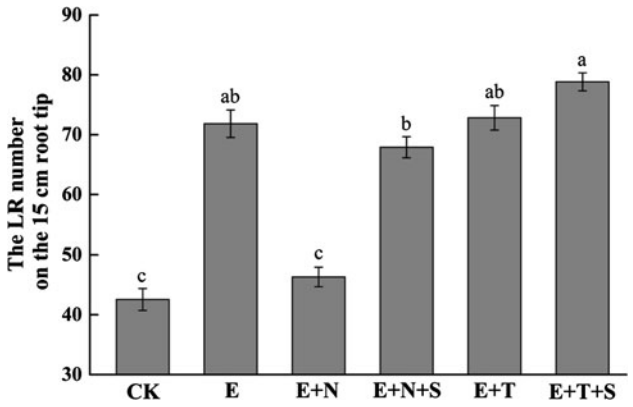

Fig. 3 Roles of NOS and NR in the formation of LRs in tomato under elevated CO_2. The LR number on the 15-cm tips of the root treated for 4 days with 0.2 mM L-NAME or 0.1 mM tungstate alone or supplement of 0.2 mM SNP under elevated CO_2. The data are mean values ± SE ($n = 6$). *Different letters* indicate significant differences ($P < 0.05$) between treatments. *CK*, ambient CO_2; *E*, elevated CO_2; *N*, L-NAME; *S*, SNP; *T*, tungstate

elevated CO_2 promoted the formation of LRs in *Arabidopsis* and tomato (Lee-Ho et al. 2007; Wang et al. 2009).

Although it has been reported that elevated CO_2 promotes the formation of LRs, the detailed mechanism is still inconclusive. Several pieces of evidence suggested that elevated CO_2 enhanced auxin production in many plants (Li et al. 2002; Teng et al. 2006; Wang et al. 2009), which promoted growth and development of LRs (Correa-Aragunde et al. 2004). NO acts downstream auxin signaling inducing the formation of LR primordia in tomato (Correa-Aragunde et al. 2004). This study confirmed that SNP increased the level of NO in LR primordia by 300 % and the formation of LRs by 100 % (Fig. 2). Elevated CO_2 increased levels of NO in LR primordia by 67 % and the formation of LRs by 75 % compared with ambient CO_2 (Fig. 2). These data suggest that NO promoted the formation of LRs and that the LR number was positively related to NO levels in the LR primordia. The conclusion is in

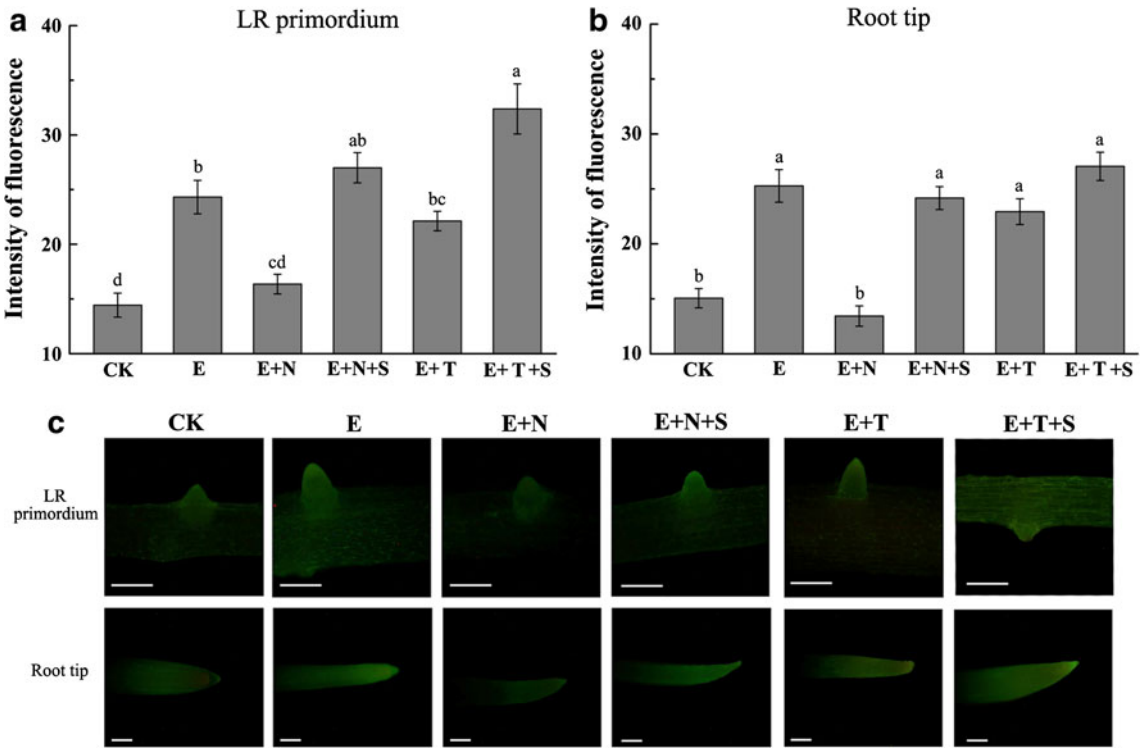

Fig. 4 Roles of NOS and NR in CO_2 elevation-induced NO accumulation. The tomato plants were treated for 2 days with 0.2 mM L-NAME or 0.1 mM tungstate alone or supplement of 0.2 mM SNP under elevated CO_2. Pixel intensity of DAF-2 DA fluorescence in the LR primordia (**a**) and root tips (**b**). The data are the mean ± SE of measurements taken from at least 18 roots for each treatment. *Different letters* indicate significant differences ($P < 0.05$) between treatments and **c** representative fluorescence images of DAF-FM DA-loaded roots. *Bars* 1 mm. *CK*, ambient CO_2; *E*, elevated CO_2; *N*, L-NAME; *S*, SNP; *T*, tungstate

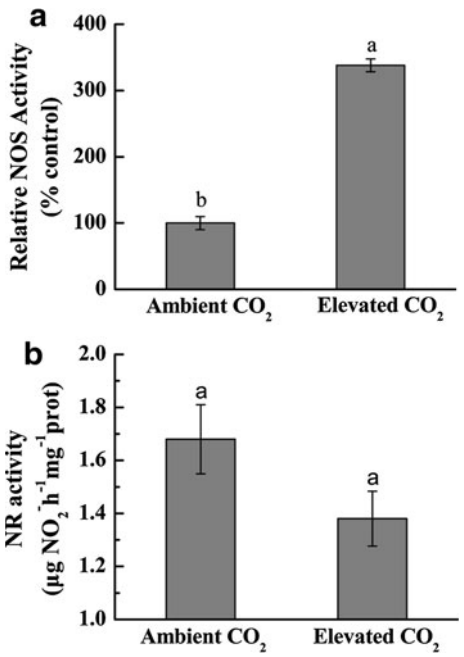

Fig. 5 Effect of elevated CO_2 on the activities of NOS (**a**) and NR (**b**) in roots. Seedlings were treated with ambient CO_2 or elevated CO_2 for 2 days, and then the roots were collected for assaying enzyme activities. The values are mean ± SE of more than six seedlings

accordance with the results that elevated CO_2 enhanced Fe-deficiency-induced responses through increased NO in tomato roots (Jin et al. 2009a). Conversely, when the roots were treated with cPTIO, NO production and LR formation were significantly inhibited under elevated CO_2 (Fig. 2). Therefore, the conclusion could be drawn from the findings that NO was involved in CO_2 elevation-induced LR formation in tomato.

It has been reported that the production of auxin is enhanced under elevated CO_2 compared with ambient CO_2 (Li et al. 2002; Wang et al. 2009). Our recent study demonstrated that a profound effect of elevated CO_2 on development of root hairs in *Arabidopsis* was attributed to increased auxin (Niu et al. 2011). In addition, it has been demonstrated that NO is located at the downstream of auxin signaling cascade in the process of auxin-induced LR formation (Lamattina et al. 2003; Bethke et al. 2007). It is reasonable to suppose that elevated CO_2 induced auxin accumulation, which increased the level of endogenous NO and promoted the formation of LRs in tomato. However, Méndez-Bravo et al. (2010) reported that NO donors did not activate the expression of auxin-responsive genes, while Fernández-Marcos et al. (2011) showed that NO affected root growth inhibiting acropetal auxin transport. It

has been shown that ROS might also be involved in the process of auxin-induced LR formation (Pasternak et al. 2005). Therefore, the cross-talk among auxin, ROS and NO in the process of CO_2 elevation-induced LR formation needs further investigation.

Until recently, there appeared to be two characterized plant enzymes capable of NO biosynthesis in plants, NOS and NR (Crawford 2006). This present study confirmed that L-NAME diminished the effect of elevated CO_2 on NO concentration and LR number, and the activity of NOS was significantly greater under elevated CO_2 than under ambient CO_2. The data suggest that NOS was involved in CO_2 elevation-induced NO production, which affected LR formation in tomato. This is consistent with the conclusion of a previous report that NOS played a crucial role in the formation of LRs in *Arabidopsis* (Flores et al. 2008). The authors showed that mutation of arginase enhanced the availability of endogenous Arg, thereby increasing the level of NO and potentiating formation of lateral roots. According to this finding, we suppose that L-Arg has a positive effect on NO production as increased NOS activity and promotes the formation of lateral roots under elevated CO_2. On the other hand, we provided evidence that tungstate did not affect NO production or LR formation under elevated CO_2, and that elevated CO_2 had no effect on NR activity in roots, which suggests that NR was not responsible for CO_2 elevation-induced NO accumulation in tomato. This conclusion is contradictory to previous findings that elevated CO_2 increased nitrate uptake and NR activity in tobacco when plants were supplied with 2 mM nitrate (Matt et al. 2001). Such a discrepancy might have resulted from the effects of NO on NR activity in tomato roots depending on levels of nitrate supply, and probably from direct interactions between NO and NR protein (Du et al. 2008; Jin et al. 2009b). It has been reported that elevated CO_2 has a stimulatory effect on NR activity in roots of tobacco irrigated with high concentration of nitrate (20 mM), but no effect is observed under low concentration of nitrate (2 mM) (Geiger et al. 1998). In our experiment, the concentration of nitrate in the rooting medium was 1.5 mM, and this concentration might not be high enough to stimulate the response of NR activity in roots of tomato to elevated CO_2. However, we cannot exclude possible effects of diurnal rhythm of NR and growth stage on the NR activity, which was observed in tobacco plants (Geiger et al. 1998; Matt et al. 2001).

In this study, we confirmed that CO_2 elevation-induced NO accumulation was involved in LR formation in tomato under elevated CO_2. Elevated CO_2 increased NOS activity, which was responsible for NO accumulation in roots, but had no significant effect on NR activity. Therefore, we proposed that NOS-generated rather than NR-generated NO was involved in LR formation in tomato grown under elevated CO_2. This finding suggests a mechanism that has not been previously described and provides a new insight into the processes of elevated CO_2-induced LR formation in plants. Furthermore, it is important to determine if elevated CO_2 is inducing lateral root initiation, lateral root maturation or both in further research.

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