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RESEARCH PAPER

Effect of elevated CO_2 on phosphorus nutrition of phosphate-deficient *Arabidopsis thaliana* (L.) Heynh under different nitrogen forms

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Abstract

Phosphorus (P) nutrition is always a key issue regarding plants responses to elevated CO_2. Yet it is unclear of how elevated CO_2 affects P uptake under different nitrogen (N) forms. This study investigated the influence of elevated CO_2 (800 μl l^{-1}) on P uptake and utilization by *Arabidopsis* grown in pH-buffered phosphate (P)-deficient (0.5 μM) hydroponic culture supplying with 2 mM nitrate (NO_3^-) or ammonium (NH_4^+). After 7 d treatment, elevated CO_2 enhanced the biomass production of both NO_3^-- and NH_4^+-fed plants but decreased the P amount absorbed per weight of roots and the P concentration in the shoots of plants supplied with NH_4^+. In comparison, elevated CO_2 increased the amount of P absorbed per weight of roots, as well as the P concentration in plants and alleviated P deficiency-induced symptoms of plants supplied with NO_3^-. Elevated CO_2 also increased the root/shoot ratio, total root surface area, and acid phosphatase activity, and enhanced the expression of genes or transcriptional factors involving in P uptake, allocation and remobilization in P deficient plants. Furthermore, elevated CO_2 increased the nitric oxide (NO) level in roots of NO_3^--fed plants but decreased it in NH_4^+-fed plants. NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) inhibited plant P acquisition by roots under elevated CO_2. Considering all of these findings, this study concluded that a combination of elevated CO_2 and NO_3^- nutrition can induce a set of plant adaptive strategies to improve P status from P-deficient soluble sources and that NO may be a signalling molecule that controls these processes.

Key words: ammonium, anthocyanin, *atnos1*, elevated CO_2, nitrate, nitrate reductase, nitric oxide, nitric oxide synthase, *nr*, phosphorus acquisition, phosphate deficiency.

Introduction

Concentrations of atmospheric CO_2 have risen from about 270 μl l^{-1} in pre-industrial times to over 380 μl l^{-1} at present, and are predicted to reach between 530 and 970 μl l^{-1} by the end of this century (IPCC, 2007). It has been demonstrated that increasing atmospheric CO_2 concentration has a profound impact on the growth and development of plants.

Abbreviations: APase, acid phosphatase; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, diaminofluorescein-FM diacetate; N, nitrogen; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; P, phosphorus; SNP, sodium nitroprusside; *AtPHR1, PHOSPHATE STARVATION RESPONSE 1; AtPHT1, PHOSPHATE TRANSPORTER 1; AtPHO1, PHOSPHATE 1; AtPHO2, PHOSPHATE 2. AtPAP2, PURPLE ACID PHOSPHATASE 2*.

Many studies have investigated plant responses to elevated CO_2 on ecosystem, community, population, physiological, and molecular scales (Gibeaut *et al.*, 2001; Teng *et al.*, 2006; Jin *et al.*, 2009; Niu *et al.*, 2011*a*). However, relatively little information exists on the effects of elevated CO_2 on nutrition acquisition and metabolism. Previous studies have shown that elevated CO_2 generally stimulates the rates of carboxylation but suppresses the rate of oxygenation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in C3 plants, thereby increasing plant growth and crop yield (Stitt *et al.*, 1991; Drake *et al.*, 1997). However, sustaining these increased rates requires increased nutrient uptake. Consequently, the role of nutrition availability is a key unresolved issue regarding plant responses to elevated CO_2.

Phosphorus is an essential element for plant growth and development. It plays important roles in energy transfer, signal transduction, photosynthesis, enzyme activation/inactivation, membrane synthesis and stability, and respiration, and is a structural component of nucleic acids and phospholipids (Abel *et al.*, 2002; Vance *et al.*, 2003; Chiou and Lin, 2011; Péret *et al.*, 2011). However, the low solubility and high sorption capacity of phosphate in soils make it relatively unavailable to plant roots. In soil solution, concentrations of available P (mainly $H_2PO_4^-$ and HPO_4^{2-}) are generally less than 10 µM, which is well below the critical level needed for the optimal performance of crops (Schachtman *et al.*, 1998; Batjes, 1997). The problem of P deficiency is mitigated by the application of concentrated fertilizers that provide soluble P to plants. This practice, however, is inherently inefficient due to chemical immobilization and surface runoff of P (Abel *et al.*, 2002). Hence, low P availability is one of the major growth-limiting factors for plants in many natural and agricultural ecosystems (Barber *et al.*, 1963; Raghothama, 1999).

When soil phosphorus availability is low, phosphorus uptake is increased by elevated CO_2; however, higher foliar phosphorus concentrations are also required to realize the maximum growth potential at elevated CO_2 (Conroy *et al.*, 1990). Although the absolute increase in growth is greatest at high nutrient availability, relatively large gains in productivity can also be achieved at lower nutrient availability (Conroy *et al.*, 1992). Notably, P deficiency reduces the assimilation of NO_3^- into the proteins, which might cause negative feedback on NO_3^- influx and/or stimulate NO_3^- efflux (Schjørring, 1986). Therefore, it seems that, under conditions of nutrition deficiency and elevated CO_2, plants sense changes in nutrition availability and trigger a set of plant adaptive responses to increase nutrition uptake and recycling. Numerous studies indicate that plants enhance P acquisition and mobilization to cope with low P availability by inducing a series of biochemical, physiological, and molecular adaptation strategies (Raghothama, 1999; Mukatira *et al.*, 2001; Abel *et al.*, 2002; Rubio *et al.*, 2009; Ramaekers *et al.*, 2010; Péret *et al.*, 2011; Jin *et al.*, 2012; Veneklaas *et al.*, 2012). However, it is unclear how plants sense and respond to P deficiency under conditions of elevated CO_2.

Nitrogen (N) has been the centre of attention not only because it is the primary limiting nutrition in most temperate ecosystems, but also because the form of N supply can modify the response of plant growth to nutrition acquisition and elevated CO_2. It has been reported that N forms (NO_3^- versus NH_4^+) can lead to contrasting responses of plant growth and metabolism to elevated CO_2. For example, elevated CO_2 increased NO_3^- uptake and nitrate reductase activity when tobacco was fed with NO_3^- but increased NH_4^+ uptake and inhibited nitrate reductase activity with an NH_4^+ supply (Matt *et al.*, 2001). Likewise, under elevated CO_2, NH_4^+-fed wheat showed greater increases in leaf area and smaller decreases in shoot protein concentration than NO_3^--fed plants (Bloom *et al.*, 2002, 2010). Pharmacological experiments and studies of mutants with low activity of *NIA* (encoding nitrate reductase) showed that nitric oxide (NO) production was induced by NO_3^- and elevated CO_2 (Gojon *et al.*, 1998), but was inhibited by ammonium, glutamine, or related downstream products of nitrate assimilation (Gojon *et al.*, 1998). Recently, Carlisle *et al.* (2012) reported that both CO_2 concentration and N form strongly affected biomass production in hydroponically grown wheat, as well as nutrient concentrations in above- and below-ground tissues. Consequently, these differences between NO_3^- and NH_4^+ nutrition led us to investigate how N form regulates the responses of P homeostasis of plants grown under elevated CO_2. Therefore, the aim of this study was to investigate the interactive effects of N form and elevated CO_2 on plant P uptake and utilization in a P-deficient medium. *Arabidopsis* was used as the test plant.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* wild-type ecotype and mutants *nr*, AT1G77760 and AT1G37130 locus (Wang *et al.*, 2004) and *atnos1*, AT3G47450 locus (Guo *et al.*, 2003), were surface-sterilized, and germinated on Petri plates containing 0.8% agar medium supplemented with one-fifth-strength nutrition solution (Niu *et al.*, 2011*a*). After 7 d, seedlings of uniform size were transferred to 1.5 ml Eppendorf tubes whose bottoms were cut about 0.7 cm from the bottom to allow root growth into the nutrition solution. The tubes were inserted in holes of plastic boxes containing 200 ml of a half-strength nutrition solution, and plants were grown for 14 d. Then, each seedling was transferred to each pot containing 300 ml of nutrition solution. The composition (µM) of the nutrition solution was as follows: 1500 KNO_3, 500 $MgSO_4$, 1000 $CaCl_2$, 500 NaH_2PO_4, 250 $(NH_4)_2SO_4$, 10 H_3BO_3, 0.5 $MnSO_4$, 0.5 $ZnSO_4$, 0.1 $CuSO_4$, 0.1 $(NH_4)_6Mo_7O_{24}$, and 25 Fe-EDTA (Hoagland and Arnon, 1938). The nutrition solution was renewed every 3 d and its pH was adjusted daily to 6.0 using 1 M NaOH. All plants were grown in controlled-environment growth chambers (Conviron E7/2, Winnipeg, Manitoba, Canada) with a humidity of 80%, a daily cycle of 22 °C/10 h day and 20 °C/14 h night. The daytime light intensity was 120 µmol photons m^{-2} s^{-1}. At least eight independent replicates were used for each treatment.

After plants were further grown in pots for 14 d, treatments commenced. Plants were grown in chambers with a CO_2 concentration of either 350 ± 50 (ambient) or 800 ± 50 µl l^{-1} (elevated CO_2). Meanwhile, N and P treatments were initiated. One set of plants were fully supplied with 2 mM NO_3^- as KNO_3, while the other was fully supplied with 2 mM NH_4^+ in each CO_2 treatment. For the treatment of NH_4^+ as the sole N source, 1 mM $(NH_4)_2SO_4$ and 1 mM K_2SO_4 were added. The low-P treatment had 0.5 µM NaH_2PO_4. The solution pH was buffered with 2 mM HEPES at pH 6.8 to minimize pH change, and was adjusted daily to 6.8 using 1 M NaOH. The treatment solutions were renewed every 2 d.

The NO-related treatments on wild-type plants were also initiated as follows: (i) ambient treatment: plants were grown continuously under ambient CO_2; (ii) elevated CO_2 treatment: plants were transferred to a chamber with a CO_2 concentration of 800 ± 50 μl l^{-1}; (iii) ambient+sodium nitroprusside (SNP) treatment: plants were transferred to the nutrition solution containing 100 μM SNP under ambient CO_2; and (iv) elevated CO_2+2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) treatment: plants were transferred to the nutrition solution containing 100 μM cPTIO under elevated CO_2.

Analysis of total P in plant tissue

After 7 d treatment with ambient or elevated CO_2, plants from various treatments were harvested, washed thoroughly with deionized water, divided into shoots and roots, and dried in an oven at 75 °C for 1 d. The samples were then weighed, digested in sulfuric acid/hydrogen peroxide, and analysed for total P concentration using the vanadium-molybdenum blue photometric method. The 'P absorbed per unit weight of roots' was calculated as the total amount of P per plant/root dry weight.

Chlorophyll concentration in leaves

After 7 d treatment, a portable chlorophyll meter (SPAD-502, Minolta, Japan) was used to measure chlorophyll content (Jin *et al*., 2009). Three leaves were selected randomly from each treatment and two SPAD readings were recorded for each leaf, avoiding the main veins during measurement.

Anthocyanins

Total anthocyanins in leaves were extracted and determined as described by Hodges *et al*. (1999) with minor modifications. Approximately 0.1 g of fresh leaves was homogenized in 3 ml of methanol-1% HCl. Samples were centrifuged and 1 ml of the supernatant appropriately diluted in methanol-1% HCl was used for analysis. The difference in absorbance at 536 and 600 nm was used to calculate total anthocyanin content. Results were expressed as cyanidin 3-glucoside equivalents (mmol g^{-1} of fresh weight) using an extinction coefficient of 0.449 mol l^{-1} at 536 nm (Fiorani *et al*., 2005).

Root morphology

After 7 d treatment, the root parameters of individual plants were measured using a root analysis instrument (WinRHIZO, Regent, Canada). The number and length of root hairs in each segment were measured using light microscopy with differential interference contrast optics. Micrographs were recorded on a CCD camera (Nikon Eclipse E600, Melville, NY, USA).

Activity of acid phosphatases (APases)

The activity of APases in shoots and roots was determined according to the method of McLachlan *et al*. (1987) with minor modifications. After 7 d in low-P treatment, the seedlings were washed thoroughly with deionized water, divided into shoots and roots, weighed, and then chilled immediately on ice. About 0.1–0.2 g of fresh samples was frozen with liquid nitrogen and homogenized in a cold mortar, and the powder was macerated with 5 ml of extraction buffer (0.2 M glacial acetic acid-sodium acetate buffer, pH 5.8). The extract was centrifuged at 27 000*g* at 4 °C for 10 min, and the supernatant was used for an assay of APase activity. APase activity was determined by measuring enzyme activity in extraction buffer. Briefly, the APase reaction mixture contained 0.05 ml of enzyme extract, 0.45 ml of buffer solution and 4.5 ml of 5 M *p*-nitrophenyl phosphate. All APase reaction mixtures were incubated at 30 °C for 30 min in the dark. The reaction mixtures were stopped by the addition of 2 ml of 2 M NaOH. After this, the concentration of *p*-nitrophenol in the APase reaction mixtures was determined in a spectrophotometer at 405 nm. The APase activity was expressed as mol substrate hydrolysed g^{-1} of fresh weight min^{-1}.

In situ *measurement of NO in the root*

NO was imaged using diaminofluorescein-FM diacetate (DAF-FM DA) and epifluorescence microscopy. DAF-FM DA has been used successfully to detect NO production in both plants and animals. Roots were loaded with 5 μM DAF-FM DA in 20 mM HEPES-NaOH buffer (pH 7.4) for 30 min, washed three times in fresh buffer and observed under a microscope (Nikon Eclipse E600, Nikon; excitation 488 nm, emission 495–575 nm). A 100 W high-pressure mercury-vapour lamp was used as a light source (HB-10103AF-Hg, Nikon). Fifteen roots of each treatment were measured each time. The signal intensities of green fluorescence in the images of the root tip and young root-hair zone were quantified using Image J.

Determination of nitric oxide synthase (NOS) activity

The activity of NOS was determined as described by Tian *et al*. (2007) with some modifications. The activity of NOS was determined with an NOS assay kit (Beyotime, Haimen, China). Briefly, 0.1 g of roots was frozen in liquid nitrogen and ground to a fine powder. The powder was homogenized in 1 ml of extraction buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl, 20 μM flavin adenine dinucleotide, 25 μM leupeptin, 5 μM Na_2MoO_4, and 1% polyvinylpyrrolidone. The contents were centrifuged at 13 000*g* for 20 min at 4 °C and 0.2 ml of clear supernatant was then added to 0.1 ml of assay mixture (containing NADPH, L-arginine, NOS assay buffer, and DAF-FM DA) and reacted at 37 °C in the dark for 1 h. The NO content was detected under a microscope as described above. The fluorescence intensity was expressed as colour level on a scale ranging from 0 to 255, and the fluorescence intensity was quantified by using Image J. Data are presented as the mean of fluorescence intensity relative to the control treatment. The control treatment was the plant grown in ambient CO_2.

Determination of maximum nitrate reductase (NR) activity

The activity of maximum NR was determined as described by Tian *et al*. (2007) with some modifications. Briefly, a total of 0.2 ml of clear supernatant prepared as described above was added into 0.4 ml of pre-warmed assay buffer containing 100 mM HEPES-KOH (pH 7.5), 5 mM KNO_3, and 0.25 mM NADPH. The mixed solution was reacted at 30 °C for 1 h, and the reaction was stopped by adding zinc acetate. The nitrite produced was measured colorimetrically at 540 nm by adding 1 ml of 1% sulfanilamide in 3 M HCl plus 1 ml of 0.2% *N*-(1-naphthyl) ethylenediamine.

Quantitative PCR analysis

Total RNA was extracted using RNAiso Plus (Takara, Otsu, Shiga, Japan) from about 70 mg of fresh root tissues. At least five plants were used for extraction. All RNA samples were checked for DNA contamination before cDNA synthesis. cDNA was synthesized, and possible residual genomic DNA contamination was verified as described by us previously (Niu *et al*., 2011*a*). The mRNA levels of the genes *PHOSPHATE STARVATION RESPONSE 1* (*AtPHR1*), *PHOSPHATE TRANSPORTER 1* (*AtPHT1*), *PHOSPHATE 1* (*AtPHO1*), *PHOSPHATE 2* (*AtPHO2*), and *PURPLE ACID PHOSPHATASE 2* (*AtPAP2*) were detected using a SYBR Green RT-PCR kit (Takara) with the following pairs of gene-specific primers: *AtPHR1*, forward: 5'-ACAGCAATAACGGAACGGGCAAG-3' and reverse: 5'-GCTCTTTCACTACCGCCAAGACTG-3'; *AtPHT1*, forward: 5'-GCCAAGGTAGACGCAGGATA-3' and reverse: 5'-AACCTCAG CCTCACCAGAGA-3'; *AtPHO1*, forward: 5'-TACGCGAGAGAAA ACAACGA-3' and reverse: 5'-TTCCGGAGAACCAAATTGTC-3'; *AtPHO2*, forward: 5'- TTTTACACAAGCCACCAAAGC-3' and reverse: 5'-TCACGAGCATGTCCAACAA-3'; *AtPAP2*, forward: 5'-CATCAAGTTCCTTTGAGAGC-3' and reverse: 5'-TTGGACCGG TGTTGTAGGAG-3'.

Real-time PCR was carried out similar to as described by Niu *et al*. (2011*a*). A pair of primers for the housekeeping gene *UBQ10* (forward: 5'-GGTTCGTACCTTTGTCCAAGCA-3', reverse: 5'-CCTTCGTTA

AACCAAGCTCAGTATC-3') was used for a control reference gene for the PCR (Gan *et al*., 2006). Melting-curve analysis and gel electrophoresis of the PCR products were used to confirm the absence of non-specific amplification products. Relative expression levels were calculated by subtracting the threshold cycle (C_t) values for *UBQ10* from those of the target gene (to give ΔC_t) and then calculating $2^{-\Delta Ct}$, where C_t was the cycle number at which the fluorescence rose above the set threshold of the quantitative PCR.

Statistical analyses

All statistical analyses were conducted with DPS software (Stirling Technologies Inc., China). Means were compared using Student's *t*-test or Fisher's least significant difference test at P=0.05 in all cases.

Results

Plant growth and P uptake in low-P culture

After 7 d growth in ambient CO_2 and P-deficient medium containing NO_3^- supplement as the sole N source, *Arabidopsis* leaves were dark green with a SPAD reading of 33. By comparison, the leaves of plants grown under the same nutrition conditions but with elevated CO_2 were a normal green colour (Fig. 1a) with SPAD readings of 26 (see Supplementary Fig. S2a at *JXB* online). Elevated CO_2 decreased the concentration of anthocyanins in leaves of NO_3^--supplied plants (see Supplementary Fig. S3a at *JXB* online), indicating that elevated CO_2 alleviated the P-deficiency-induced symptoms of the NO_3^--supplied plants. However, with a supply of NH_4^+ as the sole N source, plants grown in the low-P solution had a similar appearance under both ambient and elevated CO_2 treatments (Fig. 1a). Elevated CO_2 did not change the concentrations of chlorophyll and anthocyanins (Supplementary Figs S2a and S3a). Furthermore, ammonium toxicity was not evident in the NH_4^+-fed plants.

Elevated CO_2 enhanced shoot growth exclusively under NH_4^+ nutrition (Fig. 1b), whereas it enhanced root growth under NO_3^- nutrition (Fig. 1c). Elevated CO_2 decreased the P concentration in shoots by 27% but did not alter it in the roots of NH_4^+-supplied plants. However, elevated CO_2 increased the P concentration by 32 and 21%, respectively, in the shoots and roots of NO_3^--fed plants (Fig. 1d, e). Meanwhile, elevated CO_2 increased the amount of P absorbed per unit weight of roots of NO_3^--fed plants by 56% but decreased that of NH_4^+-fed plants by 33% (Fig. 1g), indicating that NO_3^- nutrition facilitates P uptake in roots.

After 7 d growth in P-adequate medium with either N source, the leaves were a normal green colour (data not shown). CO_2 treatment did not affect the concentration of anthocyanins in leaves, but NH_4^+ supply significantly increased SPAD readings (Supplementary Figs S2b and S3b). Elevated CO_2 increased biomass production (Supplementary Fig. S1a, b), but decreased the P concentration and the amount of P absorbed per weight of roots,

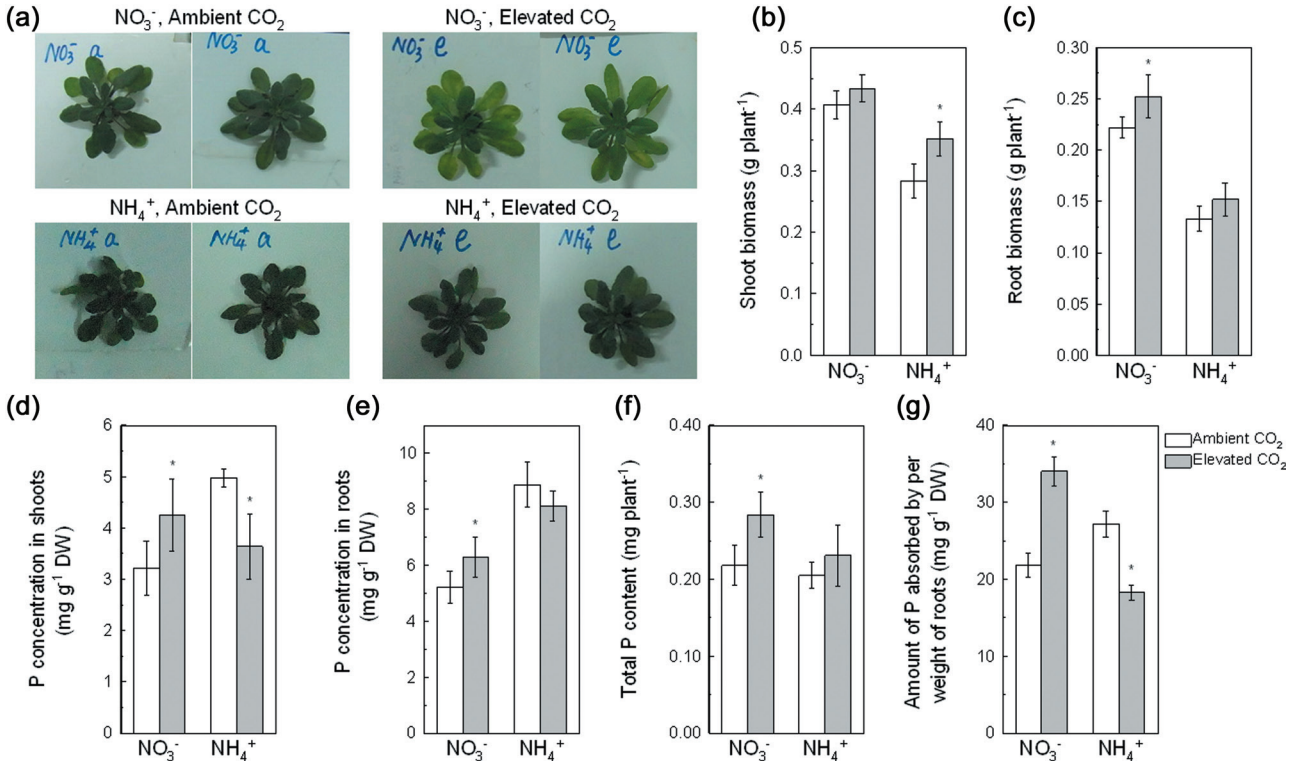

Fig. 1. Images (a), fresh biomass production of shoots (b) and roots (c), concentration of P in shoots (d) and roots (e), total P content (f), and amount of P absorbed per weight of roots (g) of 5-week-old wild-type *Arabidopsis* grown for 7 d in P-deficient nutrition solutions with NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}). Data are means ±SD (n=5). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05). DW, dry weight.

with the decrease being greater in the NO_3^- than in the NH_4^+ treatment (Fig. S1c, d).

Physiological and morphological responses to P deficiency

Elevated CO_2 greatly enhanced the number of lateral roots in the NO_3^--supplied plants but restrained it in the NH_4^+-supplied plants (Fig. 2a). The root/shoot ratio of NO_3^--fed plants was greater and that of NH_4^+-fed plants was lower under elevated compared with ambient CO_2 (Fig. 2b). Furthermore, compared with ambient CO_2, elevated CO_2 increased the total root surface area and root/shoot ratio of the NO_3^--supplied plants but not that of the NH_4^+-supplied plants (Fig. 2b). Elevated CO_2 did not affect the length or density of root hairs of plants grown in either N treatment (data not shown).

Another general response of plants to P starvation is the enhanced activity of APases. APase activity in both the shoots and roots of NH_4^+-supplied plants was similar between the ambient and elevated CO_2 treatments (Fig. 3a, b). However, elevated CO_2 strongly increased APase activity in the shoots and roots of NO_3^--fed plants (Fig. 3a, b).

Expressions of genes involved in P uptake, translocation, and allocation in P-deficient plants

Increased expression of genes involved in P uptake, transport, allocation, and remobilization is a significant adaptive response of plants to facilitate external P acquisition and internal P utilization under P-limited conditions (Rubio *et al.*, 2001; Vance *et al.*, 2003; Chiou and Lin, 2011). Elevated CO_2 enhanced the expression of the P-uptake gene *AtPHR1* and the P-transport gene *AtPHT1*; their expression in shoots were increased by 8-fold and 3.7-fold, respectively (Fig. 4a–d). By contrast, elevated CO_2 did not change the expression of *AtPHR1* and *AtPHT1* in the roots of NH_4^+-fed plants, and decreased their expression in the shoots (Fig. 4a–d).

In order to observe the allocation and retranslocation of internal P between shoots and roots, the expression of *AtPHO1* and *AtPHO2* was analysed. Elevated CO_2 increased *AtPHO1* expression in the shoots and roots of NO_3^--supplied plants by 2.6-fold and 32%, respectively. In comparison, elevated CO_2 increased *AtPHO1* expression in the shoots by 4.7-fold but decreased it by 34% in the roots of NH_4^+-fed plants (Fig. 4e, f). Elevated CO_2 increased the expression of *AtPHO2* by 8.8-fold in the shoots but decreased it by 76% in the roots of

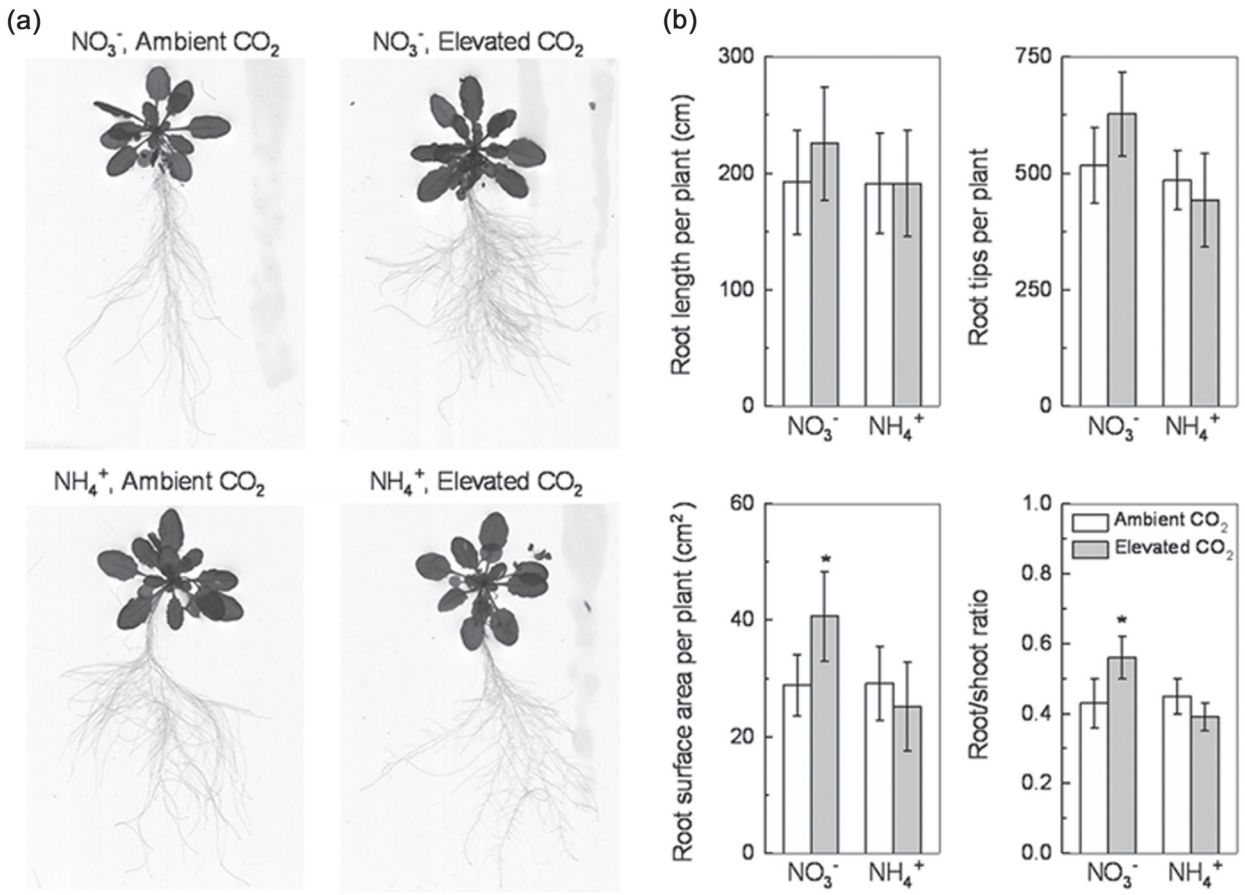

Fig. 2. Root morphology (a), and root length, number of root tips, root surface area, and root/shoot ratio (b) of 5-week-old wild-type *Arabidopsis* grown for 7 d in P-deficient nutrition solutions containing NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}). Data are means ±SD (n=8) of two independent experiments. Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05).

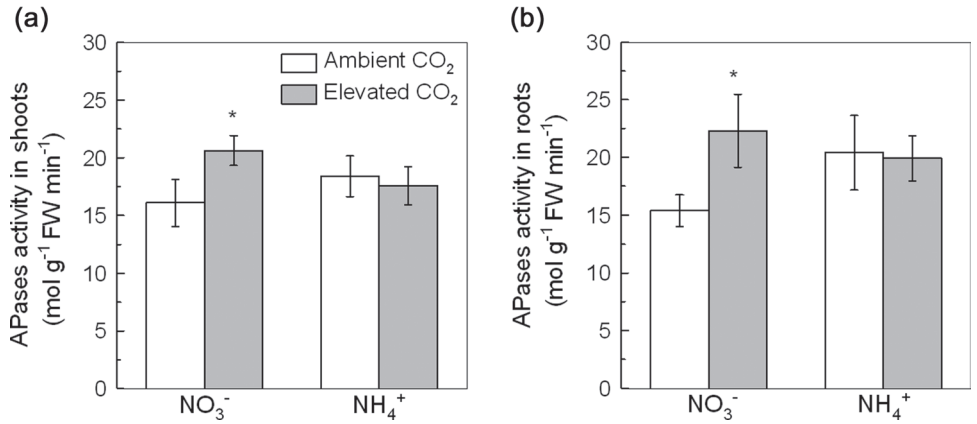

Fig. 3. APase activity in shoots (a) and roots (b) of 5-week-old wild-type *Arabidopsis* grown for 7 d in P-deficient nutrition solutions containing NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}). Data are means ±SD (n=5). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05). FW, fresh weight.

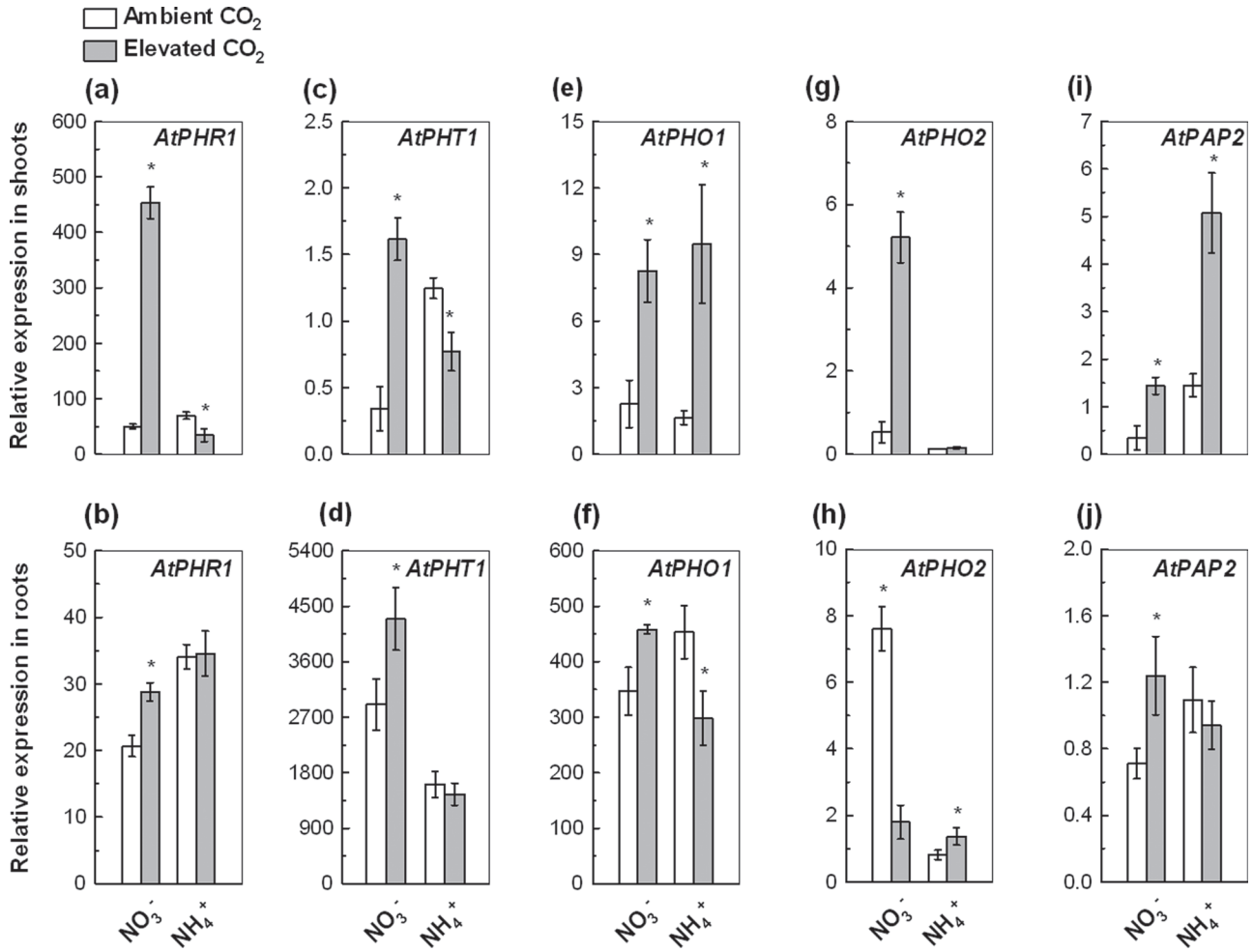

Fig. 4. Relative expression levels of the genes *AtPHR1*, *AtPHT1*, *AtPHO1*, *AtPHO2*, and *AtPAP2* in the shoots and roots of 5-week-old wild-type *Arabidopsis* grown for 7 d in P-deficient nutrition solutions with NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}). Relative expression levels were calculated and normalized with respect to expression of *UBQ10* mRNA. Data are means ±SD (n=5). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05).

NO_3^--supplied plants, whereas it did not significantly change its expression in the shoots of NH_4^+-fed plants (Fig. 4g, h).

Possible role of NO in P nutrition under P deficiency and elevated CO_2

NO was demonstrated recently to be a signal molecule involved in the regulation of root growth during P deficiency (Wang *et al*., 2010). We observed that elevated CO_2 resulted in stronger fluorescence intensity in the root tip and root-hair region in NO_3^--fed plants but decreased it in NH_4^+-fed plants, compared with results under ambient CO_2 (Fig. 5a). To confirm the observation, the fluorescence of 15 roots from each treatment was measured. As shown in Fig. 5b, elevated CO_2 significantly increased the NO level in the roots of NO_3^--supplied plants but decreased it in NH_4^+-supplied plants. A correlation was found between endogenous NO level and P concentration in the roots. Whereas the application of SNP (a NO donor) to the plants under ambient CO_2 tended to promote the P concentration to levels below those measured under elevated CO_2 (Fig. 6a), the addition of the NO scavenger cPTIO under elevated CO_2 reduced the amount of P absorbed per unit weight of roots in both NO_3^-- and NH_4^+-supplied plants and inhibited it to levels significantly below those measured under ambient CO_2 (Fig. 6c), suggesting some involvement of NO in modulating P uptake in roots. The treatment effect of total P uptake followed the same pattern (data not shown). These results implied that an appropriate concentration of endogenous NO in roots is critical for the regulation of elevated CO_2-induced P uptake in both NO_3^- and NH_4^+ medium.

To confirm the above findings further, the effects of elevated CO_2 on the activity of NR and NOS were studied (Fig. 7). In the NO_3^- treatment, elevated CO_2 increased NR activity in the shoots but decreased it in the roots (Fig. 7a, b). Elevated CO_2 also increased NOS activity in both the shoots and roots of the NO_3^--fed plants (Fig. 7c, d). In the NH_4^+ treatment, elevated CO_2 did not affect the NOS and NR activity in shoots but inhibited their activity in roots (Fig. 7).

To further verify the role of NO in mediating elevated CO_2-induced uptake of P during P deficiency, mutants *nr* (an NR-reduced mutant) and *atnos1* (an NOS-reduced mutant) were used to observe their capacity of P uptake compared with wild-type *Arabidopsis*. Overall, the P concentrations of mutants *nr* and *atnos1* grown in both NO_3^-- and NH_4^+-supplied media were similar under ambient and elevated CO_2 (Fig. 8a, b). Specially, elevated CO_2 increased the P concentration in shoots of the *nr* mutant supplied with NO_3^-. These results demonstrated that NO is the signal involved in the regulation of elevated CO_2-induced uptake of P during P deficiency.

Discussion

Previous studies have indicated that CO_2-dependent stimulation of growth is likely to enhance the requirements of plants for macronutrients such as N and P (Baxter *et al*., 1994; Kogawara *et al*., 2006; Lagomarsino *et al*., 2008). This study demonstrated that a combination of elevated CO_2 and nitrate induced a set of morphological, physiological, and molecular responses that enabled the plants to better access and utilize P and to alleviate P-deficiency symptoms.

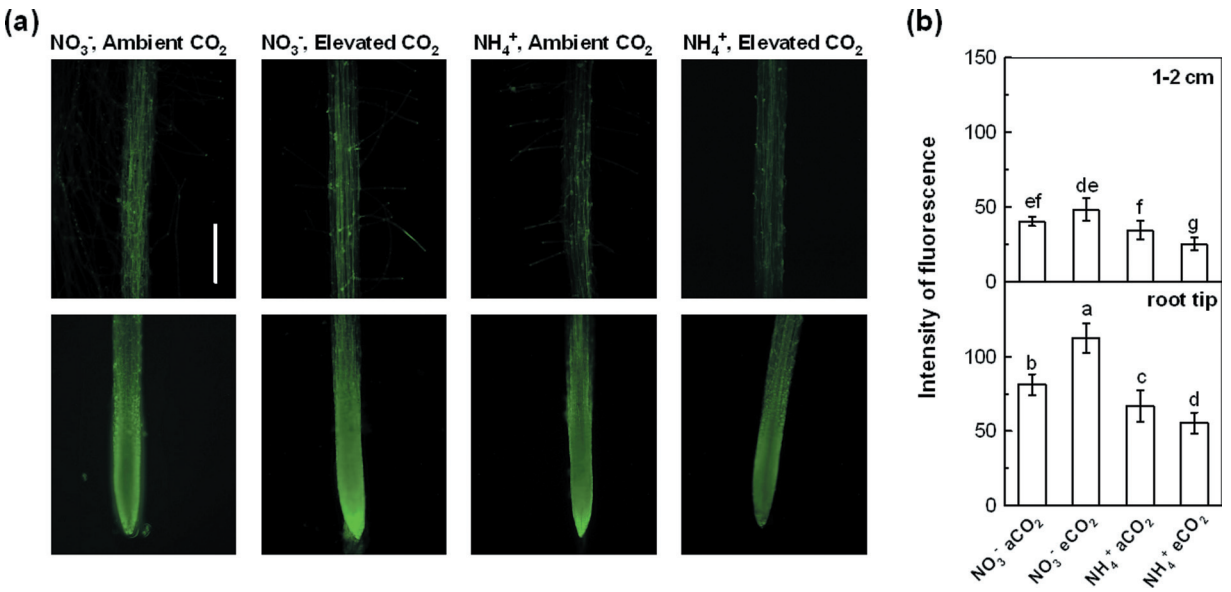

Fig. 5. NO production of 5-week-old wild-type *Arabidopsis* grown for 7 d in P-deficient nutrition solutions containing NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}). (a) Photographs of NO production shown as green fluorescence in the root tips and root-hair region of representative roots of plants supplied with NO_3^- and exposed to ambient or elevated CO_2, and with NH_4^+ exposed to ambient or elevated CO_2. Bar, 500 μm. (b) Effects of N form and CO_2 treatment on NO production expressed as relative fluorescence. NO production was visualized with DAF-FM DA dye. Data are means ±SD (*n*=15). Means followed by the same letter within a root segment are not significantly different at $P < 0.05$.

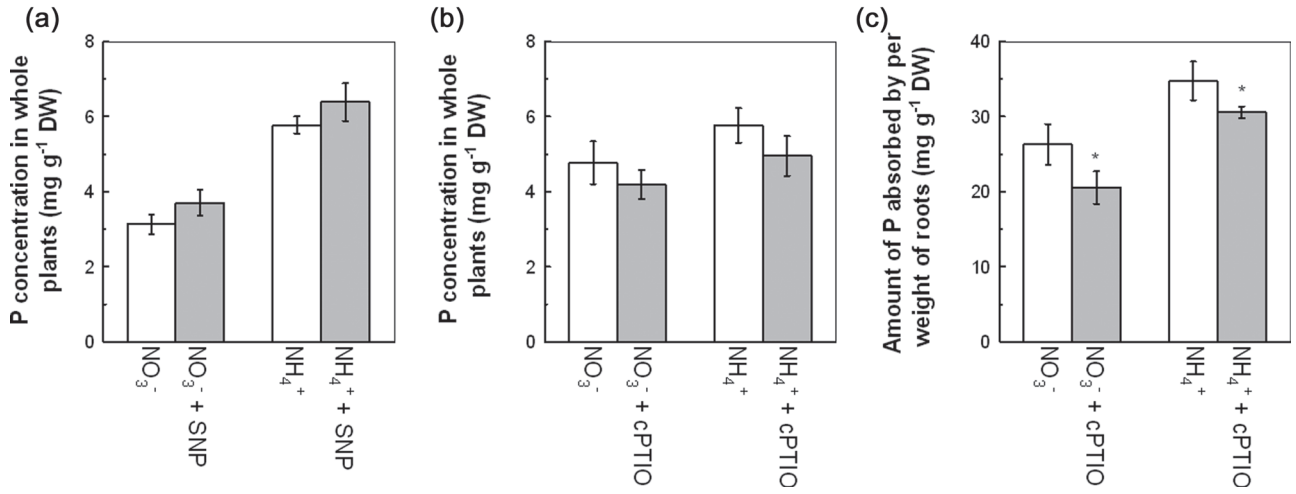

Fig. 6. Effect of SNP under ambient CO_2 (350 ± 50 μl l^{-1}) (a) and cPTIO under elevated CO_2 (800 ± 50 μl l^{-1}) (b) on P concentration per plant, and the effect of cPTIO under elevated CO_2 (800 ± 50 μl l^{-1}) (c) on the amount of P absorbed per weight of roots of 5-week-old wild-type *Arabidopsis* treated with P-deficient nutrition solutions containing NO_3^- or NH_4^+. Data are means ±SD (n=5). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05).

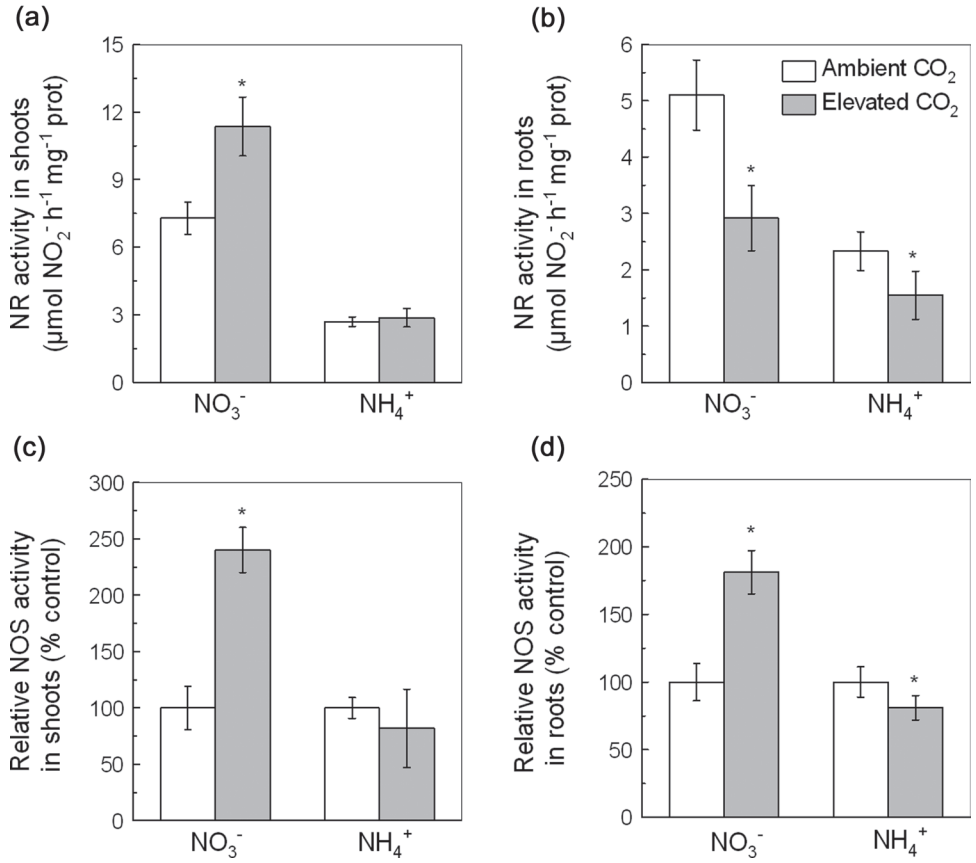

Fig. 7. NR activity in shoots (a) and roots (b) and NOS activity in shoots (c) and roots (d) of 5-week-old wild-type *Arabidopsis* treated with P-deficient nutrition solutions containing NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 μl l^{-1}) or elevated CO_2 (800 ± 50 μl l^{-1}) for 7 d. Data are means ±SD (n=5 for NR; n=10 for NOS). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05).

Elevated CO_2 improves P nutrition under a nitrate supply

The promotion of plant growth under elevated CO_2 would be associated with increased requirements for mineral nutrition to optimize the nutrition balance in plants. This was the case in a 6-year Free Air CO_2 Enrichment (FACE) experiment in which photosynthetic stimulation by elevated CO_2 was largely regulated by N and P availability in the soil (Lagomarsino

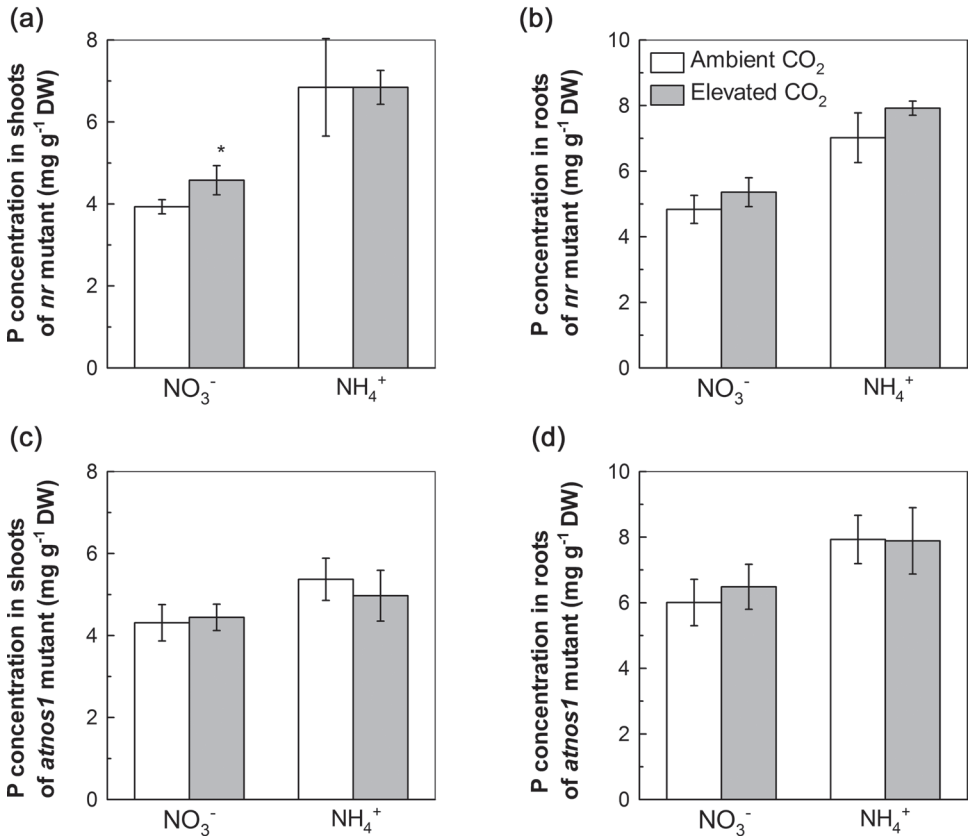

Fig. 8. P concentration in shoots (a, c) and roots (b, d) of *Arabidopsis* NR-reduced mutant *nr* and nitric oxide synthase-reduced mutant *atnos1* treated with P-deficient nutrition solutions containing NO_3^- or NH_4^+ exposed to ambient CO_2 (350 ± 50 µl l^{-1}) or elevated CO_2 (800 ± 50 µl l^{-1}) for 7 d. Data are means ±SD (n=5). Asterisks indicate that the mean values are significantly different between the ambient and elevated CO_2 treatments (P <0.05).

et al., 2008). In the present study, elevated CO_2 enhanced biomass production in both NO_3^-- and NH_4^+-fed *Arabidopsis* plants during P deficiency (Fig. 1). However, elevated CO_2 preferentially stimulated specific P (pool) concentration as well as total P concentration and reduced the accumulation of anthocyanins in NO_3^--fed plants. This result is consistent with the findings of Carlisle *et al.* (2012) that NH_4^+-supplied plants tended to have decreased nutrient concentrations with increasing CO_2 concentration, while NO_3^--supplied plants varied widely across CO_2 treatments. The present study concluded that NO_3^- nutrition facilitates the P uptake of roots under elevated CO_2.

To test whether the improved P uptake occurred only in NO_3^--fed P-deficient plants under elevated CO_2 conditions, we further examined the influence of elevated CO_2 on P nutrition of P-adequate *Arabidopsis* supplied with different N forms. Interestingly, elevated CO_2 only increased the shoot biomass, but decreased specific P uptake with the decrease being greater in the NO_3^- than the NH_4^+ treatment (Fig. S1). Numerous studies have elucidated that plants adapt to P deficiency through modifications of root architecture, carbon metabolism and membrane structure; exudation of carboxylates, protons and enzymes; and enhanced expressions of genes (Raghothama, 1999; Vance *et al*., 2003; Shenoy and Kalagudi, 2005; Shulaev *et al*., 2008; Wang *et al*., 2010). Therefore, the increase in P concentration in NO_3^--fed *Arabidopsis* plants by elevated CO_2 cannot be attributed to increased photosynthesis alone but also to the improved P nutrition of the plants through morphological, physiological, and molecular responses to P deficiency.

This study showed that NO_3^--fed plants had more lateral roots but a shorter taproot under elevated compared with ambient CO_2, whereas NH_4^+-fed plants had longer roots with fewer lateral roots under elevated CO_2 (Fig. 2). In the field, particularly with no-till systems, P concentration is great in topsoil and declines substantially with depth (Lynch and Brown, 2001; Ao *et al.*, 2010). The reduced primary root and increased lateral roots, resulting in a shallower root system, is a positive adaptive response to low-P availability by reducing inter-root competition within the same plant (Miller *et al*., 2003; Lynch, 2007). In addition, the greater root/shoot ratios seen in NO_3^-- compared with NH_4^+-supplied plants during P deficiency under high CO_2 concentrations (Fig. 2) also favour P acquisition. Nevertheless, favourable root architecture may only play a minor role in increasing P uptake of plants under the combined conditions of P limitation and elevated CO_2 in the hydroponic culture supplied with $H_2PO_4^-$.

Another general response of plants to P starvation is the enhanced activity of APases (Richardson *et al.*, 2009; Tran *et al.*, 2010). In bean plants, a high activity of APases contributes to the increased P-utilization efficiency through remobilization of P from old leaves to young tissues (Kouas *et al.*, 2009). Similarly, the physiological function of root APases has also been demonstrated by Xiao *et al.* (2006) and Wang *et al.* (2009) where transgenic plants overexpressing APase genes exhibited greater P acquisition from an organic P resource than wild-type plants. Our results showed that elevated CO_2 increased APase activity in both the shoots and roots of *Arabidopsis* plants supplied with NO_3^-, which is consistent with results reported in the literature (Lagomarsino *et al.*, 2008). The enhanced APase activity, especially in the root of NO_3^--fed plants, under elevated CO_2 conditions (Fig. 3) would be expected to facilitate hydrolysis of organic P and thus plant P uptake in the field.

Biochemical and molecular studies have identified a number of genes that are involved in plant alteration in response to P starvation. *PHR1* is a key transcriptional activator in controlling P uptake, allocation, and anthyocyanin accumulation (Rubio *et al.*, 2001; Nilsson *et al.*, 2007). The enhanced expression of *AtPHR1* under elevated CO_2 could activate numerous P-deficiency-response genes, which in turn change molecular, cellular, and physiological processes to cope with P deficiency. P assimilation is initiated by the transport of P into the roots and the number of P transporters present in the roots reflects the complexity and significance of the process. In *Arabidopsis*, the reduced P uptake activity is caused by a defect in targeting the *PHT1* transporter to the plasma membrane (González *et al.*, 2005; Jain *et al.*, 2007). Our present study showed that elevated CO_2 enhanced the expression of *AtPHT1* genes in the shoots and roots of NO_3^--fed plants with expression being much greater in the roots (Fig. 4), indicting that P transport was stimulated by elevated CO_2 in the NO_3^- medium.

Partitioning and allocation of internal P is also crucial for maintenance of P homeostasis within plants. *AtPHO1* was first identified in *Arabidopsis* as a gene playing an important role in P transport from the roots to the shoots (Poirier *et al.*, 1991), while *AtPHO2* is responsible for P translocation from the shoots to the roots. It was reported that a *pho1* mutant plant accumulated adequate P in the roots but less P in the shoots (Poirier *et al.*, 1991; Hamburger *et al.*, 2002; Stefanovic *et al.*, 2007). The downregulated expression of *AtPHO1* in the roots and the similar expression of *AtPHO2* in the shoots of NH_4^+-supplied plants in the present study (Fig. 4) probably form a negative-feedback loop against internal P recycling between shoots and roots. In contrast, the *PHO2* gene, which regulates P uptake, allocation, and remobilization, is a target gene of miR399s *pho2*, which displays P toxicity with excessive P accumulation in the shoots of *Arabidopsis* (Aung *et al.*, 2006). Accordingly, the high expression of *AtPHO1* in the roots and *AtPHO2* in the shoots of the NO_3^--supplied plants under elevated CO_2 (Fig. 4) indicated that internal P recycling between shoots and roots was stimulated by elevated CO_2 during the onset of P starvation. Furthermore, the enhanced *AtPAP2* expression in shoots under elevated CO_2 (Fig. 4) indicated that elevated CO_2 could excite intracellular P remobilization during P deficiency.

A possible role of NO

It has been shown that P deficiency significantly increases the endogenous NO concentration in roots of white lupin (Wang *et al.*, 2010). In this study, we observed that elevated CO_2 increased the NO level in roots of NO_3^--supplied plants, while the NO scavenger cPTIO inhibited specific P uptake by the root. In contrast, the NH_4^+ supply was associated with a rapid decrease in NO production under elevated CO_2, while the NO scavenger cPTIO inhibited specific P uptake (Figs 5 and 6). These results are supported by recent observations that NO_3^- but not NH_4^+ increased the NO level in the roots of tomato (*Solanum lycopersicum*) in both cadmium-free and cadmium-supplemented growth solutions (Luo *et al.*, 2012). These results suggest that NO is partly responsible for NO_3^--facilitated P accumulation in plants.

It is unclear how elevated CO_2 changes the NO levels in roots under P deficiency. It is recognized that NR and NOS are two potential enzymatic sources of NO in plants (Neill *et al.*, 2008). Although NOS genes have not yet been identified in plants (Neill *et al.*, 2008), several studies support the presence of NOS activity in plants (Corpas *et al.*, 2006; Valderrama *et al.*, 2007). As shown in Fig. 7, NR activity in the shoots of NO_3^--fed plants was greater under elevated compared with ambient CO_2, while NR activity in the roots was lower under elevated CO_2, irrespective of N form. These results are in accordance with the findings of other studies (Buchanan *et al.*, 2000; Poonnachit and Darnell, 2004). Nitrate supply has been shown to enhance NR activity (Shaner and Boyer, 1976), whereas NH_4^+ is an inhibitor of NR (Jin *et al.*, 2011). On the one hand, increasing NO_3^- availability should enhance the NR-dependent NO production. Accordingly, a higher NO level in the roots of the NO_3^--fed plants and a lower NO level in the roots of NH_4^+-fed plants under elevated CO_2 is partly due to the change in NR activity. On the other hand, elevated CO_2 enhanced the root uptake capacity for NO_3^-, but not for NH_4^+, in field-grown loblolly pine saplings (Bassirirad *et al.*, 1996). Therefore, it is reasonable to propose that stimulated NO_3^- uptake under elevated CO_2 may facilitate NO production in roots, form a positive feedback loop, and upregulate P uptake.

Elevated CO_2 also increased the NOS activity of *Arabidopsis* plants supplied with NO_3^- but decreased it in NH_4^+-fed plants (Fig. 7). It is well established that elevated CO_2 represses *NIA* in NH_4^+-fed plants and increases NH_4^+ uptake and assimilation, and the synthesis of glutamine (Gojon *et al.*, 1998; Stitt and Krapp, 1999). Thus, increased NH_4^+ uptake and assimilation under elevated CO_2 may restrain the activity of NR, and possibly of NOS. Furthermore, in mutants with low *NIA* activity, NO production was suppressed by NH_4^+ (Gojon *et al.*, 1998; Stitt and Krapp, 1999), which supports our findings.

In *Arabidopsis*, two mutants defective in NO production displayed altered phenotypes. The *nr* mutant had a deletion

of the major NR gene, *NIA2*, and an insertion in the *NIA1* NR gene (Wang *et al*., 2004), and the *atnos1* mutant has a mutation in an NOS structural gene *NOS1* (Guo *et al*., 2003). In this study, the P concentrations of both mutants, *nr* and *nos*, were similar under elevated and ambient CO_2 (Fig. 8), suggesting that regulation of P nutrition under elevated CO_2 is NO-signalling dependent. However, relative to the ambient CO_2 condition, elevated CO_2 tended to increase the P concentration in both the shoots and roots of NO_3^--fed plants. These results are consistent with a major or general role of NO in the regulation of plant development and metabolism. The results also support our hypothesis that elevated CO_2 increases P uptake in NO_3^--fed plants but suppresses it in NH_4^+-fed plants through the effect on the activity of NR and/or NOS, leading to changes in NO production and physiology. However, the details about how NO exerts its effects on P nutrition remain unclear. The studies by Neill *et al*. (2003) and Niu *et al*. (2011*b*) on interrelations between NO, Ca^{2+}, cyclic ADP ribose, cyclic GMP, salicylic acid, reactive oxygen species, hydrogen peroxide signalling, and protein kinases have provided an important focal point for future research.

Overall, although previous reports have provided other evidence concerning NH_4^+ nutrition facilitating P uptake in the roots of different plant species. Here, using wild-type *Arabidopsis* plants, we demonstrated that NO_3^- nutrition facilitates the P uptake in roots from P-deficient soluble sources under elevated CO_2 by upregulating plant adaptive strategies. The increase in NO production may be a signalling pathway controlling the above process. To our knowledge, this finding uncovers a mechanism that has not been described previously and provides a new insight into N forms regulating P nutrition under elevated CO_2, an important part of climate change in the 21st century. This study also helps to determine whether the carbon costs of P efficiency under elevated CO_2 should be prioritized in crop improvement programmes.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Effect of elevated CO_2 and N form on shoot and root fresh biomass production, P concentration in whole plants, and amount of P absorbed per weight of roots.

Supplementary Fig. S2. Effect of elevated CO_2 and N form on chlorophyll content (SPAD readings) of wild-type *Arabidopsis* grown in P-deficient (0.5 µM) (a) and P-adequate (0.5 mM) (b) nutrition solutions containing NO_3^- or NH_4^+.

Supplementary Fig. S3. Effect of elevated CO_2 and N form on anthocyanin content of wild-type *Arabidopsis* grown in P-deficient (0.5 µM) (a) and P-adequate (0.5 mM) (b) nutrition solutions containing NO_3^- or NH_4^+.

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