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A novel CO₂-responsive systemic signaling pathway controlling plant mycorrhizal symbiosis

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Summary

- Rising atmospheric carbon dioxide concentrations (eCO₂) promote symbiosis between roots and arbuscular mycorrhizal fungi (AMF), modifying plant nutrient acquisition and cycling of carbon, nitrogen and phosphate. However, the biological mechanisms by which plants transmit aerial eCO₂ cues to roots, to alter the symbiotic associations remain unknown.
- We used a range of interdisciplinary approaches, including gene silencing, grafting, transmission electron microscopy, LC-MS/MS, biochemical methodologies and gene transcript analysis to explore the complexities of environmental signal transmission from the point of perception in the leaves at the apex to the roots.
- Here we show that eCO₂ triggers apoplastic H₂O₂-dependent auxin production in tomato shoots followed by systemic signaling that results in strigolactone biosynthesis in the roots. This redox-auxin-strigolactone systemic signaling cascade facilitates eCO₂-induced AMF symbiosis and phosphate utilization.
- Our results challenge the current paradigm of eCO₂ effects on AMF and provide new insights into potential targets for manipulation of AMF symbiosis for high nutrient utilization under future climate change scenarios.

Key words: apoplastic H₂O₂, arbuscular mycorrhizal fungi (AMF), auxin, elevated CO₂, phosphate uptake, strigolactone, systematic signalling, tomato (*Solanum lycopersicum*)

Introduction

Symbiosis between plants and arbuscular mycorrhizal fungi (AMF) facilitates efficient utilization of nutrients and alters carbon (C), nitrogen (N) and phosphate (P) cycling in terrestrial ecosystems (van der Heijden *et al.*, 1998; Smith & Smith, 2011). Beneficial symbioses are predicted to play an increasingly important role in plant adaptation to projected increases in atmospheric CO₂ levels, particularly as essential nutrients become exhausted (Heimann & Reichstein, 2008). AMF development is sustained by sugars and lipids supplied by the roots (Kiers *et al.*, 2011; Jiang *et al.*, 2017; Luginbuehl *et al.*, 2017), a process that is regulated by phytohormones such as auxin and strigolactones (SLs) and nutrient availability (Akiyama *et al.*, 2005; Benjamins *et al.*, 2008; Pozo *et al.*, 2015; Lanfranco *et al.*, 2018). For example, SLs trigger fungal pre-symbiotic development, while auxin signaling promotes AM formation (Gutjahr, 2014; Guillotin *et al.*, 2017). Phosphate-deprived plants secrete SLs to initiate the establishment of symbiotic relationships with AMF (Akiyama *et al.*, 2005; Bucher, 2007). The AM symbiosis on roots is promoted by elevated atmospheric CO₂ (eCO₂) (Drigo *et al.*, 2010; Terrer *et al.*, 2016). To date, eCO₂ effects on mycorrhizal symbiosis have been explained largely in terms of an increased flux of photo-assimilates to roots (Roth & Paszkowski, 2017). However, increases in photoassimilate availability are predominantly used for root growth leading to increased root/shoot ratios as nutrients such as nitrogen and phosphorus become limiting, and there is little change in the accumulation of carbohydrates in the roots (Jauregui *et al.*, 2015). Moreover, eCO₂ exerts many local and systematic effects on plant biology, including changes in redox homeostasis, hormone signaling, root development and defense responses (Munné-Bosch *et al.*, 2013; Jin *et al.*, 2015; Shi *et al.*, 2015; Mhamdi & Noctor, 2016). Such observations suggest the probable existence

of an as yet uncovered and largely carbohydrate-independent systemic signaling pathway that underpins plant responses to eCO₂. Here we address the key question of how plants transmit eCO₂ signals systematically from the shoot to the roots in order to induce appropriate responses including effects on plant-mycorrhizal symbioses and associated nutrient uptake.

Material and methods

Plant material, plant growth and growth conditions

Wild-type tomato (*Solanum lycopersicum* L. cv. Ailsa Craig), a mutant in a cyclophilin A called *diageotropica* (*dgt*) in the Ailsa Craig background and transgenic tomato *DR5:GUS* lines in the Ailsa Craig and *dgt* backgrounds (Dubrovsky *et al.*, 2008) were obtained from M. G Ivanchenko of Oregon University, USA. The *dgt* mutant plants show a pleiotropic phenotype including lack of geotropism, abnormal xylem structure, lack of lateral roots (LRs), and higher shoot-to-root ratios with abnormal auxin fluxes (Lavy *et al.*, 2012). The AMF used in the following studies was an isolate of *Rhizophagus irregularis*, which was propagated in pot cultures with *Zea mays* L.

To generate the *RBOH*RNAi construct, a 318-bp specific DNA fragment of *SIRBOH1* was amplified with the specific primers *SIRBOH1-F* (5'-GGCCatttaaatggatccCGTTCAGCTCTCATTACC-3') and *SIRBOH1-R* (5'-TTggcgcgcctctagaCCGAAGATAGATGTGTGT-3'), which had been tailed with *Bam*HI/*Xba*I and *Swa*I/*Asc*I restriction sites at the 5' end, respectively. The amplified products were digested with *Bam*HI/*Xba*I and *Swa*I/*Asc*I and ligated into the pFGC5941 vector at the *Bam*HI/*Xba*I restriction site in the sense orientation and at the *Swa*I/*Asc*I restriction site in the antisense orientation. The resulting plasmid was

transformed into *Agrobacterium tumefaciens* strain EHA105 and transformed into cotyledon of tomato (cv. Ailsa Craig) as described (Fillatti *et al.*, 1987). Transgenic plants were identified by resistance to the herbicide Basta and then characterized by RT-qPCR.

To determine the role of SL biosynthesis and SL signaling in eCO₂-induced AMF development and P uptake, we used virus-induced gene silencing (VIGS) to suppress the transcript of *CCD7*, *CCD8*, *MAX1* and *MAX2* with the tobacco rattle virus (TRV)-based vectors (pTRV1/2) (Liu *et al.*, 2002). The cDNA fragment of *CCD7*, *CCD8*, *MAX1* and *MAX2* was PCR-amplified using the gene-specific primers listed in Table S1. The amplified fragment was digested with *EcoRI* and *XbaI*, *BamHI* and *XbaI*, respectively, and ligated into the corresponding sites of the pTRV2 vector. Empty pTRV2 vector was used as a control. All constructs were confirmed by sequencing and subsequently transformed into *Agrobacterium tumefaciens* strain GV3101. VIGS was performed by infiltration of germinated seeds, followed by infiltration into the fully expanded cotyledons of 15-d-old tomato seedlings with *A. tumefaciens* harboring a mixture of pTRV1 and pTRV2-target gene in a 1:1 ratio. Plants were grown at 23/21 °C (day/night) in a growth chamber with a 12 h day length for 30 d until control pTRV-*PDS* plants (silencing of the gene encoding phytoene desaturase) showed strong bleaching (Ekengren *et al.*, 2003). qRT-PCR analysis was performed to evaluate gene silencing efficiency.

To determine the respective role of *RBOH1* expression in the shoot and roots in the AM fungal growth, shoots of wild type (WT) and *RBOH1*-RNAi (*rboh*) plants at 3-leaf stage were self-grafted or reciprocally grafted onto roots of WT and *rboh*, respectively, which resulted in four combinations: WT/WT, *rboh/rboh*, *rboh*/WT and WT/*rboh*. Similarly, shoots of WT and *dgt* plants at 3-leaf stage were self-grafted or

reciprocally grafted onto roots of WT and *dgt* roots, respectively, which resulted in four combinations: WT/WT, *dgt/dgt*, *dgt*/WT and WT/*dgt*. After adaptation under dark for 3 d, the grafted plants were transferred to growth chambers with the following environmental conditions: 12-h photoperiod, temperature of 25/20 °C (day/night) and photosynthetic photon flux density (PPFD) of 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Tomato plants at 5-leaf stage were transplanted to pots (150 ml) filled with a sterilized soil/quartz sand/vermiculite mixture (1/1/1) or to pots containing the same mixture inoculated with *Rhizophagus irregularis* at a dose of 800 spores per plant or grown hydroponically in Hoagland's nutrient solution (Hoagland *et al.*, 1950). The content of extractable soil P was 8 mg kg⁻¹. Plants were allowed to grow under two atmospheric CO₂ conditions (aCO₂ at 380 ppmv and eCO₂ at 800 ppmv) in controlled growth chambers. CO₂ was supplied to the growth chambers using compressed CO₂ gas and automatically controlled at ± 10 ppmv. For the pot plants, seedlings were supplied with Hoagland's nutrient solution containing 1 mM KH₂PO₄ or Hoagland's nutrient solution without KH₂PO₄ but with the addition of 1mM KCl. Plants were watered daily with distilled water and fertilized with Hoagland nutrition solution every 2 d. For the hydroponically-grown plants, seedlings were exposed to P sufficient (1 mM KH₂PO₄) or P deficient (0.05mM KH₂PO₄ with the addition of 0.95 mM KCl) conditions. For the AMF inoculation, *Rhizophagus irregularis* inoculum was sandwiched between the bottom (100 g) and top (50 g) layers of the substrate in each pot. The plants were placed in a controlled environment chamber with a day/night temperature of 25/20 °C and a 12 h photoperiod. The photosynthetic photon flux density (PPFD) was 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For biomass and P content measurements, leaves, stems and roots were harvested, dried at 80 °C for 3 d before analysis.

Histological analyses and microscopy

To detect mycorrhizal colonization, root segments were rinsed by water and incubated in 10% (w/v) KOH heated to 90 °C for 1 h and then rinsed with water prior to acidification with 2% HCl solution for 5 min. The root samples were then stained with 0.05% Trypan Blue, heated to 90 °C for 30 min and decolorized 1 day by Lactic acid and glycerin (1:1, v/v). Then the root samples were observed under a Leica DM4000B microscope (Leica Microsystems, Germany) for mycorrhizal fungal colonization. A total of ca. 300 root/gridline intersects were recorded to assess the colonization level according to gridline intersect method described by Giovanetti and Mosse (1980).

To determine the auxin signaling intensity, two transgenic tomato *DR5:GUS* lines with Ailsa Craig or *dgt* backgrounds (Ekengren *et al.*, 2003) were used directly or as rootstock with shoots of *RBOH1*-RNAi and *dgt* plants as scion, respectively. GUS staining was performed as described previously (Ivanchenko *et al.*, 2006). Root samples were analyzed under a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) with differential interference contact (DIC) optics. H₂O₂ in apex leaves was visualized at the subcellular level using CeCl₃ for localization, as described previously (Thordal-Christensen *et al.*, 1997; Xia *et al.*, 2009).

Pi and H₂O₂ quantification

Metabolism was arrested in dried leaves, stems and root samples (0.1 g by grinding in 2 ml 4:1 mixtures (v/v) of 65% nitric and 70% perchloric acids. The homogenates were filtrated and diluted with distilled water. The pH value was adjusted to 7~8. Pi measurements were conducted as previously described (Delhaize and Randall, 1995). H₂O₂ was extracted from leaf tissue and measured as described previously (Xia *et al.*, 2009).

Expression analysis by qRT-PCR

Gene expression analyses were done by qRT-PCR as previously described (Wang *et al.*, 2016), with primers listed in Table S2.

Measurement of hormones levels

For the analysis of IAA, the developing leaves at the apex were used. IAA extraction and quantification were performed as described previously (Guo *et al.*, 2016). For the determination of SLs in the roots, 0.5 g of roots was blended in a blender at top speed for 1 min in 40% acetone (1:5, w/v). After centrifugation for 5 min, SLs in the residues were extracted with 50% acetone (1:5, w/v). The acetone was evaporated under reduced pressure at 35 °C. Root extracts were dissolved in methanol:water (1:1, v/v). SLs were detected using an Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source, operated in positive mode. HPLC separation was performed on a Zorbax SB C18 column (150 × 2.1 mm, 3.5 µm). The mobile phases consisted of formic acid:water (1:100, v/v; solution A) and acetonitrile (solution B). The gradient program was as follows: 20–48% B in 3min, 48–50% B in 2 min, 50–53% B in 1min, 53–65% B in 1min, 65–95% B in 0.5min, 95% B for 3.5 min. The flow rate was set at 0.3 ml min⁻¹. The SLs were detected in multiple reaction monitoring (MRM) mode (Koltai *et al.*, 2011). SLs contents were expressed as the relative content with peak area for plants under aCO₂ as 1.

Seed germination

Germination assays were performed using *Orobancha ramosa* seeds as reported previously with minor modifications (Matusova *et al.*, 2005). Seeds of *Orobancha ramosa* were preconditioned for 14 days at 21°C before the seeds became responsive to germination stimulants. Aliquots (50 µl) of 50% acetone extracts of tomato roots were added to duplicate 1-cm discs bearing approximately 50 preconditioned seeds

each. The synthetic germination stimulant GR24 (10^{-6} mM) and demineralized water were included as positive and negative controls in each bioassay. After 7 days, the germinated and ungerminated seeds were counted using a binocular microscope. Seeds were considered germinated when the radicle had protruded through the seed coat.

Statistical analysis

A completely randomized block design with at least three independent replicates was used in each experiment. Each replicate involved 15~20 plants. Data were statistically analyzed by analysis of variance (ANOVA). The significance of treatment differences was analyzed using Tukey's test ($P < 0.05$).

Results

eCO₂ induces strigolactone biosynthesis and AMF symbiosis

To gain a better understanding of how eCO₂ alters the plant-AMF symbiosis and associated P utilization efficiency, we firstly compared biomass accumulation, organ P content and AMF development in tomato plants grown in a soil/quartz sand/vermiculite mixture (1/1/1) in the absence or presence of a P supply and inoculation with the AMF *Rhizophagus irregularis* under ambient CO₂ (aCO₂, 380 ppmv) or eCO₂ (800 ppmv) conditions for 28 d. P starvation-induced decreases in plant biomass accumulation, tissue P concentrations and total plant P accumulation were greatly attenuated by inoculation with AMF and by eCO₂ (Figs 1a, S1a,b). Conversely, AMF development was suppressed by adequate P supply. Growth under eCO₂ significantly improved AMF development on the roots, especially under P starvation conditions, as shown by increased AMF colonization (Figs 1b, S1c). Significantly, the levels of transcripts encoding the SL synthesis enzymes,

CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), *CCD8* and *MORE AXILLARY BRANCHING 1 (MAX1)*, and *MAX2*, which are required for SL signaling in the roots, were induced by eCO₂, especially under P starvation and in the presence of AMF (Figs 1c, S2a). Moreover, the increase in the levels of *CCD7*, *CCD8*, *MAX1* and *MAX2* transcripts was accompanied by a large increase in the accumulation of orobanchol, didehydro-orobanchol and solanacol, which are the three principal SLs in the roots of tomato plants measured under eCO₂ conditions, regardless of the P level or the presence of AMF (Fig. S2b). Concomitant with these changes, growth under eCO₂ resulted in a higher abundance of transcripts encoding several phosphate transporters (*PT1*, *PT2*, *PT3*, *PT4* and *PT5*) (Fig. S3). This was especially marked under conditions of P starvation and in the presence of AMF. These results show that eCO₂-promoted AMF colonization and P uptake are linked to enhanced SL biosynthesis and signaling in roots.

Requirement for leaf *RBOH1* expression in the systemic induction of auxin signaling by eCO₂

Apoplastic H₂O₂ production can trigger auxin production, which hence has a potential role in the regulation of AMF growth (Etemadi *et al.*, 2014; Chen *et al.*, 2016). We, therefore, determined the local and systemic impacts of eCO₂ on H₂O₂ homeostasis and the levels of auxin in the apex, together with auxin signaling in the roots in plants grown either with sufficient P supply or under P deficient conditions. The levels of *RESPIRATORY BURST OXIDASE HOMOLOG1 (RBOH1)* transcripts encoding NADPH oxidase, which is involved in apoplastic H₂O₂ generation, increased in the apex leaves of plants grown under eCO₂ (Fig. 2a). Concurrently, the levels of *FLAVIN MONOOXYGENASE (FZY)* mRNAs that encode an enzyme catalyzing a

rate-limiting step in auxin biosynthesis, the accumulation of H₂O₂ in the apoplast and the levels of indole-3-acetic acid (IAA) increased (Figs. 2b,-c, Fig. S4a,b). These eCO₂-dependent increases were particularly marked under conditions of P deficiency, and were observed within one day of exposure to eCO₂. Histochemical analysis of GUS-activity revealed that the auxin-responsive reporter gene *DR5* was more highly expressed in root tips of plants grown under eCO₂ (Fig 2d). Similarly, the abundance of *IAA15* transcripts was increased in roots under eCO₂, especially under conditions of P starvation (Fig. S4c). Taken together, these results show that eCO₂ not only induces local changes in redox and auxin homeostasis of the apex, but also leads to enhanced auxin signaling in the roots as a result of greater polar auxin transport (PAT) from the shoots. Interestingly, the eCO₂-induced accumulation of IAA was observed in WT plants but absent from the apex of the *RBOH1*-RNAi (*rboh*) plants, regardless of P level (Fig. S5a). When *rboh* shoots were grafted onto the roots of wild type (WT) plants (*rboh*/WT), eCO₂-induced accumulation of IAA and *DR5* in the roots were attenuated (Fig. S5b,c). Moreover, exogenous application of H₂O₂ to the leaves resulted in an increase in the accumulation of IAA in the apex, a concentration of 100 μM H₂O₂ being the most effective in stimulating IAA accumulation (Fig. S5d).

By using reciprocal grafting with WT and *rboh1* as scion (S) or rootstock (R), respectively, we obtained four graft combinations (S/R), WT/WT, WT/*rboh*, *rboh*/WT and *rboh*/*rboh*. When these plants were grown in the presence of AMF under P deficient conditions, *rboh*/WT and *rboh*/*rboh* plants showed less IAA accumulation in the apex, with a lower tissue P concentration and plant dry matter and less overall P accumulation in the plants (Figs 2e, S6a-c). While eCO₂ induced significant increases in the accumulation of IAA in the apex, as well as increases in plant biomass and P accumulation in the WT/WT plants and to a lesser degree in WT/*rboh* plants, these

effects were either not observed or they were attenuated in the *rboh*/WT and *rboh/rboh* plants (Fig. S6a-c). Moreover, AMF colonization rates were decreased (Figs. 2f, S7a). In addition, the eCO₂-induced increases in AMF colonization and transcript of *PT4* and *PT5* as well as in P accumulation were attenuated in the roots of *rboh*/WT and *rboh/rboh* plants (Figs 2e,f, S6c, S7). These findings show that expression of *RBOH1* in the shoots plays a critical role in eCO₂-induced IAA biosynthesis and in systemic signaling leading to enhanced AMF symbiosis and P utilization, as well as whole plant growth under conditions of P deficiency.

Dependency of strigolactone biosynthesis on auxin signaling in leaves

Auxin perception is required for arbuscule development in the mycorrhizal symbiosis (Etemadi *et al.*, 2014). To determine whether eCO₂-dependent IAA biosynthesis in the apex is required for systemic signaling leading to greater AMF symbiosis and P utilization, we conducted a further series of grafting experiments using WT and an auxin-resistant *diageotropica* (*dgt*) mutant that has impaired PAT (Ivanchenko *et al.*, 2015) as rootstock or scion respectively. Under eCO₂ conditions, the WT/WT plants had the highest biomass, tissue P concentrations and greatest overall P accumulation, as well as the highest levels of AMF colonization in the roots, followed by the WT/*dgt*, *dgt*/WT and *dgt/dgt* plants, in the absence of P supply (Figs 3a,b, S8, S9a). The effects of eCO₂ on biomass accumulation, P concentration and accumulation, AMF colonization and the transcript of *PT4* and *PT5* were less marked in the WT/*dgt* plants than the WT/WT controls. Crucially, eCO₂ had little effect on the dry biomass, tissue P concentration and P accumulation, AMF colonization and the transcript of *PT4* and *PT5* in the *dgt*/WT and *dgt/dgt* plants (Figs. 3a,b, S8, S9). Taken together, these results demonstrate that auxin signaling in the shoots plays a vital role in

eCO₂-induced AMF growth and P uptake.

DR5-GUS staining studies showed that while *DR5* accumulation was only slightly induced in the roots as a result of P starvation, it was greatly increased in plants exposed to eCO₂ (Fig. S10a). However, the eCO₂-dependent induction in *DR5* accumulation was abolished in plants with *dgt* as scion. In contrast, *DR5* accumulation was induced in the roots of WT/*dgt* plants under eCO₂ conditions but to a lesser degree, presumably because the mobility of DGT protein could remedy DGT deficiency, at least in part, in the *dgt* roots (Spiegelman *et al.*, 2014) (Fig. S10b). Therefore, eCO₂-induced increase in auxin biosynthesis in the leaves and associated polar auxin transport (PAT) contributes the systemic induction of auxin signaling in the roots, AMF symbiosis and P utilization.

Given eCO₂-induced SL signaling and its role in AMF symbiosis in the roots (Akiyama *et al.*, 2005) (Fig. 1), we next examined whether SL biosynthesis is subject to the regulation of auxin signaling from the leaves. By using WT or *dgt* as scion, respectively, we observed decreased levels of *CCD7*, *CCD8*, *MAX1* and *MAX2* transcripts and a decreased accumulation of orobanchol, didehydro-orobanchol and solanacol in the roots of the *dgt*/WT plants compared to WT/WT plants (Figs 3c, S11). Crucially, eCO₂ failed to stimulate an increase in the expression of *CCD7*, *CCD8*, *MAX1* and *MAX2*, or the accumulation of orobanchol, didehydro-orobanchol and solanacol in the roots of the *dgt*/WT plants. Taken together, these results indicate that eCO₂ induces not only local changes in the redox-dependent auxin homeostasis in the apex, but also the induction of SL biosynthesis and expression of PT genes in the roots, as a consequence of auxin-dependent systemic signaling.

Strigolactone signaling plays a role in eCO₂-promoted AMF symbiosis and P

utilization

SLs are important signals that activate the fungal partner in the plant/AMF symbiosis, particularly with respect to the stimulation of hyphal branching (Akiyama *et al.*, 2005; Besserer *et al.*, 2006). To determine whether eCO₂-induced SL synthesis and signaling play a role in eCO₂-induced AMF colonization and P utilization, we silenced *CCD7* (pTRV-*CCD7*), *CCD8* (pTRV-*CCD8*), *MAX1* (pTRV-*MAX1*), and *MAX2* (pTRV-*MAX2*) by virus-induced gene silencing (VIGS). This resulted in a reduction of the transcripts by ca. 80% (Fig. S12a). Consistent with the role of SL in shoot and root branching (Gomez-Roldan *et al.*, 2005), silencing these genes promoted bud outgrowth with increased numbers of later branches observed under standard growth conditions (Fig. S12b). In agreement with this observation, root extracts from the pTRV-*CCD7*, pTRV-*CCD8* and pTRV-*MAX1* plants showed a decreased accumulation of the three SLs and stimulation of germination of *Orobanche ramosa* seeds compared to the empty vector control plants (pTRV). In comparison, root extracts from the pTRV-*MAX2* plants showed similar accumulation of the three SLs stimulation and caused a similar degree of germination to the pTRV controls (Fig. S12c). These findings confirm that SL synthesis was significantly suppressed in the roots of the pTRV-*CCD7*, pTRV-*CCD8* and pTRV-*MAX1* plants but not in the SL signaling silenced plants pTRV-*MAX2*. Importantly, the pTRV-*CCD7*, pTRV-*CCD8*, pTRV-*MAX1* and pTRV-*MAX2* plants showed decreased plant growth, lower levels of *PT1*, *PT2*, *PT3*, *PT4* and *PT5* transcripts, and less P accumulation in response to P starvation compared to the pTRV controls under both aCO₂ and eCO₂ conditions (Figs 4a,c, S13a-c). Crucially, eCO₂-induced stimulation of plant growth, AMF colonization and development, the levels of *PT1*, *PT2*, *PT3*, *PT4* and *PT5* transcripts and P accumulation were compromised in the pTRV-*CCD7*, pTRV-*CCD8*, pTRV-*MAX1* and

pTRV-MAX2 plants (Figs 4, S13). However, no significant differences were found in the pTRV-CCD7, pTRV-CCD8, pTRV-MAX1 and pTRV-MAX2 plants in terms of biomass accumulation, P concentration, P accumulation, AMF colonization rate, indicating that both SL biosynthesis and signaling are involved in the eCO₂-induced regulation of AMF colonization and P uptake.

Discussion

The most common mechanism by which higher plants optimize soil P uptake in response to eCO₂, is through AM symbiosis (Terrer *et al.*, 2016). In a synthesis of 135 published studies, Compant *et al.* (2010) reported that eCO₂ generally had a positive influence on AMF colonization. However, the effects of eCO₂ and P supply on the AMF varies with the host plant type, AMF species and climate factors, as well as soil biology and chemistry (Jakobsen *et al.* 2016). Here, we report that growth under eCO₂ significantly improved AMF development on tomato roots, especially under P starvation conditions (Figs 1b, S1c). Notably, enhanced AM symbiosis was followed by increased P accumulation and plant growth (Figs 1a, S1a,b). taken together these findings suggest that AM symbiosis will become increasingly important for plants in the future, as the atmospheric CO₂ continue to increase placing increasing demands on soil P availability.

To date, the mechanisms by which plants transmit eCO₂ signals systematically from the shoots to the roots have remained unclear. The simplest scenario would be that with increased CO₂ fixation, more C would be available for transfer to AMF and hence the percentage of colonization would increase. The data presented here reveals the existence of a previously unrecognized systemic redox-hormone signaling pathway and provides a critical new insights into the mechanisms underpinning

eCO₂-induced symbiosis. In the conceptual framework presented here (Fig. 5), eCO₂ triggers the apoplastic H₂O₂ dependent production of auxin in the tomato shoots followed by systemic induction of auxin signaling and resultant strigolactone biosynthesis in the tomato roots. In the absence of AMF, this signaling cascade may alter root system architecture (RSA) to enhance phosphate absorption as both auxin and SLs participate in the regulation of root system architecture (Ham *et al.* 2018). In the presence of AMF in the soil, this redox-auxin-strigolactone systemic signaling cascade can be used to facilitate mycorrhizal symbiosis and subsequent increased phosphate uptake from the soil. Moreover, our observations demonstrate that this signaling cascade is required for AMF symbiosis in response to eCO₂ regardless of the presence of enhanced carbohydrate availability.

ROS play a critical role in plant growth, development and responses to environmental stimuli (Xia *et al.*, 2015). However, up to now it has remained unknown whether they are involved in eCO₂-induced AM symbiosis and P uptake. Here, we demonstrate that leaf *RBOH1* expression is essential for eCO₂-induced AM symbiosis and associated P uptake. Growth under eCO₂ increased the levels of *RBOH1* transcripts and the accumulation of H₂O₂ in the apoplast. Conversely, suppressed expression of *RBOH1* in the shoot (*rboh*/WT) compromised eCO₂-induced AM symbiosis and P uptake (Figs 2, S6c, S7). Hence, eCO₂ alters cellular redox homeostasis as discussed previously (Mhamdi and Noctor, 2018). In addition, RBOH-mediated ROS production facilitates lateral root emergence in *Arabidopsis* (Shi *et al.*, 2015; Orman-Ligeza *et al.*, 2016). The data presented here demonstrates that *RBOH1* expression in the roots of WT/*rboh1* plants had little effect on AM symbiosis and P uptake in response to changes in the concentration of atmospheric CO₂ compared to *rboh*/WT plants (Figs 2e,f, S6c, S7). Therefore,

eCO₂ promote AM symbiosis by triggering ROS production in the shoots, rather by than in the roots.

While auxin signaling is known to promote AM development (Etemadi *et al.*, 2014; Chen *et al.*, 2016), it has remained unclear whether auxin participates in eCO₂-induced AM symbiosis. The data presented here show that there was an increase in RBOH1-dependent IAA accumulation in response to eCO₂, with the induction of transcripts encoding the auxin biosynthesis gene *FZY* (Fig. S4a). Crucially, plants with impaired PAT (*dgt*/WT and *dgt/dgt*) showed a compromised response to eCO₂ in term of auxin signaling (DR5 accumulation), AM symbiosis and P uptake, suggesting that auxin signaling is responsible for eCO₂-induced AM symbiosis (Figs 3a,b, S8-10). In addition, the data presented here show that auxin biosynthesis was subject to redox regulation. Suppression of *RBOH1* transcripts attenuated eCO₂-induced accumulation of IAA in the apex of *rboh*/WT plants (Fig. S6a). These results provide convincing evidences that redox signaling acts as a second messenger of auxin signaling in promoting AMF symbiosis and P utilization.

Auxin is involved in the regulation of SL biosynthesis in roots (Koltai, 2015). The auxin deficient *bushy* mutant of pea showed decreased expression of the SL biosynthesis gene *CCD8* and reduction in SL biosynthesis resulting in low AM colonization (Foo, 2013). Similarly, the application of IAA increased root SL accumulation, while the application of an auxin transport inhibitor decreased SL accumulation in the roots (Yoneyama *et al.*, 2015). Consistent with these findings, we show here that the roots of the *dgt*/WT plants had decreased levels of *CCD7*, *CCD8*, *MAX1* and *MAX2* transcripts with a lower accumulation of orobanchol, dihydro-orobanchol and solanacol compared to WT/WT plants (Fig. 3c, S11). Moreover, eCO₂ failed to stimulate an increase in the expression of these genes or the

accumulation of these SLs in the roots of the *dgt*/WT plants. Taken together, these results collectively indicate that eCO₂ induces a systemic redox-auxin-SL signaling cascade that promotes AMF symbiosis and P utilization.

SLs have well known roles in seed germination, plant architecture, stress responses and AM symbiosis (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; Mostofa *et al.*, 2018). Consistent with previous studies, the data presented here show that the *CCD7*, *CCD8*, *MAX1* and *MAX2*-silenced plants had low AM colonization and P uptake (Figs. 4a,b, S13b,d). Importantly, silencing these genes also compromised eCO₂-induced AM colonization, decreased the levels of PT transcripts and P uptake, suggesting that SL biosynthesis and signaling are pivotal to eCO₂-induced AM colonization and P uptake. Up to now, both induced or suppressed expression of PT genes has been observed during AM symbiosis in different plant species. This has been mostly attributed to differences in parameters such as AM or plant species, growth stage, culture system and experimental conditions applied (Nagy *et al.*, 2005; Balestrini *et al.*, 2006; Fiorilli *et al.*, 2009; Smith *et al.*, 2011). In tomato, the expression of *PT1*, *PT3* and *PT4* was differentially induced by mycorrhizal inoculum (Xu *et al.*, 2007). In addition, mutation of *PT4* resulted in decreased P accumulation, with low plant biomass accumulation in the absence of mycorrhiza inoculum, suggesting that *PT4* is not only responsive to AMF but that it is also required for appropriate P status. In our studies, we show that eCO₂ increased the induction of expression of the PT genes and that this was particularly significant for the *PT4* transcripts in the presence of AMF. These findings suggest that *PT4* is pivotal in eCO₂-induced and AMF-mediated P accumulation (Fig. S3). This dataset also raised the possibility of involvement of SLs and AMF in the regulation of PT gene expression through interactions with other P-related physiological processes.

In support of this conclusion the *max2-1* mutants displayed a lower induction of the expression of several phosphate-starvation response and phosphate-transporter genes (Mayzlish-Gati *et al.*, 2012).

In conclusion, the data presented here demonstrate the presence of a previously unrecognized redox-auxin-strigolactone systemic signaling cascade that facilitates mycorrhizal symbiosis and subsequent phosphate uptake in plants from soils. The findings have the following important implications for our current understanding plant responses to climate change. Firstly, plants have evolved a systemic signaling cascade that allows adaptation to climate change. This strategy is especially important for adaptations to increased atmospheric CO₂ levels and decreased P availability in terrestrial ecosystems, in which plants rely largely on AMF for mineral nutrients. Secondary, our studies highlight the importance of incorporating considerations of AMF-dependent effects on C, water and nutrient cycles in assessments of eCO₂ impacts in ecosystems, as well as in future climate change scenarios because eCO₂-induced AMF not only have a major impact on plant variability and productivity through plant-soil feedback processes (van der Heijden *et al.*, 1998; Clemmensen *et al.*, 2013; Teste *et al.*, 2017), but also on the biodiversity of soil microbial communities and associated turnover of C, N and P in soil (Compant *et al.*, 2010; Cheng *et al.*, 2012). Finally, our studies illustrate the potential for increasing nutrient utilization efficiency by targeting the components involved in the systemic signaling pathway described here, especially for improvement of crop productivity on marginal or infertile soils. Such considerations could also serve to reduce environmental pollution arising from high dose fertilization that is currently used in agricultural production systems.

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Author contributions

Y.Z., S.G., L.J., K.Y. and Y.W. performed the experiments; X.W. carried out mass spectrometry analysis; X.X., J.Z. and K.S. provided technical and intellectual support; C.F. and J.Y. conceived the project, designed the experiments, analyzed the data and wrote the manuscript with the help of all authors.

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Supporting Informaiton

Additional Supporting Information may be found online in the Supporting Informaiton tab for this articile:

Fig. S1 Elevated CO₂ increases biomass accumulation, P concentration and AMF symbiosis.

Fig. S2 CO₂ systemically up-regulates transcript of strigolactone signaling and accumulation in roots.

Fig. S3 CO₂ systemically up-regulates transcript of PT genes in roots.

Fig. S4 CO₂ induced-changes in redox hemostasis and auxin signaling.

Fig. S5 Shoot *RBOH1* regulates IAA accumulation and signaling.

Fig. S6 Importance for *RBOH1* expression in IAA accumulation, biomass accumulation and P content.

Fig. S7. CO₂-induced AMF colonization and transcript of PT genes were abolished in grafted plants with *rboh* mutant as scion or rootstock.

Fig. S8 Auxin signaling regulates biomass accumulation and P content in tomato.

Fig. S9. CO₂-induced AMF colonization and transcript of PT genes were abolished in grafted plants with *dgt* mutant as scion or rootstock.

Fig. S10 CO₂ systemically up-regulates auxin signaling.

Fig. S11 CO₂-induced accumulation of strigolactones was abolished in plants with *dgt* mutant as scion.

Fig. S12 Silencing efficiency, phenotype of *CCD7*, *CCD8*, *MAX1* and *MAX2*-silencing plants and the stimulation effects on the seeds of *Orobancha ramosa*

Fig. S13 Strigolactones mediate CO₂-induced biomass accumulation, P concentration and AMF symbiosis.

Table S1 Primer designed for gene silencing

Table S2 Genes and primers used in Real-time qPCR analysis

Fig. 1 Elevated CO₂ systemically induces strigolactone biosynthesis, AMF colonization and P uptake. (a) P accumulation per plant. (b) Root AMF colonization. (c) Transcript accumulation of strigolactone biosynthesis related genes in roots. Seedlings at 5-leaf stage grown in a sterilized soil/quartz sand/vermiculite mixture (1/1/1) were exposed to ambient atmospheric CO₂ (aCO₂, 380 ppmv) or elevated atmospheric CO₂ (eCO₂, 800 ppmv) for 28 d (a, b) or 10 d (c) in the presence of *Rhizophagus irregularis* AMF (+) or not (-) under P sufficient (+, 1 mM KH₂PO₄) or P deprivation (-, without KH₂PO₄ but with the addition of 1mM KCl) conditions. Data in (a) and (c) are mean±SD of three biological replicates. Different letters indicate significant difference at $P < 0.05$.

Fig. 2 Shoot *RBOH1* mediates CO₂-induced auxin signaling, AMF colonization and P uptake. (a-c) *RBOH1* transcript (a), H₂O₂ accumulation in the apoplast (b), and IAA accumulation (c) in the leaves at the shoot apex. (d) *DR5:GUS* expression in the roots. (e, f) P accumulation in plants (e) and root AMF colonization (f) in grafted plants with *RBOH1*-RNAi (*rboh*) as scion (S) or rootstock (R). For (a-d), hydroponically-grown tomato plants at the 5-leaf stage were exposed to aCO₂ and eCO₂ with either P sufficient (1 mM KH₂PO₄) or P deficient (0.05mM KH₂PO₄ with the addition of 0.95 mM KCl) conditions for 1 d in controlled growth chambers. For (e and f), the grafted plants grown in a sterilized soil/quartz sand/vermiculite mixture (1/1/1) were exposed to aCO₂ or eCO₂ under P starvation (without KH₂PO₄ but with the addition of 1mM KCl) conditions in the presence of *Rhizophagus irregularis* for 28 d. FW, fresh weight. Arrows in (b) indicates H₂O₂-CeCl₃ precipitates. Data in (a), (c) and (e) are mean±SD of three biological replicates. AMF colonization in (f) was

scored from ca. 300 root/gridline intersects. Different letters indicate significant difference at $P < 0.05$.

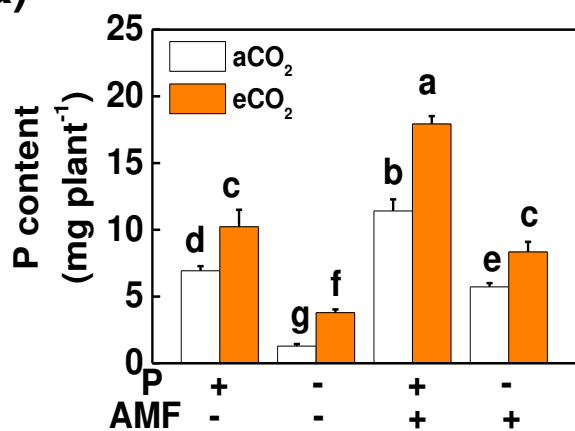
Fig. 3 Auxin signaling activates CO₂-induced biosynthesis of strigolactones, AMF colonization and P uptake. (a, b) P accumulation per plant (a) and root AMF colonization (b) in grafted plants grown in a sterilized soil/quartz sand/vermiculite mixture (1/1/1) with auxin-resistant *diageotropica* mutant (*dgt*) as scion (S) or rootstock (R) after exposure to ambient atmospheric CO₂ (aCO₂, 380 ppmv) or elevated atmospheric CO₂ (eCO₂, 800 ppmv) under P deprivation (without KH₂PO₄ but with the addition of 1mM KCl) conditions in the presence of *Rhizophagus irregularis* for 28 d. (c) Transcript accumulation of strigolactone biosynthesis and signaling related genes in roots of hydroponically-grown plants with *dgt* as scion (S) or rootstock (R) after exposure to aCO₂ or eCO₂ under P deficient (0.05 mM KH₂PO₄ with the addition of 0.95 mM KCl) conditions for 10 d. Data in (a) and (c) are mean±SD of three biological replicates. AMF colonization in (b) was scored from ca. 300 root/gridline intersects. Different letters indicate significant difference at $P < 0.05$.

Fig. 4 Strigolactones are essential for CO₂-induced AMF colonization and P uptake. (a) P accumulation per plant. (b) Root AMF colonization. (c) Transcript of PT genes in roots. The *CCD7*, *CCD8*, *MAX1* and *MAX2*-silencing plants grown in a sterilized soil/quartz sand/vermiculite mixture (1/1/1) were exposed to ambient atmospheric CO₂ (aCO₂, 380 ppmv) or elevated atmospheric CO₂ (eCO₂, 800 ppmv) in the presence of *Rhizophagus irregularis* under P deprivation (without KH₂PO₄ but with the addition of 1mM KCl) conditions for 28 d (a,b) or 10 d (c), respectively. Data in (a) and (c) are mean±SD of three replicates. AMF colonization in (b) was scored

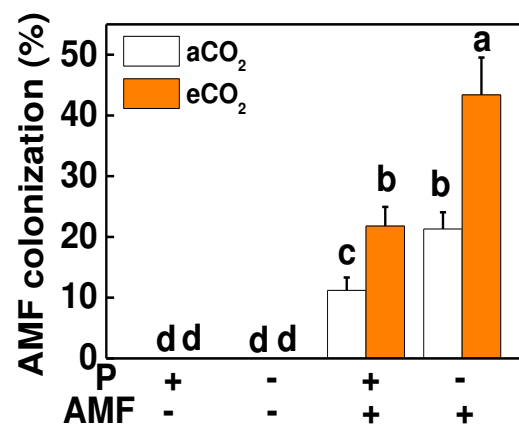
from ca. 300 root/gridline intersects. Different letters indicate significant difference at $P < 0.05$.

Fig. 5 A model for systemic eCO₂ signaling that promotes AMF symbiosis and associated P uptake. eCO₂ triggers an apoplastic H₂O₂ dependent production of auxin in the shoots followed by systemic induction of auxin signaling and resultant strigolactone biosynthesis in the roots of tomato plants. This signaling cascade enhances phosphate absorption by altering root system architecture (RSA) in the absence of AMF or facilitating mycorrhizal symbiosis in the presence of AMF.

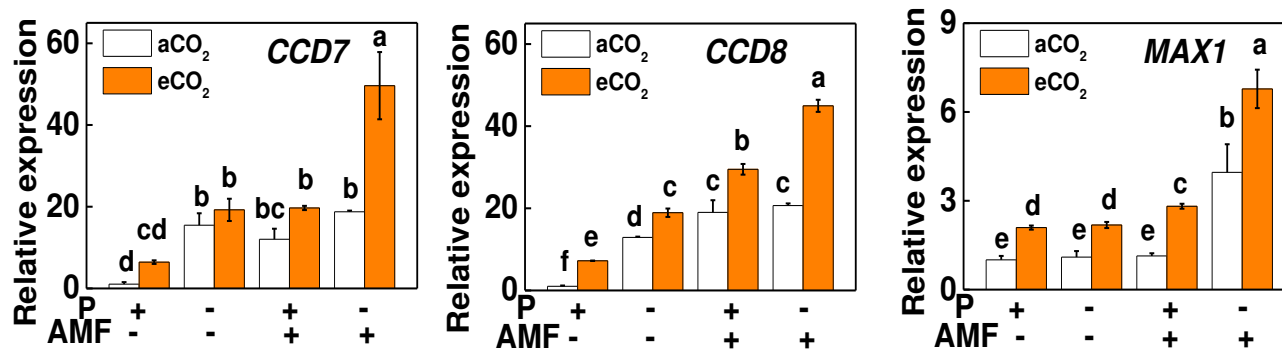
(a)

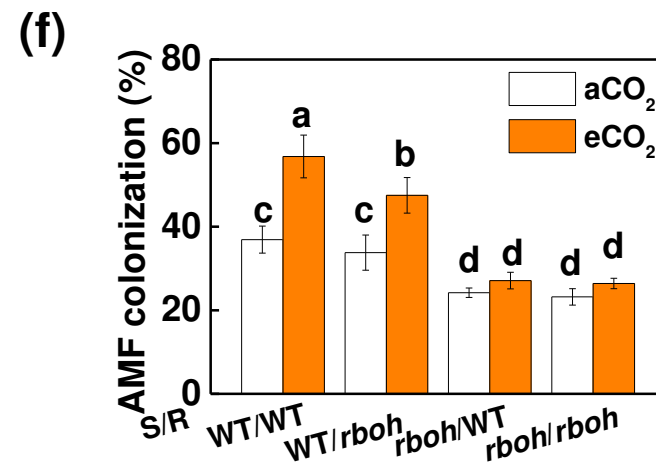
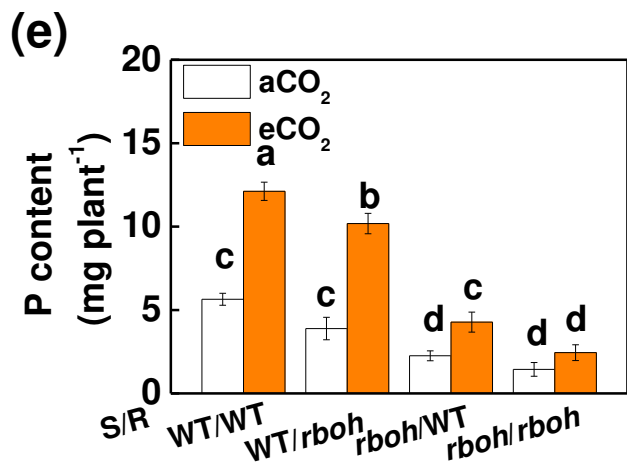
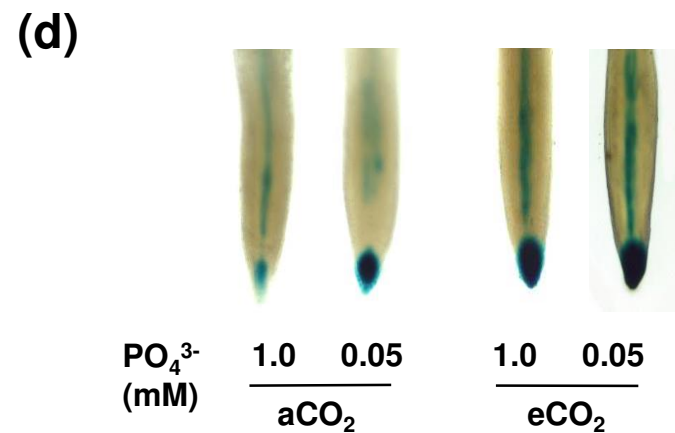
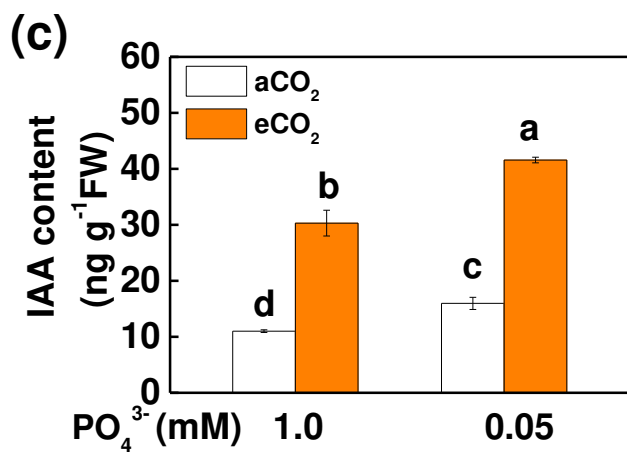
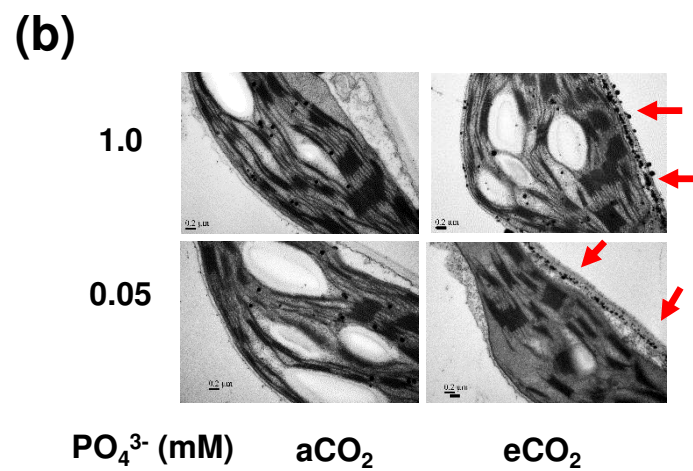
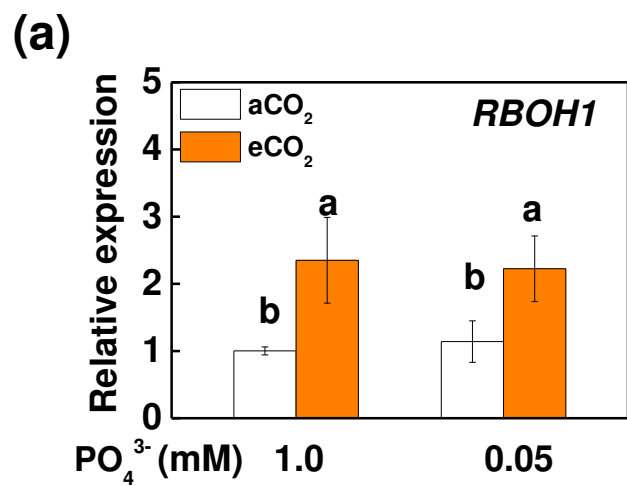


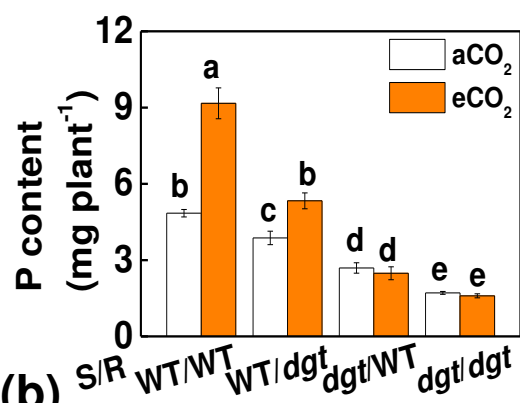
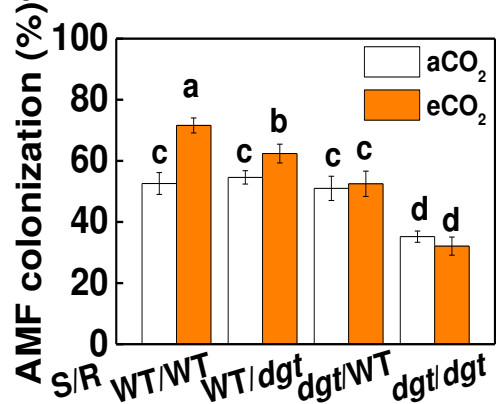
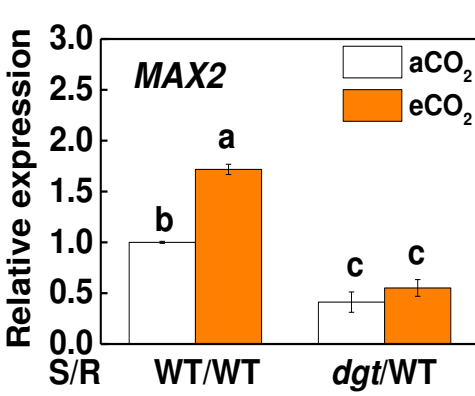
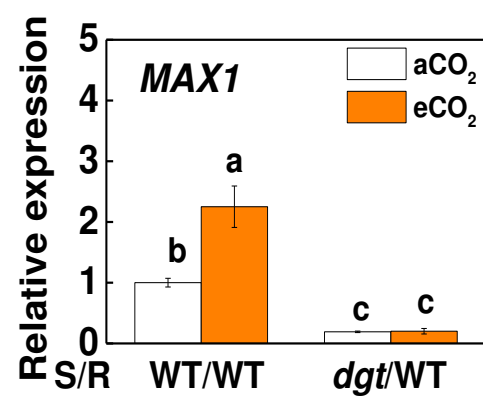
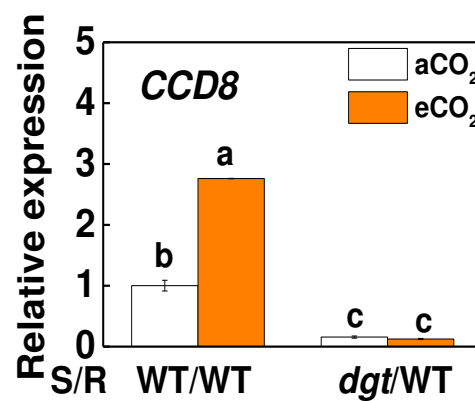
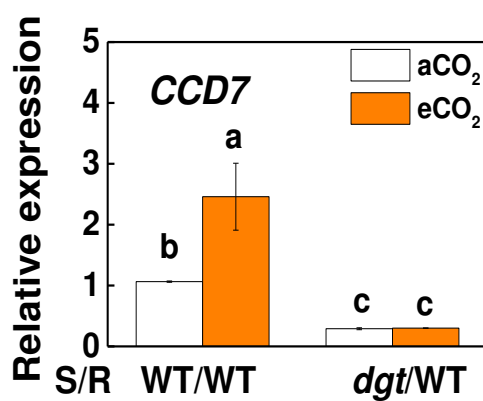
(b)



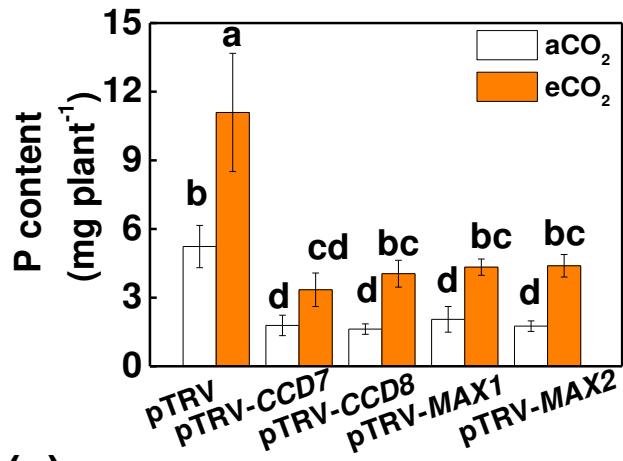
(c)



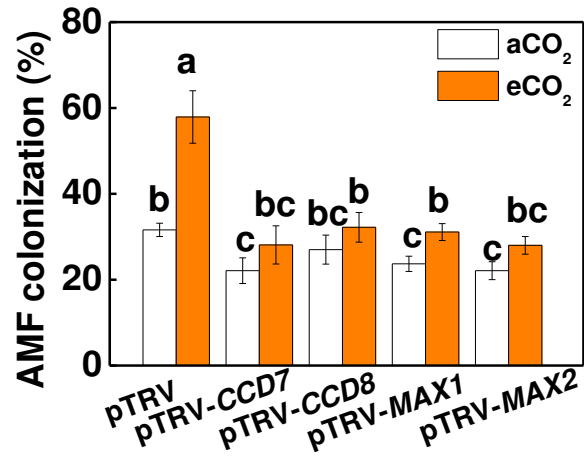


(a)**(b)****(c)**

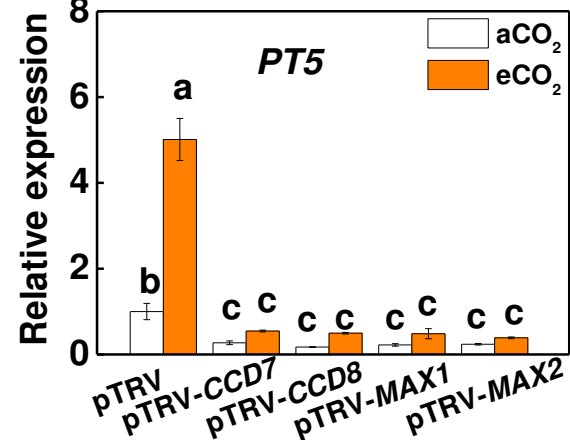
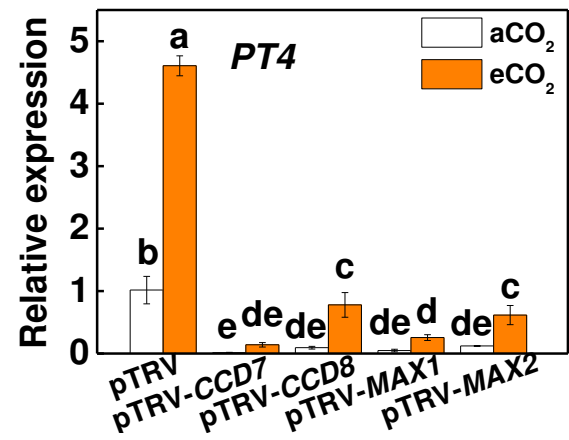
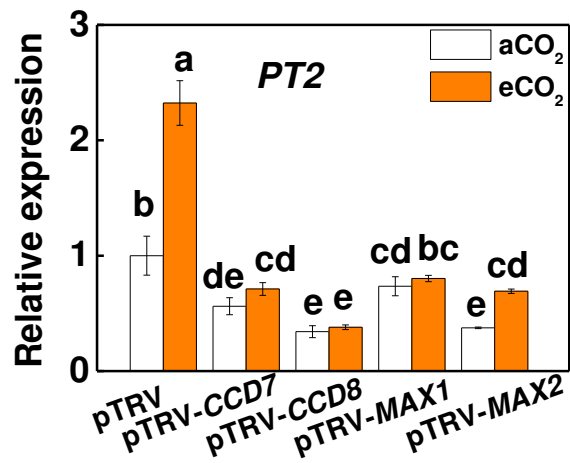
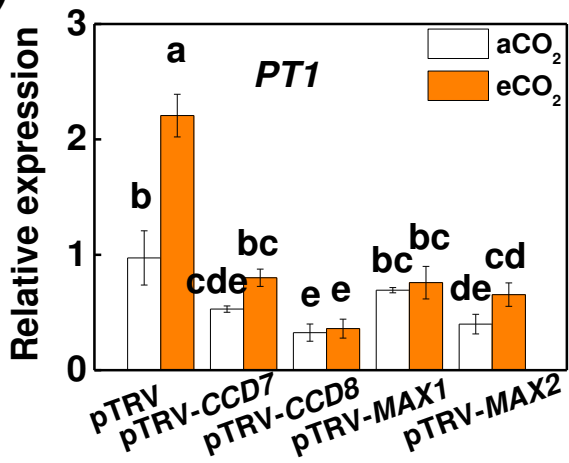
(a)



(b)



(c)



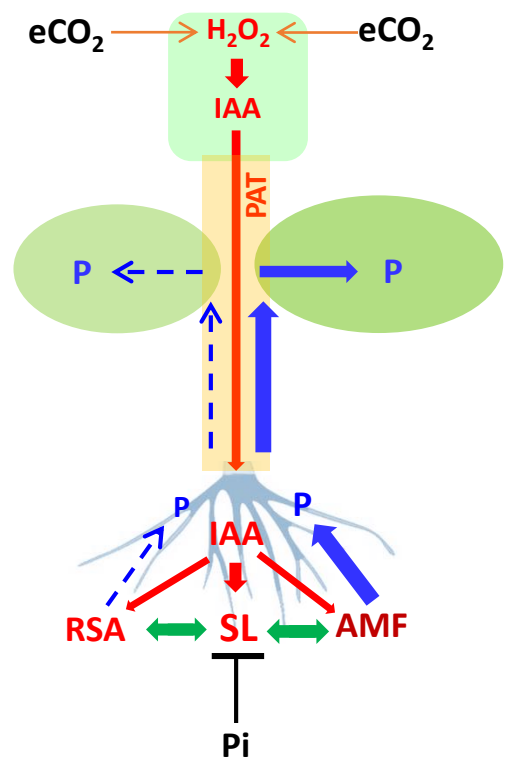


Fig. S1

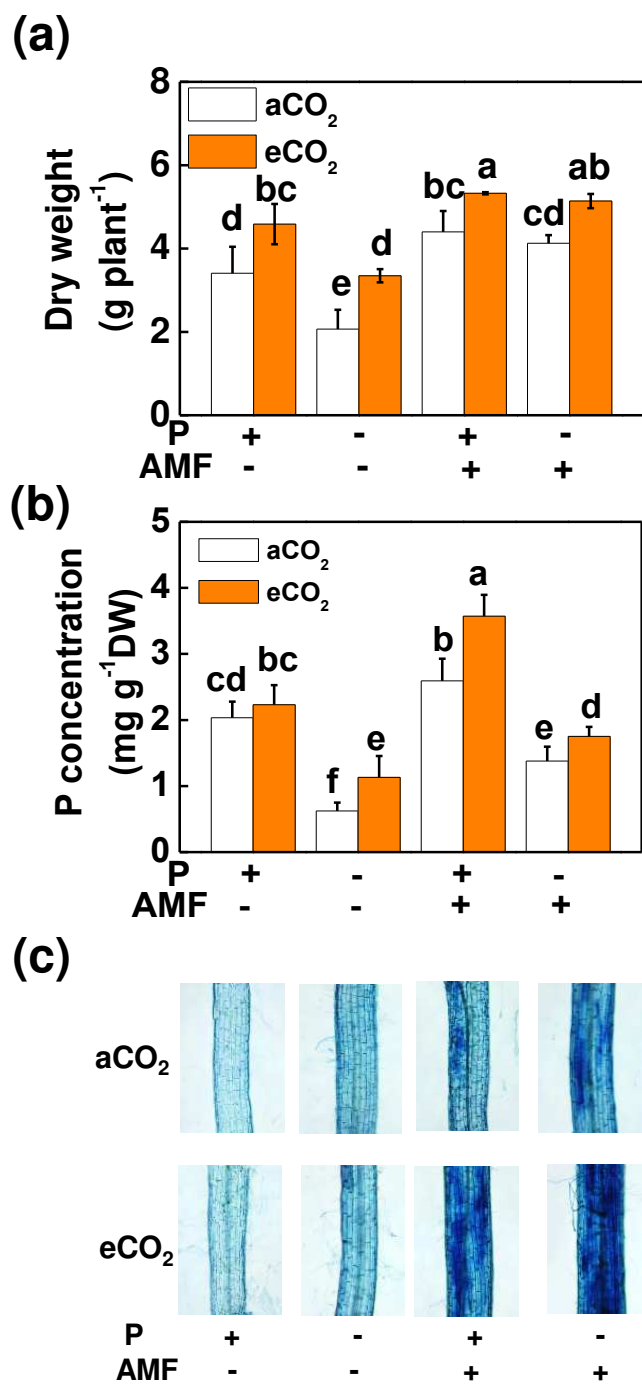


Fig. S2

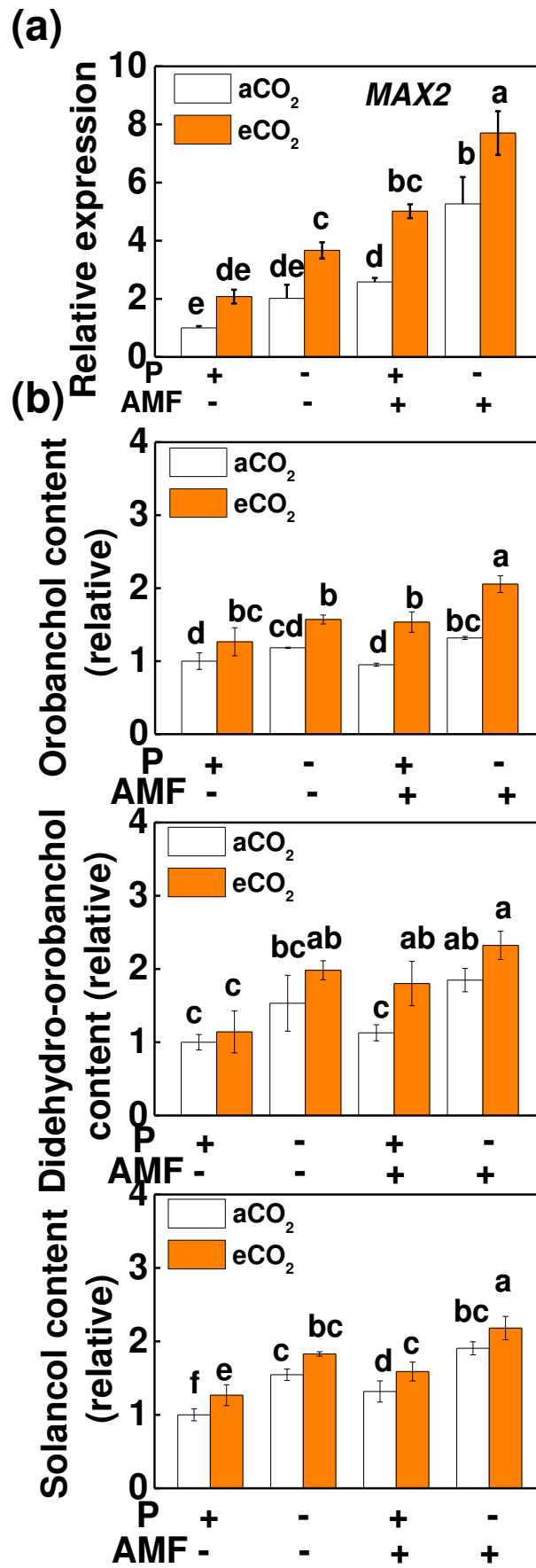


Fig. S3

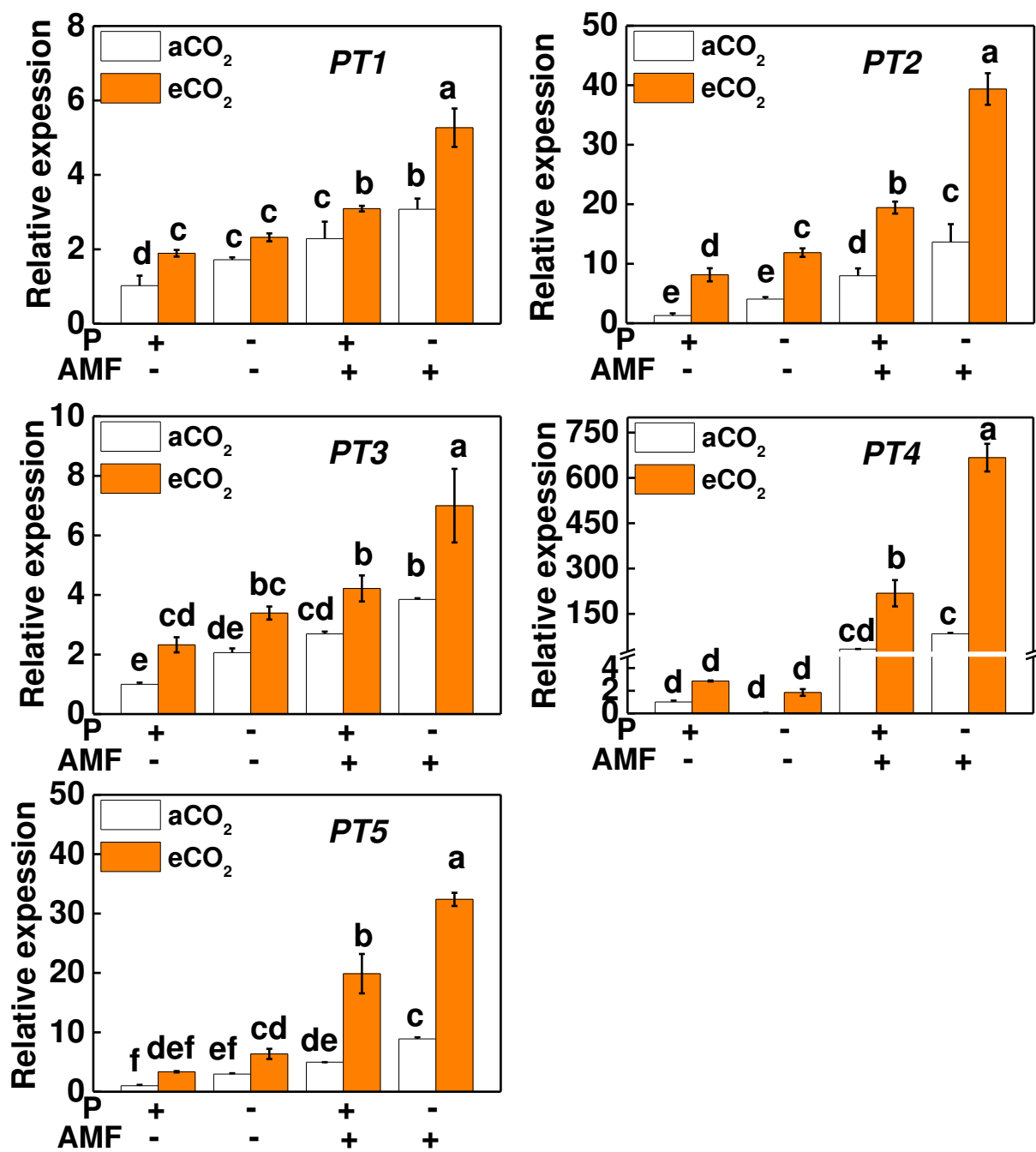


Fig. S4

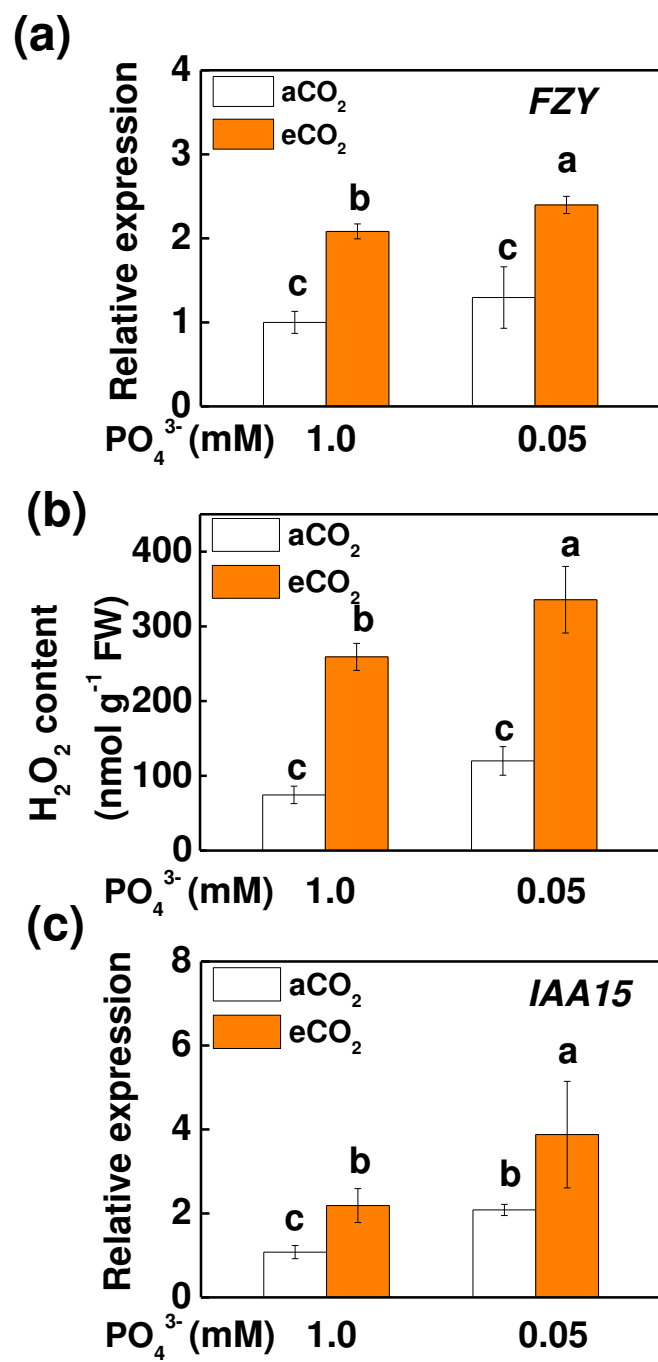


Fig. S5

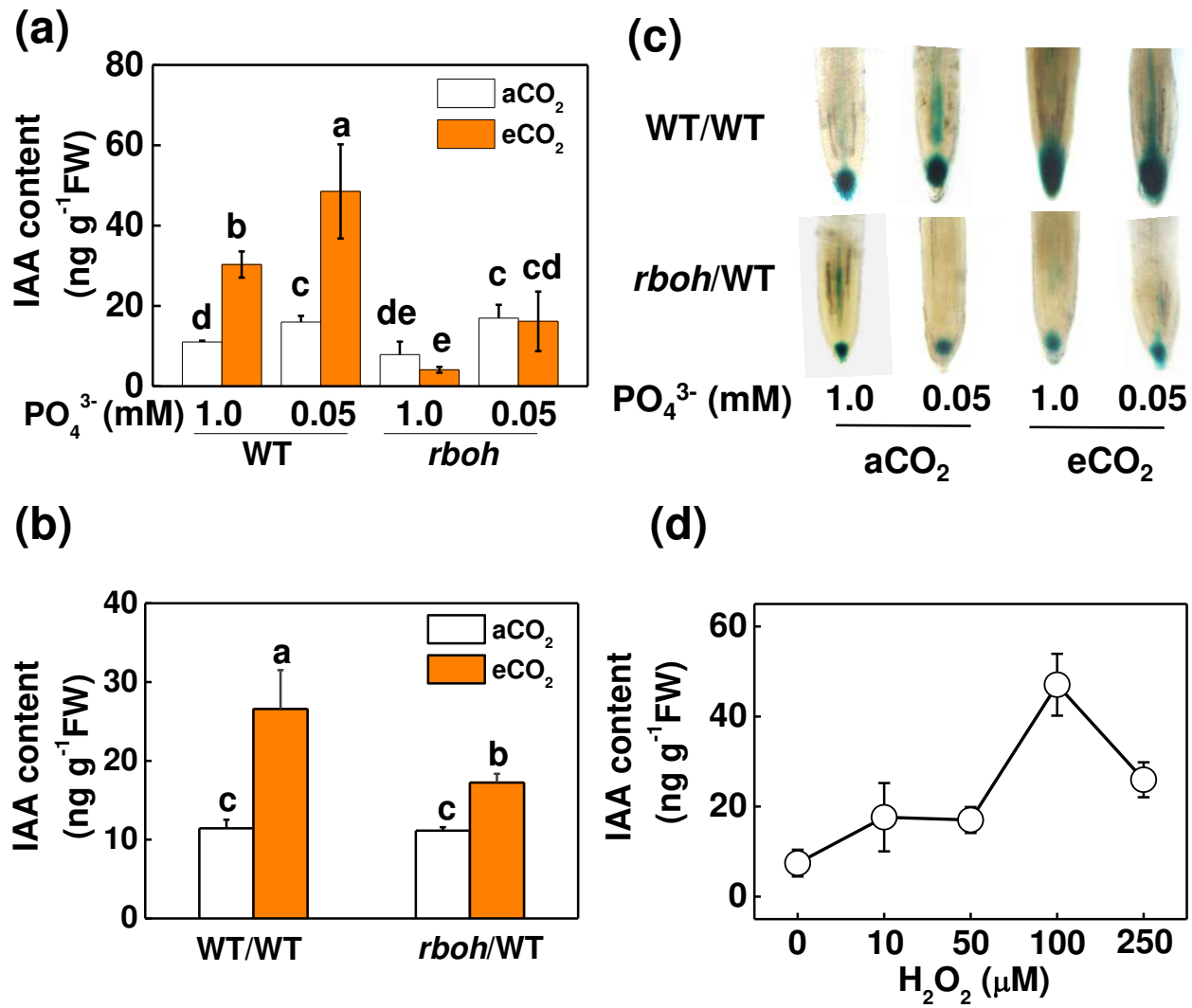


Fig. S6

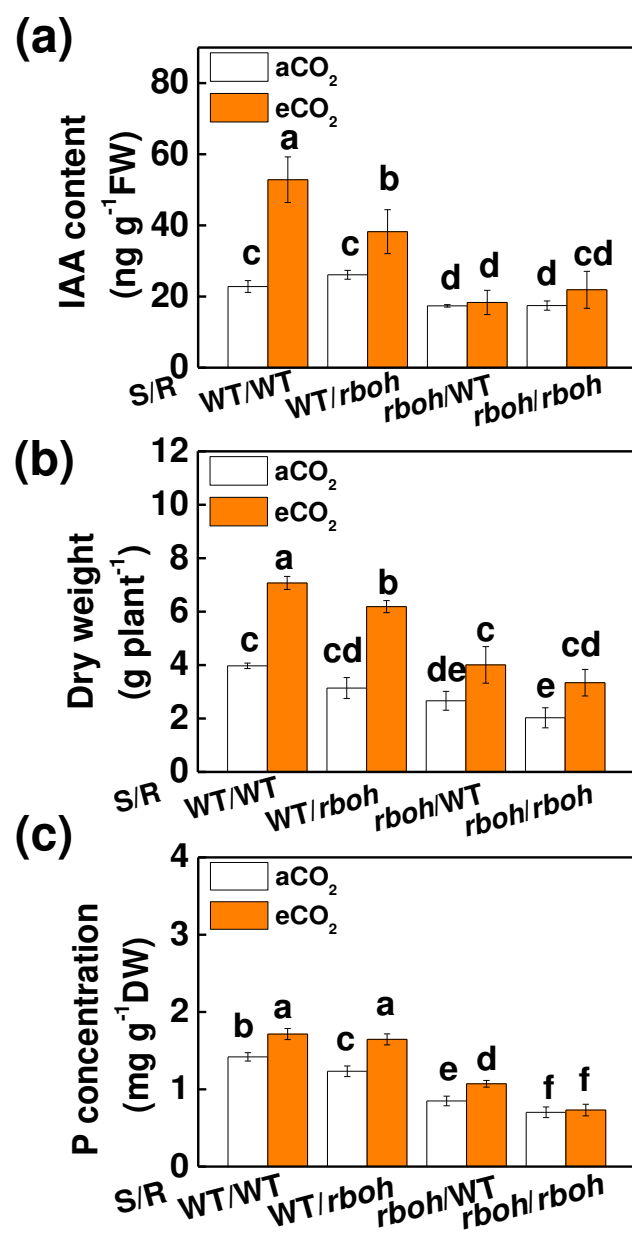


Fig. S8

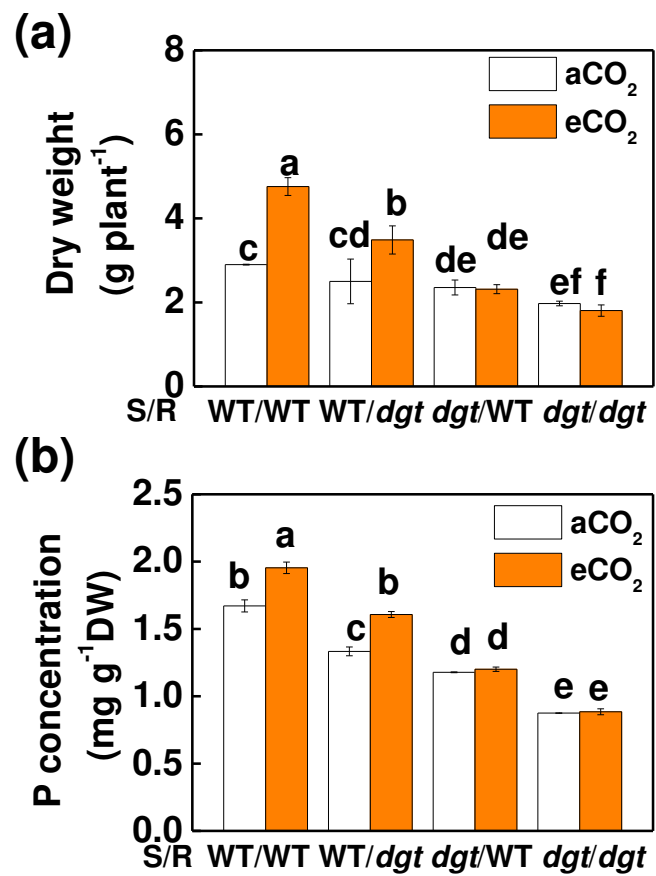


Fig. S9

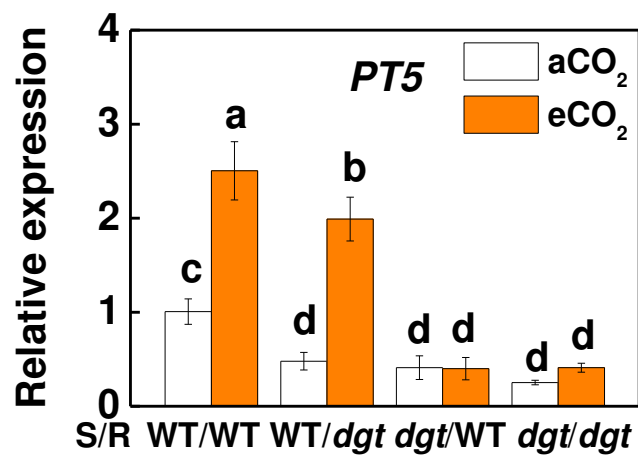
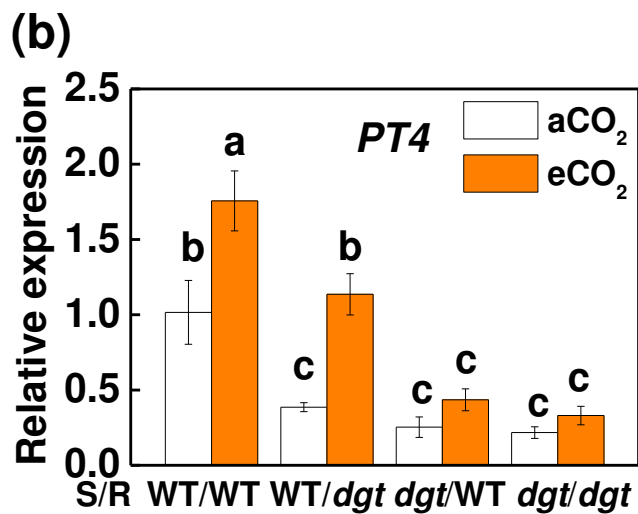
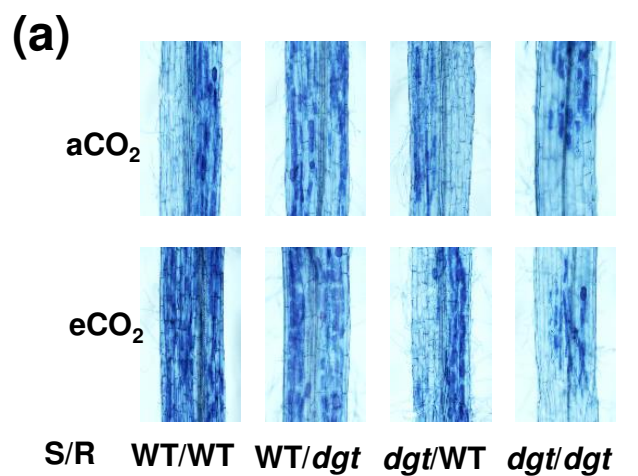


Fig. S10

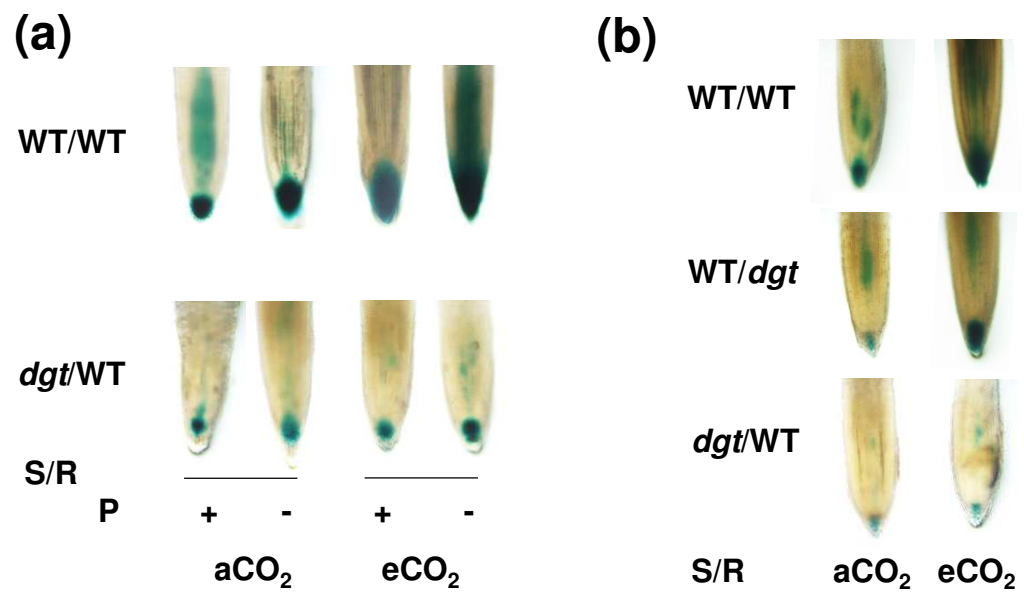


Fig. S10

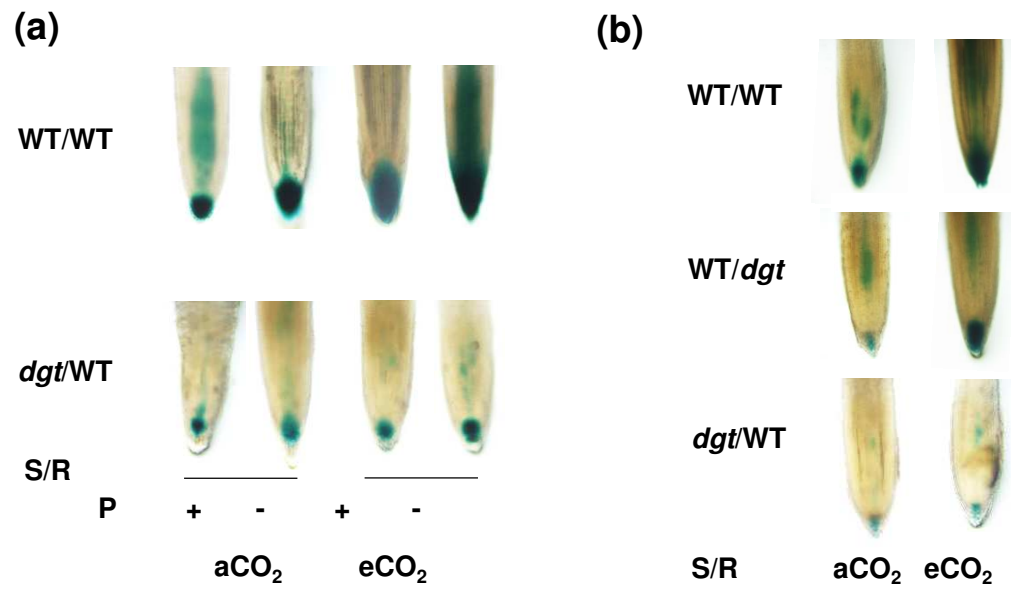


Fig. S11

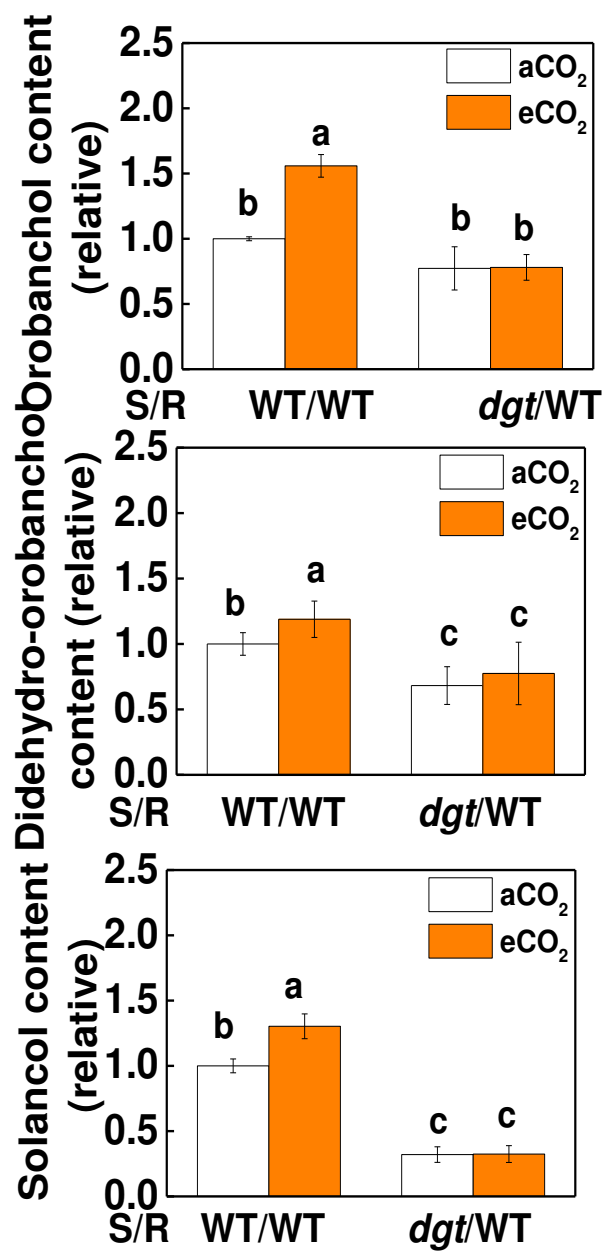
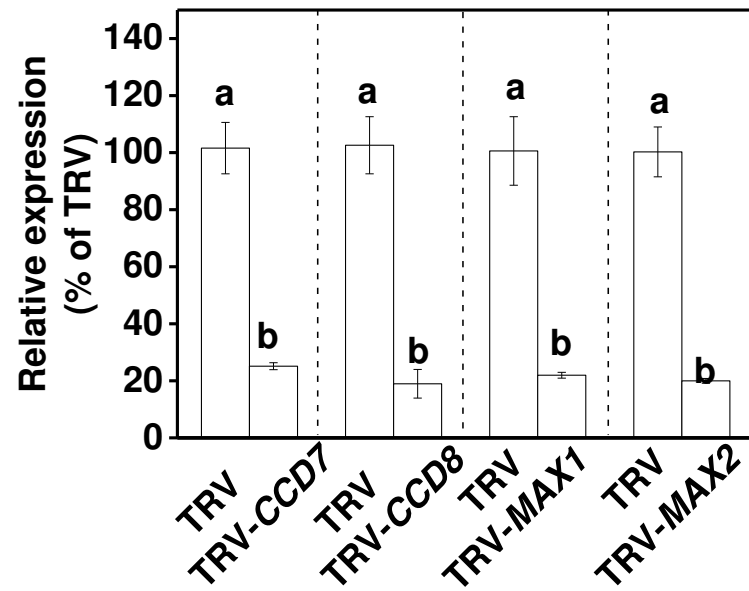
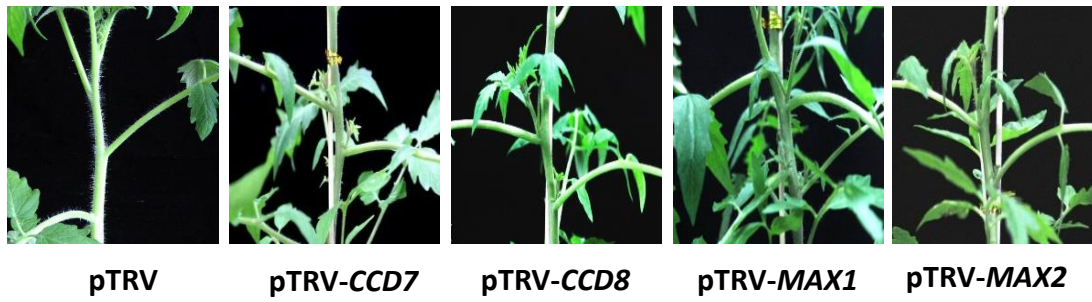


Fig. S12

(a)



(b)



(c)

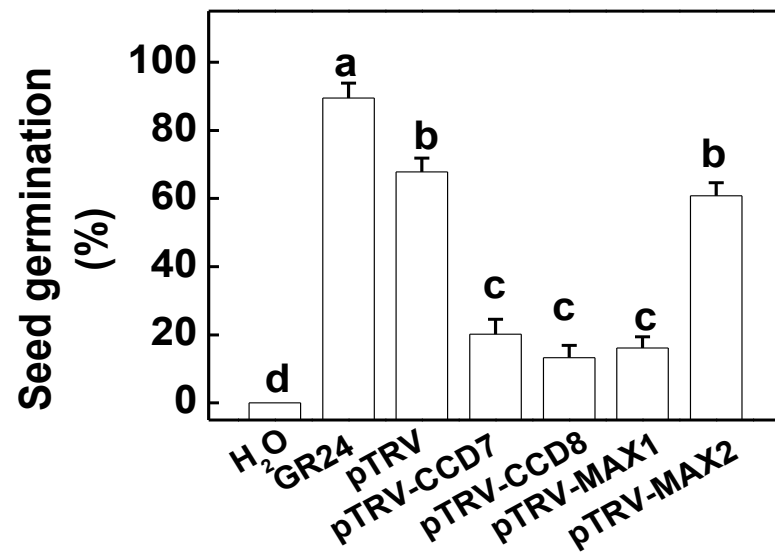


Fig. S13

