Title: *Arabidopsis PPC2* is crucial for growth at low CO₂ by involvement in photorespiratory metabolism and integration of ABI5

Running title: PPC2 and ABI5 are essential for plant growth at low CO₂

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Highlight: *PPC2* links photorespiratory metabolism with primary metabolism at low CO₂ conditions by integration of *ABI5* expression
Abstract
Phosphoenolpyruvate carboxylase (PEPC) is a pivotal enzyme that plays a key role in photosynthetic CO₂ fixation in C₄. However, the function of C₃ PEPCs and their roles at environmental CO₂ changes are still limited. Here, we report the role of PPC2 in seedling growth at low CO₂ by linking photorespiratory metabolism with primary metabolism and involvement of ABA and ABI5. Mutation of PPC2 caused seedling growth arrest, with reduced Fv/Fm, photosynthetic carbohydrates and ABA biosynthesis at low CO₂. PPC2 is induced by low CO₂ and the PEPC activity was greatly reduced in ppc2 leaves. Moreover, metabolic analyses showed the photorespiratory intermediates, glycine and serine, were greatly increased and primary metabolites were reduced. Application of sucrose, malate and ABA greatly rescued the growth arrest phenotype of ppc2. The expression of glycine/serine synthesis and metabolism related photorespiratory enzyme genes were decreased in ppc2 and regulated by ABI5 at low CO₂ conditions. ppc2 and abi5 mature plants exhibited reduced A-Ci curves at relatively low CO₂, which could be recovered by non-photorespiratory low oxygen conditions. ABI5 expression greatly rescued the growth arrest and A-Ci curves of ppc2 at low CO₂. Our findings demonstrate the important role of C₃ PEPCs in carbon fixation and metabolism.

Keywords: Arabidopsis thaliana; low CO₂; photosynthesis; photorespiration; carbon/nitrogen balance; abscisic acid; ABI5
Introduction

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous enzyme widespread in plants, algae and bacteria (Chollet et al., 1996). There are two functional forms of PEPCs in higher plants, the photosynthetic and non-photosynthetic isoforms. In CAM and C_4 plants, PEPC plays a pivotal photosynthetic role in primary CO_2 fixation, by catalyzing the irreversible \( \beta \)-carboxylation of PEP with HCO_3^- to oxaloacetate and inorganic phosphate. The photosynthetic PEPC activity in C_4 plant has been viewed as essential for carbon assimilation via prefixation of CO_2 in bundle sheath and, consequently, decreasing photorespiratory activity and resulting in higher water use efficiency (WUE) and higher photosynthetic efficiency than C_3 plant (Taylor et al., 2018). The non-photosynthetic PEPCs play key roles in plant primary metabolism by replenishing tricarboxylic acid (TCA) cycle to support carbon and nitrogen metabolism (Masumoto et al., 2010), in stomatal opening (Gehlen et al., 1996) and in supplying malate as a substrate of respiration to symbiotic N_2-fixing bacteroids in legume root nodules (Vidal and Chollet, 1997). PEPC in C_3 plant is commonly believed to play minor roles in photosynthesis or photorespiration (von Caemmerer, 2013). Recently, NMR analyses of \(^{13}\)C-fixation in sunflower indicated that PEPC activity and \(^{13}\)C-fixation was significantly increased as net CO_2 assimilation decreased at high photorespiratory conditions (low CO_2/O_2 ratio) (Abadie et al., 2017; Abadie and Tcherkez, 2019). Moreover, mutation of OsPPC4 in rice led to high accumulation of photorespiratory intermediates, such as glycine, serine and glycerate (Masumoto et al., 2010). Together that photorespiration tightly connects with primary metabolism (Mouillon et al., 1999; Rachmilevitch et al., 2004), these studies give clues that C_3 PEPC may also play roles in photorespiration or photosynthesis.

In Arabidopsis, there are four genes encoding PEPC. Mutation in either AtPPC1, AtPPC2 or AtPPC3 led to decreased fresh weight and delayed flowering time (Feria et al., 2016). Moreover, mutation of both AtPPC1 and AtPPC2 greatly reduced malate and citrate synthesis, and severely suppressed ammonium assimilation, which finally led to the growth arrest of ppc1ppc2 (Shi et al., 2015). However, how these AtPPCs
regulates plant primary metabolism and whether these AtPPCs regulate photosynthesis and plant development under different stress conditions, such as photorespiratory low CO₂ conditions, are yet unknown.

The phytohormone abscisic acid (ABA) play a prominent role in the establishment of stress tolerance. In addition, ABA regulates important aspects of plant development, including inhibiting embryo and seed development, promoting seed desiccation tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006), influencing seeds dormancy, germination and seedling establishment (Finkelstein et al., 2002), and facilitating vegetative development. ABA regulates these processes through affecting the expression levels of corresponding genes, which are modulated by various ABA-responsive trans-acting factors, such as B3-domain family proteins (e.g. ABI3, VAL1) (Giraudat et al., 1992; Suzuki et al., 2007), APETALA2 (AP2) family proteins (e.g. ABI4) (Finkelstein et al., 1998) and basic leucine zipper (bZIP) family proteins (e.g. ABI5) (Finkelstein and Lynch, 2000). ABI5 is a key component in ABA-triggered pathways during germination, and seedling establishment, as well as subsequent vegetative growth (Lopez-Molina et al., 2001). It has been reported the role of ABI5 in nitrogen assimilation and signaling. abi5 mutant seedlings displayed decreased sensitivity to nitrate inhibition (Signora et al., 2001; Yang et al., 2011). ABI5 also have been reported to positively regulate chlorophyll catabolism related genes, SGR1 and NYC1, through recognizing their upstream ABA response elements (ABREs) element (Sakuraba et al., 2014). Therefore, ABI5 is proposed to be a key player in monitoring environmental conditions during seedling growth. However, whether ABI5 is responsive to CO₂ changes and functions in low CO₂-induced plant growth are unknown.

In this study, we demonstrate the crucial role of AtPPC2 in seedling growth at limiting CO₂ conditions by linking photorespiration metabolism with primary metabolism, via integration of ABI5. ppc2 mutant showed retarded seedling growth at low CO₂ conditions and reduced carbon assimilation, in which ABI5 expression was heavily suppressed. Expression of ABI5 rescued the growth arrest phenotype of ppc2 at low CO₂ and reduced photosynthesis and biomass at ambient CO₂. Metabolic
analyses showed photorespiratory intermediates glycine and serine were accumulated, and malate were decreased in ppc2 at low CO₂ conditions. Our study demonstrates PPC2 and ABI5 are key regulators in plant growth at limiting CO₂ conditions in C₃ plants, which make positive contribution to carbon fixation and metabolism.

Materials and Methods

Plant Material and Growth Conditions

All ppc mutant lines used in this study were in the Columbia (Col-0) background. The mutant lines ppc1 (SALK_088836) (Feria et al., 2016), ppc2 (SALK_128516) (Shi et al., 2015), ppc3 (SALK_143289) (Feria et al., 2016) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu) and their homozygous were confirmed by PCR (Table S1).

All seeds were surface-sterilized and sown on 1/2 Murashinge and Skoog medium (MS) (pH 5.7). After cold treatment, seeds were germinated and grew in Percival Chambers with different CO₂ conditions (200 ppm, 400 ppm, respectively), at a light regime of 16 h light/8 h dark (light intensity 100 μmol m⁻² s⁻¹) and relative humidity (RH) of 56%.

Generation of Constructs and Transgenic Plants

The coding sequences of PPC2 and ABI5 were amplified from Arabidopsis cDNA with primers PPC2-OE-F/PPC2-OE-R, and ABI5-OE-F/ABI5-OE-R, respectively. PCR products were cloned into pGreen-35S and pEarlyGate-35S-YFP (Earley et al., 2006). The promoter region of PPC2 amplified by primers Pro-PPC2-F and Pro-PPC2-R from Arabidopsis genome was cloned into the vector pEarlyGate-100-GUS (Earley et al., 2006). All primers used in construct generation were presented in Supplemental Table 1. The constructs were introduced into Arabidopsis by Agrobacterium tumefaciens-mediated transformation using the floral dip method.

Calculation of Chlorosis Rate

15-day-old seedlings of Col-0 and ppc2 mutant which were growing at 200 ppm and 400 ppm CO₂ condition were analyzed for chlorosis rate.
Chlorosis rate (%) = number of chlorotic seedlings / number of total seedlings

**PEPC Activity Assays**

0.1 g leaves (fresh weight) of 15-day-old seedlings growing at 200 ppm and 400 ppm were harvested. Tissues were ground and extracted in 1ml extraction buffer. After centrifugation, the supernatant was immediately used for PEPC activity detection by a PEPC activity assay kit (Nanjing Jiancheng Bioengineering Institute). 1 unit of PEPC activity was defined as 1 nmol of NADH oxidation per min and per mg protein at 25°C. Total protein content was quantified by using BCA (bicinchoninic acid) protein assay kit (Sangon Biotech).

**A-C_i curve analyses**

The measurements of A-C_i curves were performed by a closed infrared gas exchange analysis system (LI-COR 6400XT). 4 to 5-week-old leaves were clamped in the 2 cm² chamber with leaf temperature at 21°C for measurement. The measurement of A-C_i curves was performed at increasing CO2 concentrations of 50, 100, 200, 300, 400, 600 and 800 ppm with photosynthetic photon flux density of 2000 μmol m⁻² s⁻¹. RH was approximately 50% in all measurements. A-C_i curves at low oxygen conditions were performed by replacement of air by pure N₂.

**Analyses of Sucrose and Starch Content**

Shoots of 15-day-old plant seedlings were harvested. Sucrose and starch were extracted and estimated by using the Sucrose Colorimetric/Spectrophotometric Assay kit (COMIN) and Starch Colorimetric/Spectrophotometric Assay kit (COMIN) according to the manufacturer's instructions, respectively.

**Analyses of Chlorophyll Content and Chlorophyll Fluorescence**

Total Chlorophyll content of leaves was extracted with 80 % acetone at 4°C for 24 h in darkness, and then the supernatant was used to measure the absorbance with a spectrophotometer (BeckMan Coulter DU730). Total chlorophyll content was calculated using the following formula:

Total chlorophyll (nmol/mL) = 19.54 × (A_{646.8−A_{720}}) + 8.29 × (A_{663.2−A_{720}})
Chlorophyll fluorescence of 15-day-old seedlings was analyzed by using a FluorCam PAM as described in a previous report (Baker, 2008).

**Quantification of Endogenous ABA**

0.1 g leaves (fresh weight) of 15-day-old seedlings growing at 200 ppm and 400 ppm were harvested, respectively. Samples were extracted in 750 μl 80:19:1 methanol-H2O-acetic acid buffer supplemented with internal standards for 6h with occasional shaking. After centrifugation, the extracts were filtered through a 0.22 μm filter and dried with N2 at room temperature. The extracts were dissolved in 200 μl methanol. Quantification of ABA were performed as described (Liu et al., 2012).

**Semi-quantitative PCR and Quantitative Real-time PCR**

Total RNA was extracted from seedlings of the wild-type Col-0 and mutants, and cDNA were reverse transcribed. Quantitative RT-PCR was performed by using a Universal SYBR® Green kit and the C1000 Touch Thermal Cycler real-time PCR detection system (Bio-Rad). The EF1α (AT5G60390) gene was used as a reference gene for mRNA normalization. Comparative cycle threshold (Ct) method was used to evaluate the relative gene expression levels. The experiment was repeated three times. The primers used for the expression analysis were listed in Supplemental Table S1.

**GUS Histochemical Analyses**

GUS histochemical analyses were carried out on transgenic lines expressing ProPPC2::GUS. Plants at various stages including emerging seedling, 7-day-old seedling, 15-day-old seedling, flowers and siliques were stained in a GUS staining solution and imaged by a Nikon microscope.

**Quantification of Amino Acid and Organic Acid**

0.2 g (fresh weight) leaves of 15-day-old seedlings growing at 200 ppm and 400 ppm were harvested, respectively. Tissues were ground and extracted in 8% (w/v) 5-sulfosalicylic acid for 1 h, then were centrifuged. The supernatants were filtered through a 0.22 μm filter. Contents of amino acid were determined by LC-MS/MS method with Agilent 1290 Infinity II and Agilent 6460 (Kowalski et al., 2017).
0.2 g (fresh weight) shoots of 15-day-old seedlings growing at 200 ppm and 400 ppm were harvested. Samples were homogenized in 3 ml 7:3 Methanol-Chloroform (-20°C) for 2 h. The water soluble metabolites were extracted from the chloroform phase by adding 2.4 ml H₂O, after shaking and centrifugation. The upper methanol-H₂O phase were transferred and dried with N₂ at room temperature. The extracts were dissolved with 200 μl H₂O and transferred to 0.45 μm cellulose acetate centrifuge tube filters. The determination of organic acid content was performed by a AB SCIEX QTRAP 6500 Plus LC-MS/MS system as described (Ma et al., 2014).

**Luciferase Assay**

Arabidopsis mesophyll protoplasts were isolated from 4 to 6-week-old plants following the method of a previous report (Yoo et al., 2007). 15 μg plasmid DNA was used for PEG-calcium transformation (PEG4000) and the protoplast transformation culture were performed as described (Yoo et al., 2007).

Cellular extracts of Arabidopsis protoplasts after transformation with different constructs for 14 hours were collected for dual-luciferase assays (Hellens et al., 2005). 30 μL of cellular extract was used to detect the firefly and Renilla luciferase activities by a Mithras LB 940 Multimode Microplate Reader. Luciferase activity was normalized to the Renilla activity. All experiments were performed at least three times.

**Accession Numbers**

The accession numbers of genes used in this study are available at TAIR (The Arabidopsis Information Resource): **PPC1** (AT1G53310), **PPC2** (AT2G42600), **PPC3** (AT3G14940), **ABI3** (AT3G24650), **ABI4** (AT2G40220), **ABI5** (AT2G36270), **GGAT1** (AT1g23310), **GGAT2** (AT1g70580), **SGAT1** (AT2G13360), **GLDT1** (AT1G11860), **GLDP1** (At4g33010), **SHMT1** (At4g37930).

**Results**

**ppc2 mutant seedlings showed growth arrest at low CO₂ conditions**

To investigate whether the *Arabidopsis* PEPCs are involved in plant growth regulation
at low CO2 conditions, we determined the growth performance of T-DNA insertion
lines of plant-type PEPCs, ppc1 (Salk_088836) (Feria et al., 2016), ppc2
(Salk_128516) (Shi et al., 2015) and ppc3 (Salk_143289) (Feria et al., 2016), on
sucrose-free 1/2 MS medium at low CO2 conditions (200 ppm) for 15 days, as well as
the control condition (400 ppm) which was considered as ambient CO2 concentration.
These single mutants were determined as knockout mutants by our analyses (Fig. 1A
and B) and previous studies (Shi et al., 2015; Feria et al., 2016). At ambient CO2
conditions, there were no obvious morphological differences among the ppc mutants
and Col-0 seedlings (Fig. 1C). At low CO2 conditions, the cotyledons of Col-0, ppc1
and ppc3 turned pale green and no big morphological difference was found among
them. Interestingly, ppc2 mutant plants failed to achieve the same growth state
compared to Col-0, ppc1 and ppc3 mutant plants (Fig. 1C). ppc2 mutant plants had
small sizes and chlorotic cotyledons, the cotyledons in 87.5% of ppc2 plants were
chlorotic, compared with that of 10% to 20% in Col-0, ppc1 and ppc3 plants (Fig. 1D).
These results suggest that PPC2 is required for seedling growth and development at
low CO2 conditions.

To determine whether the phenotypes of seedling growth arrest in the ppc2
mutant are due to the defect of seed germination, the seed-germination rates of these
lines were determined at low CO2 conditions on sucrose-free 1/2 MS medium. The
similar germination rate and status were observed among Col-0, ppc1, ppc2 and ppc3
(Fig. S1). These results revealed that the growth arrest phenotype of ppc2 was
occurred after germination developmental stage. AtPPC2 is involved in seedling
development at low CO2 conditions.

To further reveal whether PPC1 or PPC3 has effect on seedling growth at low
CO2 conditions dependent on PPC2, we crossed ppc1 or ppc3 with the ppc2 mutant.
We obtained ppc2ppc3 and ppc1ppc2 double mutant, but could not get the seeds of
ppc1ppc2 double mutant due to severely growth arrest, consistent with the previous
study (Shi et al., 2015). ppc2ppc3 double mutant showed the similar growth
phenotype as ppc2 single mutant at low CO2 conditions and has normal seed
germination (Fig. 1C and S1), suggesting AtPPC3 possibly does not participate in
To confirm that PPC2 was responsible for the phenotype of growth retardation observed in the ppc2 mutant, the CDS of PPC2 driven by CaMV 35S promoter was introduced into the ppc2 mutant. The expression levels of PPC2 in randomly selected PPC2-expressing transgenic ppc2 plants were recovered to the similar level as in Col-0 by RT-PCR analyses (Fig. 1E). When these lines together with ppc2 and Col-0 grew at low CO2 conditions, the growth arrest and cotyledon chlorotic phenotypes of ppc2 were all rescued by PPC2 expression (Fig. 1F). These results demonstrate that the seedling growth arrest is due to the dysfunction of PPC2 and PPC2 is a major regulator of seedling growth at low CO2 conditions.

PPC2 is low CO2 inducible and encodes a major PEPC in Arabidopsis leaves

We next detected the expression levels of these three PEPCs at low CO2 conditions by real-time PCR analyses, only PPC2 could be induced by low CO2 (Fig. 2A), in agreement with the previous study (Li et al., 2014). To confirm PPC2 is a functional PEPC in Arabidopsis, we measured the total PEPC activity in the ppc2 mutant and Col-0 at low and ambient CO2 conditions. Our results showed that ppc2 mutant seedlings had lost most of PEPC activity in the leaves at both 400 ppm and 200 ppm CO2 conditions (Fig. 2B). Moreover, low CO2 treatment increased PEPC activity in Col-0 but not in the ppc2 mutant leaves (Fig. 2B), further supporting that PPC2 is the major PEPC in Arabidopsis leaves and only PPC2 is in response to low CO2.

We then determined its spatial expression patterns by expressing GUS reporter gene driven by PPC2 promoter in Col-0. GUS staining showed that PPC2 was mainly expressed in leaves, hypocotyl, flowers and siliques, but not or very weak in roots (Fig. 2C). We also found PPC2 was highly expressed in guard cells. We were also interested to know the subcellular localization of PPC2, 35S::PPC2-YFP construct was transformed into the protoplasts of Col-0. YFP fluorescence of PPC2-YFP fusion protein revealed its localization in the cytoplasm, nucleus and also endoplasmic reticulum (Fig. 2D). Taken together, these results demonstrate a specific role of PPC2 in regulation of plant growth at low CO2.
ppc2 seedlings showed reduced carbon assimilation at low CO₂ conditions

To confirm whether the growth arrest and cotyledon chlorosis of ppc2 mutant at low CO₂ is due to the defect of carbohydrate accumulation, we detected starch content by iodine staining and quantification in 15-day-old ppc2 and Col-0 seedlings at the end of the illumination period (22:00 pm) and darkness period (06:00 am) at different CO₂ conditions. At ambient CO₂ conditions there was no obvious difference in starch accumulation between ppc2 and Col-0 plants (Fig. S2A and B), however, at low CO₂ conditions the starch accumulation was significantly reduced in the ppc2 cotyledons at both time points (Fig. S2A and B). Because in plant cells, starch is synthesized at daytime and degraded at night (darkness). We then measured sucrose content of ppc2 and Col-0 at these conditions. The sucrose content was lower in the ppc2 seedlings than in Col-0 at either low or ambient CO₂ conditions (Fig. S2B). Moreover, the synthetic substrates of starch and sucrose, such as G6P, F6P, G1P, ADPG, UDPG and Suc6P were decreased in ppc2 at low CO₂ conditions, but were similar at ambient CO₂ conditions compared to Col-0 (Fig. S2B), consistent with the starch and sucrose content in ppc2. These findings suggest that ppc2 mutation led to the reduced photosynthetic carbohydrate accumulation at low CO₂ conditions.

To test the reduction of photosynthetic carbohydrates in ppc2 is caused by the reduced CO₂ assimilation, we measured chlorophyll and carotenoid contents in these seedlings. At ambient CO₂ conditions, there were no significant differences in total chlorophyll and carotenoid contents (Fig. 3A and B). At low CO₂ conditions both chlorophyll and carotenoid were greatly reduced in ppc2. We next detected the maximal quantum yield of PSII (Fv/Fm) in ppc2 and Col-0 seedlings growing at 200 ppm and 400 ppm CO₂ conditions by chlorophyll florescence detector FluorCam FC800. At ambient CO₂ conditions there was no difference in Fv/Fm between Col-0 and ppc2. Low CO₂ treatment greatly reduced Fv/Fm in both Col-0 and ppc2, however a remarkable decrease was observed in ppc2 than in Col-0 (Fig. 3C and D). These results suggest PPC2 is involved in photosynthesis regulation at low CO₂.

Because PPC2 is highly expressed in guard cell (Fig. 2C), it is important to confirm if the reduced carbon assimilation rate at low CO₂ conditions was induced by
the compromised low CO2-induced stomatal opening. The CO2 shifts from 400 to 100 ppm triggered dramatic increase in stomatal conductance in both Col-0 and ppc2 mutant plants. However, there were no big difference in stomatal response to low CO2 between the ppc2 mutants and Col-0 (Fig. 3E and F), indicating minor role of PPC2 in regulating stomatal opening, and also suggesting the reduced carbon assimilation in ppc2 at low CO2 conditions is not caused by reduced CO2 uptake but by CO2 utilization.

Addition of exogenous sucrose or malate greatly rescued the seedling growth arrest of ppc2 at low CO2 conditions

Because of the reduced photosynthetic carbohydrate accumulation and carbon assimilation in the ppc2 mutant at low CO2 conditions, we would like to know whether application of sucrose, which is a photosynthetic product, could rescue the reduced seedling growth of ppc2 mutant at low CO2 conditions. Exogenous sucrose (25 mM) treatment completely recovered the seedling growth retardation and cotyledon chlorosis in ppc2 at low CO2 conditions (Fig. 4A).

PEPC catalyzes the synthesis of oxaloacetate (OAA) from PEP and HCO$_3^-$ The product OAA is rapidly converted into malate by malate dehydrogenase. Our primary metabolic analyses showed malate was reduced when PPC2 was mutated (Fig. S2B), and their differences between ppc2 mutant and Col-0 were increased at low CO2 conditions. We then explored whether the growth-arrest phenotype of ppc2 mutant at low CO2 was caused by malate defect. Application of 1.5 mM malate didn't have the effect on growth of both genotypes and could not eliminate the cotyledon phenotype of ppc2 (Fig. 4A), however the growth-arrest phenotype of ppc2 was greatly relieved by addition of 3 mM malate compared to control group at our growth conditions. These results support the function of AtPPC2 in both carbon assimilation and metabolic pathway.

Exogenous ABA partially rescued the seedling growth arrest of ppc2 at low CO2 conditions

ABA is synthesized from C$_{40}$ oxygenated carotenoids (Ruiz-Sola and
Rodriguez-Concepcion, 2012) and in the ppc2 mutant carotenoid content was seriously reduced (Fig. 3B). To determine whether ABA biosynthesis is blocked in ppc2 at low CO2 conditions. We quantified ABA level in 15-day-old ppc2 and Col-0 seedlings that growing at low and ambient CO2 conditions by UFLC-ESI-MS (Liu et al., 2012). Interestingly, ABA content in the ppc2 mutant was greatly reduced at low CO2 conditions but increased at atmospheric CO2 conditions compared to Col-0 (Fig. 4B). These results demonstrate that the reduced ABA level may contribute to the seedling growth arrest in ppc2 at limiting CO2 conditions.

To prove this, we added low concentrations of exogenous ABA (0.1 or 0.2 μM) to sucrose-free 1/2 MS medium plate to see the growth performance of Col-0 and ppc2. In Col-0, ABA treatment inhibited the growth and increased the ratio of chlorotic seedlings, while in the ppc2 mutant ABA treatment largely recovered the cotyledon chlorotic and growth arrest phenotypes (Fig. 4C and D). Taken together, our results demonstrate that ppc2 mutation affects ABA biosynthesis and the seedling growth-arrest phenotype of ppc2 mutant is at least partly due to the reduced ABA biosynthesis.

abi5 showed reduced Fv/Fm and expression of ABI5 greatly rescued the growth arrest of ppc2 seedlings at low CO2 conditions

It has been reported that ABI3, ABI4 and ABI5, downstream targets of ABA signaling pathway, are required for the ABA modulation of seed germination and postgermination development (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001). To determine whether ABI transcription factors function in the seedling growth arrest in the ppc2 mutant at low CO2 conditions, we firstly checked their expression levels by qPCR. Low CO2 treatment induced the expression levels of all these three ABIs in Col-0. The expression of ABI5 at low CO2 conditions was greatly suppressed in the ppc2 mutant (Fig. 5A), however the expression levels of ABI3 and ABI4 were comparable in Col-0 and ppc2 seedlings at both ambient and low CO2 conditions. Furthermore, exogenous ABA could rescue the ABI5 expression at low CO2 conditions in the ppc2 mutant (Fig. 5B). These results indicate the reduced expression
level of ABI5 might be a major cause of reduced seedling growth in ppc2 at low CO2 conditions. To confirm this and reveal the role of ABI5 in seedling growth at low CO2 conditions, we determined the growth performance of abi5-1 mutant and wild type Ws plants at low CO2 conditions on sucrose-free medium. No obvious morphological differences were observed between Ws and abi5-1 seedlings (Fig. 5C). However, Fv/Fm level was significantly reduced in the abi5-1 mutant compared to that in Ws at low CO2 conditions (Fig. 5D), indicating mutation of ABI5 led to reduced maximum quantum efficiency at low CO2 conditions. We further overexpressed ABI5 in the ppc2 mutant plant driven by the constitutive cauliflower 35S promoter. Expression of ABI5 greatly rescued the early seedling growth arrest phenotype of ppc2 in three randomly selected transgenic lines at low CO2 conditions (Fig. 5E and F, Fig. S3).

**Photorespiratory intermediates were increased in ppc2 at low CO2 conditions**

It is suggested that PEPC activity is linked to photorespiration by supplying malate into the TCA cycle to sustain glutamate and glutamine metabolism (Masumoto et al., 2010; Shi et al., 2015). We then determined amino acids levels in ppc2 and Col-0 at both ambient and low CO2 conditions. Our analyses showed that ppc2 mutant had reduced glutamic acid and increased glutamine, which led to increased ratio of Gln to Glu (Fig. 6A and B), increased level of β-Alanine and Arginine, and decreased level of alanine, asparatic acid and proline (Fig. S4). Glycine and serine levels have been recognized as indicators of carbon flux through photorespiration and higher ratios of glycine/serine indicate high photorespiration (Novitskaya et al., 2002). Low CO2 concentrations would increase photorespiration. ppc2 mutant had higher contents of photorespiratory intermediates glycine and serine at photorespiratory (low CO2) conditions (Fig. 6A) and higher glycine content at 400 ppm CO2 concentration (Fig. S4), which were consistent with the reduced photosynthesis and growth arrest at low CO2 conditions (Figs. 3E and 4A). We found low CO2 (photorespiratory conditions) triggered an increase in glycine/serine ratio both in the ppc2 mutant and Col-0 in contrast to ambient CO2, however no significant differences in glycine/serine ratio were found between them (Fig. 6C). At ambient CO2 conditions PPC2 didn't have
significant effect on amino acid and organic acid content, only glycine, valine and tyrosine were slightly increased in ppc2 (Fig. S2B and S4). These results suggest

PPC2 functions in both primary metabolism and photorespiratory metabolism at photorespiratory low CO2 conditions through modulation of carbon/nitrogen balance.

ABI5 regulates the expression levels of photorespiratory enzymes

To further explore whether the higher levels of serine and glycine in ppc2 at low CO2 conditions are caused by the reduced levels of those enzymes that synthesize glycine and serine during photorespiration, we checked the expression levels of GGAT1, GGAT2, SGAT1, GLDP1, GLDT1 and SHMT1 (Peterhansel et al., 2010) in ppc2 at both low and ambient CO2 conditions. In the photorespiratory pathway GGAT (glutamate:glyoxylate aminotransferase) transfers -NH3+ from glutamate into glyoxylate to generate glycine and SGAT1 (serine:glyoxylate aminotransferase, TAIR abbreviation: AGT) transfers -NH3+ from Ser, Ala and Asn into glyoxylate to generate glycine. GLDP1 and GLDT1 were enzyme components of glycine decarboxylase complex, catalyzing glycine into CH2-THF. The expression levels of these genes except GGAT2 were significantly reduced in the ppc2 mutant at low CO2 conditions (Fig. 7A). Among them, GGAT1 and SGAT1 were slightly induced by low CO2 treatment in Col-0 (Fig. 7A). The transcript of SHMT1 in the ppc2 mutant was significantly reduced at both ambient and low CO2 conditions (Fig. 7A).

ABI5 is a transcription factor that directly binds to the promoter regions of its targets to activate their expressions. ABI5 expression was reduced in ppc2 and ABI5 overexpression in ppc2 recovered the reduced growth arrest at photorespiratory low CO2 conditions, we thus hypothesized that ABI5 might regulate the expression levels of these enzymes that function in glycine and serine production, such as GGAT1, SGAT1, GLDP1, GLDT1 and SHMT1. We interestingly found there were several ABREs, ABI5 binding cis-element, in the promoter regions of these five genes (Fig. S5). The expressions of SGAT1, GLDT1 and SHMT1 were reduced in abi5-1 mutant at low CO2 conditions (Fig. 7B), suggesting these three genes could be the targets of ABI5. We interestingly found the expression levels of SGAT1, GLDT1 and SHMT1 in
three randomly selected independent transgenic lines were completely or greatly recovered (Fig. 7C). We then performed dual luciferase assays to determine the ABI5 activation of the promoters of SGAT1, GLDT1 and SHMT1 that drive LUC expression in Arabidopsis mesophyll cell protoplasts. ABI5 expression greatly activated the promoters of SGAT1 and GLDT1, but could not activate the promoter of SHMT1 (Fig. 7D and E). These results demonstrate that SGAT1 and GLDT1 could be the direct targets and SHMT1 was an indirect target of ABI5, and ABI5 regulates photorespiration by modulating the expression levels of photorespiratory enzymes.

A-C_i curve at photorespiratory low CO_2 conditions was reduced in the ppc2 mature plants

Recently, a potential role for PEPC in C_3 plant metabolism at high photorespiratory (low CO_2/high O_2) conditions has been proposed (Abadie and Tcherkez, 2019; Tcherkez and Limami, 2019). Here we also found that PPC2 is involved in seedling development by modulation of photorespiratory metabolism at low CO_2 conditions. To further investigate the function of Arabidopsis PPC2 in photorespiratory conditions, we determined the CO_2 assimilation rate under different C_i conditions in adult leaves of ppc2 and Col-0 under air condition. The ppc2 mutant exhibited a reduced CO_2 assimilation rate at low CO_2 (50-400 ppm) concentrations in the initial part of the A-C_i curve compared with Col-0, while exhibiting similar rate with Col-0 at higher CO_2 (400-800 ppm) concentrations (Fig. 8A). In addition, the maximum photosynthetic electron transport rates of Col-0 and ppc2 mutant plants which were computed from A-C_i curves exhibited no significant difference (Fig. 8B), indicating the photosynthetic capacity of the ppc2 mutant was not changed. The reduce in the initial A-C_i curve was recovered when the measurements were performed under very low oxygen conditions that restricted photorespiration (Fig. 8C). These results indicate the altered CO_2 assimilation of ppc2 mutant at low CO_2 conditions was associated with the simultaneous high photorespiratory lost in plant. Moreover, PPC2 expression rescued the reduced photosynthetic rate in response to low C_i changes in ppc2 (Fig. 8D). Together with that PEPC is an important enzyme in the glycolytic
pathway and links with photorespiration and respiration in plant, our results suggest that PPC2 is involved in photorespiration at relatively low CO2 conditions. The phenotype of ppc2 is, at least partially, due to the reduced net carbon assimilation, which may be resulted from the low capacity to utilize the photorespiratory metabolites at relatively low CO2 conditions when PPC2 is mutated.

**Overexpression of ABI5 rescued the reduced CO2 assimilation at photorespiratory low CO2 conditions in the ppc2 mature plants**

We also determined the A-Ci curves of abi5-1 30-day-old plants at air and low oxygen conditions, respectively. When measurements were performed under air condition, mutation of ABI5 greatly reduced the CO2 assimilation rate at low CO2 concentrations (50-400 ppm), but which was similar at high CO2 concentrations (600-800 ppm) compared to Col-0 (Fig. 8F). The max photosynthetic ETR of Ws and abi5-1 inferred from A-Ci curves in air condition were similar (Fig. 8E). In low oxygen conditions, the slopes of A-Ci curves of abi5-1 and Ws had no significant difference between abi5 and Ws (Fig. 8G). These results demonstrate that ABI5 is involved in photorespiratory metabolism pathway.

We next determined the A-Ci curves of ABI5-expressing ppc2 plants growing at ambient CO2 conditions. The reduced CO2 assimilation of ppc2 at low CO2 conditions was totally rescued by ABI5 expression (Fig. 8H). Moreover, ABI5-expressing not only restored the reduced dry-weight biomass of ppc2 mutant but also had some increase at ambient conditions (Fig. 8I).

**Discussion**

**PPC2 is essential for plant growth at low CO2 conditions**

CO2 is the major source for photosynthesis and is pivotal for plant growth. High CO2 often increases plant growth and reproduction, whereas low CO2 decrease plant growth by changing physiological responses (e.g. water use efficiency), reducing biomass production and delaying development (Gerhart and Ward, 2010). However, the mechanisms are still unclear. Here we report that Arabidopsis PPC2, encoding a PEPC that is involved in plant primary metabolism for producing C4-dicarboxylic
acids, is essential plant growth at low CO₂ conditions. ppc2 seedlings showed chlorosis and arrested growth at low CO₂ (200 ppm) (Fig. 1C and D). These phenotypes could be rescued by expression of PPC2 (Fig. 1E and F)). Moreover, there were no significant differences in the germination rates between ppc2 and Col-0 at both CO₂ conditions (Fig. S1), suggesting the phenotypes of ppc2 mutant occur in seedling development stage. Compared with Col-0, ppc2 mutant seedlings accumulated less photosynthetic carbohydrates in leaves, such as sucrose, starch and their upstream precursors (Fig. S2), exogenously addition of sucrose completely recovered the growth arrest phenotype of ppc2 (Fig. 4A). Mutation of PPC2 greatly decreased malate content and PEPC activity at low CO₂ conditions (Figs. 2B, Fig. S2B). Furthermore, in these three plant-type PEPCs in Arabidopsis, only PPC2 was induced by low CO₂, consistent with the previous study (Li et al., 2014), and mutation of PPC1 or PPC3 did not affect plant growth at low CO₂ conditions (Figs. 1C and 2A). (Li et al., 2014;). All these results suggest PPC2 is the major PEPC expressed in Arabidopsis leaves and is essential for plant growth at low CO₂.

**PPC2 participates in photorespiration by linking with primary metabolism at photorespiratory low CO₂ conditions**

Recent studies in sunflower have shown that the malate content closely correlates with photorespiration by metabonomics analyses and C₃ PEPC fixation increased at high photorespiratory condition (low CO₂ or high O₂) (Abadie et al., 2017; Abadie and Tcherkez, 2019), indicating non-photosynthetic PEPC may still contribute to photorespiration in C₃ plant. The studies in Arabidopsis have reported the novel function of C₃ PEPC in regulating the balance of carbon/nitrogen metabolism (Masumoto et al., 2010; Shi et al., 2015). However, the molecular mechanisms remain unclear. Here, we were the first to clarify the special role of PPC2 in photorespiration at low CO₂ conditions by regulating carbon/nitrogen balance.

Firstly, PPC2 may regulate the balance of carbon/nitrogen at photorespiratory low CO₂ conditions. The metabolites of glycolysis pathway and amino acid levels in ppc2 seedlings were greatly affected at photorespiratory low CO₂ conditions (Fig. 6A; Figs.
S2B and S4). The photorespiratory intermediates glycine and serine were significantly accumulated in ppc2 at photorespiratory CO\textsubscript{2} conditions (Fig.6A and Fig. S4), suggesting PPC2 is involved in photorespiratory metabolism at low CO\textsubscript{2} conditions. Glutamic acid plays a central signaling and metabolic role in regulating carbon and nitrogen assimilatory balance (Forde and Lea, 2007), which was greatly reduced in ppc2 mutant at photorespiratory low CO\textsubscript{2}. The increased glutamine further reduced the assimilation of ammonium released by photorespiration at glycine point, thus likely contributed to the accumulation of glycine and later serine at photorespiratory low CO\textsubscript{2} conditions. The required glutamate for photorespiration is imported from the chloroplast by exchange against malate, the reduced malate level in the ppc2 mutant is consistent with this (Fig. S2B). Addition of exogenous malate not only greatly rescued the growth arrest of ppc2 mutant at low CO\textsubscript{2} treatment (Fig. 4A), but also reduced the high accumulated photorespiratory intermediates such as glycine in the ppc1/ppc2 double mutant (Shi et al., 2015). These phenomena are in accordance with the previous report that malate dehydrogenase mutants exhibit an alteration in photorespiratory metabolism (Tomaz et al., 2010). Because of PPC2 mutation, PEP flux into glycolysis pathway was reduced, and PEP flux into shikimate and calvin cycle was increased at low CO\textsubscript{2} conditions, leading to higher levels of Phe, Tyr and Trp (Fig. 6A, Fig. S4). These results demonstrate PPC2 control the balance of carbon/nitrogen metabolisms at low CO\textsubscript{2} conditions.

Secondly, at photorespiratory low CO\textsubscript{2} conditions PPC2 affects the expression patterns of photorespiratory enzymes related with glycine and serine synthesis and metabolism. In photorespiratory pathway GGATs and SGAT1 transfers -NH\textsubscript{3}\textsuperscript{+} respectively from glutamate and serine into glyoxylate to synthesize glycine; GLDP1 and GLDT1 decarboxylate glycine into CH\textsubscript{2}-THF; and subsequently SHMT1 transfers the C1 moiety to another glycine resulting in serine formation (Peterhansel et al., 2010). Mutation either in SGAT1 or SHMT1 leads to accumulation of serine and glycine (Somerville and Ogren, 1980; Kuhn et al., 2013). Furthermore, overexpression of glycine decarboxylase results in lower glycine and serine content (Timm et al., 2015). In agreement, the expressions of SHMT1, SGAT1, GLDP1 and
GLDT1 were greatly reduced in ppc2 at low CO2 conditions, which increased the levels of glycine and serine.

Thirdly, the reduced CO2 assimilation of ppc2 mutant at low CO2 conditions was caused by the increased photorespiratory lost in plant. In air condition, ppc2 mutant exhibited declined CO2 assimilation rate in the initial part of A-Ci curve (50-400 ppm) (Fig. 8A). However, after reducing O2 to inhibit the photorespiration, the reduced CO2 assimilation of ppc2 mutant at low CO2 conditions was recovered (Fig. 8C). In agreement, the biomass of ppc2 mutant was decreased. Moreover, our results showed that the reduced carbon assimilation of ppc2 at low CO2 was not due to stomatal status at low CO2 conditions, because the stomatal conductance at the steady state and in response to low CO2 shift retained normal in ppc2 as Col-0 (Fig. 3E and F). In this sense, PPC2 is specifically involved in photorespiration at low CO2 conditions (Fig. 8D).

ABA regulates photorespiration at low CO2 conditions through ABI5

ABA induces gene expression and plays a prominent role in the establishment of stress tolerance. However, the relationships of ABA with low CO2 stress and simultaneous photorespiration are still limited. In this study, we found that ABA biosynthesis was heavily blocked at low CO2 conditions, possibly due to the decrease in carotenoid accumulation (Fig. 3B), which is the precursor for ABA biosynthesis (Ruiz-Sola and Rodriguez-Concepcion, 2012). When PPC2 was mutated, the ABA synthesis was further reduced (Fig. 4B). Addition of small amount of ABA largely recovered the ppc2 seedlings growth (Fig. 4C and D), suggesting that low concentration of ABA is required to stimulate photosynthesis and plant growth at low CO2 conditions. In addition, it was reported that exogenous ABA induced the photorespiratory rate in Barley by increasing the activity of GOX (glycolate oxidase) (Popova et al., 1987), our data also showed high ABA treatment led to leaf chlorosis in Col-0 (Fig. 4C and D). These results suggest that higher concentration of ABA in turn could reduce photosynthesis at low CO2 stress possibly by promoting carbon flux through photorespiratory cycle.
ABI5 is proposed to be a key player in monitoring environmental conditions during seedling growth (Lopez-Molina et al., 2001) and functions as an intermediate in ABA signaling to regulate seed germination and seedling growth. Our results show that ABI5 plays a key role in seedling growth at photorespiratory low CO2 conditions by regulating the expression of photorespiratory pathway enzymes and ABA regulates plant growth at low CO2 conditions through modulating ABI5 expression. abi5-1 seedlings growing at low CO2 had reduced Fv/Fm (Fig. 5C and D). A-Ci curves of mature abi5-1 mutant were reduced at low CO2 conditions, which could be fully recovered at non-photorespiratory low O2 condition (Figs. 8F and G). In ppc2 plants, ABA synthesis was reduced and ABI5 was repressed low CO2 conditions, ABA treatment greatly rescued the expression of ABI5 and growth arrest in ppc2 (Figs. 4D and 5B). Moreover, the expression levels of photorespiratory enzymes related to glycine and serine synthesis and metabolism were reduced in ppc2 and abi5 seedlings at low CO2 conditions (Fig. 7A and C). Our further experiments show that ABI5 may regulate SGAT1 and GLDT1 by directly binding to their promoters and regulates SHMT1 indirectly (Fig. 7E). ABI5 expression rescued their expressions (Fig. 7C) and reduced dry-weight biomass of ppc2 adult plants (Fig. 8H and I).

In summary, our study reveals that PPC2 is essential for plant growth at photorespiratory low CO2 conditions. It plays important role in carbon assimilation at low CO2 conditions via regulating carbon and nitrogen metabolisms during photorespiration. We also identified the important role of ABA in photorespiration and the novel function of ABI5 in photorespiratory low CO2 by regulating transcription of photorespiratory enzyme genes. Our works demonstrate the key role of C3 PEPCs in photorespiration at low CO2 conditions and may offer clues for future studies to understand the mechanism of C3 PEPCs in regulation of photosynthesis and for potential application in crop improvement against photorespiration.

Supplemental Data
Fig. S1. Seeds germination of WT and ppc mutants.
Fig. S2. Photosynthetic carbohydrates were reduced in ppc2 mutant seedlings at low
CO₂ conditions.

Fig. S3. RT-PCR analyses of ABI5 expression level in WT, ppc2 and ABI5 expressing ppc2 plants.

Fig. S4. Amino acid content in WT and ppc2 mutant seedlings.

Fig. S5. Predicted ABRE cis-elements in the promoter regions of photorespiratory enzyme genes.

Table S1. List of primers used in this study.

Acknowledgement

This work was supported by grants from the National Key Research and Development Program of China (2016YFD0100604), the National Natural Science Foundation of China (31771552) and Fundamental Research Funds for the Central Universities (2662017PY034). The authors are grateful to Dr. Mingqiu Dai (Huazhong Agricultural University) for providing the abi5-1 mutant seeds. The authors declare that they have no competing interests.
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**Figure legends**

**Fig. 1.** *ppc2* mutant seedlings show growth arrest at low CO$_2$ conditions. (A, B) Genotyping analyses (A) and expression levels (B) of *PPC1*, *PPC2* and *PPC3* in their corresponding single mutants of *ppc1*, *ppc2* and *ppc3*. *ACTIN7* (AT5G09810) was used as a control. (C, D) Phenotype (C) and statistic analyses (D) of leaf chlorosis of wild type (WT), *ppc1*, *ppc2*, *ppc3* and *ppc2ppc3* seedlings that growing on sucrose free 1/2 MS medium at 400 ppm or 200 ppm CO$_2$ conditions for 15 days. Data shown are mean ± SEM (n = 4). Each replicate had at least 60 seedlings. Asterisks indicate significant differences between genotypes (*P < 0.05; ***P <0.005 by Student’s t-test; ns, no significant difference). (E) Expression levels of *PPC2* in *PPC2* expressing *ppc2* plants by RT-PCR. RNA was extracted from leaves of 15-day-old seedlings. *ACTIN7* (AT5G09810) was used a control. (F) Growth phenotypes of seedlings of WT, *ppc2* and *PPC2* complementary lines (COM-1, COM-2 and COM-3) growing on sucrose free 1/2 MS medium at 400 ppm or 200 ppm CO$_2$ conditions for 15 days. Each replicate had at least 60 seedlings. Bars = 1 cm in C and F.

**Fig. 2.** *PPC2* is low CO$_2$ inducible and encodes a major PEPC in Arabidopsis leaves. (A) Expression levels of *PPC1*, *PPC2* and *PPC3* in Col-0 15-day-old seedlings that growing on sucrose free 1/2 MS medium at 400 ppm or 200 ppm CO$_2$ conditions. Expression levels are expressed relative to that of *EF1α* (AT5G60390). Data shown are mean ± SEM (n = 3). Asterisks indicate significant differences between genotypes (*P < 0.05; ns, no significant difference). (B) Total leaf PEPC activity analyzed in 15-day-old wild-type and *ppc2* mutant seedlings at ambient and low CO$_2$ conditions. Data shown are mean ± SEM (n = 3). Different letters indicate significant differences using Tukey's test at $P \leq 0.05$. (C) Tissue-specific expression of *PPC2* in leaves, flowers and siliques. a to c, GUS activity in leaves of 3-day-old (a), 5-day-old (b) and 15-day-old (c) seedlings. d, guard cells of cotyledon. e and f, GUS activity in calyxes. g, guard cells of calyx. h, GUS activity in siliques. i, guard cells of siliques. Bars = 50 μm in d, g. Bars = 1 mm in a to c, e, f, h and i. (D) Subcellular localization of PPC2-YFP fusion in protoplasts from 4-week-old Col-0. The yellow fluorescence from PPC2-YFP was shown in green and merged with red fluorescence from the HDEL-mCherry fusion using confocal microscopy. BF, bright field. YFP, yellow fluorescent protein. Bar = 20 μm.
**Fig. 3.** Carbon assimilation is reduced in the *ppc2* mutant at low CO₂ conditions. (A, B) Chlorophyll content (A) and carotenoid content (B) in 15-day-old seedlings of wild type (WT) and *ppc2* mutant at 400 ppm and 200 ppm CO₂ conditions. Data shown are mean ± SEM (n = 3). (C) *Fv/Fm* was monitored by Closed FluorCam FC800 in WT and *ppc2* mutant seedlings growing at 400 ppm or 200 ppm CO₂ conditions for 15 days. (D) *Fv/Fm* value comparison between WT and *ppc2* mutant. Data shown are mean ± SEM (n = 3). (E, F) Time-resolved stomatal conductance in response to CO₂ changes in WT and *ppc2* mutant plants. (E) Data shown in (F) were normalized. Different letters indicate significant differences using Tukey's test at *P* ≤ 0.05. Asterisks indicate significant differences between genotypes (**P <0.005 by Student’s t-test; ns, no significant difference**).

**Fig. 4.** Exogenous ABA greatly rescues the seedling growth arrest of *ppc2* at low CO₂ conditions. (A) Phenotype of 15-day-old seedlings of wild type (WT) and *ppc2* mutant at 200 ppm CO₂ on sucrose free 1/2 MS medium, supplemented with mock (ddH₂O), 25 mM sucrose, 1.5 mM malate or 3 mM malate. Each replicate had at least 60 seedlings. (B) ABA content in WT and *ppc2* mutant seedlings growing at different CO₂ conditions. Data shown are mean ± SEM (n = 3). FW, fresh weight. (C, D) Phenotypes (C) and statistic analyses (D) of leaf chlorosis rate of 15-day-old WT and *ppc2* mutant seedlings growing at 200 ppm CO₂ on sucrose free 1/2 MS medium with mock (ddH₂O), 0.1 μM ABA, 0.2 μM ABA, respectively. Data shown are mean ± SEM (n = 4). Each replicate had at least 60 seedlings. Asterisks indicate significant differences between genotypes (**P <0.01 by Student’s t-test**). Different letters indicate significant difference using Tukey's test at *P* ≤ 0.05. Bar = 1 cm in A and C.

**Fig. 5.** *abi5* has reduced *Fv/Fm* and expression of *ABI5* rescued the growth arrest of *ppc2* at low CO₂ concentration. (A) Relative expression levels of *ABI3*, *ABI4* and *ABI5* to *EF1α* (AT5G60390) in wild type (WT) and *ppc2* mutant growing on sucrose free 1/2 MS medium at 400 ppm or 200 ppm CO₂ conditions for 15 days. Data are shown as mean ± SEM (n= 3). (B) *ABI5* expression levels in *ppc2* and WT seedlings that treated with mock (ddH₂O), 0.1 μM ABA, 0.2 μM ABA at 200 ppm conditions, respectively. Data shown are mean ± SEM (n = 3). Different letters indicate significant differences using Tukey’s test at *P* ≤ 0.05. (C) *Fv/Fm* monitored by Closed FluorCam FC800 in Ws and *abi5-1* mutant seedlings growing at 400 ppm or 200 ppm CO₂ conditions for 15 days. (D) Maximum photosynthetic yields
($F_v/F_m$) of Ws and $abi5-1$ mutant at different CO$_2$ conditions. Data shown are mean ± SEM ($n = 3$). Asterisks indicate significant differences between genotypes (*$P < 0.05$ by Student’s t-test; ns, no significant difference). (E, F) Phenotype (E) and statistic analyses (F) of leaf chlorosis rate of WT, $ppc2$ mutant and $ABI5$ expressing $ppc2$ transgenic lines ($ppc2ABI5_{oc-7}$, $ppc2ABI5_{oc-8}$, $ppc2ABI5_{oc-9}$). Data shown are mean ± SEM ($n = 3$). Each replicate had at least 60 seedlings. Different letters indicate significant difference using Tukey’s test at $P \leq 0.05$. Bar = 1cm in C and E.

**Fig. 6.** $ppc2$ mutant has reduced carbon assimilation rate at different CO$_2$ concentrations. (A) Level changes in leaf metabolites between $ppc2$ mutant and wild type (WT) seedlings at 200 ppm CO$_2$ conditions. PEPC, phosphoenolpyruvate carboxylase. GGAT, glutamate:glyoxylate aminotransferase. GLD, glycine decarboxylase, including GLDP, GLDT and GLDH. SHMT, serine hydroxymethyltransferase. SGAT, serine:glyoxylate aminotransferase. RuBP, ribulose-1,5-disphosphate. F6P, fructose 6-phosphate. G6P, glucose 6-phosphate. G1P, glucose 1-phosphate. UDPGlc, UDP-glucose. ADPGlc, ADP-glucose. Suc6P, sucrose 6-phosphate. Pyr, pyruvate. 2-OS, 2-oxosuccinamate. OAA, oxaloacetate. PEP, phosphoenolpyruvate. (B) The ratio of glutamine to glutamic acid in 15-day-old WT and $ppc2$ seedlings at different CO$_2$ conditions. Data shown are mean ± SEM ($n = 3$). (C) The ratio of glycine to serine in 15-day-old WT and $ppc2$ seedlings at different CO$_2$ conditions. Data shown are mean ± SEM ($n = 3$). Different letters indicate significant difference using Tukey’s test at $P \leq 0.05$.

**Fig. 7.** $ABI5$ regulates the expression levels of major photorespiratory enzymes related to glycine and serine synthesis and metabolism. (A) Expression levels of photorespiratory enzyme genes in wild type (WT) and $ppc2$ mutant leaves. RNAs were extracted from the leaves of 15-day-old seedlings. $EF1\alpha$ (AT5G60390) was used as an internal control. Data shown are mean ± SEM ($n = 3$). (B) Expression levels of photorespiratory genes in WT, $ppc2$ mutant and $ABI5$ expressing $ppc2$ plants ($ppc2ABI5_{oc-7}$, $ppc2ABI5_{oc-8}$, $ppc2ABI5_{oc-9}$). RNAs were extracted from leaves of the 15-day-old seedlings at different CO$_2$ condition. $EF1\alpha$ (AT5G60390) was used an internal control. Data shown are mean ± SEM ($n = 3$). Different letters indicate significant differences using Tukey’s test at $P \leq 0.05$. (B) Expression levels of photorespiratory enzyme genes in WT (Ws) and $abi5-1$ mutant leaves. RNAs were extracted from the leaves of 15-day-old seedlings. $EF1\alpha$ (AT5G60390) was used as an internal control. Data shown are mean ± SEM ($n = 3$). (D) A schematic
representation of dual luciferase reporter system. ABI5 or GFP (control) driven by CaMV 35S as effector was co-transformed with reporter that containing 35S driving REN (Renilla Luciferase) and the tested promoter of photorespiratory enzyme genes driving LUC (Firefly Luciferase) expression. (E) Dual-luciferase reporter assays showed the transcriptional activation of ABI5 on the promoters of SGAT1, GLDT1 and SHMT1. LUC values were normalized to REN. Data shown are mean ± SEM (n = 3). Asterisks indicate significant differences between genotypes (**) \( P < 0.01 \) by Student’s t-test; ns, no significant difference).

**Fig. 8.** abi5 mutant shows reduced carbon assimilation and expression of ABI5 rescues the carbon assimilation of ppc2 mutant at photorespiratory low CO2. (A, C) A-Ci curves of 30-day-old wild type (WT) and ppc2 mutant plants under ambient air conditions (A) or low oxygen condition (C). The light intensity for measurement was set at 2000 \( \mu \)mol m\(^{-2}\) s\(^{-1}\). Data shown are mean ± SEM (n = 3). (B) Maximum photosynthetic electron transport rate (ETR) of 30-day-old wild type and ppc2 mature plants. Data shown are mean ± SEM (n = 5). (D) Expression of PPC2 complements the reduced A-Ci curves on rosette leaves of ppc2 mutant growing at 400 ppm conditions (COM-1, COM-2 and COM-3 are PPC2 complementary lines). Data shown are mean ± SEM (n = 3). (E) Maximum photosynthetic electron transport rate (ETR) of 30-day-old Ws and abi5-1 mutant plants. Data shown are mean ± SEM (n = 5). (F, G) A-Ci curves of abi5-1 and Ws rosette leaves under air (F) or low oxygen (G) conditions for 30 days. Data shown are mean ± SEM (n = 3). (H) ABI5 expression complements the reduced A-Ci curves of ppc2 plants that growing at 400 ppm conditions. Data shown are mean ± SEM (n = 3). (I) Dry weight of WT, ppc2 and ABI5 overexpression ppc2 transgenic lines (ppc2ABI5oe-7, ppc2ABI5oe-8, ppc2ABI5oe-9) growing at ambient CO2 conditions. Data shown are mean ± SEM (n = 6). Asterisks indicate significant differences between genotypes (* \( P < 0.05 \); ** \( P < 0.01 \); *** \( P <0.005 \) by Student’s t-test; ns, no significant difference). Different letters indicate significant differences using Tukey's test at \( P \leq 0.05 \).
Figure A shows the total chlorophyll content (mg/g FW) for WT and ppc2 plants under 400 and 200 ppm CO2 conditions. Figure B shows the carotenoid content (mg/g FW) for the same conditions. Figure C displays Fv/Fm images for WT and ppc2 plants at 400 and 200 ppm CO2. Figure D compares the Fv/Fm values for WT and ppc2 under 400 and 200 ppm CO2. Figure E illustrates the relative stomatal conductance over 30 minutes for WT and ppc2 plants under different CO2 concentrations. Figure F shows the stomatal conductance (mol H2O m^-2 s^-1) for WT and ppc2 over 30 minutes at various CO2 concentrations.
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