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

26 Abstract

27 Phosphoenolpyruvate carboxylase (PEPC) is a pivotal enzyme that plays a key role in
 28 photosynthetic CO₂ fixation in C₄. However, the function of C₃ PEPCs and their roles
 29 at environmental CO₂ changes are still limited. Here, we report the role of *PPC2* in
 30 seedling growth at low CO₂ by linking photorespiratory metabolism with primary
 31 metabolism and involvement of ABA and ABI5. Mutation of *PPC2* caused seedling
 32 growth arrest, with reduced F_v/F_m , photosynthetic carbohydrates and ABA
 33 biosynthesis at low CO₂. *PPC2* is induced by low CO₂ and the PEPC activity was
 34 greatly reduced in *ppc2* leaves. Moreover, metabolic analyses showed the
 35 photorespiratory intermediates, glycine and serine, were greatly increased and
 36 primary metabolites were reduced. Application of sucrose, malate and ABA greatly
 37 rescued the growth arrest phenotype of *ppc2*. The expression of glycine/serine
 38 synthesis and metabolism related photorespiratory enzyme genes were decreased in
 39 *ppc2* and regulated by *ABI5* at low CO₂ conditions. *ppc2* and *abi5* mature plants
 40 exhibited reduced A-C_i curves at relatively low CO₂, which could be recovered by
 41 non-photorespiratory low oxygen conditions. *ABI5* expression greatly rescued the
 42 growth arrest and A-C_i curves of *ppc2* at low CO₂. Our findings demonstrate the
 43 important role of C₃ PEPCs in carbon fixation and metabolism.

44

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

47

48 Introduction

49 Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) is a ubiquitous enzyme
 50 widespread in plants, algae and bacteria (Chollet *et al.*, 1996). There are two
 51 functional forms of PEPCs in higher plants, the photosynthetic and
 52 non-photosynthetic isoforms. In CAM and C₄ plants, PEPC plays a pivotal
 53 photosynthetic role in primary CO₂ fixation, by catalyzing the irreversible
 54 β-carboxylation of PEP with HCO₃⁻ to oxaloacetate and inorganic phosphate. The
 55 photosynthetic PEPC activity in C₄ plant has been viewed as essential for carbon
 56 assimilation via prefixation of CO₂ in bundle sheath and, consequently, decreasing
 57 photorespiratory activity and resulting in higher water use efficiency (WUE) and
 58 higher photosynthetic efficiency than C₃ plant (Taylor *et al.*, 2018). The
 59 non-photosynthetic PEPCs play key roles in plant primary metabolism by
 60 replenishing tricarboxylic acid (TCA) cycle to support carbon and nitrogen
 61 metabolism (Masumoto *et al.*, 2010), in stomatal opening (Gehlen *et al.*, 1996) and in
 62 supplying malate as a substrate of respiration to symbiotic N₂-fixingbacteroids in
 63 legume root nodules (Vidal and Chollet, 1997). PEPC in C₃ plant is commonly
 64 believed to play minor roles in photosynthesis or photorespiration (von Caemmerer,
 65 2013). Recently, NMR analyses of ¹³C-fixation in sunflower indicated that PEPC
 66 activity and ¹³C-fixation was significantly increased as net CO₂ assimilation decreased
 67 at high photorespiratory conditions (low CO₂/O₂ ratio) (Abadie *et al.*, 2017; Abadie
 68 and Tcherkez, 2019). Moreover, mutation of *OsPPC4* in rice led to high accumulation
 69 of photorespiratory intermediates, such as glycine, serine and glycerate (Masumoto *et al.*,
 70 2010). Together that photorespiration tightly connects with primary metabolism
 71 (Mouillon *et al.*, 1999; Rachmilevitch *et al.*, 2004), these studies give clues that C₃
 72 PEPC may also play roles in photorespiration or photosynthesis.

73 In *Arabidopsis*, there are four genes encoding PEPC. Mutation in either *AtPPC1*,
 74 *AtPPC2* or *AtPPC3* led to decreased fresh weight and delayed flowering time (Feria
 75 *et al.*, 2016). Moreover, mutation of both *AtPPC1* and *AtPPC2* greatly reduced malate
 76 and citrate synthesis, and severely suppressed ammonium assimilation, which finally
 77 led to the growth arrest of *ppc1ppc2* (Shi *et al.*, 2015). However, how these *AtPPCs*

78 regulates plant primary metabolism and whether these *AtPPCs* regulate
79 photosynthesis and plant development under different stress conditions, such as
80 photorespiratory low CO₂ conditions, are yet unknown.

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

102 In this study, we demonstrate the crucial role of *AtPPC2* in seedling growth at
103 limiting CO₂ conditions by linking photorespiration metabolism with primary
104 metabolism, via integration of *ABI5*. *ppc2* mutant showed retarded seedling growth at
105 low CO₂ conditions and reduced carbon assimilation, in which *ABI5* expression was
106 heavily suppressed. Expression of *ABI5* rescued the growth arrest phenotype of *ppc2*
107 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 Murashige 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$) 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.

137 Chlorosis rate (%) = number of chlorotic seedlings / number of total seedlings

138

139 *PEPC Activity Assays*

140 0.1 g leaves (fresh weight) of 15-day-old seedlings growing at 200 ppm and 400 ppm
141 were harvested. Tissues were ground and extracted in 1ml extraction buffer. After
142 centrifugation, the supernatant was immediately used for PEPC activity detection by a
143 PEPC activity assay kit (Nanjing Jiancheng Bioengineering Institute). 1 unit of PEPC
144 activity was defined as 1 nmol of NADH oxidation per min and per mg protein at 25°C.
145 Total protein content was quantified by using BCA (bicinchoninic acid) protein assay
146 kit (Sangon Biotech).

147 *A-C_i curve analyses*

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

155 *Analyses of Sucrose and Starch Content*

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

160 *Analyses of Chlorophyll Content and Chlorophyll Fluorescence*

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

165 Total chlorophyll (nmol/mL) = 19.54 × (A_{646.8} - A₇₂₀) + 8.29 × (A_{663.2} - A₇₂₀)

166 Chlorophyll fluorescence of 15-day-old seedlings was analyzed by using a FluorCam
167 PAM as described in a previous report (Baker, 2008).

168 *Quantification of Endogenous ABA*

169 0.1 g leaves (fresh weight) of 15-day-old seedlings growing at 200 ppm and 400 ppm
170 were harvested, respectively. Samples were extracted in 750 μ l 80:19:1
171 methanol-H₂O-acetic acid buffer supplemented with internal standards for 6h with
172 occasional shaking. After centrifugation, the extracts were filtered through a 0.22 μ m
173 filter and dried with N₂ at room temperature. The extracts were dissolved in 200 μ l
174 methanol. Quantification of ABA were performed as described (Liu *et al.*, 2012).

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

176 Total RNA was extracted from seedlings of the wild-type Col-0 and mutants, and
177 cDNA were reverse transcribed. Quantitative RT-PCR was performed by using a
178 Universal SYBR[®] Green kit and the C1000 Touch Thermal Cycler real-time PCR
179 detection system (Bio-Rad). The *EF1 α* (AT5G60390) gene was used as a reference
180 gene for mRNA normalization. Comparative cycle threshold (C_t) method was used to
181 evaluate the relative gene expression levels. The experiment was repeated three times.
182 The primers used for the expression analysis were listed in Supplemental Table S1.

183 *GUS Histochemical Analyses*

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

188 *Quantification of Amino Acid and Organic Acid*

189 0.2 g (fresh weight) leaves of 15-day-old seedlings growing at 200 ppm and 400 ppm
190 were harvested, respectively. Tissues were ground and extracted in 8% (w/v)
191 5-sulfosalicylic acid for 1 h, then were centrifuged. The supernatants were filtered
192 through a 0.22 μ m filter. Contents of amino acid were determined by LC-MS/MS
193 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), *GLDPI* (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

252 seedling growth regulation at low CO₂ conditions.

253 To confirm that *PPC2* was responsible for the phenotype of growth retardation
254 observed in the *ppc2* mutant, the CDS of *PPC2* driven by CaMV 35S promoter was
255 introduced into the *ppc2* mutant. The expression levels of *PPC2* in randomly selected
256 *PPC2*-expressing transgenic *ppc2* plants were recovered to the similar level as in
257 Col-0 by RT-PCR analyses (Fig. 1E). When these lines together with *ppc2* and Col-0
258 grew at low CO₂ conditions, the growth arrest and cotyledon chlorotic phenotypes of
259 *ppc2* were all rescued by *PPC2* expression (Fig. 1F). These results demonstrate that
260 the seedling growth arrest is due to the dysfunction of *PPC2* and *PPC2* is a major
261 regulator of seedling growth at low CO₂ conditions.

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

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

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

281 *ppc2* seedlings showed reduced carbon assimilation at low CO₂ conditions

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

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

309 Because *PPC2* is highly expressed in guard cell (Fig. 2C), it is important to
310 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 HCO₃⁻. 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 C₄₀ 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 F_v/F_m 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, F_v/F_m 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, aspartic 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 CO₂ conditions was reduced in the ppc2 mature plants

Recently, a potential role for PEPC in C₃ plant metabolism at high photorespiratory (low CO₂/high O₂) 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 CO₂ conditions. To further investigate the function of *Arabidopsis* PPC2 in photorespiratory conditions, we determined the CO₂ assimilation rate under different C_i conditions in adult leaves of *ppc2* and Col-0 under air condition. The *ppc2* mutant exhibited a reduced CO₂ assimilation rate at low CO₂ (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 CO₂ (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 CO₂ assimilation of *ppc2* mutant at low CO₂ 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-C_i 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-C_i curves in air condition were similar (Fig. 8E). In low oxygen conditions, the slopes of A-C_i 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-C_i 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.

514 S2B and S4). The photorespiratory intermediates glycine and serine were significantly
 515 accumulated in *ppc2* at photorespiratory CO₂ conditions (Fig.6A and Fig. S4),
 516 suggesting PPC2 is involved in photorespiratory metabolism at low CO₂ conditions.
 517 Glutamic acid plays a central signaling and metabolic role in regulating carbon and
 518 nitrogen assimilatory balance (Forde and Lea, 2007), which was greatly reduced in
 519 *ppc2* mutant at photorespiratory low CO₂. The increased glutamine further reduced
 520 the assimilation of ammonium released by photorespiration at glycine point, thus
 521 likely contributed to the accumulation of glycine and later serine at photorespiratory
 522 low CO₂ conditions. The required glutamate for photorespiration is imported from the
 523 chloroplast by exchange against malate, the reduced malate level in the *ppc2* mutant is
 524 consistent with this (Fig. S2B). Addition of exogenous malate not only greatly
 525 rescued the growth arrest of *ppc2* mutant at low CO₂ treatment (Fig. 4A), but also
 526 reduced the high accumulated photorespiratory intermediates such as glycine in the
 527 *ppc1/ppc2* double mutant (Shi *et al.*, 2015). These phenomena are in accordance with
 528 the previous report that malate dehydrogenase mutants exhibit an alteration in
 529 photorespiratory metabolism (Tomaz *et al.*, 2010). Because of *PPC2* mutation, PEP
 530 flux into glycolysis pathway was reduced, and PEP flux into shikimate and calvin
 531 cycle was increased at low CO₂ conditions, leading to higher levels of Phe, Tyr and
 532 Trp (Fig. 6A, Fig. S4). These results demonstrate PPC2 control the balance of
 533 carbon/nitrogen metabolisms at low CO₂ conditions.

534 Secondly, at photorespiratory low CO₂ conditions *PPC2* affects the expression
 535 patterns of photorespiratory enzymes related with glycine and serine synthesis and
 536 metabolism. In photorespiratory pathway GGATs and SGAT1 transfers -NH₃⁺
 537 respectively from glutamate and serine into glyoxylate to synthesize glycine; GLDP1
 538 and GLDT1 decarboxylate glycine into CH₂-THF; and subsequently SHMT1 transfers
 539 the C1 moiety to another glycine resulting in serine formation (Peterhansel *et al.*,
 540 2010). Mutation either in *SGAT1* or *SHMT1* leads to accumulation of serine and
 541 glycine (Somerville and Ogren, 1980; Kuhn *et al.*, 2013). Furthermore,
 542 overexpression of glycine decarboxylase results in lower glycine and serine content
 543 (Timm *et al.*, 2015). In agreement, the expressions of *SHMT1*, *SGAT1*, *GLDP1* and

544 *GLDT1* were greatly reduced in *ppc2* at low CO₂ conditions, which increased the
545 levels of glycine and serine.

546 Thirdly, the reduced CO₂ assimilation of *ppc2* mutant at low CO₂ conditions was
547 caused by the increased photorespiratory lost in plant. In air condition, *ppc2* mutant
548 exhibited declined CO₂ assimilation rate in the initial part of A-C_i curve (50-400 ppm)
549 (Fig. 8A). However, after reducing O₂ to inhibit the photorespiration, the reduced CO₂
550 assimilation of *ppc2* mutant at low CO₂ conditions was recovered (Fig. 8C). In
551 agreement, the biomass of *ppc2* mutant was decreased. Moreover, our results showed
552 that the reduced carbon assimilation of *ppc2* at low CO₂ was not due to stomatal
553 status at low CO₂ conditions, because the stomatal conductance at the steady state and
554 in response to low CO₂ shift retained normal in *ppc2* as Col-0 (Fig. 3E and F). In this
555 sense, *PPC2* is specifically involved in photorespiration at low CO₂ conditions (Fig.
556 8D).

557 *ABA regulates photorespiration at low CO₂ conditions through ABI5*

558 ABA induces gene expression and plays a prominent role in the establishment of
559 stress tolerance. However, the relationships of ABA with low CO₂ stress and
560 simultaneous photorespiration are still limited. In this study, we found that ABA
561 biosynthesis was heavily blocked at low CO₂ conditions, possibly due to the decrease
562 in carotenoid accumulation (Fig. 3B), which is the precursor for ABA biosynthesis
563 (Ruiz-Sola and Rodriguez-Concepcion, 2012). When *PPC2* was mutated, the ABA
564 synthesis was further reduced (Fig. 4B). Addition of small amount of ABA largely
565 recovered the *ppc2* seedlings growth (Fig. 4C and D), suggesting that low
566 concentration of ABA is required to stimulate photosynthesis and plant growth at low
567 CO₂ conditions. In addition, it was reported that exogenous ABA induced the
568 photorespiratory rate in Barley by increasing the activity of GOX (glycolate oxidase)
569 (Popova *et al.*, 1987), our data also showed high ABA treatment led to leaf chlorosis
570 in Col-0 (Fig. 4C and D). These results suggest that higher concentration of ABA in
571 turn could reduce photosynthesis at low CO₂ stress possibly by promoting carbon flux
572 through photorespiratory cycle.

573 *ABI5* is proposed to be a key player in monitoring environmental conditions
 574 during seedling growth (Lopez-Molina *et al.*, 2001) and functions as an intermediate
 575 in ABA signaling to regulate seed germination and seedling growth. Our results show
 576 that *ABI5* plays a key role in seedling growth at photorespiratory low CO₂ conditions
 577 by regulating the expression of photorespiratory pathway enzymes and ABA regulates
 578 plant growth at low CO₂ conditions through modulating *ABI5* expression. *abi5-1*
 579 seedlings growing at low CO₂ had reduced F_v/F_m (Fig. 5C and D). A-C_i curves of
 580 mature *abi5-1* mutant were reduced at low CO₂ conditions, which could be fully
 581 recovered at non-photorespiratory low O₂ condition (Figs. 8F and G). In *ppc2* plants,
 582 ABA synthesis was reduced and *ABI5* was repressed low CO₂ conditions, ABA
 583 treatment greatly rescued the expression of *ABI5* and growth arrest in *ppc2* (Figs. 4D
 584 and 5B). Moreover, the expression levels of photorespiratory enzymes related to
 585 glycine and serine synthesis and metabolism were reduced in *ppc2* and *abi5* seedlings
 586 at low CO₂ conditions (Fig. 7A and C). Our further experiments show that *ABI5* may
 587 regulate *SGAT1* and *GLDT1* by directly binding to their promoters and regulates
 588 *SHMT1* indirectly (Fig. 7E). *ABI5* expression rescued their expressions (Fig. 7C) and
 589 reduced dry-weight biomass of *ppc2* adult plants (Fig. 8H and I).

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

599 **Supplemental Data**

600 Fig. S1. Seeds germination of WT and *ppc* mutants.

601 Fig. S2. Photosynthetic carbohydrates were reduced in *ppc2* mutant seedlings at low

602 CO₂ conditions.
 603 Fig. S3. RT-PCR analyses of *ABI5* expression level in WT, *ppc2* and *ABI5* expressing
 604 *ppc2* plants.
 605 Fig. S4. Amino acid content in WT and *ppc2* mutant seedlings.
 606 Fig. S5. Predicted ABRE cis-elements in the promoter regions of photorespiratory
 607 enzyme genes.
 608 Table S1. List of primers used in this study.

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616

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Figure legends

Fig. 1. *ppc2* mutant seedlings show growth arrest at low CO₂ 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 CO₂ conditions for 15 days. Data shown are mean $\pm$ 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 CO₂ conditions for 15 days. Each replicate had at least 60 seedlings. Bars = 1 cm in C and F.

Fig. 2. *PPC2* is low CO₂ 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 CO₂ conditions. Expression levels are expressed relative to that of *EF1 α* (AT5G60390). Data shown are mean $\pm$ 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 CO₂ conditions. Data shown are mean $\pm$ 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 μ m 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 μ m.

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 $\pm$ SEM (n = 3). (C) F_v/F_m 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) F_v/F_m value comparison between WT and *ppc2* mutant. Data shown are mean $\pm$ 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 $P \leq 0.05$. 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 $\pm$ 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 μ M ABA, 0.2 μ M ABA, respectively. Data shown are mean $\pm$ 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 $P \leq 0.05$. Bar = 1 cm in A and C.

Fig. 5. *abi5* has reduced F_v/F_m and expression of *ABI5* rescued the growth arrest of *ppc2* at low CO₂ concentration. (A) Relative expression levels of *ABI3*, *ABI4* and *ABI5* to *EF1 α* (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 $\pm$ SEM (n = 3). (B) *ABI5* expression levels in *ppc2* and WT seedlings that treated with mock (ddH₂O), 0.1 μ M ABA, 0.2 μ M ABA at 200 ppm conditions, respectively. Data shown are mean $\pm$ SEM (n = 3). Different letters indicate significant differences using Tukey's test at $P \leq 0.05$. (C) F_v/F_m 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 CO₂ conditions. Data shown are mean $\pm$ 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_{oe-7}*, *ppc2ABI5_{oe-8}*, *ppc2ABI5_{oe-9}*). Data shown are mean $\pm$ 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 CO₂ concentrations. (A) Level changes in leaf metabolites between *ppc2* mutant and wild type (WT) seedlings at 200 ppm CO₂ 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 CO₂ conditions. Data shown are mean $\pm$ SEM (n = 3). (C) The ratio of glycine to serine in 15-day-old WT and *ppc2* seedlings at different CO₂ conditions. Data shown are mean $\pm$ 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 α* (AT5G60390) was used as an internal control. Data shown are mean $\pm$ SEM (n = 3). (B) Expression levels of photorespiratory genes in WT, *ppc2* mutant and *ABI5* expressing *ppc2* plants (*ppc2ABI5_{oe-7}*, *ppc2ABI5_{oe-8}*, *ppc2ABI5_{oe-9}*). RNAs were extracted from leaves of the 15-day-old seedlings at different CO₂ condition. *EF1 α* (AT5G60390) was used as an internal control. Data shown are mean $\pm$ SEM (n = 3). Different letters indicate significant differences using Tukey's test at $P \leq 0.05$. (C) 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 α* (AT5G60390) was used as an internal control. Data shown are mean $\pm$ 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 $\pm$ 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-C_i 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Data shown are mean $\pm$ SEM (n = 3). (B) Maximum photosynthetic electron transport rate (ETR) of 30-day-old wild type and *ppc2* mature plants. Data shown are mean $\pm$ SEM (n = 5). (D) Expression of *PPC2* complements the reduced A-C_i 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 $\pm$ SEM (n = 3). (E) Maximum photosynthetic electron transport rate (ETR) of 30-day-old *Ws* and *abi5-1* mutant plants. Data shown are mean $\pm$ SEM (n = 5). (F, G) A-C_i curves of *abi5-1* and *Ws* rosette leaves under air (F) or low oxygen (G) conditions for 30 days. Data shown are mean $\pm$ SEM (n = 3). (H) *ABI5* expression complements the reduced A-C_i curves of *ppc2* plants that growing at 400 ppm conditions. Data shown are mean $\pm$ SEM (n = 3). (I) Dry weight of WT, *ppc2* and *ABI5* overexpression *ppc2* transgenic lines (*ppc2ABI5*_{oe-7}, *ppc2ABI5*_{oe-8}, *ppc2ABI5*_{oe-9}) growing at ambient CO₂ conditions. Data shown are mean $\pm$ 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 $P \leq 0.05$.

A **B** **C** **D** **E** **F**

A **B** **C** **D**

A **B** **C** **D**

A **B** **C** **D** **E***ppc2* **F**

A

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ppc2 vs WT at 200 ppm

B

C