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1 **Glyceraldehyde-3-phosphate dehydrogenase subunits A and B are**
2 **essential to maintain photosynthetic efficiency**

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29 **Short title:** Evaluation of GAPDH subunits A & B in Arabidopsis

30 **One-sentence summary:** A reduction in the level of either the GAPA or GAPB proteins in
31 *Arabidopsis thaliana* results in significant reductions in photosynthetic efficiency and biomass.

32 *In vivo* data is presented showing that the A₂B₂ form of GAPDH is necessary to maintain
33 maximum photosynthetic capacity and growth.

34

35 **ABSTRACT**

36 In plants, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) reversibly
37 converts 1,3-bisphosphoglycerate to glyceraldehyde-3-phosphate coupled with the reduction
38 of NADPH to NADP⁺. The GAPDH enzyme that functions in the Calvin Benson Cycle is
39 assembled either from four glyceraldehyde-3-phosphate dehydrogenase A subunits (GAPA)
40 proteins forming a homotetramer (A₄) or from two GAPA and two glyceraldehyde-3-phosphate
41 dehydrogenase B subunit (GAPB) proteins forming a heterotetramer (A₂B₂). The relative
42 importance of these two forms of GAPDH in determining the rate of photosynthesis is
43 unknown. To address this question, we measured the photosynthetic rates of Arabidopsis
44 (*Arabidopsis thaliana*) plants containing reduced amounts of the GAPDH A and B subunits
45 individually and jointly, using T-DNA insertion lines of GAPA and GAPB and transgenic
46 GAPA and GAPB plants with reduced levels of these proteins. Here we show that decreasing
47 the levels of either the A or B subunits decreased the maximum efficiency of CO₂ fixation,
48 plant growth, and final biomass. Finally, these data showed that the reduction in GAPA protein
49 to 9% wild-type levels resulted in a 73% decrease in carbon assimilation rates. In contrast,
50 eliminating GAPB protein resulted in a 40% reduction in assimilation rates. This work
51 demonstrates that the GAPA homotetramer can compensate for the loss of GAPB, whereas
52 GAPB alone cannot compensate fully for the loss of the GAPA subunit.

53

54 **Key words:** Glyceraldehyde-3-phosphate dehydrogenase; GAPDH; Photosynthesis; biomass

55

56

57 INTRODUCTION

58 In recent years there has been a focus to develop strategies to increase crop yields in
59 order to feed the growing world population against the backdrop of climate change (IPCC,
60 2014; Pereira, 2017; IPCC, 2019; NASA, 2020). The photosynthetic capacity of a crop over
61 the season determines the rate of growth and hence yield potential. A number of reports have
62 now been published demonstrating that under glasshouse and field conditions improvements
63 in photosynthesis, including the Calvin-Benson Cycle (CBC) can improve the productivity and
64 yield of the plant (Driever et al., 2017; Kubis and Bar-Even, 2019; Simkin, 2019; Simkin et al.,
65 2019; Burgess et al., 2022; De Souza et al., 2022; Raines et al., 2022). In the CBC,
66 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyses the conversion of 1,3-
67 bisphosphoglycerate to the triose phosphate, glyceraldehyde 3-phosphate (GAP) (Cséke and
68 Buchanan, 1986). Previous work has shown that the antisense suppression of the GAPDH gene
69 had no effect on the rate of CO₂ assimilation until GAPDH activity had decreased to 30-40%
70 of WT levels (Price et al., 1995; Ruuska et al., 2000). However, more recently a study showed
71 that overexpression of GAPDH in rice (*Oryza sativa*) resulted in increased photosynthetic CO₂
72 assimilation under elevated [CO₂] conditions (Suzuki et al., 2021), raising the possibility that
73 GAPDH could be a target for future manipulations to improve photosynthesis.

74 The CBC GAPDH is highly regulated and in plants is comprised of two distinct
75 subunits, the glyceraldehyde-3-phosphate dehydrogenase A subunits (GAPA) and the
76 glyceraldehyde-3-phosphate dehydrogenase B subunits (GAPB) that function as either as a
77 homotetramer A₄ or heterotetramer A₂B₂ (Cerff, 1979; Iadarola et al., 1983; Howard et al.,
78 2011). The primary structures of these two subunits show considerable similarity and are
79 produced from separate nuclear genes (*GapA1*, *GapA2* and *GapB*) (Cerff, 1995). The GAPA
80 subunits share 92.6% identity and the major difference in the primary sequence between the
81 GAPA and GAPB subunits is a C-terminal extension (CTE) on the GAPB, with substantial

82 similarity to the C-terminus of Chloroplast protein of 12 kDa (CP12) (Baalmann et al., 1996;
83 Pohlmeier et al., 1996), This CTE contains cysteine residues which have been shown to confer
84 thioredoxin-mediated redox regulatory capacity onto the GAPDH A₂B₂ complex (Baalmann et
85 al., 1996; Scheibe et al., 1996; Sparla et al., 2002; Marri et al., 2005; Fermani et al., 2007).

86 In the chloroplast of vascular plants, the predominant active form of GAPDH is
87 believed to be the A₂B₂, however, leaves of many species contain other, less abundant forms
88 of GAPDH including the A₄ form and the 2(A₂B₂) and 4(A₂B₂) multimers involved in
89 deactivation of the enzyme (Wolosiuk and Buchanan, 1976; Scagliarini et al., 1998; Sparla et
90 al., 2005; Fermani et al., 2007). The homotetramer A₄ form of GAPDH has been termed ‘non-
91 regulatory’ (GAPDH_N), firstly because of the absence of the CTE identified in GAPB and
92 secondly, it fails to aggregate into larger oligomers and the A₂B₂ regulatory form (GAPDH_R)
93 (Scagliarini et al., 1998). In the absence of the CTE, GAPDH_N is thought to be regulated by
94 the formation of the CP12/GAPDH/PRK (Phosphoribulokinase) complex (Trost et al., 2006;
95 Lopez-Calcagno et al., 2014). Although the regulation of the two tetramers of GAPDH are
96 different, results of Scagliarini et al (1998) showed that the kinetic properties of GAPDH_N are
97 similar to GAPDH_R. The activity data for the GAPDH A₄ and the A₂B₂ showed that both of
98 these isoforms have similar kinetic parameters, with a V_{max} (NADPH) of 130 and 114 μmol
99 $\text{min}^{-1} \text{mg}^{-1}$ respectively and a K_m (BPGA) of 2.0 and 2.3 μM respectively. Based on these data
100 it was proposed that that the B-subunits are mostly responsible for regulation of the enzyme
101 (Sparla et al., 2005) and the A-subunits for catalytic activity (Scagliarini et al., 1998). Over and
102 above the multimers of the A₂B₂ complex a further level of redox regulation of GAPDH activity
103 occurs through the formation of a high molecular weight complex which includes the CBC
104 enzyme PRK and the small regulatory protein CP12 (Howard et al., 2008; Carmo-Silva et al.,
105 2011; Lopez-Calcagno et al., 2014; Lopez-Calcagno et al., 2017).

106 The A₄ homotetramer has been shown in spinach (*Spinacia oleracea*) chloroplast
107 preparations to constitute 15–20% of the total GAPDH activity (Scagliarini et al., 1998).
108 Howard et al. (2011) examined stromal extracts from dark-adapted leaves of species from
109 Leguminosae (pea (*Pisum sativum* ‘Onwards’), Medicago (*Medicago truncatula* ‘Jemalong’),
110 broad bean (*Vicia faba* ‘The Sutton’), French bean (*Phaseolus vulgaris* ‘Vilbel’)), Solanaceae
111 (potato (*Solanum tuberosum* ‘Desiree’), tomato (*Solanum lycopersicon* ‘Gardener’s Delight’),
112 and tobacco (*Nicotiana tabacum* ‘Samson’)), Amaranthaceae (spinach), and the Brassicaceae
113 (*Arabidopsis*). This study revealed that the relative amounts of the A₂B₂ and the A₄ complexes
114 vary among species. Whereas all species were found to accumulate the A₂B₂ heterotetramer,
115 in contrast, in some plant species the A₄ tetramer was not detected (Howard et al., 2011). This
116 raises the question of the role of the A₄ form for the activity of GAPDH in the CB cycle. To
117 date the relative importance of the A₄ versus the A₂B₂ form of plastid GAPDH, in determining
118 the rate of CO₂ assimilation, has not been elucidated. In this manuscript, to explore this
119 question we have used insertion mutants in both the *GapB* and *GapA-1* genes together with
120 transgenic lines where the relative amounts of the GAPA and GAPB proteins have been
121 decreased individually.

122

123 **RESULTS**

124 **Identification and analysis of Arabidopsis lines with reductions in GAPDH A and B** 125 **transcript and protein levels**

126 We identified T-DNA insertion mutants for *gapa-1* (SAIL_164_D01) and *gapb*
127 (SAIL_308_A06) from The Arabidopsis Information Resource (TAIR) database
128 (<http://www.arabidopsis.org/>). All T-DNA insertion sites were confirmed using PCR analysis
129 of genomic DNA followed by sequencing of the T-DNA/gene junctions. The positions of the
130 T-DNA inserts are presented in **Fig. 1**. Homozygous plants (identified by PCR) were used to

131 assess the effect of each T-DNA insertion on the expression of the GAPDH transcripts. RT-
132 qPCR analysis confirmed that the transcript abundance encoding the GAPA subunit in the
133 *gapa-1* mutant was reduced by approx. 45%, the remaining transcript being produced by the
134 *gapa-2* gene (**Fig. 2A**). The transcript for the GAPB subunit in the *gapb* mutant was not
135 detected in the T-DNA insertion line, evidencing the *gapb* mutant as a true knockout (KO)
136 (**Fig. 2B**). In order to obtain additional independent mutant lines, GAPA and GAPB expression
137 levels were downregulated using antisense constructs (Supplemental **Fig. S1, Fig. 2A and B**).
138 Additionally, two GAPA co-suppressed lines were identified from plants transformed with a
139 GAPA over-expression construct using the Arabidopsis sequence in the sense orientation
140 (Supplemental **Fig. S1C**). In *GapA* co-suppressed transformants, we identified two lines with
141 5% (cA1) and 11% (cA2) of total *GapA* (*GapA-1* and *GapA-2*) transcript levels and antisense
142 lines with 35% (aA1) and 11% (aA2) of *GapA* transcript (**Fig. 2A**). In anti-sense *GapB*
143 transformants, we identified three lines with 11% (aB1), 15% (aB2) and 41% (aB2) levels of
144 the *GapB* transcript (**Fig. 2B**). Western blot analysis of these mutant lines was used to
145 determine changes in GAPDH protein levels, which showed a reduction in bands at 37.6 kDa
146 representing GAPA and 47.7 kDa (**Fig. 2A and B**). In the GAPA1 insertion line and the
147 GAPA1 antisense lines the level of the GAPA protein was reduced to 51-43% of the CN plants
148 and in the GAPA co-suppressed lines only 9% of the GAPA protein was detected (**Table 1**). In
149 the GAPB insertion line no band was detected indicating the absence of the B subunit in this
150 mutant (**Fig.2A**). The level of protein in the GAPB antisense lines was between 15 and 40% of
151 the CN plants (**Fig.2B and Table 1**).

152

153 **Chlorophyll fluorescence imaging reveals that PSII efficiency is maintained in plants**
154 **showing a significant reduction in GAPA protein levels.**

155 In order to explore the impact of a decrease in the subunits of the GAPDH enzyme on
156 photosynthetic capacity, the quantum efficiency of PSII (F_q'/F_m'), chlorophyll *a* fluorescence
157 was analysed (Baker, 2008; Murchie and Lawson, 2013). No significant decrease in F_q'/F_m'
158 was observed in the *gapa-1* insertion line 164 which had a 45% reduction in *GapA* transcript
159 levels and 46% reduction in GAPA protein or in the *gapb* insertion line 308 with no detectable
160 level of the GAPB protein (**Table 1**). The *GapA* antisense lines, containing 46% of WT protein
161 levels, maintained equivalent PSII photosynthetic efficiency to controls. *GapB* antisense lines
162 also showed no significant differences in F_q'/F_m' consistent with the observed result in the
163 *gapb* insertion line 308, suggesting that in the absence of GAPB, the presence of the GAPDH
164 A subunit is sufficient to maintain the photosynthetic capacity of these plants.

165 A small decrease of 12% in F_q'/F_m' was found in the *GapA* co-suppressed lines that
166 had the lowest level of GAPA protein (**Table 1**). To investigate further the impact of a
167 combined reduction of both the GAPA and GAPB protein levels, double mutants *gapa-1/gapb*
168 (164/308) were generated. Homozygous plants with insertions in both *gapa-1* (164) and *gapb*
169 (308) were grown as described above. Interestingly, double mutants of *gapa-1/gapb* (164/308)
170 showed a significant reduction in F_q'/F_m' suggesting that a GAPA protein level of 52% is
171 insufficient to maintain photosynthetic efficiency in the absence of GAPB.

172

173 **Photosynthetic CO₂ assimilation and electron transport rates are reduced in lines with** 174 **reduced GAPDH protein level**

175 To assess the impact on photosynthesis of changes in the levels of GAPDH protein,
176 CO₂ assimilation rates were determined as a function of internal CO₂ concentration (C_i) (A/C_i'
177 curve). Plants were grown in environmentally controlled chambers under short day conditions
178 as described in materials and methods. The gas exchange measurements were made on mature
179 leaves on plants six weeks after germination. A/C_i curves were determined for the *gapa-1*

180 insertion line (164), *gapb* insertion line (308), co-suppressed line of *GapA* (cA), *GapA*
181 antisense line (aA), *GapB* antisense line (aB) and *gapa-1/gapb* crossed line (164/308)
182 compared to the controls (CN) plants (**Fig. 3**). From these A/C_i curves, the maximum rate of
183 CO₂ assimilation (A_{max}) in all mutant lines tested was shown to be significantly lower than for
184 the CN plants. The plants with the lowest levels of the GAPA protein had the greatest decrease
185 in assimilation rate (**Fig. 2A**), with maximum assimilation rates attained in these plants being
186 approx. 27% of that observed in the CN (**Fig. 3A; Table 1**). Furthermore, in the *gapa-1* mutant
187 (164), an approx. 50% reduction in GAPA protein levels resulted in a 40% reduction in
188 maximum assimilation (**Fig. 3A**).

189 Plants with no detectable level of GAPB protein (**Fig 2B**) had a 30% decrease in
190 assimilation rates compared to the 73% reduction observed in a line with 9% GAPA proteins
191 (cA) (**Fig. 3B**). Finally, in line 164/308, representing the double mutant *gapa-1/gapb*,
192 containing no GAPB protein and only 51% of the levels of GAPA protein, the assimilation
193 rates are similar to the single *gapa-1* (164), and *gapb* (308) insertion mutants. This result
194 suggests that the double mutant shows no cumulative impact on assimilation rates under these
195 conditions as long as 51% GAPA protein remains (**Fig. 3C**).

196 Further analysis of the A/C_i curves using the equations published by von Caemmerer
197 and Farquhar (1981) illustrated that the maximum rate of carboxylation by Rubisco (V_{Cmax}) and
198 maximum electron transport rate (J_{max}), were reduced in some lines (Sharkey et al., 2007;
199 Sharkey, 2016) (Table 1). The results for V_{Cmax} showed that lines with a reduction in GAPA
200 displayed a significant decrease compared to CN. No significant difference in V_{Cmax} was
201 observed in plants with a reduction in GAPB.

202 Furthermore, the results showed that the lines with reductions in either GAPA or GAPB
203 had a lower rate of photosynthetic electron transport (J_{max}), needed to sustain Ribulose 1,5-
204 biphosphate (RuBP) regeneration, when compared to control plants (Table 1). As previously

205 noted, the maximum rate of CO₂ assimilation (A_{max}) was significantly lower in all lines
206 compared to CN, however, A_{max} was significantly lower in cA, where *GapA* transcript and
207 GAPA protein levels were at the lowest levels. No significant differences in A_{max} were observed
208 between the single mutants *gapa-1* (164) and *gapb* (308) compared to the double mutant *gapa-*
209 *1/gapb* (164/308).

210

211 **Growth and vegetative biomass are reduced in both GAPA and GAPB reduced lines**

212 Growth analysis of *GapA* co-suppressed and insertion lines was carried out on
213 homozygous plants grown in growth chambers at 22 °C under short day length (130 μ mol m⁻²
214 s⁻¹ in an 8 h/16 h light/dark cycle) and relative humidity (RD) 50%. The growth rate of these
215 plants was determined using image analysis of total leaf area over a period of 52 days from
216 planting. Observations of the growth rates of *GapA* co-suppressed lines (cA), CN Columbia
217 (Col-0), and the *gapa-1* and *gapb* insertion lines (164 and 308) showed a statistically significant
218 reduction in all growth parameters (**Fig. 4**; Supplemental **Fig. S2**). The co-suppressed and
219 insertion lines were shown to have a statistically significantly slower growth rate when
220 compared to the CN plants at 40 days post planting (**Fig. 4A and B**). By 52 days post planting,
221 this growth trend continued (**Fig. 4B**) and the final leaf area was reduced compared to controls
222 (**Fig. 4B**).

223 A growth analysis of the *gapa-1* insertion mutant (164), the *GapA* co-suppressed (cA)
224 and the *GapA* antisense lines (aA) showed a significant reduction in dry weight and leaf number
225 (**Fig 5A**) compared to the CN. Significant reductions in the leaf number and final biomass were
226 seen in the *gapb* insertion mutant (308) and *GapB* antisense lines was also observed when
227 compared to CN (**Fig. 5B**). A comparative analysis of the single insertion mutants *gapa-1* and
228 *gapb* with the double mutants *gapa-1/gapb* showed that reduction in both the A and B subunits
229 resulted in a greater decrease in leaf area, biomass and leaf number after 46 days of growth

230 (Fig. 5C5B), even in the absence of a larger decrease in assimilation rates observed in Fig. 3C
231 (see Table 1).

232

233 DISCUSSION

234 A reduction in GAPA protein levels inhibits CO₂ assimilation and reduces biomass yield

235 Previous research showed that a 60-70% reduction in GAPDH activity was needed to
236 affect growth and development in tobacco (*N. tabacum* 'cv W38') antisense GAPDH lines and
237 that no severe impact on photosynthesis was observed until levels were reduced to less than
238 35% of wild-type levels (Price et al., 1995). The results presented in this study clearly showed
239 a slow growth phenotype in *A. thaliana* following reductions in GAPA protein levels by 50%.
240 *GapA* co-suppressed lines, with more than a 90% reduction in *GapA* transcript levels and a
241 barely detectable GAPA protein content showed the most statistically significant impact on
242 photosynthetic efficiency (-73%) even with GAPB being present at wild-type levels. The
243 principal form of GAPDH in plant chloroplast has been proposed to be the heterotetrameric
244 A₂B₂. In some plants, including spinach, an A₄ homotetramer has also been detected
245 representing up to 20% of total GAPDH activity (Scagliarini et al., 1998). This A₄
246 homotetramer was not detected in Arabidopsis by previous studies (Howard et al., 2011),
247 providing evidence that under normal circumstances the A₂B₂ tetramer is the principal active
248 form in Arabidopsis. In this study, plants showing an absence of GAPB protein in the *gapb*
249 mutant lines-maintained photosynthesis rates at 66% of wild type levels suggesting that under
250 conditions where GAPB is limiting, or absent, that the A₄ form of GAPDH can maintain
251 photosynthesis.

252 Importantly, the work here also allowed a comparative analysis between plants with
253 different levels of the GAPA and GAPB subunits under the same environmental conditions.
254 When the *gapa-1* (164) and *gapb* (308) mutant lines were crossed to form the double mutant

255 *gapa-1/gapb* (164/308), the combined effects resulted in a cumulative reduction in biomass (-
256 60%), which was significantly greater than the reductions observed in the *gapa-1* (-35%) or
257 *gapb* (-16%) mutants alone. Interestingly, the assimilation rates for the *gapa-1/gapb* double
258 mutant showed no further reductions compared to *gapa-1*, and *gapb* single mutants. Firstly,
259 suggesting that GAPA, even though reduced in level, is able to maintain the assimilation rate
260 even in the absence of GAPB and secondly, that the decrease in biomass observed in the double
261 mutant may be due to impacts early in development leading to a cumulative effect on growth.

262 Recent reviews of the literature have shown that over-expressing of some CBC enzymes
263 can lead to increases in photosynthesis and biomass and that a multi-target approach can result in
264 cumulative yield gains in some plants (Simkin, 2019; Simkin et al., 2019; Raines, 2022). The co-
265 overexpression of *GapA* and *GapB* in transgenic rice increased GAPDH activity to more than 3.2-
266 fold of the wild-type levels; under elevated [CO₂], CO₂ assimilation increased by approximately
267 10% demonstrating that the overproduction of the chloroplast GAPDH proteins is effective at
268 improving photosynthesis at least under elevated [CO₂]. However, under these conditions, no
269 statistical significant differences in biomass were observed compared to wild-type plants, although
270 a small increase in starch accumulation was observed. (Suzuki et al., 2021). In contrast, no
271 statistical significant difference in CO₂ assimilation was observed in ambient [CO₂] (Suzuki et al.,
272 2021). These results suggest that the manipulation of GAPDH activity may have more importance
273 as atmospheric [CO₂] increases due to current climate change models where [CO₂] increases from
274 416 ppm to 550 by 2050 and 700 ppm by 2100 (Le Quéré et al., 2009; IPCC, 2019; NASA,
275 2020). Furthermore, given that no increase in growth rate or final biomass was observed at
276 ambient [CO₂], increasing GAPDH may have more value in a multi-target approach, such as
277 targeting additional CBC enzymes, photorespiratory elements and photosynthetic electron
278 transport in combination with GAPDH in the same plants.

279

280 CONCLUSION

281 Our results have shown that both GAPA and GAPB are essential for normal growth
282 and development in Arabidopsis plants and that the A₂B₂ form of the enzyme is required for
283 maximum photosynthetic efficiency. The phenotypes described in this manuscript provide *in*
284 *vivo* evidence of the relative importance of the individual subunits of the GAPDH complex on
285 photosynthetic carbon assimilation. In this study we also show that the suppression of GAPA
286 to almost undetectable levels resulted in a 73% decrease in carbon assimilation compared to
287 34% reduction in photosynthesis in the absence of GAPB providing direct evidence of the
288 importance of GAPA in maintain photosynthetic capacity.

289

290 MATERIALS AND METHODS

291 Identification and analysis of T-DNA GAPDH mutants and production of double mutants

292 The *gapa-1* and *gapb* mutants in Arabidopsis (*Arabidopsis thaliana*) were identified in
293 the Arabidopsis Information Resource (TAIR) database (*gapa-1*: SAIL_164_D01 and *gapb*:
294 SAIL_308_A06). The mutant insertion sites were identified by PCR and the location of each
295 T-DNA insertion was determined by sequencing the PCR products spanning the junction site
296 (**Fig. 1**). The *GapA-1* was amplified with forward primers GapA1 Fwd
297 (5'gagagcatgtgacataacggg'3) and reverse primer GapA1 Rev (5'accttaagcttggcctcagtc'3) in
298 conjunction with primer Sail_LB3 (5'tagcatctgaatttcataaccaatctcgatacac'3). The *GapB* was
299 amplified with forward primers GapB Fwd (5'cgacgatgtctcctctcagc'3) and reverse primer
300 GapB Rev (5'gaccgggattcttgagagc'3) in conjunction with primer Sail_LB3. Double mutants
301 *gapa-1/gapb* (164/308) was obtained by crossing homozygous plants of *gapa-1*
302 (SAIL_164_D01) and *gapb* (SAIL_308_A06) and segregating the double homozygous plants.

303

304 Construct Generation

305 *GAPA and GAPB antisense constructs*

306 A partial-length coding sequence of glyceraldehyde-3-phosphate dehydrogenase A subunit
307 (*GapA-1*: At3g26650) and the glyceraldehyde-3-phosphate dehydrogenase B subunit (*GapB*:
308 At1g42970) were amplified by RT-PCR using primers AtGAPAf
309 (5'cacctatcgaaggaaccggagtgtt'3) and AtGAPAr (5'tcctgtagatggtggaacaatg'3) and AtGAPBf
310 (5'caccttgatggttaagctcatcaaagtt'3) and AtGAPBr (5'ggtgtaggagtgtgtggtt'3) respectively. The
311 resulting amplified products were cloned into pENTR/D (Invitrogen, UK) to make pENTR-
312 GAPA1; pENTR-*antiGAPA* and pENTR-*antiGAPB*. The cDNA's were introduced into the
313 pGWB2 gateway vector (Nakagawa et al., 2007; AB289765) by recombination from the
314 pENTR/D vector to make pGWB2-AntiGAPA and pGWB2-AntiGAPB (Supplemental **Fig.**
315 **S1**). cDNA are under transcriptional control of the 35s tobacco mosaic virus promoter, which
316 directs constitutive high-level transcription of the transgene, and followed by the *nos* 3'
317 terminator.

318

319 *GAPA-1 sense constructs*

320 Destination vector pGWPTS1 was generated as described in Simkin et al., (2017). The
321 full-length coding sequencer of *GapA-1* was amplified using primers AtFwd
322 (5'caccatggttcggttactttctctgtcc'3) and AtRev (5'ttgatgaaatcacttccagttgttgg'3). The resulting
323 amplified product was cloned into pENTR/D (Invitrogen, UK) to make pENTR-AtGAPA-1
324 and the sequence was verified and found to be identical. The full-length cDNA was introduced
325 into destination vector pGWPTS1 by recombination from the pENTR/D vector to make
326 pGWPTS1-AtGAPA-1 (PTS1-GAPA-1) (Supplemental **Fig. S1**). The transgene was under the
327 control of the *rbcS2B* (1150bp; At5g38420) promoter. In this instance the expression of the
328 cDNA was under transcriptional control of the Rubisco small subunit 2B (*rbcS2B*) promoter

329 (At5g38420), which directs high-level photosynthetic tissue specific transcription of the
330 transgene and followed by the *nos* 3' terminator.

331

332 **Generation of transgenic plants**

333 The recombinant plasmid pGWB2-AntiGAPA, pGWB2-AntiGAPB and pGWPTS1-
334 GAPA-1, were introduced into wild type Arabidopsis by floral dipping (Clough and Bent,
335 1998) using *Agrobacterium tumefaciens* GV3101. Positive transformants were regenerated on
336 MS medium containing kanamycin (50mg L⁻¹). Kanamycin resistant primary transformants
337 (T1 generation) with established root systems were transferred to soil and allowed to self-
338 fertilize. Full details of pGWB2-AntiGAPA, pGWB2-AntiGAPB and PTS1-GAPA-1,
339 construct assembly can be seen in the Supplemental **Fig. S1**.

340

341 **Plant Growth Conditions**

342 For experimental study, T3 progeny seeds from selected lines were germinated on soil
343 in controlled environment chambers at an irradiance of 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 22°C, relative
344 humidity of 60%, in an 8h/16h square-wave photoperiod. Plants were sown randomly, and
345 trays rotated daily. Leaf areas were calculated using standard photography and ImageJ software
346 (imagej.nih.gov/ij). Wild type plants and null segregants (azygous) used in this study were
347 evaluated independently. Once it was determined that no significant differences were observed
348 between these two groups, wild type plants and null segregants were combined (null segregants
349 from the transgenic lines verified by PCR for non-integration of the transgene) and used as a
350 combined “control” group (CN) (Supplemental **Fig. S3**). Four leaf discs (0.6 cm diameter) from
351 two individual leaves, were taken and immediately plunged into liquid nitrogen, and stored at
352 -80°C for determination of transcript levels by qRT-PCR and protein content by western blot.

353

354 cDNA generation and RT-PCR

355 Total RNA was extracted from Arabidopsis leaf using the NucleoSpin® RNA Plant Kit
356 (Macherey-Nagel, Fisher Scientific, UK). cDNA was synthesized using 1 µg total RNA in 20
357 µl using the oligo-dT primer according to the protocol in the RevertAid Reverse Transcriptase
358 kit (Fermentas, Life Sciences, UK). cDNA was diluted 1 in 4 to a final concentration of 12.5ng
359 µL⁻¹. For semi quantitative RT-PCR, 2 µL of RT reaction mixture (100 ng of RNA) in a total
360 volume of 25 µL was used with DreamTaq DNA Polymerase (Thermo Fisher Scientific, UK)
361 according to manufacturer's recommendations. For qPCR, the SensiFAST SYBR No-ROX Kit
362 was used according to manufacturer's recommendations (Bioline Reagents Ltd., London, UK).
363 GAPA-1 (At3g26650) and GAPA-2 (At1g12900) transcript were amplified using primers
364 GAPA-F (5'atggttatgggatgatgatgg'3) and GAPA-R (5'ttattggcaacaatgtcagcc'3) and GAPB-F
365 (5'ttcaggtgctctgatgtctctacc'3) and GAPB-R (5' tagccactaggtgagccaaatccacc'3) respectively.

366

367 Protein Extraction and Western Blotting

368 Total protein was extracted in extraction buffer (50 mM 4-(2-Hydroxyethyl)piperazine-
369 1-ethanesulfonic acid (HEPES) pH 8.2, 5 mM MgCl₂, 1 mM Ethylenediaminetetraacetic Acid
370 Tetrasodium Salt (EDTA), 10% Glycerol, 0.1% Triton X-100, 2 mM Benzamidine, 2 mM
371 Aminocaproic acid, 0.5 mM Phenylmethanesulfonyl fluoride (PMSF) and 10 mM DTT). Any
372 insoluble material was removed by centrifugation at 14000 g for 10 min (4°C) and protein
373 quantification was determined as previously described (Harrison et al., 1998; Simkin et al.,
374 2017). Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE,
375 transferred to polyvinylidene difluoride membrane, and probed using antibodies raised against
376 GAPDH (Pohlmeyer et al., 1996). Proteins were detected using horseradish peroxidase
377 conjugated to the secondary antibody and ECL chemiluminescence detection reagent
378 (Amersham, Buckinghamshire, UK).

379

380 Chlorophyll fluorescence imaging screening in seedlings

381 Measurements were performed on 2-week-old Arabidopsis seedlings that had been
382 grown in a controlled environment chamber at $130 \mu\text{mol mol}^{-2}\text{s}^{-1}$ PPFD and ambient
383 CO_2 . Chlorophyll fluorescence parameters were obtained using a chlorophyll fluorescence (CF)
384 imaging system (Technologica, Colchester, UK (Barbagallo et al., 2003; von Caemmerer et al.,
385 2004)). The operating efficiency of photosystem two (PSII) photochemistry, F_q'/F_m' , was
386 calculated from measurements of steady state fluorescence in the light (F') and maximum
387 fluorescence in the light (F_m') since $F_q'/F_m' = (F_m' - F')/F_m'$. Images of F' were taken when
388 fluorescence was stable at $130 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, whilst images of maximum fluorescence
389 were obtained after a saturating at 600 ms pulse of $6200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD (Oxborough and
390 Baker, 2000; Baker et al., 2001; Lawson et al., 2008; Simkin et al., 2017).

391

392 Gas Exchange Measurements

393 The response of net photosynthesis (A) to intracellular CO_2 (C_i) was measured using a
394 portable gas exchange system (CIRAS-1, PP Systems Ltd, Ayrshire, UK) as previously
395 described (Simkin et al., 2017). Leaves were illuminated with an integral red-blue LED light
396 source (PP systems Ltd, Ayrshire, UK) attached to the gas-exchange system, and light levels
397 were maintained at saturating photosynthetic photon flux density (PPFD) of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$
398 for the duration of the A/C_i response curve. Measurements of A were made at ambient CO_2
399 concentration (C_a) at $400 \mu\text{mol mol}^{-1}$, before C_a was decreased to 550, 350, 215, 60 $\mu\text{mol mol}^{-1}$
400 before returning to the initial value and increased to 740, 900, 1140, 1340, 1640 $\mu\text{mol mol}^{-1}$.
401 Measurements were recorded after A reached a new steady state (1-2 minutes). Leaf
402 temperature and vapour pressure deficit (VPD) were maintained at 25°C and $1 \pm 0.2 \text{ kPa}$
403 respectively. The maximum rates of Rubisco- ($V_{C_{max}}$) and the maximum rate of electron

404 transport for RuBP regeneration (J_{max}) were determined and standardized to a leaf temperature
405 of 25°C based on equations from von Caemmerer (1981), Bernacchi et al. (Bernacchi et al.,
406 2001) and Sharkey (2016). All points below 200 ppm were assigned as rubisco-limited, points
407 above 300 ppm as RuBP-regeneration limited as described (Sharkey, 2016).

408

409 **Statistical Analysis**

410 All statistical analyses were done by comparing ANOVA, using Sys-stat, University of
411 Essex, UK. The differences between means were tested using the Post hoc Tukey test (SPSS,
412 Chicago).

413

414 **Accession numbers**

415 Sequence data from this article can be found in the GenBank/EMBL data libraries under
416 accession numbers_At3g26650 (NM113576) and At1g42970 (AY039961).

417

418 **Supplemental Data**

419 **Supplemental Figure S1. Schematic representation constructs used for floral dipping.**

420

421 **Supplemental Figure S2. Original photos for Figure 4. Growth analysis of control and**
422 **experimental lines grown in low light.**

423

424 **Supplemental Figure S3. Growth analysis of non-transformed (WT) and Azygous (Azy)**
425 **experimental lines grown in low light.**

426

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436

437 **Author Contributions:** A.J.S and M.A generated transgenic plants and performed molecular
438 and biochemical experiments and carried out plant phenotypic and growth analysis. P.E.L.C
439 and L.R.H screened and identified GPADH insertion mutants and assisted in experimental
440 design. M.A performed gas exchange measurement on Arabidopsis. A.J.S and M.A carried out
441 data analysis on their respective contributions. C.A.R conceived and designed the research and
442 C.A.R and A.J.S supervised the research. C.A.R, A.J.S wrote the manuscript with input from
443 all authors.

444

445

446 **Tables**

447 **Table I.** Maximum electron transport rate (J_{max}), the maximum rate of carboxylation by Rubisco (V_{Cmax})
 448 and maximum assimilation (A_{max}) in wild-type and GAPDH lines.

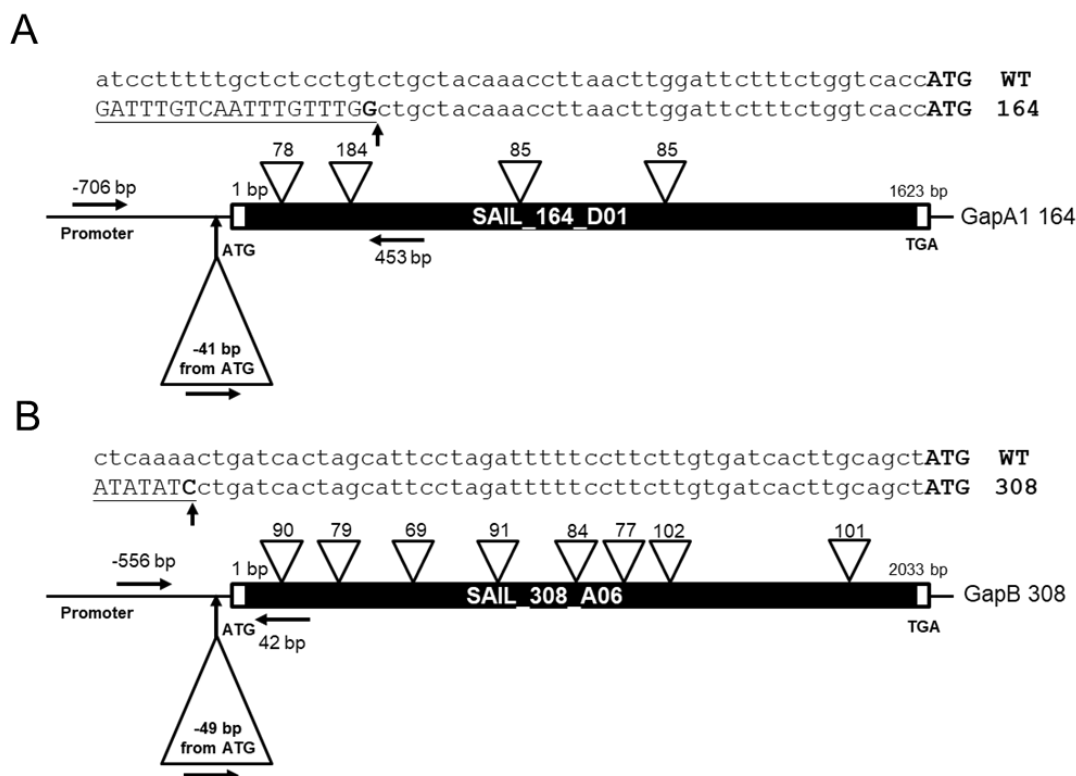
449 Plants were grown in short days at $130 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 8 h light/16 h dark cycle. Values represent 4-
 450 6 plants independent lines (6-8 plants) for group. A_{max} and J_{max} derived from A/C_i response curves shown in Figure
 451 3 using the equations published by von Caemmerer and Farquhar (1981) using the spreadsheet provided by
 452 Sharkey, (2016). ND = not detected. Statistical differences are shown in boldface ($P < 0.05$). SE are shown. WT
 453 = plants containing Wild Type levels of the transcript and protein subunit. Protein quantities are shown in italics.

Line	Relative % <i>GapA</i> transcript and protein	Relative % <i>GapB</i> transcript and protein	Fq'/Fm' 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$	J_{max}	V_{Cmax}	A_{max}
CN	WT	WT	0.478 +/- 0.014	145.5 +/- 15.75	55.1 +/- 5.2	28.3 +/- 1.15
164	55.2 +/- 15.5 <i>51.6 +/- 1.9</i>	WT	0.479 +/- 0.004	89.0 +/- 11.12	40.2 +/- 5.62	16.2 +/- 2.71
cA	9.4 +/- 4.4 <i>9.1 +/- 3.7</i>	WT	0.420 +/- 0.014	51.9 +/- 4.41	37.6 +/- 3.19	7.67 +/- 0.91
aA	23.3 +/- 11.8 <i>43.6 +/- 5.1</i>	WT	0.449 +/- 0.006	119.7 +/- 7.90	40.2 +/- 2.67	23.4 +/- 1.38
308	WT	ND	0.469 +/- 0.003	101.8 +/- 8.91	56.4 +/- 1.12	18.8 +/- 2.27
aB	WT	22.3 +/- 9.34 <i>26.1 +/- 13.8</i>	0.453 +/- 0.004	110.5 +/- 17.12	52.3 +/- 5.92	18.2 +/- 2.34 ***
164/ 308	55.2 +/- 15.5 <i>51.6 +/- 1.9</i>	ND	0.450 +/- 0.012	101.1 +/- 4.44	38.4 +/- 3.49	20.1 +/- 0.54

454 CN = control. *gapa-1* insertion (164); *gapb* insertion (308); co-suppressed GapA (cA); antisense GapA
 455 (aA); antisense GapB (aB);

456

457

458 **Figure Legends****Figure 1**

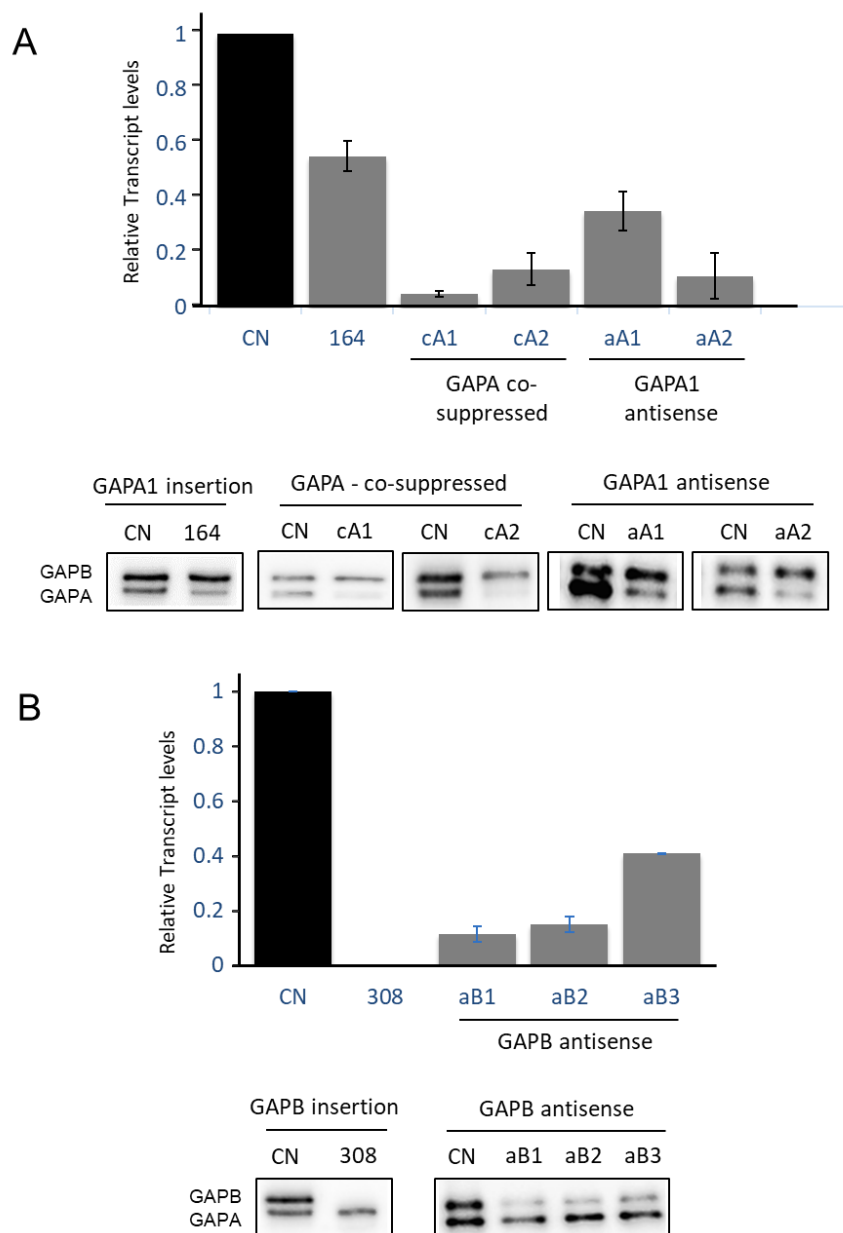
459

460 **Figure 1. Molecular analysis of homozygous GAPDH T-DNA insertion mutants.** Structure
 461 of the two GAPDH genes and the location of T-DNA insertions in the (A) *gapa-1* (At3g26650;
 462 SAIL_164_D01) and (B) *gapb* (At1g42970; SAIL_308_A06) mutants. Protein-coding exons
 463 are represented by black and intron locations are displayed as inverted white triangles above
 464 the coding sequence. Location of genomic PCR-screening primers are shown by black arrows
 465 on each gene model. T-DNA insertion sites are indicated by triangles below the sequence and
 466 the precise position is given as the number of base pairs from the ATG. ATG, translation
 467 initiation codon; TGA, translation termination codon. Bolded G (Panel A) and C (Panel B)
 468 indicate the point of sequence insertion into the promoter region of the SAIL_164_D01 and
 469 SAIL_308_A06 mutants.

470

471

Figure 2

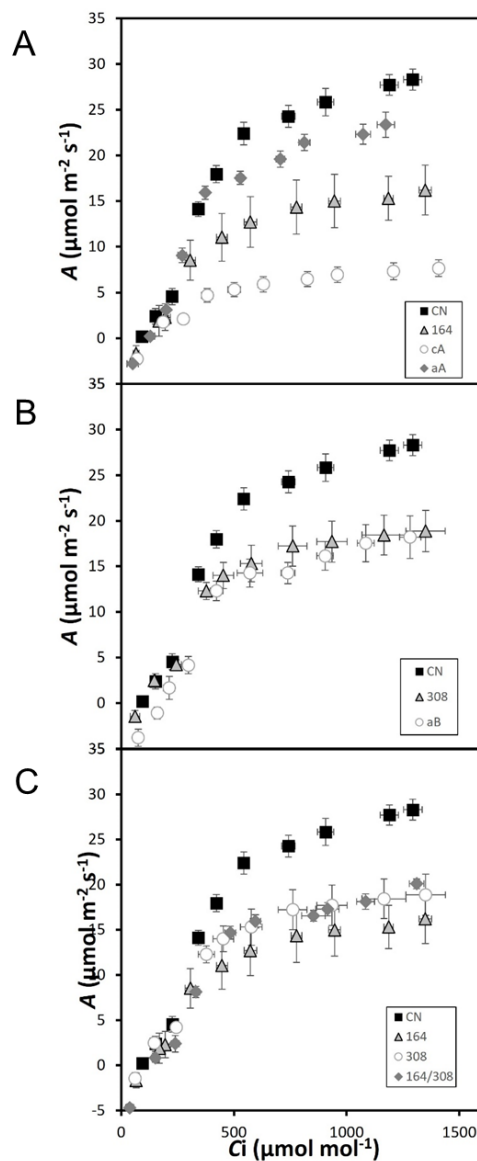


472

473 **Figure 2. RT-qPCR and Immunoblot analysis of leaf proteins of wild type and**474 **experimental GAPDH plants. (A) Transcript and Protein levels in *gapa-1* (At3g26650;**475 **SAIL_164_D01), GAPA co-suppressed lines (cA), GAPA antisense lines (aA) and control**476 **(CN). (B) Transcript and Protein levels in *gapb* (At1g42970; SAIL_308_A06) and GAPB**477 **antisense lines (aB). Protein (6 μ g) extracts from leaf discs taken from two leaves per plant and**478 **separated on a 12% acrylamide gel, transferred to membranes and probed with antibodies to**479 **GAPDH which recognises both GAPA and GAPB subunits. Error bars represent SE.**

480

Figure 3



481

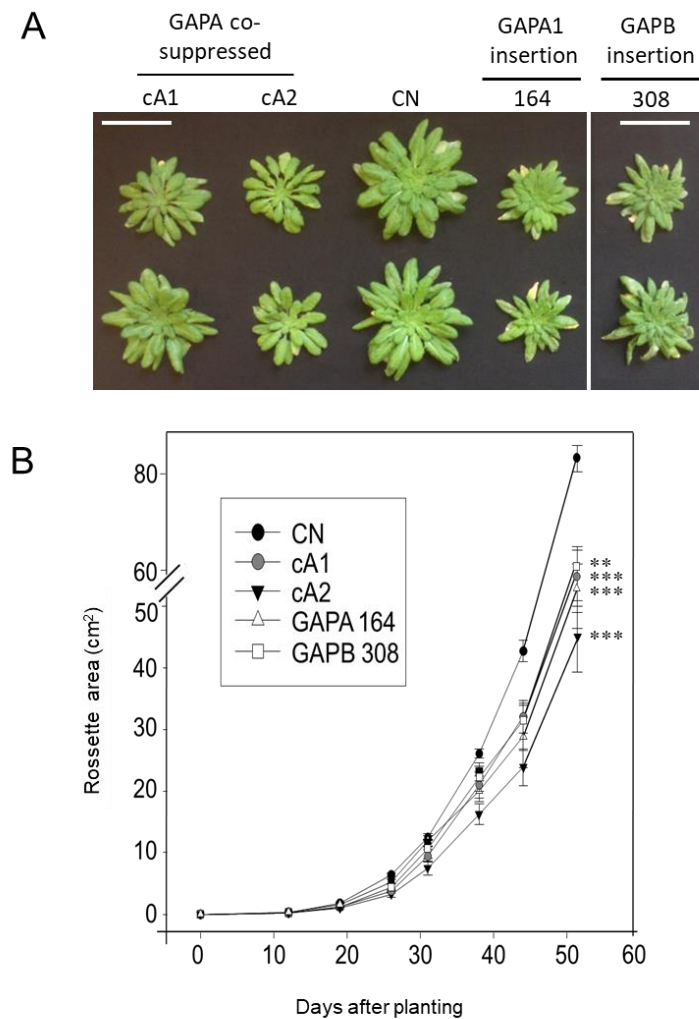
482

483 **Figure 3. Photosynthetic carbon fixation rate determined as a function of increasing CO₂**484 **concentrations (A/C_i) at saturating-light levels ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$).** (A) Controls (CN)485 compared to *gapa-1* insertion line (164), GAPA co-suppressed (cA) and GAPA antisense (aA)486 lines. (B) CN compared to *gapb* insertion line (308) and GAPB antisense (aB) line lines and487 (C) Photosynthetic carbon fixation of CN compared to single insertion mutants *gapa-1* (164)488 and *gapb* (308) and the double mutant *gapa-1/gapb* (164/308). Extrapolated data are in Table

489 1. Error bars represent SE of 6 plants per line).

490

Figure 4



491

492

493 **Figure 4. Growth analysis of control and experimental lines grown.** (A) Plants were grown494 at $130 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity in short days (8h/16h days) for 52 days. White bar represents

495 a size of 6cm. (B) Plant growth rate evaluated over the first 52 days. Lines co-suppressing

496 GAPA (cA), Controls (CN), *gapa-1* insertion mutant (164) and *gapb* insertion mutant (308) are

497 represented. Results are representative of 9 to 12 plants per line (CN plants include azygous

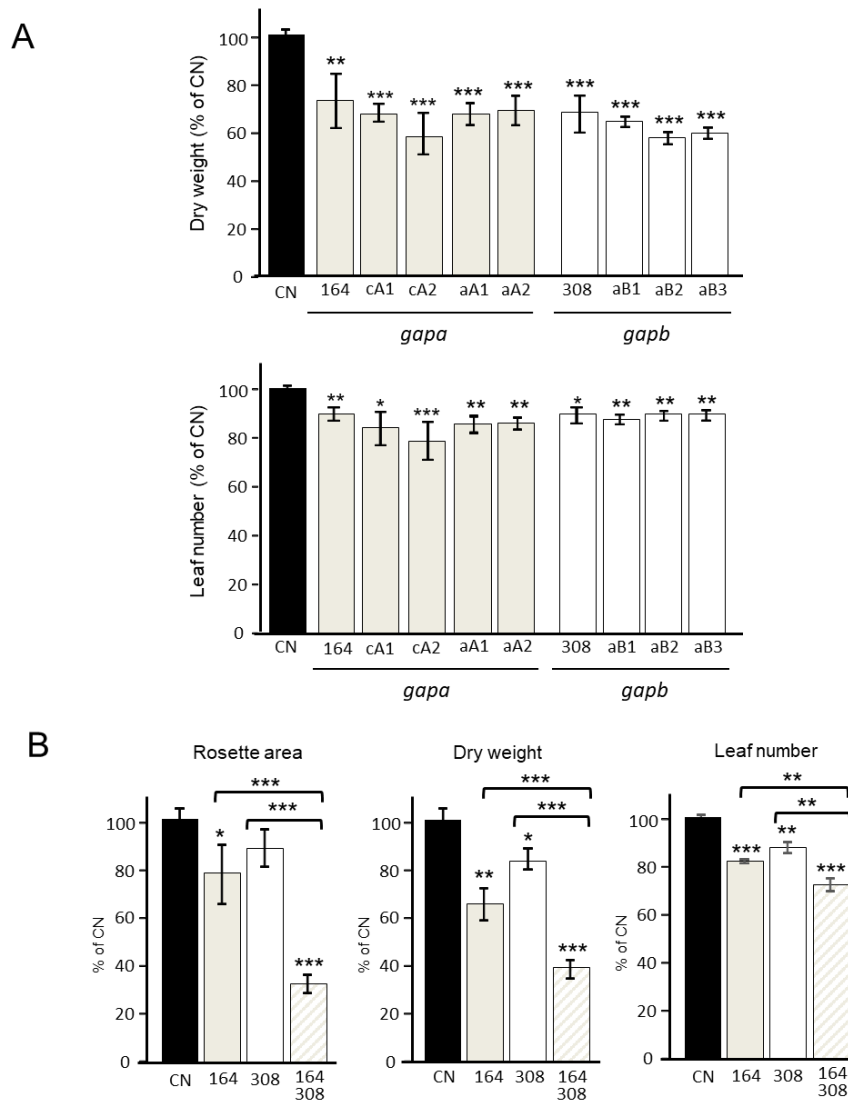
498 lines segregated from primary transformants). Data were statistically analysed using 2-way

499 ANOVA. Significant differences * ($p < 0.10$); ** ($p < 0.05$); *** ($p < 0.01$) are indicated. Unless

500 indicated, results are presented as a percentage of CN (CN = 100%). Error bars represent SE.

501

Figure 5



502

503

504 **Figure 5. Growth analysis of control and experimental lines grown in low light.** (A) *gapa-*505 *l* (164) and *gapb* (308) insertion mutants and GAPA co-suppressed (cA), GAPA antisense (aA)

506 and GAPB (aB) antisense lines were analyzed in parallel. Results are representative of 8 plants

507 per line. (B) *gapa-l* (164) and *gapb* (308) insertion mutants and the double mutant *gapa-l/gapb*

508 (164/308) crosses were evaluated. Results are representative of 9 to 12 plants per line. Plants

509 were grown at $130 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity in short days for 46 days. (CN plants include

510 azygous lines segregated from primary transformants). Data were statistically analysed using

511 2-way ANOVA. Significant differences * ($p < 0.10$); ** ($p < 0.05$); *** ($p < 0.01$) are indicated.

512 Results are presented as a percentage of CN (CN = 100%). Error bars represent SE.

513

514

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