

1 Short title:

2 ***ca1pase* decreases Rubisco abundance and grain yield**

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9 **Overexpression of *ca1pase* decreases Rubisco abundance and grain yield in wheat**

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22 **One Sentence Summary:**

23 *ca1pase* overexpression decreased the content of Rubisco inhibitors and the amount of
24 Rubisco active sites in wheat leaves, with consequent decreases in biomass and grain yield.

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26

27 **Keywords:**

28 CA1Pase, crop yield, gene expression, inhibition, regulation, Rubisco, tight-binding, wheat

29 **FOOTNOTES:**

30 **List of Author Contributions**

31 ECS conceived, designed and supervised the research; PJA and MAJP contributed to the
32 conception of the research; PJA developed the CA1Pase assay; CAS generated the
33 transgenic lines; AKML, DJO and MOG contributed to the experimental design and performed
34 the experiments; AKML analysed the data; AKML and ECS wrote the manuscript with
35 contributions from all authors.

36

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44 **Abstract**

45 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the fixation of CO₂
46 into organic compounds that are used for plant growth and production of agricultural products.
47 Specific sugar-phosphate derivatives bind tightly to the active sites of Rubisco, locking the
48 enzyme in a catalytically inactive conformation. 2-carboxy-D-arabinitol-1-phosphate
49 phosphatase (CA1Pase) dephosphorylates such tight-binding inhibitors, contributing towards
50 maintaining Rubisco activity. The hypothesis that overexpressing *ca1pase* would decrease
51 the abundance of inhibitors, thereby increasing the activity of Rubisco and enhancing
52 photosynthetic performance and productivity was investigated in wheat. Plants of four
53 independent wheat transgenic lines showed up to 30-fold increases in *ca1pase* expression
54 compared to wild-type (WT). Plants overexpressing *ca1pase* had lower quantities of Rubisco
55 tight-binding inhibitors and higher Rubisco activation states than WT, however the amount of
56 Rubisco active sites decreased by 17-60% in the four transgenic lines compared to WT. The
57 lower Rubisco content in plants overexpressing *ca1pase* resulted in lower initial and total
58 carboxylating activities measured in flag leaves at the end of the vegetative stage, and lower
59 aboveground biomass and grain yield measured in fully mature plants. Hence, contrary to
60 what would be expected from our theory, *ca1pase* overexpression caused decreased Rubisco
61 content and compromised wheat grain yields. These results support a possible role for
62 Rubisco inhibitors in protecting the enzyme and maintaining an adequate content of Rubisco
63 active sites available to support carboxylation rates *in planta*.

64 **Introduction**

65 Rates of yield increase for major food crops have recently slowed and in some cases
66 stagnated, spurring efforts to identify novel approaches to reverse this trend (Long et al.,
67 2015). Despite the benefits brought about by breeding programs, together with better farming
68 practices implemented in the last century, current predictions suggest that an increase in
69 agricultural production of 70% will be required to support the projected demand over the
70 coming decades (Ray et al., 2013; Tilman et al., 2011). Global food security will also be
71 increasingly challenged by fluctuations in crop production resulting from climate change (Ray
72 et al., 2015; Tilman & Clark, 2015), for example through altered soil- and plant-atmosphere
73 interactions (Dhankher & Foyer, 2018). The development of high yielding and climate resilient
74 food crops is thus emerging as one of the greatest global challenges to humankind (Long et
75 al., 2015; Paul et al., 2017).

76 Plant growth and biomass production are determined by photosynthetic CO₂
77 assimilation, a process with scope for significant improvement (Zhu et al., 2010). In recent
78 years, improving photosynthesis has emerged as a promising strategy to increase crop yields
79 without enlarging the area of cultivated land (Ort et al., 2015). A number of recent studies have
80 been successful in the use of genetic manipulation of photosynthetic enzymes to improve
81 genetic yield potential by increasing carbon assimilation and biomass production (Nuccio et
82 al., 2015; Simkin et al., 2015; Kromdijk et al., 2016; Driever et al., 2017).

83 Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) catalyses the
84 first step in the Calvin-Benson-Bassham cycle, fixing CO₂ through the carboxylation of RuBP.
85 Modulation of Rubisco activity is complex and involves interaction with many cellular
86 components (see reviews by Andersson, 2008; Parry et al., 2008). We have postulated that
87 the regulation of the carboxylating enzyme in response to the surrounding environment is not
88 optimal for crop production (Carmo-Silva et al., 2015). Estimates from modelling and *in vivo*
89 experimentation suggest that improving the regulation of Rubisco activity has the potential to
90 improve carbon assimilation by as much as 21% (Reynolds et al., 2009; Taylor & Long, 2017).

91 Certain phosphorylated compounds bind tightly to Rubisco active sites, locking the
92 enzyme in a catalytically inactive conformation (see Bracher et al., 2017). These inhibitors
93 include 2-carboxy-D-arabinitol-1-phosphate (CA1P), a naturally occurring Rubisco inhibitor
94 that is produced in the leaves of some plant species under low light or darkness (Gutteridge
95 et al., 1986; Moore & Seeman, 1992). In addition, catalytic misfire (i.e. the low frequency but
96 inexorable occurrence of side reactions within the catalytic site of Rubisco, described by
97 Pearce, 2006) occurs during the multistep carboxylase and oxygenase reactions catalysed by
98 Rubisco. These side reactions lead to production of phosphorylated compounds that resemble
99 the substrate RuBP and/or reaction intermediates. Misfire products, including xylulose-1,5-
100 bisphosphate (XuBP) and D-glycero-2,3-pentodiulose-1,5-bisphosphate (PDBP), bind tightly

101 to either carbamylated or uncarbamylated active sites inhibiting Rubisco activity (Parry et al.,
102 2008; Bracher et al., 2017).

103 Inhibitor-bound Rubisco active sites are reactivated by the combined activities of
104 Rubisco activase (Rca) and specific phosphatases, such as CA1P phosphatase (CA1Pase)
105 and XuBP phosphatase (XuBPase), in a light-dependent manner. Rca remodels the
106 conformation of active sites to facilitate the release of inhibitors; CA1Pase and XuBPase
107 convert the sugar-phosphate derivatives into non-inhibitory compounds by removing the
108 phosphate group (Andralojc et al., 2012; Bracher et al., 2015).

109 Of all the naturally occurring Rubisco inhibitors, CA1P is the only one known to be
110 actively synthesised, the others being by-products of Rubisco activity. The light/dark regulation
111 of Rubisco activity by CA1P has received considerable attention in a number of studies since
112 the nocturnal inhibitor was first described (Gutteridge et al., 1986; Berry et al., 1987; Holbrook
113 et al., 1992; Moore & Seemann, 1994). Non-aqueous subcellular fractionation (Parry et al.,
114 1999) and metabolic studies (Andralojc et al., 1994, 1996, 2002) have shown that CA1P is
115 produced in the chloroplast by the phosphorylation of 2-carboxy-D-arabinitol (CA) during low
116 light or darkness, whilst CA is derived from the light dependent reactions: $\text{CO}_2 \rightarrow$ (Calvin cycle)
117 \rightarrow FBP (chloroplastic fructose bisphosphate) \rightarrow HBP (hamamelose bisphosphate) \rightarrow 2Pi + H
118 (hamamelose / 2-hydroxymethylribose) \rightarrow CA. CA1P binds tightly to carbamylated Rubisco
119 active sites (Moore & Seemann, 1994). In an ensuing period of illumination, CA1P is released
120 from Rubisco by the action of Rubisco activase, and is then dephosphorylated by CA1Pase in
121 a pH- and redox-regulated process (Salvucci & Holbrook, 1989; Andralojc et al., 2012) to yield
122 the non-inhibitory products, CA and Pi.

123 Some plant species contain only modest amounts of CA1P. For example, Moore et al.
124 (1991) showed that dark-adapted leaves of wheat contain sufficient CA1P to inhibit no more
125 than 7% of the available Rubisco active sites. By contrast, comparable leaves of species from
126 the genera *Petunia* and *Phaseolus* contain sufficient CA1P to occupy all available Rubisco
127 catalytic sites (Moore et al., 1991). Even so, both wheat and *Phaseolus vulgaris* (and all other
128 higher plant species so far investigated) possess the gene for CA1Pase (Andralojc et al.,
129 2012). The presence of the capacity to synthesise and remove CA1P, even in species which
130 do not produce sufficient CA1P to significantly influence whole leaf Rubisco activity, implies
131 that CA1P may be more than a simple regulator of Rubisco activity.

132 Daytime inhibitors of Rubisco activity present in wheat leaves have proven too unstable
133 for detailed study (Keys et al., 1995). However, Andralojc et al. (2012) showed that CA1Pase
134 efficiently dephosphorylates sugar-phosphate derivatives closely related to CA1P, such as 2-
135 carboxy-D-arabinitol 1,5-bisphosphate (CABP) and 2-carboxy-D-ribitol 1,5-bisphosphate
136 (CRBP), and that CA1Pase also appears to dephosphorylate the main contender for diurnal
137 inhibition of Rubisco, PDBP (Kane et al., 1998).

138 *In vitro* experiments provide evidence that CA1P may protect Rubisco from proteolytic
139 breakdown under stress conditions (Khan et al., 1999), in addition to any role it may play as a
140 reversible regulator of Rubisco catalytic activity. However, the *in vivo* significance of this
141 potential protective role is unknown. Most published studies have focused on the *in vitro*
142 regulation of Rubisco activity by inhibitors and CA1Pase (Berry et al., 1987; Parry et al., 1997;
143 Kane et al., 1998; Khan et al., 1999; Andralojc et al., 2012). Charlet et al. (1997) showed that
144 CA1Pase abundance is species-specific but generally represents less than 0.06% of the leaf
145 total protein concentration.

146 In the present study, we investigated the hypothesis that overexpression of *ca1pase*
147 would lower the content of Rubisco inhibitors and, consequently, increase Rubisco activation
148 state, Rubisco activity, CO₂ assimilation and grain yield production. We demonstrate that
149 *ca1pase* overexpression does decrease the quantity of Rubisco inhibitors *in vivo*, but also
150 decreases the number of Rubisco active sites in wheat leaves, together with decreased
151 biomass production and grain yield. These results imply that the multiple elements involved in
152 the regulation of Rubisco activity must be carefully balanced during attempts to improve crop
153 productivity by genetically engineering this complex photosynthetic enzyme.

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155

156 **Results**

157 *Transgenic wheat lines overexpressing ca1pase*

158 Wheat transgenic lines overexpressing the native gene for 2-carboxy-D-arabinitol-1-
159 phosphate phosphatase (CA1Pase) were produced. Based on results from a preliminary
160 experiment with 15 independent lines overexpressing (OE) *ca1pase* (first generation, T₁) to
161 test for presence of the transgene and enhanced CA1Pase activity (data not shown), four lines
162 (OE1-OE4) were selected for further analysis and grown alongside WT plants (Fig. 1A). Based
163 on the presence of the transgene in all the plants investigated, lines OE1 and OE3 were
164 identified as likely homozygous, while lines OE2 and OE4 were verified to be heterozygous
165 (Table 1). For the subsequent analyses, a total of 7-10 plants containing the gene of interest
166 were used for each OE line. The five plants that were negative for the presence of the
167 transgene (azygous, AZY) were used as an additional negative control and showed a
168 phenotype similar to the WT plants.

169 The expression of *ca1pase* relative to WT strongly increased in wheat transgenic lines
170 engineered to overexpress the native gene (OE1-OE4), and was greatest in the OE3 plants
171 (31-fold increase; Fig. 1B). The activity of CA1Pase was greater in both OE3 and OE4 plants
172 compared to WT, by 58% and 36%, respectively (Fig. 2A). In OE1 and OE2 plants, whilst the
173 mean value of CA1Pase activity was higher compared to WT plants, this difference was not

174 statistically significant (Fig. 2A). On the other hand, the quantity of Rubisco tight-binding
 175 inhibitors present in the leaves was significantly lower in OE1, OE3 and OE4 compared to WT
 176 plants (with decreases of 35-50%), while no significant difference was observed between OE2
 177 and WT plants (Fig. 2B).

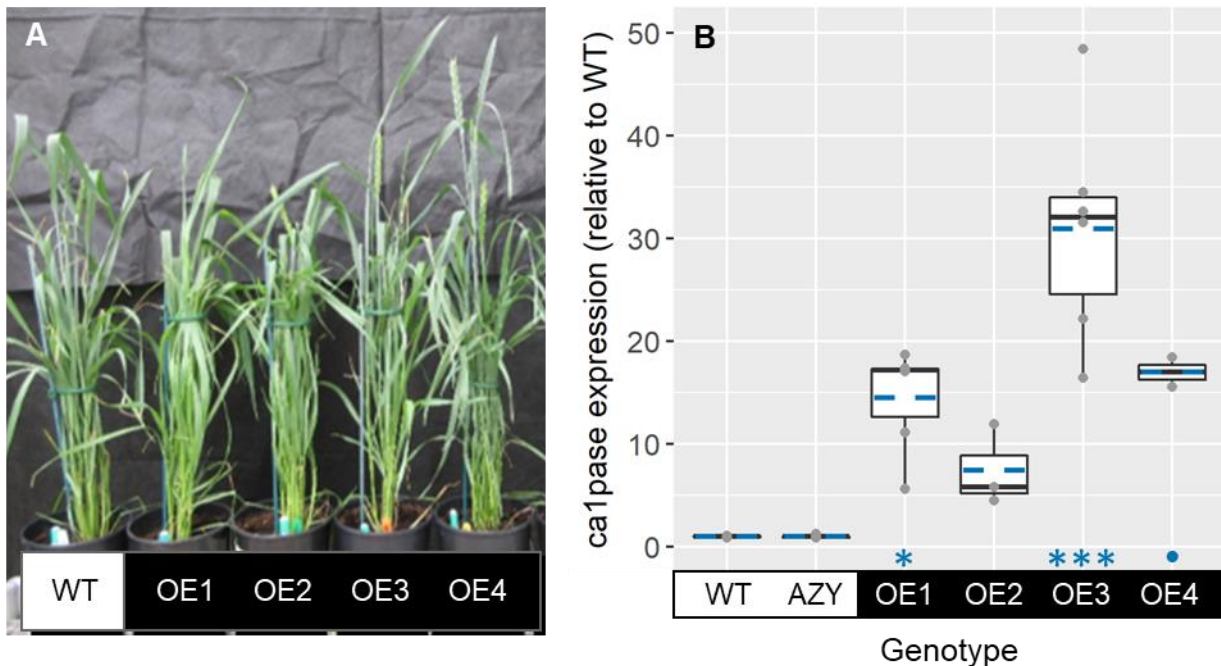
178

179 **Table 1.** Qualitative PCR analysis to verify the presence of the transgene for overexpression
 180 of *ca1pase*. In addition to the experiment described in this manuscript (experiment 2), a
 181 previous experiment was conducted and showed identical results (experiment 1). Of the 10
 182 plants investigated per line, the transgene was present in all plants in lines OE1 and OE3
 183 (likely homozygous), while it was only present in 6-8 plants of the lines OE2 and OE4
 184 (heterozygous).

Transgenic line	Number of plants containing the transgene		Zygoty
	Experiment 1	Experiment 2	
WT	0/10	0/10	Negative control
OE1	10/10	10/10	Likely homozygous
OE2	6/10	7/10	Heterozygous
OE3	10/10	10/10	Likely homozygous
OE4	7/10	8/10	Heterozygous

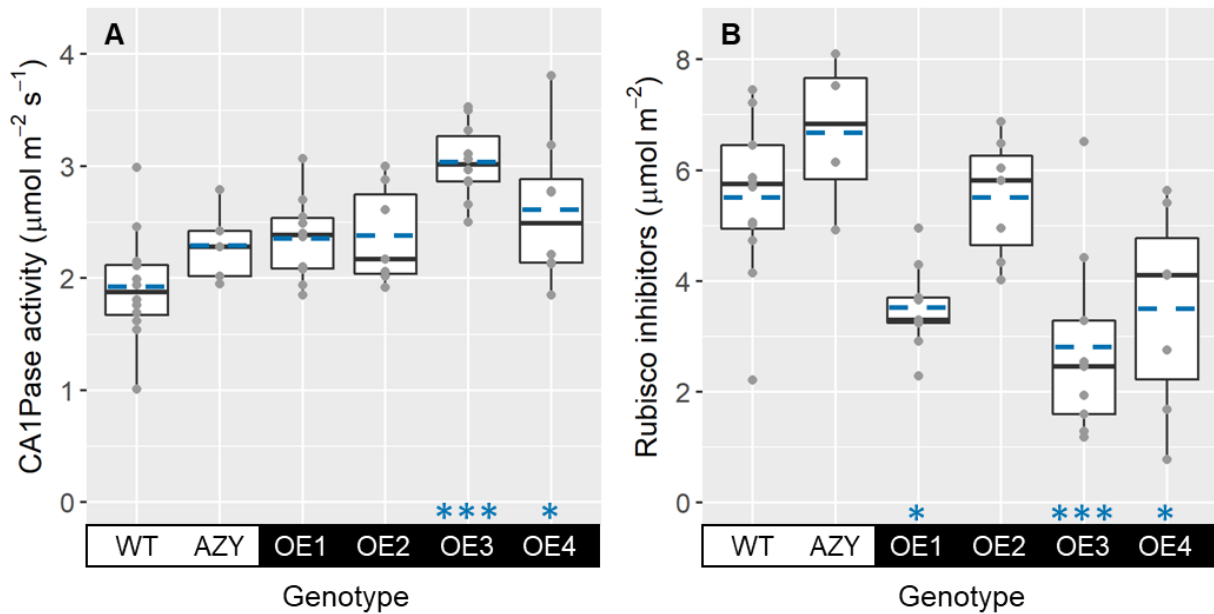
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187

188 **Figure 1.** Wheat transgenic lines overexpressing *ca1pase*. (A) Plants grown under well-
 189 watered conditions in a greenhouse. Measurements and pictures were taken before
 190 anthesis. (B) Relative expression of *ca1pase* in wild type plants (WT), negative controls
 191 (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the
 192 median, first and third quartiles, whiskers represent the range; symbols represent individual
 193 samples and dashed blue lines represent the mean (n = 2-6 biological replicates). There was
 194 a significant effect of genotype on *ca1pase* expression (ANOVA, p < 0.001). Significant
 195 differences between each OE line and WT are denoted as: • p ≤ 0.1; * p ≤ 0.05; *** p ≤ 0.001
 196 (Tukey HSD).



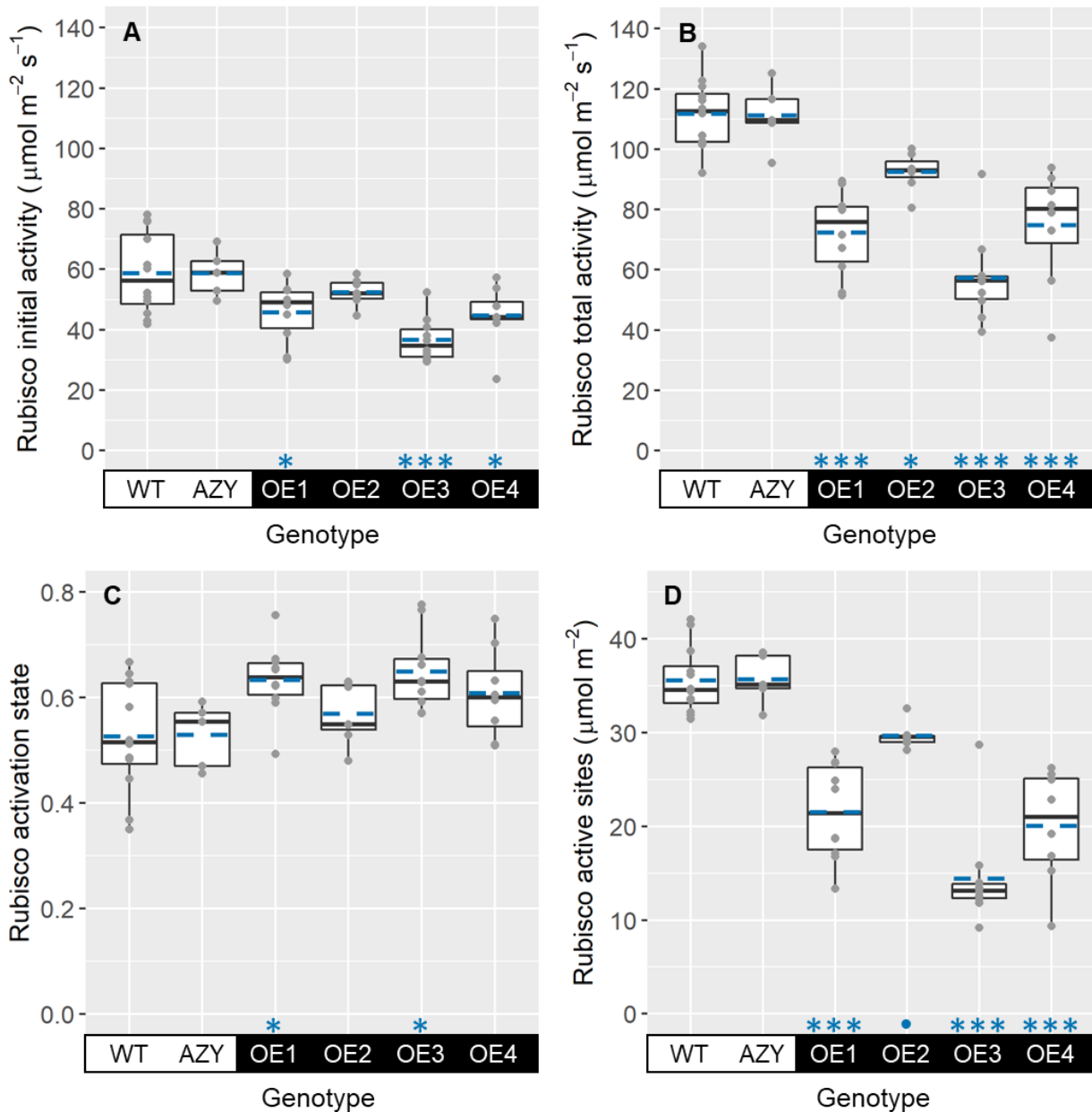
197 **Figure 2.** CA1Pase activity (A) and quantity of Rubisco tight-binding inhibitors (B) in flag
 198 leaves of wheat wild type plants (WT), negative controls (AZY), and transgenic lines
 199 overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and third quartiles,
 200 whiskers represent the range; symbols represent individual samples and dashed blue lines
 201 represent the mean (n = 4-12 biological replicates). There was a significant effect of genotype
 202 on CA1Pase activity and Rubisco inhibitors (ANOVA, p < 0.001). Significant differences
 203 between each OE line and WT are denoted as: * p ≤ 0.05; *** p ≤ 0.001 (Tukey HSD).
 204
 205

206
 207 *Overexpression of ca1pase decreased Rubisco amount and activity, and affected plant*
 208 *biomass and grain yield*

209 The activity of Rubisco measured immediately upon extraction of the enzyme from flag leaves
 210 (initial activity) and after incubation of the enzyme with CO₂ and Mg²⁺ to allow for carbamylation
 211 of active sites (total activity) was significantly lower in plants overexpressing *ca1pase*
 212 compared to WT (Fig. 3A, 3B). The decrease in activity compared to WT plants was most
 213 marked in the transgenic line with highest expression of *ca1pase*, OE3 (Fig. 1B). Moreover,
 214 total activity decreased to a greater extent than initial activity; Rubisco initial activity in OE3
 215 plants decreased by 38% compared to WT, while total activity showed a more marked 49%
 216 decrease. Consequently, the activation state of Rubisco, as measured by the ratio of initial
 217 and total activities was 23% higher in OE3 plants compared to WT plants (Fig. 3C); a similar
 218 increase in Rubisco activation state was observed for the other homozygous line
 219 overexpressing *ca1pase*, OE1 (Table 1).

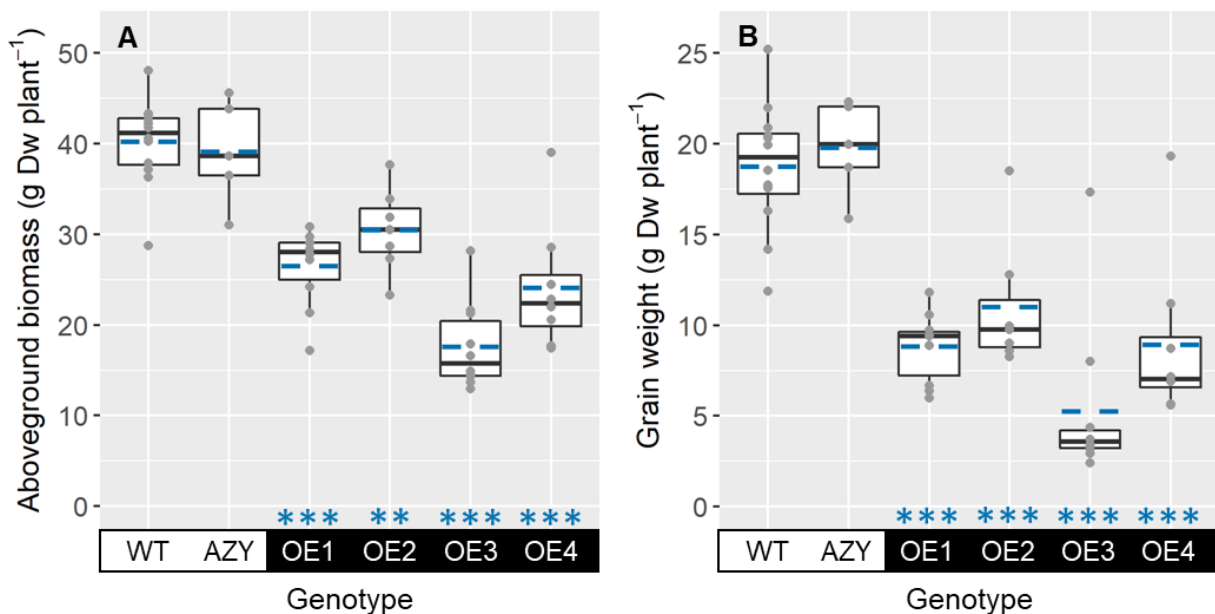
220 The amount of Rubisco protein (Supplementary Fig. S1A), and consequently the
 221 amount of Rubisco active sites (Fig. 3D) decreased in all lines overexpressing *ca1pase*
 222 compared to the WT, with the decrease being greatest in OE3 plants (60% lower than WT).
 223 These results imply that Rubisco activity (Fig. 3A, 3B) was negatively regulated primarily by

224 its reduced amount in plants with higher CA1Pase activity and lower amounts of inhibitors of
 225 Rubisco activity (Fig. 2). The decrease in the amount of Rubisco in *ca1pase* overexpressing
 226 plants was accompanied by decreases in total soluble protein (up to 25% lower than WT;
 227 Supplementary Fig. S1B).
 228



229 **Figure 3.** Rubisco initial (A) and total (B) activities, Rubisco activation state (C) and Rubisco
 230 active sites content (D) in flag leaves of wheat wild type plants (WT), negative controls (AZY),
 231 and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first
 232 and third quartiles, whiskers represent the range; symbols represent individual samples and
 233 dashed blue lines represent the mean (n = 5-12 biological replicates). There was a significant
 234 effect of genotype on Rubisco initial activity (ANOVA, $p < 0.001$), total activity (ANOVA, $p <$
 235 0.001), activation state (ANOVA, $p < 0.01$), and active sites content (ANOVA, $p < 0.001$).
 236 Significant differences between each OE line and WT are denoted as: * $p \leq 0.1$; * $p \leq 0.05$; **
 237 $p \leq 0.01$; *** $p \leq 0.001$ (Tukey HSD).
 238
 239

240 In addition to the downregulation of Rubisco content and activity in wheat flag leaves
 241 in plants overexpressing *ca1pase* (Fig. 3), significant genotypic effects were also observed for
 242 total aboveground biomass and grain yield at full maturity (Fig. 4). All the transgenic lines
 243 overexpressing *ca1pase* had significantly reduced aboveground biomass and grain yield
 244 compared to WT plants. OE3 plants showed the greatest decreases in biomass (56% lower
 245 than WT) and grain yield (72% lower than WT). The proportion of biomass allocated to the
 246 grain, which is represented by the harvest index, was highly variable (large standard deviation)
 247 and not significantly different in the OE lines compared to the WT (Fig. S2A). However, grain
 248 produced by plants overexpressing *ca1pase* was lighter than in WT plants, as evidenced by
 249 the significant decrease in the weight of a thousand grains in all OE lines (TGW; Fig. S2B),
 250 and more markedly in OE3 (50% lower than WT).
 251



252 **Figure 4.** Aboveground biomass (A) and grain weight (B) in wheat wild type plants (WT),
 253 negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes
 254 represent the median, first and third quartiles, whiskers represent the range; symbols
 255 represent individual samples and dashed blue lines represent the mean (n = 5-12 biological
 256 replicates). There was a significant effect of genotype on aboveground biomass and grain
 257 weight (ANOVA, p < 0.001). Significant differences between each OE line and WT are denoted
 258 as: ** p ≤ 0.01; *** p ≤ 0.001 (Tukey HSD).
 259
 260

261 In keeping with the observations for OE3 (Fig. 1-4), a correlation analysis across wild-
 262 type, azygous and transgenic plants highlighted significant correlations between *ca1pase*
 263 expression, Rubisco biochemistry and plant productivity (Fig. S3). As predicted by our
 264 hypothesis, the expression of *ca1pase* in wheat wild-type and transgenic CA1Pase lines was
 265 positively correlated with CA1Pase activity and Rubisco activation state, and negatively
 266 correlated with Rubisco inhibitor content. However, a negative correlation with *ca1pase*

267 expression was also observed for Rubisco active site content, Rubisco initial and total activity,
268 aboveground biomass and grain yield.

269

270

271 **Discussion**

272 Wheat plants with increased expression of CA1Pase were generated and their impact on the
273 regulation and abundance of Rubisco and on crop yield was investigated. We had expected
274 that reducing the abundance of Rubisco inhibitors (by overexpressing *ca1pase*) would
275 increase the activity of Rubisco and positively impact crop productivity. Our results show the
276 contrary: that overexpression of *ca1pase* downregulates Rubisco activity *in planta* by
277 decreasing the amount of this enzyme, and that this negatively affects wheat yield.

278 The greatest level of *ca1pase* overexpression was observed in transgenic plants of the
279 line OE3 (Fig. 1), which was one of the two lines likely to be homozygous for this trait (Table
280 1). OE3 plants also showed a highly significant increase in CA1Pase activity and a highly
281 significant decrease in the content of inhibitors of Rubisco activity in the light (Fig. 2). CA1P
282 has been shown to be present in very small amounts in dark-adapted leaves of wheat,
283 especially when compared to CA1P accumulating leaves of French bean (Moore et al., 1991).
284 In contrast, the measured content of alternative inhibitors of Rubisco activity known to occur
285 during the day was equivalent in wheat and French bean (Keys et al., 1995). Given the ability
286 of CA1Pase to dephosphorylate compounds other than CA1P, including diurnal inhibitors of
287 Rubisco activity (Andralojc et al., 2012) it is likely that the lower content of Rubisco inhibitors
288 in illuminated leaves of OE3 plants was a consequence of increased CA1Pase activity
289 dephosphorylating both CA1P and other sugar-phosphate derivatives (Fig. 2, Supplementary
290 Figure S3).

291 In agreement with our hypothesis, OE3 plants had lower amounts of Rubisco inhibitors
292 and a higher Rubisco activation state than WT plants. However, and contrary to our prediction,
293 the amount and measurable activity of Rubisco was greatly reduced, and grain yield was
294 negatively impacted. In fact, all four *ca1pase* overexpression lines showed significant
295 decreases in Rubisco active sites and total activity in the wheat flag leaf (Fig. 3), as well as
296 significant decreases in aboveground biomass and grain yield (reduced by up to 72%
297 compared to WT plants, Fig. 4). Moreover, a strong negative correlation was observed
298 between *ca1pase* expression, Rubisco active sites content and grain yield (Fig. S3). Increased
299 Rubisco activation state in some of the *ca1pase* overexpression lines partially compensated
300 for the decrease in the content of Rubisco active sites, such that Rubisco initial activity did not
301 significantly correlate with *ca1pase* expression. A negative correlation between Rubisco
302 activation state and amount has been reported in multiple studies (see Carmo-Silva et al.,
303 2015 and references therein). For example, this negative correlation was observed in the flag

304 leaves of 64 UK field-grown UK wheat cultivars (Carmo-Silva et al., 2017). In that study,
305 Rubisco accounted for over 50% of the total soluble leaf protein, and the amount of Rubisco
306 and soluble protein in the leaves decreased as leaves aged, consistent with Rubisco becoming
307 a source of fixed nitrogen for the developing grain (Hirel & Gallais, 2006).

308 The amount of a given protein in a leaf reflects the balance between its synthesis and
309 degradation (Li et al., 2017). Rubisco is synthesised at fast rates compared to other leaf
310 proteins (Piques et al., 2009). In rice, Rubisco synthesis has been shown to occur at fast rates
311 while degradation is minimal until just before the leaf reaches full expansion (Mae et al., 1983;
312 Makino et al., 1984; Suzuki et al., 2001). In wheat plants under normal metabolic conditions,
313 i.e. in the absence of stress and before the onset of senescence, Rubisco is continuously
314 degraded at a slow rate compared to other leaf proteins (Esquível et al., 1998). The
315 degradation of Rubisco in *Arabidopsis thaliana* rosettes has been estimated to occur at a
316 similar rate (0.03-0.08 d⁻¹) to that of the total pool of leaf proteins, with a resulting similar
317 protein half-life of ~3.5 d (Ishihara et al., 2015; Li et al., 2017). A mathematical model
318 developed by Irving & Robinson (2006) suggested that, in cereal leaves, Rubisco degradation
319 is a simple process that follows first-order kinetic principles and is unlikely to be tightly
320 regulated. On the other hand, translation of both the large and small subunits of Rubisco is
321 tightly coordinated and rapidly adjusted in response to environmental cues (Winter &
322 Feierabend, 1990). This would suggest that the synthesis, rather than degradation of Rubisco,
323 could be impaired in wheat plants overexpressing *ca1pase* (Irving & Robinson, 2006; Hirel &
324 Gallais, 2006).

325 Evidence suggests that altering the interactions between Rubisco and its molecular
326 chaperone Rubisco activase would be a credible strategy to optimise the regulation of Rubisco
327 for enhanced biomass production in the model plant *Arabidopsis thaliana* grown under
328 fluctuating light environments (Carmo-Silva & Salvucci, 2013). In wheat, the response of
329 Rubisco activation to increases in irradiance has been predicted to limit carbon assimilation in
330 light fluctuating environments by up to 21% (Taylor & Long, 2017). These studies indicate that
331 speeding the adjustment of Rubisco activity when a leaf transitions from being shaded to being
332 fully illuminated by sunlight in a canopy could result in significant crop yield increases. Similar
333 to the results reported herein for wheat plants overexpressing *ca1pase*, rice plants
334 overexpressing Rubisco activase had higher Rubisco activation state but lower Rubisco
335 quantity than WT (Fukayama et al., 2012; 2018). The decreased amounts of Rubisco in rice
336 were not due to changes in the transcription of genes encoding the Rubisco subunits (*rbcL*,
337 and *RbcS*) or genes encoding chaperones that assist in Rubisco folding and assembly (*RAF1*,
338 *RAF2*, *BSD2*, *RbcX*), suggesting that Rubisco amount was modulated by post-translational
339 factors (Fukayama et al., 2012; 2018). Further research is warranted to examine the
340 hypothesis that the lower amounts of tight-binding phosphorylated compounds in the OE

341 plants may render Rubisco more susceptible to proteolytic breakdown (Khan et al., 1999),
342 thereby enhancing the rate of degradation of the enzyme when plants reach full maturity or
343 experience environmental stress (Suzuki et al., 2001; Ishida et al., 2014).

344 CA1Pase has been shown to represent a very small proportion of the total leaf protein
345 fraction, even in *Phaseolus vulgaris*, a species which has some of the highest amounts of
346 CA1P and of CA1Pase among the plant species studied to date (Moore et al., 1995; Charlet
347 et al., 1997). The same authors showed that measurable CA1Pase activity in wheat (*T.*
348 *aestivum*) is less than 10% of that observed in *P. vulgaris* (Charlet et al., 1997). The negative
349 effects of *ca1pase* overexpression reported herein suggest that the low abundance of
350 CA1Pase in wheat may have been selected for alongside the relatively large allocation of N
351 to Rubisco in wheat leaves (Carmo-Silva et al., 2015, 2017; Evans & Clarke, 2019). Significant
352 natural variation in the amount of CA1P and CA1Pase activity has been reported between
353 species and within genera (Vu et al., 1984; Seeman et al., 1985; Moore et al., 1991). Of
354 particular interest in terms of crop improvement is that even amongst cultivars of soybean and
355 rice as much as 50% variation has been reported in Rubisco inhibition attributed to CA1P
356 binding (Bowes et al., 1990). This raises the prospect that similar genetic variation in the extent
357 of Rubisco inhibition by phosphorylated compounds may exist in wheat.

358 That *ca1pase* overexpression diminished the amount of Rubisco active sites in wheat
359 suggests that genetic manipulation of enzymes involved in the regulation of Rubisco may have
360 unexpected consequences, such as downregulation of Rubisco active sites content. Further
361 studies to better understand the complexity of Rubisco regulation and genetic variation in the
362 underlying components that affect the activity and content of the carboxylating enzyme will
363 enable a more targeted approach to improve crop yields and resilience to climate change.

364

365

366 **Materials and Methods**

367 *Production of wheat CA1Pase transgenic lines*

368 Wheat (*Triticum aestivum* L. cv Cadenza) was used for overexpression (OE) of 2-carboxy-D-
369 arabinitol-1-phosphate phosphatase (CA1Pase). Plant transformation was carried out by
370 biolistics, as described by Sparks & Jones (2014). To produce the CA1Pase OE construct, the
371 full-length *ca1pase* cDNA of the wheat D genome was cloned into a vector containing a maize
372 ubiquitin promoter plus intron, previously shown to drive strong constitutive expression in
373 wheat (Christensen & Quail, 1996) and nopaline synthase (*nos*) terminator sequences, to give
374 pRRes14.ca1pase (Supplementary Fig. S4).

375 The OE construct was co-bombarded with a construct carrying the *bar* selectable
376 marker gene under control of the maize ubiquitin promoter plus intron with a nopaline synthase
377 (*nos*) terminator sequence, pAHC20 (Christensen & Quail, 1996). Transformed calli were

378 selected in tissue culture using phosphinothricin (PPT), the active ingredient of glufosinate
379 ammonium-based herbicides. Surviving plants were transferred to soil and grown to maturity.
380 The presence of the transgene was confirmed by PCR using primers as described in
381 Supplementary Table S1. The transformation process generated 15 OE lines; resulting T₁
382 plants of each transgenic line were allowed to self-pollinate to produce the T₂ generation,
383 which was used in this study. Transformed plants were selected by screening for gene
384 presence and expression using qualitative PCR analysis (Supplementary Table S1). Four
385 independent T₂ lines (OE1-OE4) were selected based on enhanced CA1Pase activity in earlier
386 experiments with T₁ and T₂ plants.

387

388 *Plant growth conditions*

389 Plants were grown in semi-controlled conditions in a glasshouse at the Lancaster Environment
390 Centre with minimum temperatures set to 24°C day / 18°C night. The observed maximum daily
391 temperatures were typically higher than 24°C and occasionally exceeded 30°C on very sunny
392 days. Photoperiod was set to 16 h, with supplemental lighting provided when external light
393 levels fell below 200 μmol m⁻² s⁻¹. Seeds were sown on 27th June 2017 into 3 L round pots,
394 with a 3:1 mixture of special wheat mix growth media (Petersfield compost, Hewitt & Son Ltd.,
395 Cosby, UK) and silver sand (Kelkay Horticultural Silver Sand, RHS, UK). Initial experiments
396 tested the pot size and medium composition, enabling optimization of the growth conditions.
397 Plants including 12 wild type (WT) and 10 of each transgenic line (OE1-OE4) were distributed
398 according to a split-plot design with equal replicates per genotype. All pots were kept well-
399 watered throughout the experiment.

400 Leaf samples for genotyping were taken from 3-week-old plants. Samples for
401 biochemical analyses were taken from the flag leaf of the main tiller of each plant prior to
402 complete ear emergence (Zadoks 4.5-5.5; Zadoks et al., 1974), collected 4-5 h after the
403 beginning of the photoperiod and rapidly snap-frozen in liquid nitrogen followed by storage at
404 -80°C until analysis.

405

406 *Genotyping to evaluate presence/absence of DNA of interest*

407 Leaf samples were taken from 3-week-old plants, placed directly into wells of a deep 96-well
408 plate (Life Technologies, Paisley, UK) and freeze-dried for two days. Leaf material was ground
409 using a Tissue Lyser (Retsch MM200, Qiagen, Manchester, UK) with two 5 mm ball bearings
410 per well. DNA was extracted following the protocol described by Van Deynze & Stoffel (2006).
411 PCR was completed in 20 μL reactions (as per manufacturer's instructions; GoTaq DNA
412 Polymerase, Promega, Southampton, UK). Primers and PCR conditions are listed in
413 Supplementary Table S1. Positive controls using the plasmid were included. PCR fragments
414 were separated in 0.8% (w/v) agarose gels and visualised in the presence of SYBR safe DNA

415 gel stain (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA). This enabled verification
416 of homozygous lines (OE1 and OE3), and identification of positive versus negative plants for
417 presence of the transgene in the heterozygous lines (OE2 and OE4). The five plants that
418 showed no evidence of presence of the transgene (azygous, AZY) were subsequently used
419 as negative controls alongside the wild type (WT).

420

421 *Quantitative real-time PCR (qRT-PCR)*

422 To evaluate the expression of *ca1pase*, mRNA was extracted using a NucleoSpin® Tri Prep
423 kit (Macherey-Nagel, Düren, Germany) including DNase treatment. RNA concentration and
424 quality were determined via a spectrometer (SpectraStar Nano, BMG Labtech, Aylesbury,
425 UK). A subsample of 1 µg RNA was used for cDNA synthesis using the Precision nanoScript
426™ 2 Reverse Transcription kit (Primer design Ltd., Camberley, UK) according to the
427 manufacturer's instructions. qRT-PCR was performed with the Precision®PLUS qPCR Master
428 Mix kit (Primer design Ltd.) containing cDNA (1:5 dilution) and the primer pair (Supplementary
429 Table S1) in a Mx3005P qPCR system (Stratagene, Agilent Technologies, Stockport, UK).
430 Melting curves were also completed. Primer efficiency was analysed based on a cDNA dilution
431 series with mean primer efficiency estimated using the linear phase of all individual reaction
432 amplification curves and calculated according to Pfaffl (2001). The succinate dehydrogenase
433 (UniGene Cluster ID Ta.2218) and ADP-ribosylation factor (Ta.2291) genes were used as
434 reference genes to normalise gene expression (Paolacci et al., 2009; Evens et al., 2017). The
435 normalized relative quantity (NRQ) of expression was calculated in relation to the cycle
436 threshold (CT) values and the primer efficiency (E) of the target gene (X) and the reference
437 genes (N), based on (Rieu & Powers, 2009): $NRQ = (EX)^{-CT, X} / (EN)^{-CT, N}$.

438

439 *Protein extraction and enzyme activity assays*

440 Total soluble proteins (TSP) were extracted according to Carmo-Silva et al. (2017) with slight
441 modifications. Flag leaf samples were ground in an ice-cold mortar and pestle in the presence
442 extraction buffer (50 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 1 mM EDTA, 2 mM benzamidine,
443 5 mM ε-aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM DTT, 1% (v/v) protease inhibitor
444 cocktail (Sigma-Aldrich Co., St Louis, USA), 1 mM phenylmethylsulphonyl fluoride and 5%
445 (w/v) polyvinylpolypyrrolidone). The homogenate was clarified by centrifugation at 14,000 g
446 for 1 min and 4°C. The supernatant was used to measure Rubisco activities and amount,
447 CA1Pase activity, and TSP concentration (Bradford, 1976).

448 Rubisco activities were determined immediately upon extraction via incorporation of
449 ¹⁴CO₂ into stable sugars as described by Carmo-Silva et al. (2017). The initial activity was
450 initiated by adding supernatant to the reaction mixture: 100 mM Bicine-NaOH pH 8.2, 20 mM
451 MgCl₂, 10 mM NaH¹⁴CO₃ (9.25 kBq µmol⁻¹), 2 mM KH₂PO₄, and 0.6 mM RuBP. For the total

452 activity, extract was incubated with the assay buffer (without RuBP) for 3 min prior to assaying,
453 and the reaction started by addition of 0.6 mM RuBP to the mixture. Reactions were performed
454 at 30°C and quenched after 30 s by addition of 100 µl of 10 M formic acid. To quantify the
455 acid-stable ¹⁴C, assay mixtures were dried at 100°C, the residue re-dissolved in deionized
456 water and mixed with scintillation cocktail (Gold Star Quanta, Meridian Biotechnologies Ltd.,
457 Surrey, UK) prior to liquid scintillation counting (Packard Tri-Carb, PerkinElmer Inc., Waltham,
458 US). All assays were conducted with two analytical replicates. Rubisco activation state was
459 calculated from the ratio (initial activity / total activity) x 100. The amount of Rubisco was
460 quantified in the same supernatant by a [¹⁴C]CABP [carboxyarabinitol-1,5-bisphosphate]
461 binding assay (Whitney et al., 1999).

462 CA1Pase activity was measured by the formation of Pi following the method described
463 by Van Veldhoven & Mannaerts (1987) with modifications as in Andralojc et al. (2012). The
464 assay was initiated by adding supernatant to the reaction mixture: 50 mM Bis-tris propane
465 (BTP) pH 7.0, 200 mM KCl, 1 mM EDTA, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 10
466 mM CaCl₂, 0.5 mg/mL BSA, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 0.5 mM
467 2-carboxy-D-ribitol-1,5-bisphosphate (CRBP). A negative control without CRBP was included.
468 After 60 min, the activity assay was quenched with 1 M trichloroacetic acid (TCA), the mixture
469 was centrifuged at 14,000 g for 3 min to sediment protein residues and the supernatant was
470 mixed with 0.44% (w/v) ammonium molybdate in 1.6 M H₂SO₄ and, after 10 min, 0.035% (w/v)
471 malachite green in 0.35% (w/v) poly(vinyl) alcohol. After 60 min at room temperature, the
472 absorbance at 610 nm was determined and the quantity of Pi calculated based on a standard
473 curve with K₂H₂Pi.

474

475 *Quantification of Rubisco inhibitors*

476 Tight-binding inhibitors of Rubisco activity were quantified as described by Carmo-Silva et al.
477 (2010). Leaf samples were ground to a fine powder in liquid nitrogen and inhibitors extracted
478 following further grinding with 0.45 M trifluoroacetic acid (TFA). After thawing and
479 centrifugation (14,000 g for 5 min at 4°C), a sub-sample of the supernatant (20 µL) was
480 incubated for 5 min with 10 µg of activated wheat Rubisco (previously purified as described
481 by Orr & Carmo-Silva, 2018) in 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂ and 10 mM
482 NaH¹²CO₃. The extent of Rubisco activity inhibition was measured in presence of complete
483 assay buffer with 100 mM Bicine-NaOH pH 8.2, 20 mM MgCl₂, 10 mM NaH¹⁴CO₃ (18.5 kBq
484 µmol⁻¹) and 0.4 mM RuBP. The inhibitor content was determined by reference to a standard
485 curve with known quantities of CA1P in TFA, which had been incubated with activated Rubisco
486 exactly as described above and had been prepared alongside the sample reactions.

487

488 *Biomass and yield traits*

489 Plant aboveground biomass was determined at full physiological maturity (Zadoks 9.1-9.2;
490 Zadoks et al., 1974). Tillers and spikes were counted, vegetative biomass (leaves and stems)
491 were dried at 65°C until constant weight was attained. Ears were threshed (Haldrup LT-15,
492 Haldrup GmbH, Ilshofen, Germany) and a seed subsample of ~3 g was used to determine
493 water content and to estimate the number of seeds using the phone app *SeedCounter*
494 (Komyshev et al., 2017) to calculate the thousand-grain weight (TGW). The harvest index was
495 estimated by the ratio between the dry weights of grain and aboveground biomass per plant.

496

497 *Statistical analysis*

498 One-way analysis of variance (ANOVA) was used to test statistical significance of differences
499 between means of each trait for the six genotypes. Where a significant genotype effect was
500 observed, a Tukey post-hoc test was used for multiple pairwise comparisons. Statistical
501 analyses were performed in R (version 3.3.3; R Core Team, 2016) and RStudio (version
502 1.0.153; RStudio Team, 2015). Box and whiskers plots were prepared using ggplot2
503 (Wickham, 2016): boxes show medians, first and third quartiles (25th and 75th percentiles), and
504 whiskers extend from the hinge to the largest or smallest value, no further than 1.5 * IQR from
505 the hinge (where IQR is the inter-quartile range, or distance between the first and third
506 quartiles). Symbols represent individual data points and dashed lines represent the mean
507 values.

508

509

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512 overexpression in wheat; Dr Rhiannon Page for technical assistance with qPCR; and Prof
513 Christine Foyer for useful discussions on the turnover of Rubisco protein in the chloroplast.

514 **Figure Legends**

515 **Figure 1.** Wheat transgenic lines overexpressing *ca1pase*. (A) Plants grown under well-
516 watered conditions in a greenhouse. Measurements and pictures were taken before anthesis.
517 (B) Relative expression of *ca1pase* in wild type plants (WT), negative controls (AZY), and
518 transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and
519 third quartiles, whiskers represent the range; symbols represent individual samples and
520 dashed blue lines represent the mean (n = 2-6 biological replicates). There was a significant
521 effect of genotype on *ca1pase* expression (ANOVA, $p < 0.001$). Significant differences
522 between each OE line and WT are denoted as: • $p \leq 0.1$; * $p \leq 0.05$; *** $p \leq 0.001$ (Tukey
523 HSD).

524
525 **Figure 2.** CA1Pase activity (A) and quantity of Rubisco tight-binding inhibitors (B) in flag
526 leaves of wheat wild type plants (WT), negative controls (AZY), and transgenic lines
527 overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and third quartiles,
528 whiskers represent the range; symbols represent individual samples and dashed blue lines
529 represent the mean (n = 4-12 biological replicates). There was a significant effect of genotype
530 on CA1Pase activity and Rubisco inhibitors (ANOVA, $p < 0.001$). Significant differences
531 between each OE line and WT are denoted as: * $p \leq 0.05$; *** $p \leq 0.001$ (Tukey HSD).

532
533 **Figure 3.** Rubisco initial (A) and total (B) activities, Rubisco activation state (C) and Rubisco
534 active sites content (D) in flag leaves of wheat wild type plants (WT), negative controls (AZY),
535 and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first
536 and third quartiles, whiskers represent the range; symbols represent individual samples and
537 dashed blue lines represent the mean (n = 5-12 biological replicates). There was a significant
538 effect of genotype on Rubisco initial activity (ANOVA, $p < 0.001$), total activity (ANOVA, $p <$
539 0.001), activation state (ANOVA, $p < 0.01$), and active sites content (ANOVA, $p < 0.001$).
540 Significant differences between each OE line and WT are denoted as: • $p \leq 0.1$; * $p \leq 0.05$; **
541 $p \leq 0.01$; *** $p \leq 0.001$ (Tukey HSD).

542
543 **Figure 4.** Aboveground biomass (A) and grain weight (B) in wheat wild type plants (WT),
544 negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes
545 represent the median, first and third quartiles, whiskers represent the range; symbols
546 represent individual samples and dashed blue lines represent the mean (n = 5-12 biological
547 replicates). There was a significant effect of genotype on aboveground biomass and grain
548 weight (ANOVA, $p < 0.001$). Significant differences between each OE line and WT are denoted
549 as: ** $p \leq 0.01$; *** $p \leq 0.001$ (Tukey HSD).

550

551 **Supplemental Materials**

552 **Figure S1.** Rubisco (A) and total soluble protein content (B) in flag leaves of wheat wild type
553 plants (WT), negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-
554 OE4). Boxes represent the median, first and third quartiles, whiskers represent the range;
555 symbols represent individual samples and dashed blue lines represent the mean (n = 5-12
556 biological replicates). There was a significant effect of genotype on Rubisco and total soluble
557 protein contents (ANOVA, $p < 0.001$). Significant differences between each OE line and WT
558 are denoted as: • $p \leq 0.1$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Tukey HSD).

559

560 **Figure S2.** Harvest index (A) and thousand grain weight (B) in wheat wild type plants (WT),
561 negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes
562 represent the median, first and third quartiles, whiskers represent the range; symbols
563 represent individual samples and dashed blue lines represent the mean (n = 5-12 biological
564 replicates). There was a significant effect of genotype on harvest index and thousand grain
565 weight (ANOVA, $p < 0.001$). Significant differences between each OE line and WT are denoted
566 as: • $p \leq 0.1$; ** $p \leq 0.01$; *** $p \leq 0.001$ (Tukey HSD).

567

568 **Figure S3.** Correlation matrix showing the significance of pairwise linear correlations between
569 *ca1pase* expression, Rubisco biochemistry and plant productivity traits for wild-type, azygous
570 and transgenic wheat plants. Pearson correlation coefficients (r) were computed and
571 visualised in R using the packages *Hmisc* (Harrell et al., 2018) and *corrplot* (Wei & Simko,
572 2017). Circle size and colour intensity are proportional to the correlation coefficient ($p \leq 0.01$;
573 n = 24-52).

574

575 **Figure S4.** Construct used for wheat plant transformation to overexpress *ca1pase*
576 (pRRes14.ca1pase).

577

578 **Table S1.** Primers and PCR conditions used to evaluate presence/absence of the DNA to
579 overexpress *ca1pase* and the selectable marker gene *bar*, and to quantify gene expression.

580

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