Short title:

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

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

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One Sentence Summary:

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

Keywords:

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

FOOTNOTES:

List of Author Contributions

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

Funding

This work was funded by an N8 AgriFood Resilience Programme Pump Priming award from Lancaster University to ECS. Production of the wheat CA1Pase lines was supported by the Rothamsted Research Institute Strategic Program 20:20 Wheat® (BBSRC BB/J/00426X/1).

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Abstract

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the fixation of CO_2 into organic compounds that are used for plant growth and production of agricultural products. Specific sugar-phosphate derivatives bind tightly to the active sites of Rubisco, locking the enzyme in a catalytically inactive conformation. 2-carboxy-D-arabinitol-1-phosphate phosphatase (CA1Pase) dephosphorylates such tight-binding inhibitors, contributing towards maintaining Rubisco activity. The hypothesis that overexpressing *ca1pase* would decrease the abundance of inhibitors, thereby increasing the activity of Rubisco and enhancing photosynthetic performance and productivity was investigated in wheat. Plants of four independent wheat transgenic lines showed up to 30-fold increases in *ca1pase* expression compared to wild-type (WT). Plants overexpressing *ca1pase* had lower quantities of Rubisco tight-binding inhibitors and higher Rubisco activation states than WT, however the amount of Rubisco active sites decreased by 17-60% in the four transgenic lines compared to WT. The lower Rubisco content in plants overexpressing *ca1pase* resulted in lower initial and total carboxylating activities measured in flag leaves at the end of the vegetative stage, and lower aboveground biomass and grain yield measured in fully mature plants. Hence, contrary to what would be expected from our theory, *ca1pase* overexpression caused decreased Rubisco content and compromised wheat grain yields. These results support a possible role for Rubisco inhibitors in protecting the enzyme and maintaining an adequate content of Rubisco active sites available to support carboxylation rates *in planta*.

Introduction

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

Plant growth and biomass production are determined by photosynthetic CO_2 assimilation, a process with scope for significant improvement (Zhu et al., 2010). In recent years, improving photosynthesis has emerged as a promising strategy to increase crop yields without enlarging the area of cultivated land (Ort et al., 2015). A number of recent studies have been successful in the use of genetic manipulation of photosynthetic enzymes to improve genetic yield potential by increasing carbon assimilation and biomass production (Nuccio et al., 2015; Simkin et al., 2015; Kromdijk et al., 2016; Driever et al., 2017).

Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) catalyses the first step in the Calvin-Benson-Bassham cycle, fixing CO_2 through the carboxylation of RuBP. Modulation of Rubisco activity is complex and involves interaction with many cellular components (see reviews by Andersson, 2008; Parry et al., 2008). We have postulated that the regulation of the carboxylating enzyme in response to the surrounding environment is not optimal for crop production (Carmo-Silva et al., 2015). Estimates from modelling and *in vivo* experimentation suggest that improving the regulation of Rubisco activity has the potential to improve carbon assimilation by as much as 21% (Reynolds et al., 2009; Taylor & Long, 2017).

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

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

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

Of all the naturally occurring Rubisco inhibitors, CA1P is the only one known to be actively synthesised, the others being by-products of Rubisco activity. The light/dark regulation of Rubisco activity by CA1P has received considerable attention in a number of studies since the nocturnal inhibitor was first described (Gutteridge et al., 1986; Berry et al., 1987; Holbrook et al., 1992; Moore & Seemann, 1994). Non-aqueous subcellular fractionation (Parry et al., 1999) and metabolic studies (Andralojc et al., 1994, 1996, 2002) have shown that CA1P is produced in the chloroplast by the phosphorylation of 2-carboxy-D-arabinitol (CA) during low light or darkness, whilst CA is derived from the light dependent reactions: CO_2 → (Calvin cycle) → FBP (chloroplastic fructose bisphosphate) → HBP (hamamelose bisphosphate) → 2Pi + H (hamamelose / 2-hydroxymethylribose) → CA. CA1P binds tightly to carbamylated Rubisco active sites (Moore & Seemann, 1994). In an ensuing period of illumination, CA1P is released from Rubisco by the action of Rubisco activase, and is then dephosphorylated by CA1Pase in a pH- and redox-regulated process (Salvucci & Holbrook, 1989; Andralojc et al., 2012) to yield the non-inhibitory products, CA and Pi.

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

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

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

In the present study, we investigated the hypothesis that overexpression of *ca1pase* would lower the content of Rubisco inhibitors and, consequently, increase Rubisco activation state, Rubisco activity, CO_2 assimilation and grain yield production. We demonstrate that *ca1pase* overexpression does decrease the quantity of Rubisco inhibitors *in vivo*, but also decreases the number of Rubisco active sites in wheat leaves, together with decreased biomass production and grain yield. These results imply that the multiple elements involved in the regulation of Rubisco activity must be carefully balanced during attempts to improve crop productivity by genetically engineering this complex photosynthetic enzyme.

Results

Transgenic wheat lines overexpressing ca1pase

Wheat transgenic lines overexpressing the native gene for 2-carboxy-D-arabinitol-1-phosphate phosphatase (CA1Pase) were produced. Based on results from a preliminary experiment with 15 independent lines overexpressing (OE) *ca1pase* (first generation, T_1) to test for presence of the transgene and enhanced CA1Pase activity (data not shown), four lines (OE1-OE4) were selected for further analysis and grown alongside WT plants (Fig. 1A). Based on the presence of the transgene in all the plants investigated, lines OE1 and OE3 were identified as likely homozygous, while lines OE2 and OE4 were verified to be heterozygous (Table 1). For the subsequent analyses, a total of 7-10 plants containing the gene of interest were used for each OE line. The five plants that were negative for the presence of the transgene (azygous, AZY) were used as an additional negative control and showed a phenotype similar to the WT plants.

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

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

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

Transgenic line	Number of plants containing the transgene		Zygosity
	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

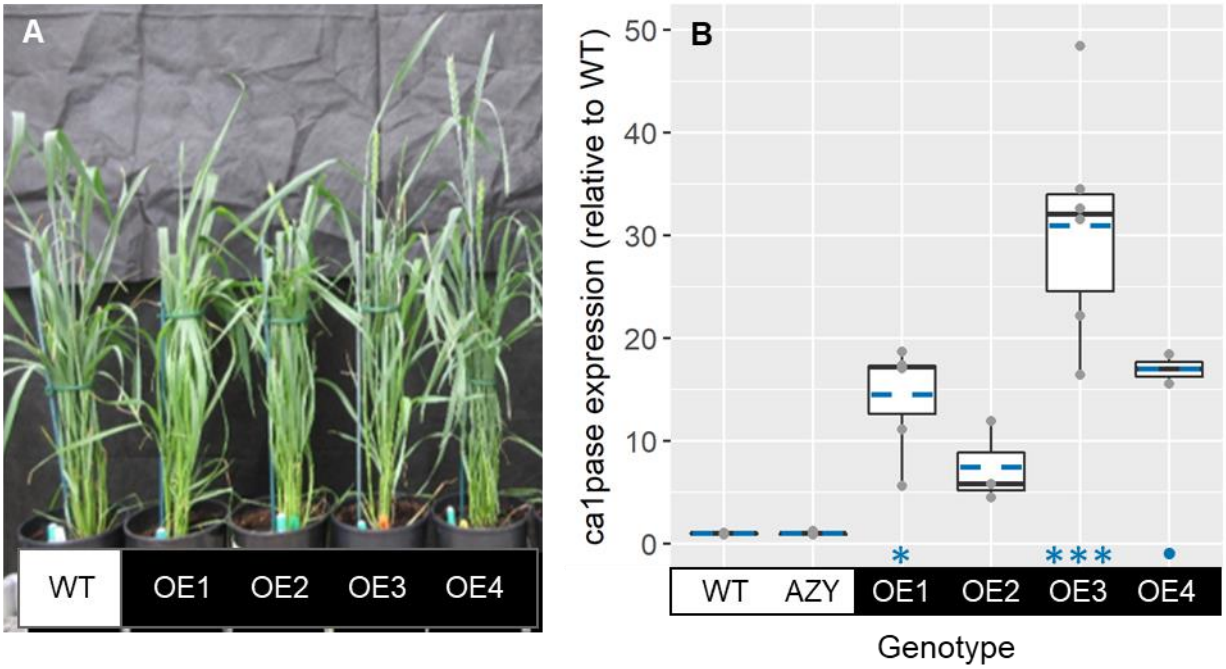

Figure 1. Wheat transgenic lines overexpressing *ca1pase*. (A) Plants grown under well-watered conditions in a greenhouse. Measurements and pictures were taken before anthesis. (B) Relative expression of *ca1pase* in wild type plants (WT), negative controls (AZY), and transgenic lines overexpressing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

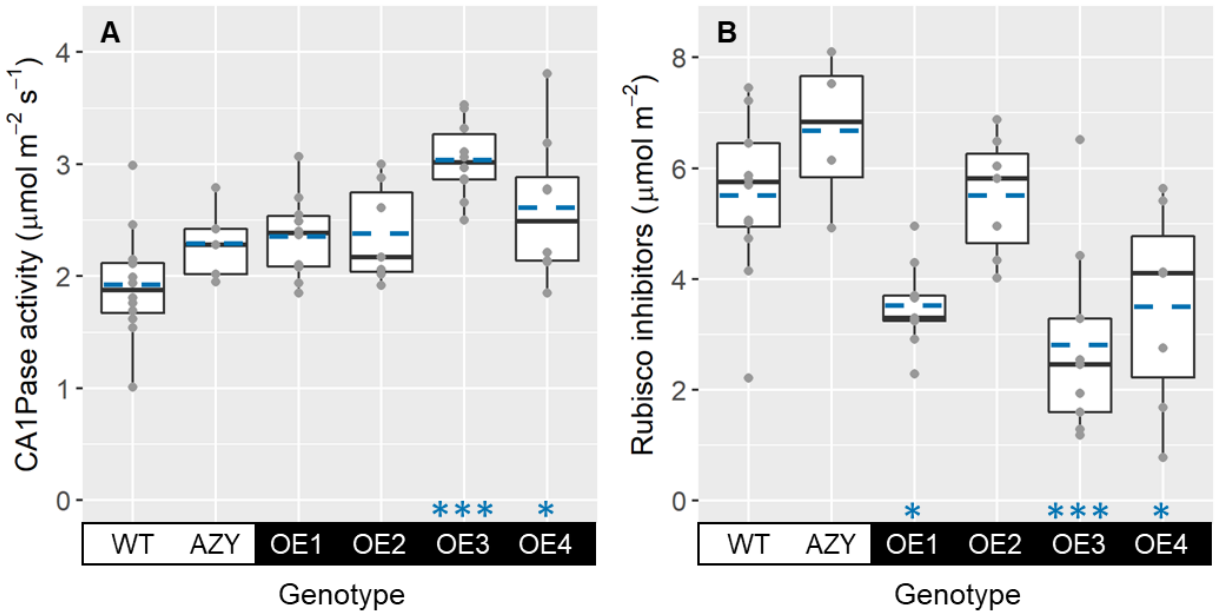

Figure 2. CA1Pase activity (A) and quantity of Rubisco tight-binding inhibitors (B) in flag leaves of wheat wild type plants (WT), negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and third quartiles, whiskers represent the range; symbols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

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

The activity of Rubisco measured immediately upon extraction of the enzyme from flag leaves (initial activity) and after incubation of the enzyme with CO_2 and Mg^{2+} to allow for carbamylation of active sites (total activity) was significantly lower in plants overexpressing *ca1pase* compared to WT (Fig. 3A, 3B). The decrease in activity compared to WT plants was most marked in the transgenic line with highest expression of *ca1pase*, OE3 (Fig. 1B). Moreover, total activity decreased to a greater extent than initial activity; Rubisco initial activity in OE3 plants decreased by 38% compared to WT, while total activity showed a more marked 49% decrease. Consequently, the activation state of Rubisco, as measured by the ratio of initial and total activities was 23% higher in OE3 plants compared to WT plants (Fig. 3C); a similar increase in Rubisco activation state was observed for the other homozygous line overexpressing *ca1pase*, OE1 (Table 1).

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

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

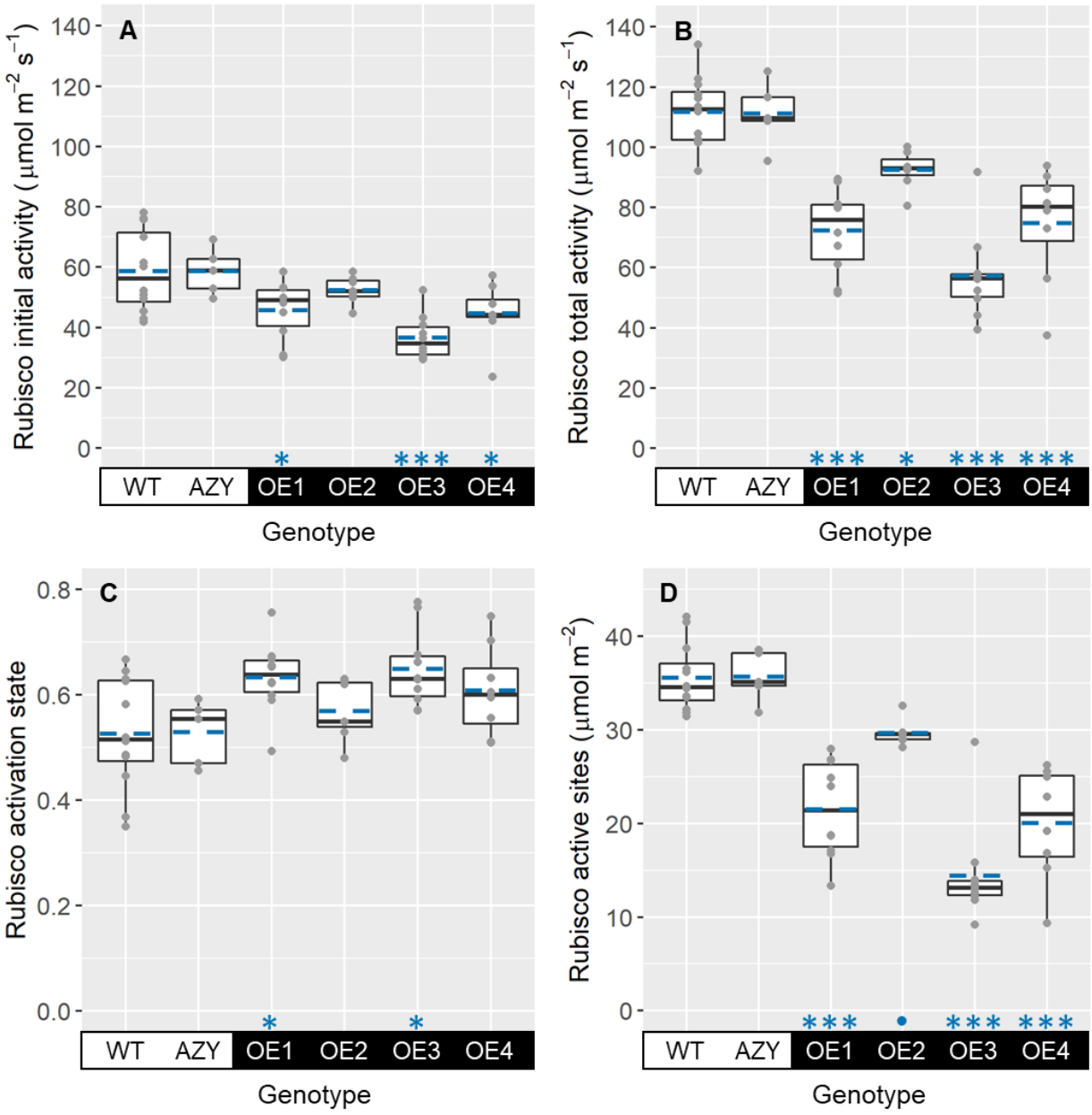

Figure 3. Rubisco initial (A) and total (B) activities, Rubisco activation state (C) and Rubisco active sites content (D) in flag leaves of wheat wild type plants (WT), negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and third quartiles,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

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

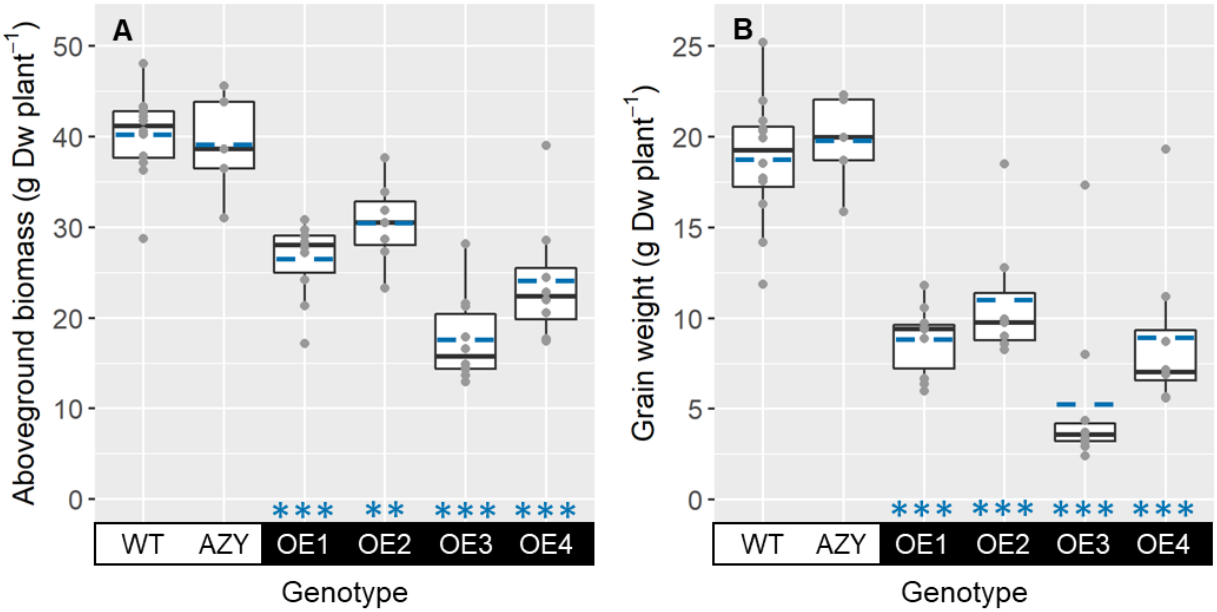

Figure 4. Aboveground biomass (A) and grain weight (B) in wheat wild type plants (WT), negative controls (AZY), and transgenic lines overexpressing *ca1pase* (OE1-OE4). Boxes represent the median, first and third quartiles, whiskers represent the range; symbols represent individual samples and dashed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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Materials and Methods

Production of wheat CA1Pase transgenic lines

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

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

selected in tissue culture using phosphinothricin (PPT), the active ingredient of glufosinate ammonium-based herbicides. Surviving plants were transferred to soil and grown to maturity. The presence of the transgene was confirmed by PCR using primers as described in Supplementary Table S1. The transformation process generated 15 OE lines; resulting T_1 plants of each transgenic line were allowed to self-pollinate to produce the T_2 generation, which was used in this study. Transformed plants were selected by screening for gene presence and expression using qualitative PCR analysis (Supplementary Table S1). Four independent T_2 lines (OE1-OE4) were selected based on enhanced CA1Pase activity in earlier experiments with T_1 and T_2 plants.

Plant growth conditions

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

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

Genotyping to evaluate presence/absence of DNA of interest

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

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

Quantitative real-time PCR (qRT-PCR)

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

Protein extraction and enzyme activity assays

Total soluble proteins (TSP) were extracted according to Carmo-Silva et al. (2017) with slight modifications. Flag leaf samples were ground in an ice-cold mortar and pestle in the presence extraction buffer (50 mM Bicine-NaOH pH 8.2, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM benzamidine, 5 mM ε-aminocaproic acid, 50 mM 2-mercaptoethanol, 10 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich Co., St Louis, USA), 1 mM phenylmethylsulphonyl fluoride and 5% (w/v) polyvinylpolypyrrolidone). The homogenate was clarified by centrifugation at 14,000 *g* for 1 min and 4°C. The supernatant was used to measure Rubisco activities and amount, CA1Pase activity, and TSP concentration (Bradford, 1976).

Rubisco activities were determined immediately upon extraction via incorporation of $^{14}CO_2$ into stable sugars as described by Carmo-Silva et al. (2017). The initial activity was initiated by adding supernatant to the reaction mixture: 100 mM Bicine-NaOH pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (9.25 kBq µmol^{-1}), 2 mM KH_2PO_4, and 0.6 mM RuBP. For the total

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

CA1Pase activity was measured by the formation of Pi following the method described by Van Veldhoven & Mannaerts (1987) with modifications as in Andralojc et al. (2012). The assay was initiated by adding supernatant to the reaction mixture: 50 mM Bis-tris propane (BTP) pH 7.0, 200 mM KCl, 1 mM EDTA, 1 mM ε-aminocaproic acid, 1 mM benzamidine, 10 mM $CaCl_2$, 0.5 mg/mL BSA, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 0.5 mM 2-carboxy-D-ribitol-1,5-bisphosphate (CRBP). A negative control without CRBP was included. After 60 min, the activity assay was quenched with 1 M trichloroacetic acid (TCA), the mixture was centrifuged at 14,000 *g* for 3 min to sediment protein residues and the supernatant was mixed with 0.44% (w/v) ammonium molybdate in 1.6 M H_2SO_4 and, after 10 min, 0.035% (w/v) malachite green in 0.35% (w/v) poly(vinyl) alcohol. After 60 min at room temperature, the absorbance at 610 nm was determined and the quantity of Pi calculated based on a standard curve with K_2HPi.

Quantification of Rubisco inhibitors

Tight-biding inhibitors of Rubisco activity were quantified as described by Carmo-Silva et al. (2010). Leaf samples were ground to a fine powder in liquid nitrogen and inhibitors extracted following further grinding with 0.45 M trifluoroacetic acid (TFA). After thawing and centrifugation (14,000 *g* for 5 min at 4°C), a sub-sample of the supernatant (20 µL) was incubated for 5 min with 10 µg of activated wheat Rubisco (previously purified as described by Orr & Carmo-Silva, 2018) in 100 mM Bicine-NaOH pH 8.2, 20 mM $MgCl_2$ and 10 mM $NaH^{12}CO_3$. The extent of Rubisco activity inhibition was measured in presence of complete assay buffer with 100 mM Bicine-NaOH pH 8.2, 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (18.5 kBq μmol^{-1}) and 0.4 mM RuBP. The inhibitor content was determined by reference to a standard curve with known quantities of CA1P in TFA, which had been incubated with activated Rubisco exactly as described above and had been prepared alongside the sample reactions.

Biomass and yield traits

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

Statistical analysis

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

Acknowledgments

The authors thank Dr Pippa Madgwick for designing the construct for CA1Pase overexpression in wheat; Dr Rhiannon Page for technical assistance with qPCR; and Prof Christine Foyer for useful discussions on the turnover of Rubisco protein in the chloroplast.

Figure Legends

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

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

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

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

Supplemental Materials

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

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

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

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

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

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