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An isoleucine residue acts as a thermal and regulatory switch in wheat Rubisco activase

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SUMMARY

The regulation of Rubisco, the gatekeeper of carbon fixation into the biosphere, by its molecular chaperone Rubisco activase (Rca) is essential for photosynthesis and plant growth. Using energy from ATP hydrolysis, Rca promotes the release of inhibitors and restores catalytic competence to Rubisco-active sites. Rca is sensitive to moderate heat stress, however, and becomes progressively inhibited as the temperature increases above the optimum for photosynthesis. Here, we identify a single amino acid substitution (M159I) that fundamentally alters the thermal and regulatory properties of Rca in bread wheat (*Triticum aestivum* L.). Using site-directed mutagenesis, we demonstrate that the M159I substitution extends the temperature optimum of the most abundant Rca isoform by 5°C *in vitro*, while maintaining the efficiency of Rubisco activation by Rca. The results suggest that this single amino acid substitution acts as a thermal and regulatory switch in wheat Rca that can be exploited to improve the climate resilience and efficiency of carbon assimilation of this cereal crop as temperatures become warmer and more volatile.

Keywords: Rubisco, Rubisco activase, heat stress, photosynthesis, food security, wheat, *Triticum aestivum*.

INTRODUCTION

Photosynthesis is the entry point of carbon into the biosphere. Several processes of carbon fixation have been identified as operating below their theoretical maximum, however, thereby limiting the efficiency of photosynthesis (Ort *et al.*, 2015). Therefore, strategies to optimize or redesign photosynthesis represent a promising opportunity to increase crop yields sustainably. Enhancing the efficiency of photosynthesis would lead to increased biomass production with equal or lower resource inputs, thereby helping to meet the rising food demands of a growing world population and the shift to higher calorific diets (Asseng *et al.*, 2015), while minimising the negative impact of agriculture on the environment.

Triticum aestivum L. (bread wheat) is one of the world's most important crops, providing over 20% of the calories consumed by humanity (Ray *et al.*, 2013), and in 2017 accounted for 15% of the global harvested agricultural area (FAOSTAT, 2019). Modern domesticated wheat is a temperate hexaploid cereal crop that emerged as a result of the natural hybridization of three grasses (Mayer *et al.*, 2014). It grows optimally at 22°C, and temperatures exceeding 25°C have been found to negatively impact wheat growth and productivity (Porter and Gawith, 1999). Decreased carbon assimilation has also been reported in wheat above this thermal threshold (Silva-Pérez *et al.*, 2017). Worryingly, climate resilience in European wheat has declined in recent years (Kahiluoto *et al.*, 2019) and climate change has already affected global wheat yields negatively (Asseng *et al.*, 2013; Ray *et al.*, 2019). Thus, efforts to increase wheat crop yields must be accompanied by improvements in yield stability during environmental oscillations (Pennacchi *et al.*, 2018).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) plays a central role in carbon assimilation. Prior to the binding of ribulose-1,5-bisphosphate (RuBP) and subsequent reaction with the gaseous substrate, CO_2 or O_2, Rubisco catalytic sites must be carbamylated, via binding of non-substrate CO_2 and Mg^{2+} to form a stable carbamate (Lorimer and Miziorko, 1980). In addition, Rubisco activity *in vivo* is constrained by naturally occurring inhibitory phosphorylated compounds, including the substrate RuBP when it binds to uncarbamylated active sites. A number of other sugar-phosphate derivatives have been described to inhibit Rubisco, including D-xylulose-1,5-bisphosphate (XuBP), a product of RuBP misprotonation that accumulates at elevated temperatures (Zhu and Jensen, 1991; Schrader *et al.*, 2006). To restore catalytic competence to Rubisco, inhibitory compounds must be removed from active sites by Rubisco activase (Rca). Rca is a

member of the AAA+ protein family and uses energy from ATP hydrolysis to promote the release of such inhibitors (Salvucci *et al.*, 1985; Salvucci and Ogren, 1996). The activity of Rca is regulated by the redox status, the ADP:ATP ratio and the Mg^{2+} concentration in the chloroplast (Robinson and Portis, 1989; Zhang and Portis, 1999; Hazra *et al.*, 2015), thereby adjusting Rubisco activity to the prevailing irradiance level, e.g. during photosynthetic induction when leaves transition from low to high light levels (Taylor and Long, 2017). Post-translational modifications have also been shown to influence Rca activity and are likely to contribute to the regulation of carbon assimilation *in vivo* (Hartl *et al.*, 2017; Kim *et al.*, 2019).

The activity of Rubisco *in planta* declines as temperatures exceed the species-specific optimum temperature for plant growth (Carmo-Silva and Salvucci, 2012). For example, in the warm-adapted crop cotton (*Gossypium hirsutum*), the activation state of Rubisco remains stable up to 35°C, whereas in the temperate crop wheat, the Rubisco activation state decreases at temperatures above 30°C (Eckardt and Portis, 1997; Crafts-Brandner and Salvucci, 2000). *In vitro* experiments have shown that Rubisco itself is stable and remains active at temperatures of up to 50°C (Crafts-Brandner and Salvucci, 2000). Conversely, Rca has been shown to be sensitive to elevated temperatures (Eckardt and Portis, 1997; Salvucci and Crafts-Brandner, 2004). In the warm-adapted species tobacco (*Nicotiana tabacum*), Rca can hydrolyse ATP at temperatures of up to 40°C, which is 20°C below the denaturing temperature of Rubisco (Salvucci *et al.*, 2001). Hence, photosynthesis at high temperatures is not constrained by Rubisco itself, but by the inability of Rca to remove inhibitors and maintain Rubisco activity *in vivo*. It has been postulated that the thermolabile nature of Rca might act as a thermal fuse to limit carbon assimilation, and that tight-binding inhibitors might protect Rubisco from proteolysis during short periods at supra-optimal temperatures (Sage *et al.*, 2008), although this hypothesis remains to be tested.

Species from contrasting thermal environments have Rca isoforms with different thermal optima: i.e. Rca activated Rubisco best at 20°C in Antarctic hairgrass (*Deschampsia antarctica*) and at 30°C in the desert-dwelling creosote bush (*Larrea tridentata*) (Salvucci and Crafts-Brandner, 2004). In warm-adapted wild rice (*Oryza australiensis*), Rca has low catalytic activity but can promote Rubisco activation at warm temperatures when cultivated rice (*Oryza sativa*) Rca is largely inactive (Scafaro *et al.*, 2016). Importantly, maximum rates of ATP hydrolysis by the Rca isoforms present in these species were observed at temperatures 5–7°C higher than the maximum rates of Rubisco activation by the same Rca isoforms (Salvucci and Crafts-Brandner, 2004; Scafaro *et al.*, 2016). This indicates that the two reactions catalysed by Rca, ATP hydrolysis and Rubisco activation have different temperature profiles, and that both reactions must be taken into account to describe the temperature response of Rca activity.

Most plant species have two Rca isoforms, a shorter β isoform (approximately 42 kDa) and a longer α isoform (approximately 46 kDa), that are produced via alternative splicing of a single gene, whereas other species only have the short isoform or encode the long and short isoforms on separate genes (Nagarajan and Gill, 2018). In wheat, two genes encode three Rca isoforms: Rca1β, Rca2β and Rca2α (Carmo-Silva *et al.*, 2015). Recently, Scafaro *et al.* (2019b) showed that Rca1β was more thermostable by using differential scanning fluorimetry. In the same study, 11 amino acid residues that are present in Rca isoforms from warm-adapted species increased Rca thermostability by 7°C. The effect of the 11 amino acid residues on the temperature response of Rubisco activation by Rca was not tested, however, and it remained unclear whether all 11 residues were required for improved thermostability. In Arabidopsis, expression of thermostable Rca isoforms resulted in faster photosynthetic rates as well as increased plant biomass and seed yield during moderate heat stress (Kurek *et al.*, 2007; Kumar *et al.*, 2009), highlighting the importance and scope of increasing the temperature optimum of Rubisco activation by Rca to enhance plant resilience to global warming.

Wheat Rca isoforms have also been shown to differ in their response to increasing ADP:ATP ratios (Perdomo *et al.*, 2019; Scafaro *et al.*, 2019a), which are likely to change in wheat at high temperatures through reduced photosystem-II efficiency and increased cyclic electron flow (Sharkey, 2005; Haque *et al.*, 2014). Combined, these observations suggest that Rca thermotolerance and ADP sensitivity might be co-regulated. Here, we identify a single isoleucine residue in the α/β-subdomain of Rca that increases the thermostability of Rubisco activation in wheat. Characterization of the temperature response of both ATP hydrolysis and Rubisco activation for the three wheat Rca isoforms showed that Rca1β was the most thermostable but was less efficient at activating Rubisco. Substitution of a methionine residue by an isoleucine in the wheat Rca2β isoform (Rca2β-M159I) improved the thermal optimum without affecting the efficiency of Rubisco activation.

RESULTS

Identification of a residue putatively associated with the temperature optimum of Rca

The wheat Rca amino acid sequence residue 159 (numbering of wheat Rca2β lacking the chloroplast transit peptide) was identified as putatively associated with the temperature optimum of Rca (Figure 1). The Rca sequences from two warm-adapted rice species and the wheat Rca1β

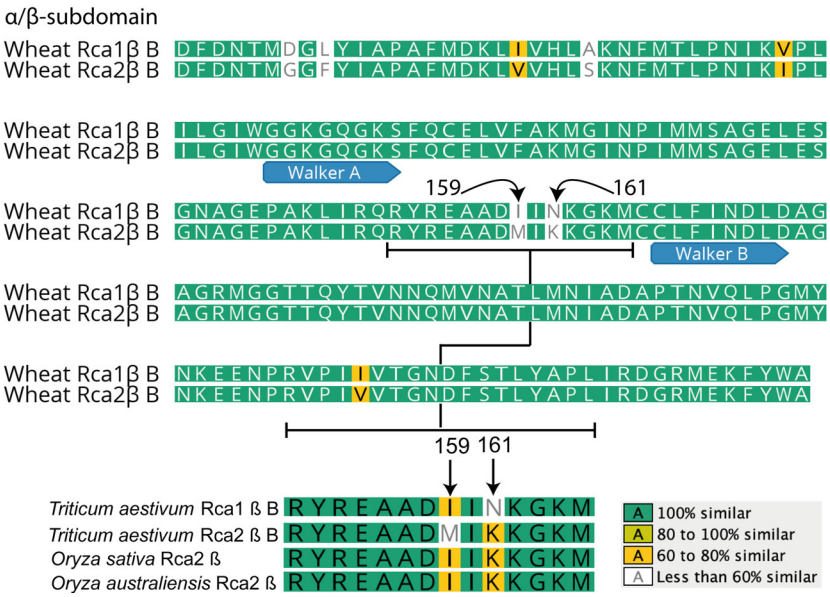

Figure 1. Selection of residues putatively involved in the temperature response of Rubisco activase (Rca). Upper panel: amino acid sequence alignment of the α/β-subdomain of *Triticum aestivum* (wheat) Rca1β and Rca2β from the B genome (numbered according to wheat Rca2β lacking the chloroplast transit peptide). The Walker A and Walker B motifs and two residues (159 and 161) differing between the two sequences are identified. Lower panel: amino acid alignment of a short sequence within the α/β-subdomain of the two Rca isoforms from wheat and the Rca isoforms from two *Oryza* (rice) species that are adapted to warmer temperatures.

isoform, which was recently shown to be more thermostable than the Rca2β isoform (Scafaro *et al.*, 2019b), have an isoleucine at position 159, whereas the wheat Rca2β contains a methionine at position 159 (Figure 1). This residue is located in the α/β subdomain of the Rca AAA+ module, between the Walker A and Walker B nucleotide binding motifs (Stotz *et al.*, 2011; Miller and Enemark, 2016).

To test the hypothesis that replacing the wheat Rca2β methionine at position 159 with an isoleucine increases the temperature optimum of Rca activity, the single mutant Rca2β-M159I was generated by site-directed mutagenesis. A double mutant of wheat Rca2β with two residues changed to the corresponding amino acids in Rca1β, including the residue change at position 159, was recently described (Rca2β-M159I/K161N; Perdomo *et al.*, 2019). The mutation M159I was also present, whereas K161N was absent, in two recently characterized mutant proteins of wheat Rca containing 8- and 11-residue changes and characterized by enhanced thermal stability (Scafaro *et al.*, 2019b). These results suggest that the residue change M159I, rather than K161N, might be associated with the temperature optimum of Rca.

Wheat Rca2β-M159I exhibits higher temperature optima of ATP hydrolysis and Rubisco activation

In order to verify that the residue change M159I is associated with the temperature optimum of Rca, the temperature response of ATP hydrolysis and Rubisco activation were determined for the Rca2β-M159I single mutant and compared with the native wheat Rca isoforms and the double mutant Rca2β-M159I/K161N (Figure 2). ATP hydrolysis peaked at lower temperatures for Rca2β, at intermediate temperatures for Rca2β-M159I and Rca2β-M159I/K161N, and at higher temperatures for Rca1β (Figure 2a), consistent with the higher thermal stability of Rca1β (Scafaro *et al.*, 2019b). Rca2α hydrolysed ATP at slower rates (Figure S1a) but showed a similar temperature-response profile to that of Rca2β at 20–40°C (Figure S1b). The maximum ATPase activity was highest for Rca1β, Rca2β-M159I had a maximum rate of ATP hydrolysis that was comparable with that of Rca2β, and Rca2β-M159I/K161N was intermediate to Rca2β and Rca1β (Table 1). The temperature at which Rca2β-M159I and Rca2β-M159I/K161N reached maximum rates of ATP hydrolysis (T_{max}) was approximately 3°C higher than in Rca2β, but not as high as in Rca1β (Table 1).

The temperature optimum of ATPase activity of Rca2β-M159I shifted towards higher temperatures compared with Rca2β, with the mutant showing lower rates at temperatures up to 35°C and higher rates at 35°C –45°C (Figure 2a; Table S2). Shifts in temperature optimum are best visualized by considering the activity of each protein relative to the maximum activity (Figure 2b). Relative ATPase activities did not significantly differ between Rca2β-M159I and Rca2β-M159I/K161N at temperatures between 20°C and 37.5°C; however, above 40°C Rca2β-M159I presented significantly higher relative ATPase activity compared with Rca2β-M159I/K161N (Figure 2b; Table S2). M159I also presented a shift towards a higher optimum temperature range (T_{opt}) and a higher temperature at which 50% of the maximum ATPase activity of remains ($T_{0.5}$), compared with Rca2β-M159I/K161N (Table 1).

The rate of Rubisco activation by Rca2β-M159I was intermediate to the slower Rca1β and the faster Rca2β at 20–25°C (Figure 2c; Table S3). The native wheat isoforms Rca2β and Rca2α activated Rubisco at comparable rates across the temperature range of 20–45°C (Figure S1c; Table S3), showing a similar temperature response profile

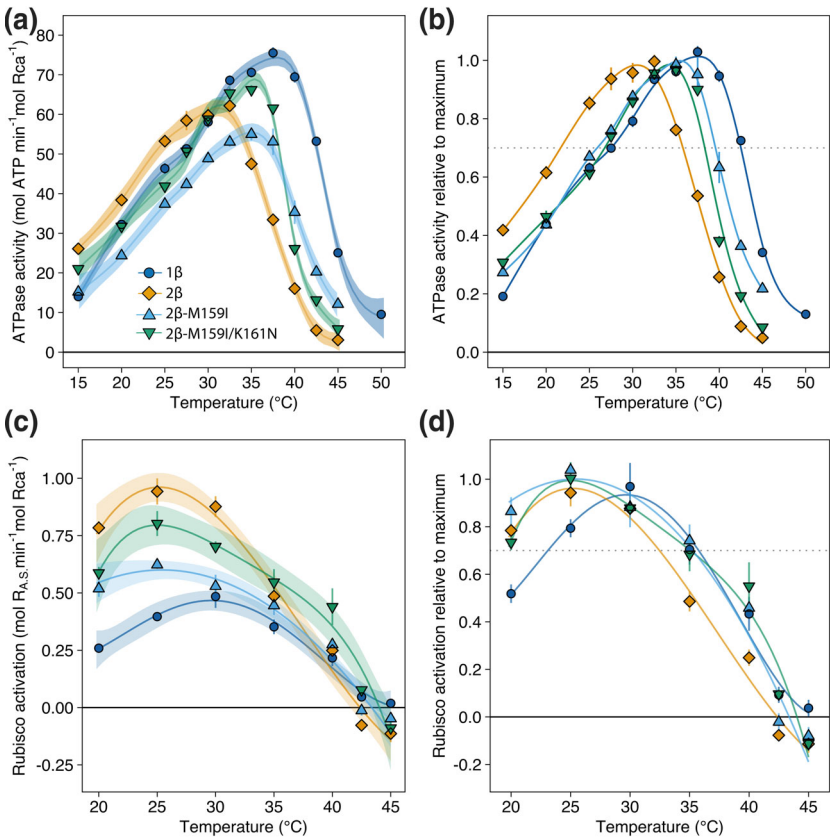

Figure 2. Temperature response of ATP hydrolysis and Rubisco activation by Rubisco activase (Rca). (a, c) Rates of ATP hydrolysis and Rubisco activation by *Triticum aestivum* (wheat) Rca1β (dark blue), Rca2β (orange), and the mutant proteins Rca2β-M159I (light blue) and Rca2β-M159I/K161N (green). Assays were performed at the indicated temperatures using 2 μM Rca monomer and 5 mM ATP. Activation of pre-inhibited Rubisco used 6 μM Rubisco active sites ($R_{A.S.}$) in the ER form (1:3 Rca: $R_{A.S.}$). Values are means ± standard errors (n = 5–19 technical replicates in a and n = 3–15 technical replicates in c). Lines represent the best fit for each enzyme (Akaike information criterion; Table S1) and coloured areas denote the 95% confidence interval for each fit. (b, d) ATP hydrolysis and Rubisco activation relative to estimated maximum activities (Table 1).

(Figure S1d). The ER complex (inhibited Rubisco enzyme with RuBP bound to active sites), consisting of uncarbamylated Rubisco bound to RuBP, is very stable at 20–25°C and remains inactive throughout the assay; however, as temperatures increase, RuBP spontaneously releases from Rubisco catalytic sites (Figure S2). The rate of Rca-dependent Rubisco reactivation was determined by the difference between the rates of Rubisco reactivation in the presence and the absence of Rca measured in parallel at each temperature (Figure S2a; Carmo-Silva and Salvucci, 2011), to account for the increase in the spontaneous release of inhibitors from Rubisco active sites at high temperatures (Schrader *et al.*, 2006). At 42.5–45°C, the rate of spontaneous Rubisco activation in assays without Rca is significant and wheat Rca isoforms become unable to reactivate Rubisco, resulting in very low or even negative rates of Rubisco activation by Rca (Figures 2 and S1).

Importantly, the temperature response of Rubisco activation by both Rca2β-M159I and Rca2β-M159I/K161N was characterized by a broader peak compared with the temperature response of Rca2β, which peaked at 25.2°C (Figure 2d). The T_{opt} of Rubisco activation by Rca2β-M159I and Rca2β-M159I/K161N extended approximately 5°C above Rca2β and approximately 5°C below Rca1β (Table 1). The maximum rates of Rubisco activation by Rca2β-M159I and Rca2β-M159I/K161N were intermediate to the lower rates measured for Rca1β and the higher rates measured for Rca2β and Rca2α, and occurred at a temperature comparable with that for Rca2β (Table 1). At 35°C, however, Rca2β-M159I had significantly higher relative Rubisco activation activity compared with Rca2β (Figure 2d; Table S3), with a consequent increase in $T_{0.5}$ (Table 1).

M159I alters the regulatory properties of Rca

In addition to the increased temperature optimum of ATP hydrolysis and the broader temperature optimum of Rubisco activation, the residue substitution M159I also caused changes in the relative rates of Rca activity. To test the hypothesis that M159I might affect the regulation as well as the thermotolerance of Rca, the rates of ATP hydrolysis and Rubisco activation by Rca2β-M159I were determined at 25°C in the presence of increasing ratios of ADP:ATP. Rubisco activation by Rca2β was previously shown to be insensitive to inhibition by ADP, in contrast to Rca1β, which shows inhibition of both ATP hydrolysis and Rubisco activation as the ADP:ATP ratio increases (Perdomo *et al.*, 2019). Rubisco activation by Rca2β-M159I was inhibited to a greater extent than ATP hydrolysis in the presence of increasing ADP:ATP ratios (Table 2), indicating that this amino acid substitution causes Rubisco activation by Rca to become more thermotolerant, but also more sensitive to inhibition by ADP.

The efficiency of Rubisco activation by Rca was estimated as the quantity of Rubisco active sites activated per

Table 1 Optimum temperature of Rubisco activase (Rca) activity. Maximum rate of ATP hydrolysis and Rubisco activation and corresponding temperature (T_{max}), optimum temperature range (T_{opt}, above 70% activity) and temperature above the optimum at which 50% of the maximum activity remains ($T_{0.5}$), as estimated from the best-fit models applied to describe the temperature response of each Rca protein (Table S1). The mutant protein Rca2β-M159I is highlighted in bold

Rca	Maximum ATPase activity (mol ATP min^{-1} mol Rca^{-1})	T_{max} (°C)	T_{opt} (°C)	$T_{0.5}$ (°C)
1β	73.4 ± 4.2	38.9 ± 0.0	25–42.5	44.3 ± 0.2
2β	61.6 ± 4.2	31.6 ± 0.0	20–35	37.8 ± 0.3
2α	33.0 ± 4.7	30.8 ± 0.0	20–35	38.6 ± 0.6
2β-M159I	**55.5 ± 4.6**	**35.0 ± 0.0**	**25–40**	**41.0 ± 0.4**
2β-M159I/K161N	68.4 ± 3.9	35.3 ± 0.0	20–37.5	39.5 ± 0.1

Rca	Maximum Rubisco activation rate (mol $R_{A.S.}$ min^{-1} mol Rca^{-1})	T_{max} (°C)	T_{opt} (°C)	$T_{0.5}$ (°C)
1β	0.47 ± 0.09	29.7 ± 0.3	25–35	38.6 ± 1.0
2β	0.96 ± 0.13	25.2 ± 0.0	20–30	35.6 ± 0.9
2α	0.90 ± 0.25	26.0 ± 0.1	20–30	37.3 ± 1.8
2β-M159I	**0.60 ± 0.08**	**25.4 ± 2.2**	**20–35**	**37.9 ± 0.9**
2β-M159I/K161N	0.80 ± 0.18	25.0 ± 1.2	20–35	39.2 ± 1.8

Table 2 ADP sensitivity of Rca2β-M159I. The rate of ATP hydrolysis and Rubisco activation by Rca was measured at 25°C with 2 μM Rca monomer and (for Rubisco activation) 6 μM Rubisco active sites ($R_{A.S.}$) (i.e. 1:3 Rca:$R_{A.S.}$). Adenine nucleotide concentration was maintained at 5 mM. The 0.00 ADP:ATP control (v_c, 5 mM ATP and 0 ADP:ATP) contained the ATP regeneration system, but this was absent in assays with 0.11 or 0.33 ADP:ATP (v_i)

ADP:ATP	ATP hydrolysis(v_i/v_c)	Rubisco activation(v_i/v_c)
0.00	1.00 ± 0.007[a]	1.00 ± 0.010[a]
0.11	0.83 ± 0.029[b]	0.62 ± 0.048[b]
0.33	0.71 ± 0.019[c]	0.53 ± 0.004[b]

Values are means ± standard errors (n = 3 technical replicates) of rates normalized to the 0.00 ADP:ATP control. Different letters denote significant differences (Tukey's honestly significant difference, $P < 0.05$).

quantity of ATP consumed, i.e. the rate of Rubisco activation divided by the rate of ATP hydrolysis for each enzyme at each temperature and nucleotide treatment. Rca1β was the least efficient, especially as temperatures increased above 30°C (Figure 3a) and in the presence of ADP (Figure 3b). Rca2β-M159I and Rca2β-M159I/K161N showed comparable efficiency to Rca2β at 20–35°C (Figure 3a). The apparent increase in Rca efficiency at 40°C for Rca2β and Rca2β-M159I/K161N resulted from a more marked decrease in ATP hydrolysis than Rubisco activation activity at this temperature (Figure 2). The efficiency of Rca2β was also increased in the presence of ADP (Figure 3b) through the inhibition of ATP hydrolysis, with no effect on Rubisco activation (Perdomo *et al.*, 2019). Rca2β-M159I and Rca2β-M159I/K161N maintained efficiency independently of ADP presence, because both ATP hydrolysis and Rubisco activation activities were inhibited (Rca2β-M159I; Table 2) or very little affected (Rca2β-M159I/K161N; Perdomo *et al.*, 2019) as ADP:ATP ratios increased. The response of Rca2β-M159I to ADP contrasts with that observed for Rca2β and shows that the single mutation significantly affects both the regulatory properties and the temperature optimum of Rubisco activation by Rca.

DISCUSSION

A single amino acid residue substitution in the wheat Rca2β isoform (M159I) extended the temperature optimum while maintaining the efficiency of Rubisco activation by Rca. A previous study (Kurek *et al.*, 2007) showed an increase in the rate of ATP hydrolysis by Arabidopsis Rca at elevated temperatures in a mutant protein with one single residue substitution. To the best of our knowledge, however, this is the first report of a specific amino acid residue in Rca that increases the thermostability of Rubisco activation specifically. Importantly, this residue substitution also altered the regulatory properties of Rca while resulting in a more efficient activation of Rubisco compared with the naturally occurring thermostable wheat Rca1β.

Of the three native wheat isoforms, Rca1β activated Rubisco at slower rates but exhibited high ATPase and Rubisco activation activities at higher temperatures compared with Rca2β and Rca2α. These results confirm and expand a recent observation that Rca1β is more thermostable, obtained by incubating Rca at high temperatures and measuring at 25°C, and by inferring thermal midpoints from differential scanning fluorimetry (Scafaro *et al.*, 2019b). The higher temperature optimum of ATPase activity compared with Rubisco activation activity observed for all isoforms (Figure 2) suggests that the Rca–Rubisco interaction itself is sensitive to temperature and demonstrates that both Rca activities must be considered when assessing thermostability.

Wheat Rca1β and the Rca isoforms in cultivated rice and in a wild rice species adapted to warm environments, for which Rca has been characterized as thermotolerant, contain an isoleucine residue at position 159, whereas a methionine is present in wheat Rca2β (Figure 1). The two Rca genes in monocots are organized in tandem and are likely to have resulted from gene duplication (Nagarajan and Gill, 2018). Unlike wheat and other monocots, the *Rca1* gene in rice is not functional because of multiple deletions (Nagarajan and Gill, 2018), and only the products

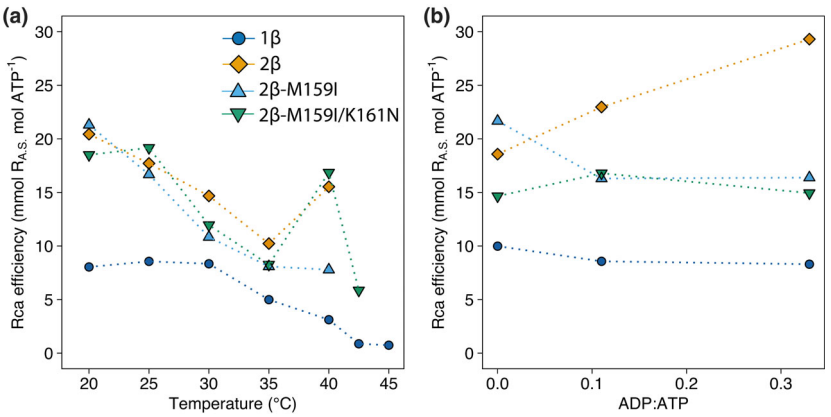

Figure 3. The efficiency of Rubisco activation by Rubisco activase (Rca). The efficiency of Rubisco activation by wheat Rca1β (dark blue), Rca2β (orange), and the mutant proteins Rca2β-M159I (light blue) and Rca2β-M159I/K161N (green). Rca efficiency was estimated by dividing the rate of Rubisco activation by the rate of ATP hydrolysis for each Rca isoform at each temperature (a; data from Figure 2) and ADP:ATP ratio (b, data for M159I from Table 2; for other Rca isoforms, see Perdomo *et al.*, 2019).

of *Rca2* expression are present. The presence of an isoleucine at position 159 in rice Rca2 (Scafaro *et al.*, 2016) may reflect an adaptation to warm environments. A switch from methionine to isoleucine would occur when the third base in the methionine-coding ATG codon mutates to any of A, C or T, suggesting that this residue switch could occur relatively frequently. Thus, it could be speculated that the ancestral Rca of monocots contained a methionine residue at this position, as the reverse switch would be far less likely.

Two mutant wheat Rca2β proteins containing eight and 11 residue substitutions to amino acids found in warm-adapted species, including the mutation M159I, showed 5–7°C increases in thermostability (Scafaro *et al.*, 2019b). It is likely that some of the other amino acid changes may have contributed to increased Rca thermostability. The results presented here show that the single isoleucine residue substitution at position 159 is likely to have contributed the majority of the thermostability increase observed in the more complex mutants, however. The increased thermostability in Rca2β-M159I might result from increased hydrophobicity, as this amino acid change results in an increase of the hydropathy index (Kyte and Doolittle, 1982) from 1.9 to 4.5. Increased thermostability in response to a residue substitution causing increased hydrophobicity has previously been observed in the CutA1 protein from *Escherichia coli* (Matsuura *et al.*, 2010; Matsuura *et al.*, 2015).

In wheat, photosynthesis and Rubisco activation start to decline at leaf temperatures above 30°C (Feller *et al.*, 1998; Perdomo *et al.*, 2017). Although gene expression of *Rca1β* has recently been shown to increase with temperature (Scafaro *et al.*, 2019b), the rate of Rubisco activation by Rca1β is considerably slower than by Rca2β (Figure 2), suggesting that the level of active Rca at high temperatures would be insufficient to maintain Rubisco activity. Furthermore, Rca1β is less efficient than Rca2β because of its high ATPase activity (Figure 3), meaning that this isoform hydrolyses ATP at high rates at elevated temperatures. Rca2β-M159I on the other hand was much more efficient than Rca1β, even at elevated temperatures (Figure 3), and was less sensitive to ADP inhibition than Rca1β (Table 2, Perdomo *et al.*, 2019). ADP inhibition may not constitute a bottle neck, however, as cyclic electron transport has been shown to increase at elevated temperatures (Havaux *et al.*, 1991; Bukhov *et al.*, 1999; Bukhov and Carpentier, 2000). Measuring cyclic electron flow in plants expressing a genetically engineered Rca2β-M159I would enable the investigation of changes in ATP demand as the temperature increases.

The redox-sensitive isoform Rca2α exhibited considerably lower ATPase activity but comparable Rubisco activation activity to that of Rca2β (Figure S1), suggesting that the C-terminal extension reduces ATP hydrolysis specifically, making Rca2α more efficient (less ATP consumed per Rubisco activated). The difference in ATPase activity between Rca2α and Rca2β was greater in rice (Scafaro *et al.*, 2016), which might be associated with the presence of a glutamic acid (E) at the C-terminal extension position 392. Substitution of this glutamic acid in the rice Rca2α by an alanine (which is present in wheat Rca2α; Figure S3) to produce a mutant E392A increased ATPase activity by approximately 30% (Portis *et al.*, 2008), indicating that this residue may influence Rca2α efficiency.

In addition to broadening the thermal optimum of Rubisco activation by 5°C, Rca2β-M159I activated Rubisco at lower rates at 20–30°C and showed increased sensitivity of Rubisco activation to inhibition by ADP compared with Rca2β (Table 2, Perdomo *et al.*, 2019). Recently, a single lysine residue in the C-terminal extension of Rca was also shown to decrease ADP sensitivity (Scafaro *et al.*, 2019a). Importantly, the double mutant Rca2β-M159I/K161N was much less inhibited by ADP than was Rca2β-M159I, suggesting that the residue substitution K161N counteracts the ADP sensitivity induced by M159I. The efficiency of Rubisco activation by both Rca2β-M159I and Rca2β-M159I/K161N was unaffected in the presence of ADP, as both ATP hydrolysis and Rubisco activation were similarly affected.

In summary, we show that a single isoleucine residue substitution significantly alters the function of wheat Rca, a

key regulator of Rubisco activity and carbon assimilation. Rca2β-M159I showed a broadened temperature optimum compared with the native wheat Rca2β, while maintaining a rate of Rubisco activation that is higher, less inhibited by ADP and much more efficient than the thermostable native wheat Rca1β. The findings suggest that the isoleucine residue at position 159 of Rca acts as a thermal and regulatory switch that can be exploited to improve the efficiency and climate resilience of wheat carbon fixation.

EXPERIMENTAL PROCEDURES

Protein sequences

Rubisco activase (Rca) protein sequences from *T. aestivum* (wheat) (Carmo-Silva *et al.*, 2015), *O. australiensis* and *O. sativa* (Scafaro *et al.*, 2016) were aligned using GENEIOUS 9.1.8 (https://www.geneious.com) and edited with ILLUSTRATOR 22.0 (Adobe, https://www.adobe.com). The MUSCLE algorithm (Edgar, 2004) was used and colour coding represents similarity (Henikoff and Henikoff, 1992). Sequence data for wheat Rca can be found in the GenBank data library under accession numbers LM992844 (Rca1β), LM992846 (Rca2β) and LM992845 (Rca2α), corresponding to EnsemblPlants gene numbers TraesCS4B02G140200 (Rca1) and TraesCS4B02G140300 (Rca2).

Site-directed mutagenesis

Site-directed mutagenesis (SDM) was performed using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, https://www.agilent.com) according to the manual (https://www.agilent.com/cs/library/usermanuals/public/210513.pdf). Primers were designed based on the coding sequence of Rca2β from the B genome of wheat using the QuickChange Primer Design website (https://www.agilent.com/store/primerDesignProgram.jsp). The Rca2β coding sequence was ligated into the pET-23(+) vector and used as a template to create the double mutant Rca2β-M159I/K161N, as described previously(Perdomo *et al.*, 2019), and the single mutant Rca2β-M159I using the primer 5′-ACCGTGAGGCTGCAGACAT**A**ATCAAGAAGGGTAAG-3′ (the base change is highlighted in bold). After validation by sequencing of the Rca coding region, the plasmid was cloned into BL21(DE3)pLysS cells for protein expression.

Purification of enzymes

Rubisco activase (Rca) enzymes were expressed in *E. coli* and purified as described by Barta, Carmo-Silva and Salvucci (2011a), with the following modifications. The frozen cell pellets from 10-L cultures were lysed and thawed by suspending the pellet in 130 ml of cell extraction buffer (50 mM HEPES-KOH, pH 7.0, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (w/v) Triton X-100, 2 mM ATP, 5 mM dithiothreitol (DTT), 20 mM ascorbate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μM leupeptin) for 30 min on ice. In addition, after adding ammonium sulphate to 37.5%, collecting the precipitated material and suspending in 10 ml of cell extraction buffer, the solution was centrifuged at 200 000 ***g*** for 30 min in a Beckman centrifuge (Beckman Coulter, https://www.beckmancoulter.com) using a swinging bucket ultracentrifuge rotor SW 31 Ti. Rubisco was purified from wheat leaves according to the method described by Orr and Carmo-Silva (2018). Wheat (*T. aestivum* cultivar Cadenza) was grown in a glasshouse at approximately 22°C and a photosynthetic photon flux density of approximately 500 μmol m^{-2} sec^{-1} (16 h day, 8 h night) for 2 weeks. Leaf material was flash frozen in liquid nitrogen and stored at −80°C until purification. Protein concentration was determined by the Bradford method (Bradford, 1976). Polypeptides present in the Rca and Rubisco preparations were separated by SDS-PAGE on 12% gels (Bio-Rad, https://www.bio-rad.com) and visualized by staining with Coomassie Blue (Figure S4). The molecular weight of proteins was calculated from the respective amino acid sequence using THE SEQUENCE MANIPULATION SUITE (Stothard, 2000; https://www.bioinformatics.org/sms/prot_mw.html). Two independent preparations of four of the recombinant Rca proteins showed no significant differences in ATPase activity (Figure S5, Table S4). Consequently, only one purification was used for the single mutant Rca2β-M159I and only one preparation per Rca was used for the Rubisco reactivation assays.

Rubisco activase activity: ATP hydrolysis

The rate of ATP hydrolysis by Rca isoforms was assayed using the method described by Chifflet *et al.* (1988), with the following modifications. ATP hydrolysis was measured at a range of temperatures (15–50°C) over 5 min in an assay mix (50 μl) containing 100 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$ and 5 mM ATP. The reaction was initiated by adding Rca (to a final concentration of 2 μM Rca monomer) and quenched after 5 min by adding 12% sodium dodecyl sulphate (SDS). Blanks were prepared by adding SDS to the reaction tube before adding Rca. Phosphate standards for a calibration curve were prepared by adding 0, 2.5, 5, 10, 15, 20, 32.5 and 50 mM potassium phosphate (KPi) (final concentrations) to the reaction mixture described above (in the absence of Rca). Tubes containing the assay mix were incubated in a temperature block (Echotherm; Torrey Pines Scientific, https://www.torreypinesscientific.com) until the desired temperature was reached before initiating the assay (typically 3 min). The temperature of 50 μl H_2O incubated alongside the reaction tubes was monitored using a thermocouple (Omega Thermometer RDXL4SD; Omega, https://www.omega.co.uk). After the addition of SDS, all reactions and standards were kept in the fridge until Pi determination in a spectrophotometer (BMG Labtech, https://www.bmglabtech.com), as detailed by Perdomo *et al.* (2019).

Rubisco activase activity: Rubisco reactivation

The rate of Rubisco reactivation by Rca was measured as described by Barta, Carmo-Silva and Salvucci (2011b) and Perdomo *et al.* (2019). To prepare inhibited Rubisco complexes (ER), purified Rubisco was first incubated in activation mix for 1 h at 4°C, followed by spin desalting, protein concentration determination and incubation of uncarbamylated enzyme with 4 mM RuBP at 4°C overnight. The assay for Rubisco activation consisted of two stages (Barta, Carmo-Silva and Salvucci, 2011b). In the first stage, Rubisco (ER) was reactivated by Rca at a range of temperatures (20–45°C), and in the second stage, aliquots were taken from the first-stage assay to measure Rubisco activity at 30°C by the incorporation of $^{14}CO_2$ into acid-stable compounds. The temperature of Rubisco activation was controlled as described above for the ATP hydrolysis assay. First-stage assays (90 μl final volume) contained 50 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM DTT, 5 mM ATP, an ATP regenerating system consisting of 40 U ml^{-1} phosphocreatine kinase, 4 mM phosphocreatine, 5% (w/v) PEG-3350, 6 μM Rubisco active sites (ER), 2 μM Rca monomer, 5 mM ATP plus an ATP regenerating system and 6 mM RuBP. Three variants of assays were performed for determination of: fully activated Rubisco activity (ECM, this contained no RuBP

in the first-stage assay), Rca-dependent Rubisco activation (contained all components) and the spontaneous activation of ER (contained no Rca, negative control). Second-stage assays to assess Rubisco activity in aliquots taken at time intervals from the first-stage assays (Figure S2a) contained 100 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (0.5 Ci mol^{-1}) and 0.4 mM RuBP. The rate of Rubisco activation (fraction of Rubisco sites activated per minute) in the Rca and ER reactions was calculated from the difference in Rubisco activity measured 1.5 and 0.5 min after starting the first stage assay, taking into account the activity of fully activated Rubisco (Carmo-Silva and Salvucci, 2011). The rate of Rubisco activation by Rca was calculated as the difference between the rate of increase in Rubisco activity measured at each temperature in the presence and absence of Rca (Rca reaction minus spontaneous activation in the ER reaction; Figure S2), and corrected for the concentrations of Rubisco active sites and Rca in the assay.

Data analysis and modelling

Data was processed using R 3.5.1 and RSTUDIO 1.1.463, and graphs were prepared using the GGPLOT 2 package (Wickham, 2017). Outliers were detected using the GGSTATSPLOT package using Tukey's fences method, where outliers are defined as extreme values that are 1.5 times the interquartile range (1.5 IQR) below the first quartile or 1.5 IQR above the third quartile. To estimate maximum ATP hydrolysis and Rubisco activation activities and the corresponding temperatures at which the maximum values are attained, as well as the temperature above the peak at which half of the activity remains ($T_{0.5}$), second- to fourth-order polynomials and generalized additive models (GAM) were fitted to the experimental data using the gam function from the MGCV 1.8–24 package in R (Wood, 2017). The model that best fit the experimental data was selected based on the Akaike information criterion (Akaike, 1974) using the AIC function (Table S1). The 'predict' function was used to estimate maximum activity values and corresponding temperatures for each Rca isoform (T_{max}), and the 'approximately' function was used to estimate the optimum temperature range (T_{opt}, above 70% activity) and the temperature above the peak at which 50% of activity remains ($T_{0.5}$). The standard error calculated for each model was used to predict 95% confidence intervals for each fit (dashed lines in the graphs). One-way analysis of variance ANOVA followed by Tukey's *post-hoc* tests were used to test the significance of differences between Rca isoforms at each temperature and nucleotide treatment (Tables S2–S4).

ACCESSION NUMBERS

Wheat Rca sequence data can be found in the GenBank data library under accession numbers LM992844 (Rca1β), LM992846 (Rca2β) and LM992845 (Rca2α).

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AUTHOR CONTRIBUTIONS

GED and ECS designed the research, GED performed the research with help from DW, ECS supervised the research, GED analysed the data, and GED and ECS wrote the article.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data presented in this publication is being deposited in the data repository used by Lancaster University and will be made publicly available.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Temperature response of ATP hydrolysis and Rubisco activation by Rca.

Figure S2. The spontaneous release of RuBP from inhibited Rubisco (ER) increases with temperature.

Figure S3. Amino acid sequence alignment of Rca isoforms from warm-adapted wild rice (*Oryza australiensis*, Oa), cultivated rice (*Oryza sativa*, Os) and bread wheat (*Triticum aestivum*, Ta).

Figure S4. Separation of purified Rca and Rubisco proteins by SDS-PAGE.

Figure S5. Temperature response of ATP hydrolysis by Rca proteins.

Table S1. Modelling the temperature response of ATP hydrolysis and Rubisco activation by wheat Rca isoforms.

Table S2. Result of a Tukey's multiple comparison test of Rca ATPase activity.

Table S3. Result of a Tukey's multiple comparison test of Rubisco activation activity.

Table S4. Two-way ANOVA showed no significant differences between the results obtained for ATPase activity using different preparations of Rca ($P > 0.05$).

OPEN RESEARCH BADGE

This article has earned an Open Data Badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at http://www.research.lancs.ac.uk/portal/en/ with project number PRJNA598053.

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