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Research Article

Rubisco activation by wheat Rubisco activase isoform 2β is insensitive to inhibition by ADP

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Rubisco activase (Rca) is a catalytic chaperone that remodels the active site, promotes the release of inhibitors and restores catalytic competence to Rubisco. Rca activity and its consequent effect on Rubisco activation and photosynthesis are modulated by changes to the chloroplast environment induced by fluctuations in light levels that reach the leaf, including redox status and adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio. The *Triticum aestivum* (wheat) genome encodes for three Rca protein isoforms: 1β (42.7 kDa), 2β (42.2 kDa) and 2α (46.0 kDa). The regulatory properties of these isoforms were characterised by measuring rates of Rubisco activation and ATP hydrolysis by purified recombinant Rca proteins in the presence of physiological ADP/ATP ratios. ATP hydrolysis by all three isoforms was sensitive to inhibition by increasing amounts of ADP in the assay. In contrast, Rubisco activation activity of Rca 2β was insensitive to ADP inhibition, while Rca 1β and 2α were inhibited. Two double and one quadruple site-directed mutants were designed to elucidate if differences in the amino acid sequences between Rca 1β and 2β could explain the differences in ADP sensitivity. Changing two amino acids in Rca 2β to the corresponding residues in 1β (T358K & Q362E) resulted in significant inhibition of Rubisco activation in presence of ADP. The results show that the wheat Rca isoforms differ in their regulatory properties and that amino acid changes in the C domain influence ADP sensitivity. Advances in the understanding of Rubisco regulation will aid efforts to improve the efficiency of photosynthetic CO_2 assimilation.

Introduction

Wheat is the most widely grown crop in the world, it provides 20% of the daily protein and food calories for 4.5 billion people [1]. Currently, wheat yields are not increasing at a sufficient rate to meet increasing food demand, set to double by 2050 [2,3]. One viable strategy to improve crop production is to optimise photosynthetic productivity in current and projected climates, with most current efforts focusing on steady-state photosynthesis. However, plants in an agricultural setting are normally exposed to non-steady-state conditions, experiencing rapid transitions from shade to full sunlight, due to changes in cloud cover or simply as a consequence of the natural movement of leaves in the canopy [4]. In soybean and wheat, the slow adjustment of Rubisco activation, one of the factors contributing to photosynthetic induction in shade to sun transitions, is predicted to limit potential CO_2 assimilation in fluctuating environments by up to 20% [5,6].

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) assimilates CO_2 through the carboxylation of ribulose-1,5-bisphosphate (RuBP). Although Rubisco is an essential enzyme for life on earth [7], it is characterised by a number of limitations, and its activity is highly regulated [8]. One of the limitations of Rubisco is its susceptibility to inhibition by the binding of sugar-phosphate derivatives that lock active sites into a closed conformation. Rubisco activase (Rca) is an AAA+ catalytic chaperone that uses the energy from ATP hydrolysis to remodel the active site of Rubisco [9]. This activity restores the catalytic competence of Rubisco by removing inhibitory sugar-phosphate derivatives from Rubisco's active sites [10,11]. In many of the plant species studied to date, there are two Rca isoforms:

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a shorter redox-insensitive β-isoform, and a longer α-isoform that contains a redox-sensitive C-terminal extension [12]. In wheat, two genes that encode for three Rca protein isoforms have been described [8], expression of *TaRca1* produces a short isoform 1β (42.7 kDa), whereas alternative splicing of *TaRca2* produces either a short isoform 2β (42.2 kDa) or a long isoform 2α (46.0 kDa).

Rca plays a primary role in the light-induction of Rubisco activity and photosynthetic CO_2 assimilation (e.g. [13,14]). Regulation of Rca activity and its effects on Rubisco activation are linked to the irradiance level via changes in redox status, ADP/ATP ratio and Mg^{2+} concentration of the chloroplast [15,16]. Studies with Arabidopsis showed that at physiological ratios of ADP to ATP, Rubisco activation by the Rca α isoform activity was significantly inhibited, while the β isoform was insensitive to inhibition by ADP [12]. Subsequently, Carmo-Silva and Salvucci [17] used leaf extracts to demonstrate that Arabidopsis plants expressing only the Rca β isoform showed a lack of down-regulation of Rubisco activity at low light and faster light induction of photosynthesis than in wild-type plants containing the ADP-sensitive Rca α isoform. The physiological significance of Rubisco down-regulation at low light is yet to be understood; however, this mechanism appears conserved. In the Solanaceous species tobacco, which contains only the redox-insensitive Rca β isoform, Rubisco activation is considerably more sensitive to inhibition by ADP than the Arabidopsis Rca β isoform [17], and the light response is similar to that observed in wild-type Arabidopsis.

We set out to investigate the regulatory properties of the three Rca isoforms present in the important cereal crop wheat. We hypothesised that the two wheat Rca isoforms encoded by the same alternatively spliced gene *TaRca2*, i.e. Rca 2β and 2α, would show a response to inhibition by ADP similar to that observed for the Arabidopsis β and α isoforms [17] while Rca 1β resulting from a non-spliced gene would be more like tobacco Rca β. This would make it possible to compare the sequences of these Rca isoforms grouped by ADP sensitivity, identify residues possibly involved in the interaction with adenine nucleotides, and test these predictions using site-directed mutagenesis (SDM).

Material and methods

Purification of recombinant Rca isoforms

The *Escherichia coli* competent cell strain BL21(DE3)pLysS was transformed with the pET-23a(+) (Novagen, U.S.A.) plasmid harbouring the wheat B genome coding regions for the mature Rca protein isoforms [8]. Recombinant wheat Rca isoforms were prepared from 6–10 L *E. coli* cultures as described by Barta et al. [18]. Purified Rca protein was supplemented with 0.2 mM ATP, flash frozen in liquid N_2 and stored at −80°C.

Design and production of Rca mutants

SDM was performed using the QuickChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies, U.K.) according to the manufacturer's instructions. A pET-23a(+) vector containing the coding region for the mature wheat Rca 2β isoform (B genome) was used as the PCR template to create three modified pET-23a(+)-Rca2β expression plasmids. SDM primers were designed using the Rca 2β sequence and the QuickChange Primer Design website (https://www.agilent.com/store/primerDesignProgram.jsp). The target residue changes and respective primer sequences used to create the two double mutants and one quadruple mutant are listed in Table 1. The SDM changes were verified by DNA sequencing of the entire Rca coding region for each expression construct. Plasmids were sub-cloned into *E. coli* strain BL21(DE3)pLysS for expression and each mutant protein purified following the procedure described above.

Gel electrophoresis and immunoblotting of Rca

Polypeptides present in the Rca and Rubisco preparations and in a wheat leaf extract prepared as described by Perdomo et al. [19] were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) on 12% gels (Bio-Rad, U.K.) and either visualised by staining with Coomassie Blue, or subject to immunoblotting. For the latter, proteins were transferred from the gel to a nitrocellulose membrane (iBlot, Thermofisher, U.K.), probed with a primary antibody anti-Rca produced in rabbit against cotton Rca [20] and a fluorescent secondary antibody for visualisation of Rca using an Odyssey Fc imaging (LI-COR, Lincoln, U.S.A.).

Rubisco activation by recombinant Rca proteins

The rate of activation of pre-inhibited Rubisco by recombinant Rca proteins was measured using a two-stage assay according to Carmo-Silva and Salvucci [17]. An aliquot of purified Rubisco was incubated with activation

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Table 1 Target residue changes and primers used for SDM of wheat Rca 2β (B genome)

Rca 2β residue changes	Primer sequence for SDM
M159**I**/K161**N**	5′-TACCGTGAGGCTGCAGACAT**A**ATCAA**T**AAGGGTAAGATGTG-3′
T358**K**/Q362**E**	5′-CGTGTGCAGCTTGCTGACA**AG**TACATGAGC**G**AGGCAGCTCT-3′
Combined M159**I**/K161**N**-T358**K**/Q362**E**	Both primers used.

The nucleotides targeted for mutagenesis and resulting amino acids are highlighted in bold.

mix containing 100 mM Tricine-NaOH pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, and 50 mM DL-dithiothreitol (DTT) for 1 h on ice to fully carbamylate the enzyme. Columns of 2 ml Sephadex G-50 superfine (GE Healthcare, U.S.A.) were equilibrated with at least three column volumes of Rubisco ER desalting buffer containing 50 mM Tricine-NaOH pH 8.0 and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Columns were centrifuged at 400 ***g*** and 4°C for 2 min in a swing-out bucket rotor (TX-150, Thermo Scientific, U.K.) to remove the excess buffer. Carbamylated Rubisco was added to the dry column bed and centrifuged at 400 ***g*** for 2 min at 4°C to recover the desalted sample. The uncarbamylated desalted Rubisco was supplemented with 4 mM RuBP and incubated at 4°C overnight to form the inactive Rubisco-RuBP (ER) complex.

In the first stage of the assay, Rubisco (ER) was reactivated by Rca at 25°C and, in the second stage of the assay, aliquots taken from the first-stage assay were used to measure Rubisco activity at 30°C. For Rubisco reactivation (first stage), the basic assay mix contained 50 mM Tricine-NaOH pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM DTT, 5% (w/v) PEG-3350, and 4 mM RuBP (final concentrations in 90 μl assays). Rca and Rubisco were maintained at a 1 : 5 ratio by using 0.2 mg ml^{-1} Rca and 1.0 mg ml^{-1} Rubisco (ER), or 0.1 mg ml^{-1} Rca and 0.5 mg ml^{-1} Rubisco (ER). The rate of Rubisco reactivation by Rca was assayed in presence of 5 mM ATP and an ATP-regenerating system consisting of 40 U ml^{-1} phosphocreatine kinase and 4 mM phosphocreatine. The maximum activity of Rubisco was determined in the presence of all components except RuBP. Negative control assays with no ATP were performed to estimate the rate of spontaneous release of RuBP from the ER complex [21]. Assays in presence of 5 mM ADP + ATP at a ratio of 0.055, 0.11 or 0.33 ADP/ATP were performed in the absence of the ATP-regenerating system. The reaction was initiated by adding ER; at 0.5, 1.5 and 3 min after the addition of ER, an aliquot of the first-stage assay was transferred to the second stage assay to measure Rubisco activity.

In the second stage assay, Rubisco activity was measured at 30°C to assess the extent of reactivation. The assay mix 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 (final concentrations in 500 μl assays). The reaction was initiated by adding a 25 μl aliquot from the first-stage assay and quenched 30 s later by adding 100 μl of 4 N HCOOH.

The rate of Rubisco reactivation by each Rca isoform in presence of ATP or ADP/ATP ratios was calculated as the fraction of sites reactivated per min, taking into account the maximum activity of Rubisco in each experiment and subtracting the rate of spontaneous Rubisco reactivation [21].

Two experiments were conducted, one comparing the three recombinant purified wheat Rca proteins and one comparing these proteins with three mutant proteins. For each experiment, assays were performed for all three or six isoforms using one biological replicate (one independent preparation of each Rca isoform) and a minimum of three technical replicates. The two experiments used two independent sets of protein preparations (Rubisco and Rca).

ATP hydrolysis of recombinant Rca proteins

The rate of ATP hydrolysis by recombinant purified wheat Rca and mutant proteins was assayed at 25°C using the method of Chifflet et al. [22]. ATP hydrolysis was measured in 50 μl assays containing 100 mM Tricine-NaOH pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM ATP or 5 mM ADP + ATP at a ratio of 0.11 or 0.33 ADP/ATP. Reactions were initiated by adding Rca at a final concentration of 0.05 mg ml^{-1}, and quenched after 5 min by adding 12% SDS. Inorganic phosphate (Pi) released during ATP hydrolysis was determined by measuring the absorbance of molybdenum blue at 850 nm in a spectrophotometer (SpectroStar Nano, BMG Labtech, Germany). To form molybdenum blue, the reactions were first incubated with 100 μl of (final concentrations) 3% ascorbic acid, 0.5 N HCl, and 0.5% ammonium molybdate, at room temperature for 10 min.

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Subsequently, these mixtures were added with 150 μl of 0.9% ascetic acid, 0.9% sodium meta arsenite and 0.9% of sodium citrate, and incubated for 20 min at room temperature. Within the subsequent 30 min, 200 μl were used to determine the absorbance of each sample at 850 nm. The Pi content was calculated by reference to a KPi standard curve and the rate of ATP hydrolysis was determined taking into account the quantity of Rca in the assay and the reaction time. Assays were performed for all isoforms using two biological replicates (two independent preparations of each Rca isoform) and two to three technical replicates.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the statistical significance of differences between mean values of Rca activity for each Rca isoform in presence of ATP. Two-way ANOVA was used to assess significance of differences between Rca isoforms and adenine nucleotide treatments (ATP and ADP/ATP ratios), and the interaction between Rca isoforms and adenine nucleotide treatments. Where a significant main effect or interaction of effects was observed, a Tukey post-hoc test was used for multiple pairwise comparisons. Statistical analyses were performed in R (version 3.3.3; [23]) and RStudio (version 1.0.153; [24]). Data are presented as means ± standard error of the mean (SEM).

Results

Rubisco activation by wheat Rca 2β is insensitive to ADP inhibition

To characterise the regulatory properties of the three Rca isoforms encoded by the wheat B genome, each gene was individually expressed in *E. coli* and the respective proteins were purified. The quality of each protein preparation was assessed by SDS–PAGE and immunoblotting of the recombinant wheat Rca isoforms (1β, 2β and 2α). Rubisco purified from wheat leaves was also analysed by SDS–PAGE. Both Rca and Rubisco showed clear bands, and the preparations contained a majority of the target protein (Figure 1). The two shorter Rca isoforms, 1β and 2β, have predicted molecular mass of 42.7 and 42.2 kDa [8]. This results in a similar migration when the two proteins are separated by SDS–PAGE (Figure 1A) and visualised after immunoblotting using a specific primary antibody against Rca (Figure 1B). The proteins extracted from a wheat flag leaf were also subject to SDS–PAGE followed by immunoblotting, which showed the presence of both the longer α and

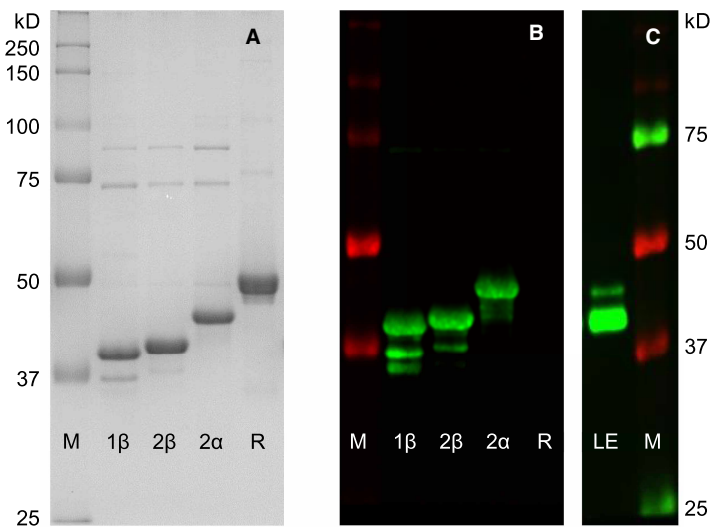

Figure 1. Wheat Rca isoforms and Rubisco.

Wheat Rca isoforms (1β, 2β, 2α) were purified after expression in *E. coli*, and Rubisco (R) was purified from wheat leaves. Polypeptides in each preparation were separated by SDS–PAGE and stained with Coomassie Blue (**A**) or used for immunoblotting with Rca antibody (**B**). Polypeptides in a wheat leaf extract (LE) were subjected to the same procedure (**C**). The positions of apparent molecular mass markers (M) are indicated to the left and right of the gel and blot images, respectively. Each lane was loaded with 1 μg (Rca and LE) or 2 μg (Rubisco) of total soluble protein.

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shorter β Rca isoforms. The relative abundance of the 1β and 2β Rca isoforms in wheat leaves cannot be determined from this data (Figure 1B,C).

Inhibited Rubisco in the form of uncarbamylated catalytic sites bound to RuBP (complex ER) was reactivated by all three native wheat Rca isoforms (Figure 2A). The lack of Rubisco activity in assays performed in the absence of ATP shows the Rca-dependent nature of Rubisco reactivation in the assay. The rate of Rubisco reactivation by Rca was determined using the Rubisco activity values at 0.5 and 1.5 min after the start of the reactivation assay. In the presence of ATP, the wheat Rca isoforms 2β and 2α activated pre-inhibited Rubisco (ER) at faster rates than 1β (Table 2), in agreement with a recent report [25].

To determine the ADP sensitivity of the recombinant Rca isoforms, Rubisco reactivation was measured in the presence of physiological ratios of ADP to ATP [26] (Figure 2B). Rubisco activation by Rca 1β and 2α was inhibited by increasing ADP/ATP ratios, with a 12–23% decrease in presence of 0.055 ADP/ATP, 31% decrease with 0.11 ADP/ATP, and 76–86% decrease with 0.33 ADP/ATP. By comparison, Rca 2β activity remained unaffected by increasing ADP/ATP ratios, suggesting that Rubisco activation by wheat Rca 2β is insensitive to ADP inhibition.

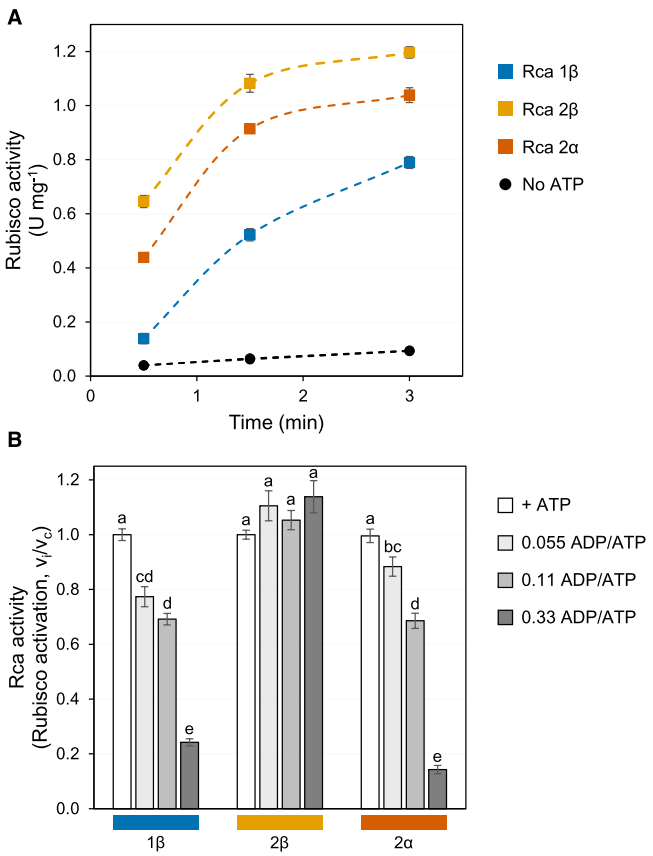

Figure 2. Rubisco activation by recombinant wheat Rca isoforms.

(**A**) Time-course of Rubisco activation by Rca in the absence (circles) and presence of 5 mM ATP plus an ATP-regenerating system (squares). Wheat Rubisco was pre-inhibited by incubation with RuBP in the absence of CO_2 and Mg^{2+}. Rubisco activation assays used 0.2 mg ml^{-1} Rca and 1.0 mg ml^{-1} Rubisco (ER) and were performed at 25°C. Rubisco activity was measured at 30°C at the indicated times. Values are means ± SEM ($n = 12$). (**B**) Rates of Rubisco activation by Rca were determined in presence of 5 mM ATP (v_c) or 5 mM ADP + ATP at the indicated ratios (0.055, 0.11, 0.33; v_i). Rca activity is expressed relative to the control (v_i/v_c), i.e. the rate determined in presence of ADP relative to the rate in presence of ATP alone (see Table 2 for absolute values of control rates). Values are means ± SEM ($n = 6$–12). Two-way ANOVA showed significant effects of Rca isoform ($P < 0.001$), adenine nucleotide treatment ($P < 0.001$), and a significant interaction between isoform and treatment ($P < 0.001$). Different letters denote significant differences (Tukey HSD, $P < 0.05$).

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Table 2 Rubisco activation by recombinant wheat Rca isoforms

Rca isoform	Rubisco activation (Fraction of sites activated min^{-1})
1β	0.248 ± 0.007 b
2β	0.305 ± 0.008 a
2α	0.284 ± 0.010 a

Rates of Rubisco activation were determined in the presence of 5 mM ATP using 0.2 mg ml^{-1} Rca and 1.0 mg ml^{-1} Rubisco (ER). Values are means ± SEM (n = 10–12). One-way ANOVA showed a significant effect of isoform ($P < 0.001$). Different letters denote significant differences (Tukey HSD, $P < 0.05$).

Targeted amino acid changes in Rca2β increase the ADP sensitivity of Rubisco activation

To gain a better understanding of the ADP sensitivity of the Rca isoforms present in wheat, the amino acid sequences were aligned and compared with the sequences of the Rca isoforms present in Arabidopsis and tobacco (Figure 3A). Comparing the sequences of the wheat and the dicot Rca isoforms differing in ADP sensitivity (i.e. wheat Rca 1β versus 2β, and tobacco versus Arabidopsis β), two pairs of amino acid polymorphisms were identified as having a putative role in conferring ADP sensitivity to Rca. This included two residues in the α/β subdomain of the AAA+ module (M159 & K161) and two residues downstream of the final α-helix (V333–D357 [27]) in the α-helical subdomain (T358 & Q362) of wheat Rca 2β (Figure 3B). To test this hypothesis, two double mutants and the corresponding quadruple mutant were generated by SDM of wheat Rca 2β, to replace these amino acids with those present in 1β: M159I/K161N, T358K/Q362E and M159I/K161N-T358K/Q362E.

The rate of ATP hydrolysis by the three mutant proteins was largely comparable to the rates observed for the native proteins, except that the rate of ATP hydrolysis by the quadruple mutant was significantly faster than the rate observed for the native proteins 1β and 2α (Table 3). There were no significant differences in the rates of ATP hydrolysis by the native proteins; however, the rates of Rubisco activation by wheat Rca 2β and 2α were faster than by wheat Rca 1β (Table 3). The mutant Rca protein T358K/Q362E activated Rubisco at a similar rate to wheat Rca 2β, while the mutants containing two residue changes in the α/β subdomain (M159I/K161N and M159I/K161N-T358K/Q362E) activated Rubisco at a rate significantly slower than wheat Rca 2β and comparable to wheat Rca 1β (Table 3).

The sensitivity of the ATP hydrolysis and Rubisco activation activities to inhibition by ADP was determined for each mutant and the native isoforms by assays in presence 0.11 and 0.33 ADP/ATP ratios (Figure 4). The decrease in the relative rate of ATP hydrolysis with 0.33 ADP/ATP was less pronounced for Rca 2β than for Rca 1β or 2α (Figure 4A). The two double mutants and the quadruple mutant showed a significant decrease in the relative rate of ATP hydrolysis in presence of increasing ADP/ATP ratios, but the sensitivity of M159I/K161N was reduced compared with Rca 1β and 2β in presence of either 0.11 or 0.33 ADP/ATP. The sensitivity of T358K/Q362E to inhibition by ADP was comparable to Rca 2β and the quadruple mutant showed significantly lower sensitivity to ADP inhibition compared with Rca 2β at 0.33 ADP/ATP only.

Different responses of Rubisco activation activity to inhibition by ADP were observed in the three mutants (Figure 4B). In M159I/K161N, which contains two residue changes in the α/β subdomain of Rca, and in the quadruple mutant, which combines the two changes in the α/β subdomain and two changes downstream of the final α-helix in the AAA+ module, Rubisco activation was not significantly inhibited by ADP at any of the ADP/ATP ratios, showing a similar response to the native Rca 2β isoform. The mutant T358K/Q362E had significantly decreased Rubisco activation activity at 0.33 ADP/ATP, showing a response to ADP that was intermediate between the two wheat Rca isoforms 2β and 1β.

Discussion

The rate of Rubisco activation by Rca 2β in bread wheat (*Triticum aestivum*) was insensitive to ADP inhibition. All three wheat Rca isoforms showed progressively inhibited rates of ATP hydrolysis in presence of increasing ADP/ATP ratios, while only the isoforms 1β and 2α showed inhibition of Rubisco activation in presence of increasing ADP/ATP ratios. Rca 2β activated Rubisco at identical rates in the absence of ADP and in presence

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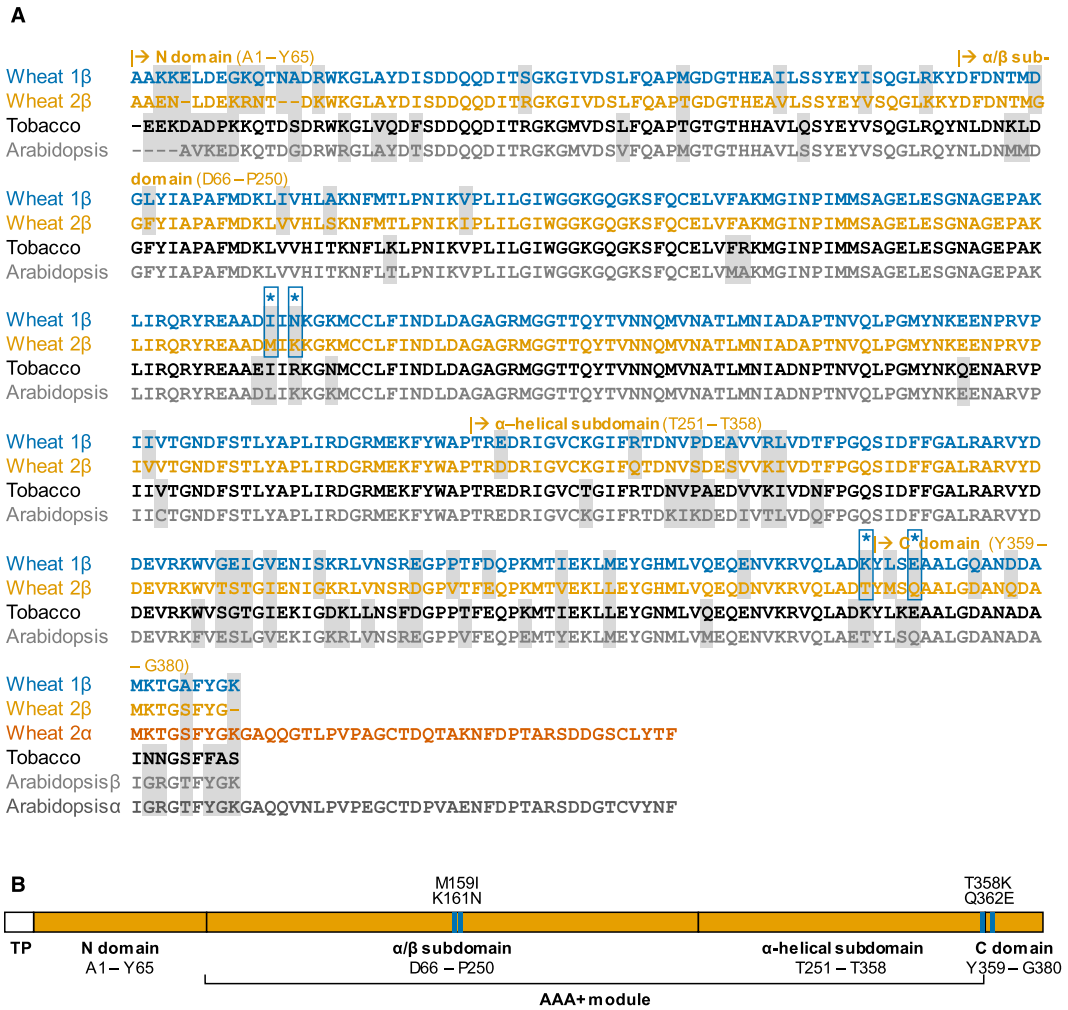

Figure 3. Selection of amino acid residues putatively involved in the ADP sensitivity of Rca.

(**A**) Multiple amino acid sequence alignment for the predicted mature Rca isoforms from wheat (B genome 1β, 2β and 2α [8]), tobacco and Arabidopsis [17] after the chloroplast transit peptide (TP) is removed. Shaded residues differ in the pairwise comparison between wheat 1β and 2β, or between tobacco and Arabidopsis. Based on the similarity of the changes observed for the two pairs of sequences, four residues (M159, K161, T358, Q362) of wheat Rca 2β were selected for SDM (selected residues are marked by boxes and asterisks). (**B**) Schematic representation of wheat Rca 2β. The AAA+ module consists of an N-terminal α/β subdomain and a C-terminal α-helical subdomain, and is flanked by a 65-residue N-terminus (N domain) and a 21-residue C-terminus (C domain) [27]. The relative positions of the mutations M159I/K161N and T358K/Q362E are indicated.

of ADP/ATP ratios up to 0.33. A mutant Rca protein generated by SDM of Rca 2β to change two amino acids to the same as those present in Rca 1β (T358K & Q362E) showed an intermediary response between Rca 2β and 1β, suggesting that one or both of these residues influence ADP sensitivity of Rubisco activation by Rca in wheat.

Regulatory properties of wheat Rca isoforms

Rca uses energy from ATP hydrolysis to promote the release of inhibitors from Rubisco active sites, thereby promoting Rubisco activation. The three recombinant wheat Rca isoforms (1β, 2β and 2α, [8]) hydrolysed ATP at comparable rates, but Rubisco activation by Rca 2β and 2α was faster than rates measured for Rca 1β (Table 3). Rca 2α represents ∼12% of the total Rca protein in wheat leaf extracts prepared from unstressed plants (Figure 1C; [28]), and *TaRca1* is not expressed in significant amounts in wheat plants grown at 22°C [25], suggesting that in the absence of heat stress 2β is the most abundant Rca isoform in wheat leaves. The

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Table 3 Activity of recombinant wheat and mutant Rca isoforms

Rca isoform	ATP hydrolysis (μmol ATP min^{-1} mg^{-1} Rca)	Rubisco activation (Fraction of sites activated min^{-1})
1β	0.95 ± 0.10 b	0.121 ± 0.014 c
2β	1.21 ± 0.12 ab	0.278 ± 0.028 a
2α	0.83 ± 0.05 b	0.248 ± 0.006 ab
M159I/K161N	1.26 ± 0.15 ab	0.184 ± 0.013 bc
T358K/Q362E	1.42 ± 0.21 ab	0.273 ± 0.015 a
M159I/K161N-T358K/Q362E	1.56 ± 0.11 a	0.186 ± 0.012 bc

Rates of ATP hydrolysis and Rubisco activation were determined in presence of 5 mM ATP using 0.05 mg ml^{-1} Rca (ATP hydrolysis) or 0.1 mg ml^{-1} Rca and 0.5 mg ml^{-1} Rubisco (Rubisco activation). Values are means ± SEM (n = 3–6). One-way ANOVA showed a significant effect of isoform for both ATP hydrolysis and Rubisco activation rates ($P < 0.001$). Different letters denote significant differences (Tukey HSD, $P < 0.05$).

recent report of higher thermostability of wheat Rca 1β compared with 2β [25] poses the question as to whether there might be a link between thermostability and a role for sensitivity to ADP inhibition, implying that regulation of 1β by ADP could become more relevant under future climate scenarios [29].

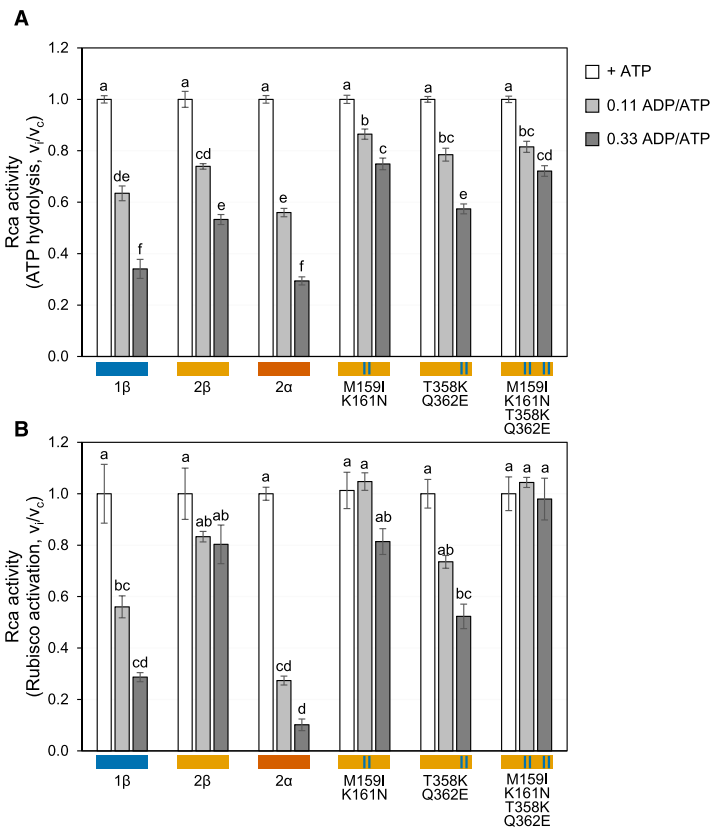

Figure 4. Effect of ADP on the activity of recombinant wheat Rca isoforms and three mutant proteins.
Rca 2β was subject to SDM to introduce two residue changes (M159I/K161N or T358K/Q362E), or all four residue changes combined. Rates of ATP hydrolysis (**A**) and Rubisco activation (**B**) were determined with 0.05 mg ml^{-1} Rca (**A**) or 0.1 mg ml^{-1} Rca and 0.5 mg ml^{-1} Rubisco (**B**), and are expressed relative to the control (v_i/v_c; see Table 3 for absolute values of control rates). Values are means ± SEM (n = 3–6). Two-way ANOVA showed significant effects of Rca isoform ($P < 0.001$), adenine nucleotide treatment ($P < 0.001$), and interaction of isoform and treatment ($P < 0.001$) for both ATP hydrolysis and Rubisco activation activities. Different letters denote significant differences (Tukey HSD, $P < 0.05$).

Photosynthetic efficiency and productivity of a crop canopy depend to a large extent on the speed a leaf can acclimate to a constantly changing light environment [5,6]. As leaves transition between shade and full illumination, changes in the chloroplast stroma, including altered redox status, ADP/ATP ratios, and the concentration of Mg^{2+} modulate the activity of Rca [15,16]. Rca α and β isoforms have been shown to exhibit varying sensitivity to inhibition by ADP across different plant species [12,17,30]. Here we show that the rate of ATP hydrolysis by the three recombinant wheat Rca isoforms was sensitive to increases in ADP (Figure 4A), as previously reported for Arabidopsis, tobacco, spinach and cotton [12,16,17,30]. In contrast, the rate of Rubisco activation by Rca was inhibited by increasing ADP/ATP ratios for the recombinant wheat Rca isoforms 1β and 2α, and insensitive to ADP inhibition for recombinant wheat Rca 2β (Figures 2B and 4B). Insensitivity to inhibition of Rubisco activation rates by ADP was previously reported for the Arabidopsis Rca β isoform using leaf extracts of an Arabidopsis transformant expressing only this isoform (rwt43) [17]. In that study, the rate of Rubisco activation by Rca in leaf extracts of Arabidopsis rwt43 remained unchanged in the absence or presence of ADP, while inhibition of Rubisco activation with increasing ADP/ATP ratios was observed in leaf extracts of wild-type plants of Arabidopsis, tobacco and camelina.

In Arabidopsis, redox regulation of the Rca α isoform, which contains the redox-sensitive C-terminal extension, has been shown to affect the activity of the β isoform, suggesting co-regulation of the two isoforms in the holoenzyme [12,31,32,33]. In our hands, assays with combinations of the wheat isoforms at different ratios (e.g. Rca 2α and 2β at 3:1, 1:1, 1:3 ratios) showed no evidence of regulation of Rca 2α by ADP affecting the activity of Rca 2β (data not shown).

To the best of our knowledge, most studies on the sensitivity of Rca to inhibition by ADP have focused on ATP hydrolysis. Differences between Rca isoforms in the relative rates of ATP hydrolysis and Rubisco activation have been previously reported [17,30,34,35,36]. Although ATP hydrolysis is required for Rubisco activation, the two Rca activities are not strictly coupled because ATP hydrolysis can proceed with or without the presence of Rubisco. In addition, the absolute rates of ATP hydrolysis and Rubisco activation per quantity of Rca (i.e. specific activities) change upon self-association of the enzyme; therefore changes in protein aggregation affect Rca activity and measured rates are dependent on the Rca concentration [37,38,39,40,41].

As in Arabidopsis, wheat Rca 2β and 2α are the product of alternative splicing of a single gene (*TaRca2*), whereas wheat Rca 1β is encoded by a separate gene (*TaRca1*) [8]. In some species such as cotton [30] and soybean [42], the α and β isoforms are encoded by separate genes. It is possible that Rca β isoforms resulting from alternative splicing are insensitive, while β isoforms resulting from expression of non-alternatively spliced genes are sensitive to ADP inhibition of Rubisco activation, however current evidence is limited to studies with Arabidopsis [12,32] and wheat (this study). Rubisco activation by Rca 1β and 2α was sensitive to ADP inhibition (Figures 2 and 4). In addition to the two cysteine residues that confer redox regulation to Rca 2α, it is possible that the C-terminal extension in this isoform may also provide a regulatory sensor of the chloroplast ADP/ATP ratio [33,43]. Both wheat Rca 1β and 2α share a C domain lysine residue (K381, wheat 2α numbering), which is lacking in the ADP insensitive Rca 2β isoform (Figure 3A). Replacing this residue with glutamine in Rca β from Arabidopsis has been shown to reduce ADP sensitivity, although this was only shown for ATP hydrolysis [44]. The presence of this lysine residue in wheat Rca 1β and 2α might partly explain ADP sensitivity in the Rubisco activation assays with these two isoforms.

Amino acid changes downstream of the final α-helix in the AAA+ module of Rca affect ADP sensitivity of Rubisco activation

Site-directed mutagenesis has been used to study the affinity of Rca for ATP and identify residues involved in the recognition of Rubisco [43,45]. Four amino acid polymorphisms were identified herein (Figure 3) for SDM by comparing the sequences of Rca isoforms differing in the sensitivity of Rubisco activation, i.e. wheat Rca 1β (sensitive) versus 2β (insensitive), and tobacco (sensitive) versus Arabidopsis (insensitive). Two of these amino acid residues were located in the α/β subdomain (M159I & K161N) and the other two downstream of the final α-helix in the AAA+ module (T358K & Q362E; Figure 3B). Two double mutants and one quadruple mutant were subsequently produced in Rca 2β to make changes equivalent to the amino acids present in Rca 1β and test the hypothesis that these might be affecting Rca sensitivity to inhibition by ADP. The three mutant proteins displayed a decrease in the rate of ATP hydrolysis in presence of increasing ADP/ATP ratios (Figure 4A), albeit to a lesser extent in mutants containing the residue changes M159I & K161N compared with either of the recombinant wheat Rca isoforms.

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Similar to other AAA+ proteins, Rca has a modular structure consisting of an N-terminal α/β subdomain and a C-terminal α-helical subdomain, flanked by a ∼65-residue N-terminus (N domain) and a ∼21-residue C-terminus (C domain) [9,27,45]. The insensitivity of Rubisco activation by Rca 2β to inhibition by ADP was unaltered in mutants containing the residue changes M159I & K161N (Figure 4B), but a significant decrease in the rate of Rubisco activation was observed for the mutant T358K/Q362E in presence of 0.33 ADP/ATP. The two residues are located downstream of the last α-helix of AAA+ module and in close proximity to the critical C domain tyrosine (Y359, wheat Rca 2β numbering). Mutation of this residue in tobacco Rca (Y361A, tobacco Rca numbering) has been shown to cause complete loss of ATP hydrolysis and Rubisco activation activities, suggesting a possible involvement of Y361 in nucleotide binding or allosteric regulation of ATP hydrolysis [27].

Rubisco activation by T358K/Q362E was inhibited to a lesser extent than Rca 1β, which implies that additional residue changes are likely to be involved. In addition, Rubisco activation by the quadruple mutant containing both pairs of residue changes was insensitive to inhibition by ADP suggesting that, when combined, the two pairs of residue changes cancel the inhibitory effect observed for the double mutant T358K/Q362E. Future studies will aim to identify additional polymorphisms likely to affect the interaction between Rca and adenine nucleotides.

The C domain of Rca is located next to the nucleotide-binding pocket [9,45], and thus it would be plausible that mutations in this region could affect the interaction with ADP and ATP. In addition, certain AAA+ proteins use specific C-terminal domains for substrate recognition [46,47,48]. In the case of Rca, experiments with tobacco and spinach have shown that mutations in the C domain affect the rate of Rubisco reactivation and ATP hydrolysis by Rca and the species specificity of Rca for Rubisco [27,39,43]. In the present study, the double mutant T358K/Q362E had comparable rates of Rubisco activation and ATP hydrolysis in relation to Rca 2β (Table 3). The inhibition of Rubisco activation by this mutant protein in presence of 0.33 ADP/ATP suggests that, at least in wheat, one or both of these residue changes (T358K & Q362E) affect ADP sensitivity of Rubisco activation by Rca.

Conclusion

The results presented here show that wheat Rca isoforms respond differently to physiological ratios of ADP to ATP. The three Rca isoforms present in wheat were sensitive to ADP inhibition in their ATPase activity, but only Rca 2β was insensitive to ADP when reactivating Rubisco at increasing ADP levels. SDM revealed that the double mutant Rca 2β-T358K/Q362E exhibited increased sensitivity to ADP, suggesting that these residues are involved in regulating nucleotide binding.

Abbreviations

ADP, adenosine diphosphate; ANOVA, One-way analysis of variance; ATP, adenosine triphosphate; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, inhibited complex Rubisco-RuBP; LDH, lactate dehydrogenase; NADH, β-Nicotinamide adenine dinucleotide; PEG, polyethylene glycol; Rca, Rubisco activase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SDM, site-directed mutagenesis; SDS–PAGE, sodium dodecyl Sulfate polyacrylamide gel electrophoresis.

Author Contributions

E.C.S. designed the experiments and supervised the project. J.A.P. and G.D. carried out the experiments. D.W. prepared recombinant enzyme expression constructs. D.W., J.A.P. and G.D. purified recombinant proteins. J.A.P. and E.C.S. analysed the data. J.A.P. and E.C.S. wrote the manuscript with contributions from all authors. All authors discussed the results and provided critical feedback.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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