

In Vitro Characterization of Thermostable CAM Rubisco Activase Reveals a Rubisco Interacting Surface Loop^{1[OPEN]}

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To maintain metabolic flux through the Calvin-Benson-Bassham cycle in higher plants, dead-end inhibited complexes of Rubisco must constantly be engaged and remodeled by the molecular chaperone Rubisco activase (Rca). In C3 plants, the thermolability of Rca is responsible for the deactivation of Rubisco and reduction of photosynthesis at moderately elevated temperatures. We reasoned that crassulacean acid metabolism (CAM) plants must possess thermostable Rca to support Calvin-Benson-Bassham cycle flux during the day when stomata are closed. A comparative biochemical characterization of rice (*Oryza sativa*) and *Agave tequilana* Rca isoforms demonstrated that the CAM Rca isoforms are approximately 10°C more thermostable than the C3 isoforms. *Agave* Rca also possessed a much higher in vitro biochemical activity, even at low assay temperatures. Mixtures of rice and *agave* Rca form functional hetero-oligomers in vitro, but only the rice isoforms denature at nonpermissive temperatures. The high thermostability and activity of *agave* Rca mapped to the N-terminal 244 residues. A Glu-217-Gln amino acid substitution was found to confer high Rca activity to rice Rca. Further mutational analysis suggested that Glu-217 restricts the flexibility of the α 4- β 4 surface loop that interacts with Rubisco via Lys-216. CAM plants thus promise to be a source of highly functional, thermostable Rca candidates for thermal fortification of crop photosynthesis. Careful characterization of their properties will likely reveal further protein-protein interaction motifs to enrich our mechanistic model of Rca function.

Rubisco is responsible for all higher plant photosynthetic CO₂ assimilation but exhibits slow and error-prone catalytic properties (Spreitzer and Salvucci, 2002; Tcherkez et al., 2006; Bracher et al., 2017). To support the enormous flux of carbon entering the biosphere, the enzyme is highly overexpressed in leaf tissue, at times comprising up to 50% of the total soluble protein (Parry et al., 2013). An additional unusual property of this enzyme concerns a high affinity for its substrate ribulose 1,5-bisphosphate and similar sugar phosphates, when the active site has not been primed by the cofactors CO₂ and Mg²⁺ (Jordan and Chollet, 1983; Parry et al., 2008). In addition, the activated holoenzyme can also bind a range of molecules nonproductively (Andralojc et al., 2012). This situation leads to the formation of dead-end inhibited complexes that need to be remodeled by dedicated molecular chaperones known as Rubisco activases (Rcas; Portis, 2003; Mueller-Cajar et al., 2014; Carmo-Silva et al., 2015). Molecular motors exhibiting the Rca activity have evolved multiple times convergently in diverse autotrophic

lineages to deal with the near universal property of Rubisco inhibition (Salvucci et al., 1985; Mueller-Cajar et al., 2011; Tsai et al., 2015; Mueller-Cajar, 2017).

In higher plants, the necessity of Rca to constantly clear blocked Rubisco active sites has been co-opted into a regulatory checkpoint, and the green-type Rcas are stimulated and inhibited by signals that correspond to high light (reducing equivalents) and low energy levels (ADP), respectively (Portis et al., 2008). It has been known for a long time that photosynthesis is inhibited at temperatures only slightly above the optimum (Berry and Bjorkman, 1980; Sage and Kubien, 2007; Yamori et al., 2014) and that this effect is correlated with a reduction in Rubisco activity (Weis, 1981; Kobza and Edwards, 1987). In the past two decades, numerous studies have linked an unusual thermolability of Rca to the reduction of photosynthesis at moderately elevated temperatures (Feller et al., 1998; Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001; Salvucci and Crafts-Brandner, 2004a, 2004b; Carmo-Silva and Salvucci, 2011). The notion that photosynthesis could be heat fortified by engineering Rca received significant experimental support, when *Arabidopsis* (*Arabidopsis thaliana*) plants expressing more thermostable Rca variants were shown to exhibit improved growth and biomass accumulation at supraoptimal temperatures (Kurek et al., 2007; Kumar et al., 2009). However, it should be noted that temperature stress does not solely affect Rca and photosynthesis, but also interferes with other processes in plants, such as anthesis and pollen tube growth (Wahid et al., 2007).

Rice (*Oryza sativa*) is the world's most important food crop (Khush, 1997), and thus exploring the possibility of

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enhancing its thermotolerance is critical to ensure food security, especially when considering the anticipated effects of global climate change (Sage et al., 2008). Similar to other plants, photosynthesis in rice has also been shown to be Rubisco limited at temperatures over 33°C (Scafaro et al., 2012), and its Rca is thermolabile (Scafaro et al., 2016). Like most plants, rice possesses two isoforms of Rca, which are the product of alternative mRNA splicing (To et al., 1999) and yield a 46-kD Rca α and a 43-kD Rca β gene product, which differ at the C termini. In rice, the two isoforms are not equally expressed; Rca β is more abundant than Rca α in both *O. sativa* (Wang et al., 2010) and the wild rice *Oryza meridionales* (Scafaro et al., 2010). These studies also reported that in both species, heat stress resulted in a relative increased abundance of Rca α ; however, thermostability of the purified isoforms was found to be similar (Scafaro et al., 2016). In contrast to *O. sativa*, the wild rice *Oryza australensis* was shown to maintain photosynthetic activity at 45°C, which correlated with presence of Rca that, when measured in leaf extracts, maintained full activity up to 42°C compared to 36°C for *O. sativa* Rca (Scafaro et al., 2016). Interestingly, the recombinant *O. australensis* Rca presented with identical thermostability to the *O. sativa* Rca.

One hypothesis regarding the thermolability of Rubisco activase suggests that the enzyme may function as a fuse (Sharkey et al., 2001; Sharkey, 2005). As leaf temperatures rise, the deactivation of the activase will result in reduced Rubisco activity and thus a reduction in metabolic flux through the Calvin-Benson-Bassham cycle. In addition, plants from a high temperature environment may not require a particularly thermostable activase, since they often possess powerful leaf cooling mechanisms, especially transpiration (Wise et al., 2004; Carmo-Silva et al., 2012; Sage, 2013). We note that such a mechanism would not be available to plants using crassulacean acid metabolism (CAM) growing in a desert environment, where Calvin-Benson-Bassham cycle function will be taking place in the light, with the stomata closed to minimize water loss (Borland et al., 2014). Consequently, we reasoned that, like other enzymes required during daytime, CAM activases should be relatively thermostable (Brandon, 1967; Lutge, 2004). Any potential temperature fuse function should not be conserved in CAM Rca proteins.

While the lack of Rca thermostability and its physiological ramifications has received abundant attention, the molecular mechanism of Rca function was less amenable in the past, due to a lack of structural information. Only recently have atomic models of Rca become available, providing a structural framework to interpret earlier biochemical results (Henderson et al., 2011; Stotz et al., 2011; Hasse et al., 2015). Nevertheless, details of the green-type Rca mechanism remain mostly obscure (Wachter et al., 2013; Hauser et al., 2015). A current model based on biochemical results involves the disc-shaped Rca hexamer engaging inhibited Rubisco at the exposed β C- β D loop of the Rubisco large subunit N-terminal domain via the specificity helix H9 of the

Rca's α -helical subdomain (Larson et al., 1997; Ott et al., 2000; Li et al., 2005). In addition, the Rca N-terminal domain of approximately 60 residues and the pore loops of the hexamer have also been implicated in Rubisco remodeling (Esau et al., 1996; van de Loo and Salvucci, 1996; Stotz et al., 2011). It is evident that generating a more detailed mechanistic model requires the identification of additional residues involved in the Rca-Rubisco interaction.

Here we have utilized an in vitro biochemical approach to characterize the Rca isoforms from the CAM plant *Agave tequilana* and compared their properties to those from *O. sativa*. In addition to being highly thermostable, the CAM enzymes exhibit a high specific Rca activity due to enhanced affinity for rice Rubisco. Identification of a single residue change conferring this phenotype to rice Rca implicates a flexible loop in the Rca nucleotide binding domain as a new Rubisco interacting motif. Our results suggest that agave Rca is a suitable candidate to rigorously evaluate the effect of thermostable Rca function in rice, which may lead to enhanced productivity at moderately elevated temperatures.

RESULTS

Rubisco Activase from Agave Is Highly Functional at Activating Rice Rubisco

We aimed to explore our outlined hypothesis that CAM Rca proteins would need to be highly thermostable to maintain Rubisco function in high temperature environments in the absence of transpirational cooling. A sequence alignment of selected C3 and CAM Rca sequences did not yield systematic group-specific residue changes that could indicate parallel adaptive evolution as for instance seen in the phosphoenolpyruvate carboxylase of C4 plants (Supplemental Fig. S1; Christin et al., 2007). We therefore decided to test our hypothesis experimentally. We produced the agave (sequences mined from transcriptome data; Gross et al., 2013) and rice long (α) and short (β) Rca isoforms in *Escherichia coli*, followed by purification to homogeneity (Fig. 1A). The transcripts encoding the agave Rca isoforms result in amino acid sequences that are only 91% identical to each other and not consistent with alternative splicing of a single transcript. This indicates the existence of two separate Rca genes as described in cotton (*Gossypium hirsutum*; Salvucci et al., 2003). Both agave isoforms are 86% identical to the respective rice isoform.

Similar to that recently reported (Scafaro et al., 2016), the ATPase activity of the short rice Rubisco activase isoform (OsRca β) at $23 \pm 3.2 \text{ min}^{-1}$ protomer activase $^{-1}$ was approximately 3-fold higher than that observed for the long isoform (OsRca α ; Fig. 1C). In contrast both *A. tequilana* isoforms displayed a relatively high ATPase activity of approximately 32 min^{-1} (Fig. 1C). These numbers are very similar to those reported earlier for activases from other plant species (Hazra et al., 2015). All Rca

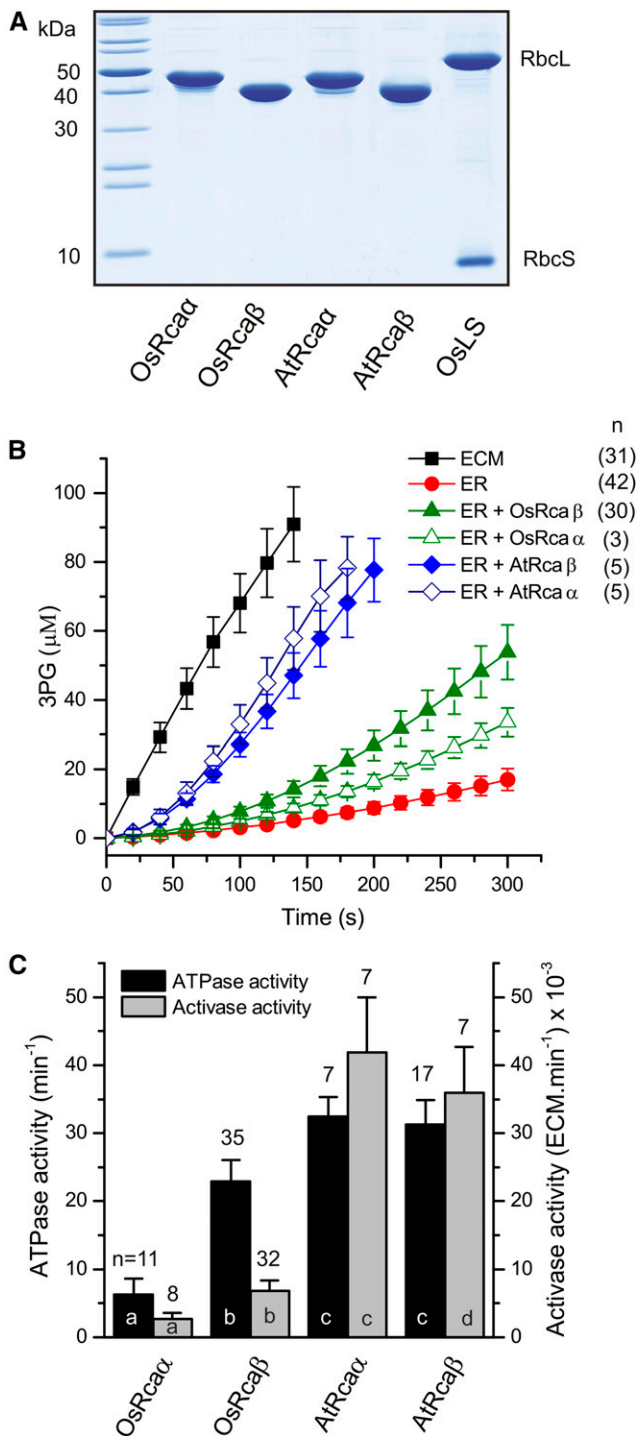


Figure 1. Biochemical properties of Rubisco activase isoforms from rice and agave. A, SDS-PAGE analysis of purified activases and rice Rubisco. A total of 4 μ g of protein loaded per lane. B, Rubisco activation assays of RuBP-inhibited rice Rubisco (ER) performed at 25°C (0.5 μ M Rubisco active sites, 5 μ M activase protomer). C, ATPase activity assays (5 μ M protomer, 25°C) and quantified Rubisco activase activity of the different activase isoforms. Error bars show mean and SD of independent experiments with n as indicated. Significant differences ($P < 0.05$) are denoted by different letters at the base of each bar.

isoforms presented with the concentration-dependent poly-disperse oligomeric state typical for green-type Rca when analyzed by size exclusion chromatography (Supplemental Fig. S2; Blayney et al., 2011; Keown et al., 2013).

Plant activases tend to be broadly compatible, and so far incompatibility has only been demonstrated between activases and Rubiscos derived from Solanaceae and non-Solanaceae species (Wang et al., 1992). Consistent with this notion, the CAM activases were functional at catalyzing the conversion of inhibited rice Rubisco Enzyme-RuBP (ER) complexes to the holoenzyme (ECM). Strikingly, their comparative ability to do so was approximately 5-fold that of OsRca β under our assay conditions, with an activation rate of approximately $40 \times 10^{-3} \text{ ECM} \cdot \text{min}^{-1}$ protomer activase $^{-1}$ (Fig. 1, B and C). Supplemental Figure S3A illustrates an Rca activity quantification using our adaptation of the spectrophotometric Rubisco activase assay (Lan and Mott, 1991). The ability of Rca to function in vitro has previously been enhanced by inclusion of the macromolecular crowding agent polyethylene glycol (Salvucci, 1992), which increases the affinity between macromolecules due to excluded volume effects (Ellis, 2001). Inclusion of 5% v/v polyethylene glycol 3350 in the Rca assay almost eliminated the difference in Rca activity between OsRca β and AtRca β (Supplemental Fig. S3B). We therefore conclude that the enhanced Rca function exhibited by the agave isoforms are largely a consequence of an increased affinity between Rca and the carboxylase.

The Agave Activases Are Thermostable but Maintain High Activity at Lower Temperatures

Next we tested the thermostability of the different isoforms by incubating them at temperatures ranging from 25°C to 55°C for 10 mins, followed by an ATPase activity assay at 25°C. Consistent with our hypothesis, agave isoforms were around 10°C more thermostable than those from rice. AtRca β still maintained some functionality after incubation at 52°C, whereas OsRca β was completely inactive after incubation at 45°C (Fig. 2A). For both rice and agave, the long (α) isoforms were slightly less thermostable than the short (β) isoforms (Fig. 2A). In earlier work, it has been repeatedly found that bound ligands can increase the thermostability of Rca, for example (Salvucci et al., 2001; Henderson et al., 2013). In particular, the nucleotide analog ATP γ S has an extraordinary effect on spinach (*Spinacia oleracea*) Rca α , increasing the denaturation temperature from a low 30°C to 55°C (Crafts-Brandner et al., 1997; Keown and Pearce, 2014). An effect of this magnitude has not been described for any other Rca so far, and its physiological significance is unknown. For our isoforms, inclusion of Mg-ATP γ S had only a very modest effect on thermostability (Supplemental Fig. S3C). Hence, the remainder of our thermostability characterizations were performed using the apo forms of our Rca proteins.

It is commonly believed that increased thermostability of proteins in general trades off with functionality

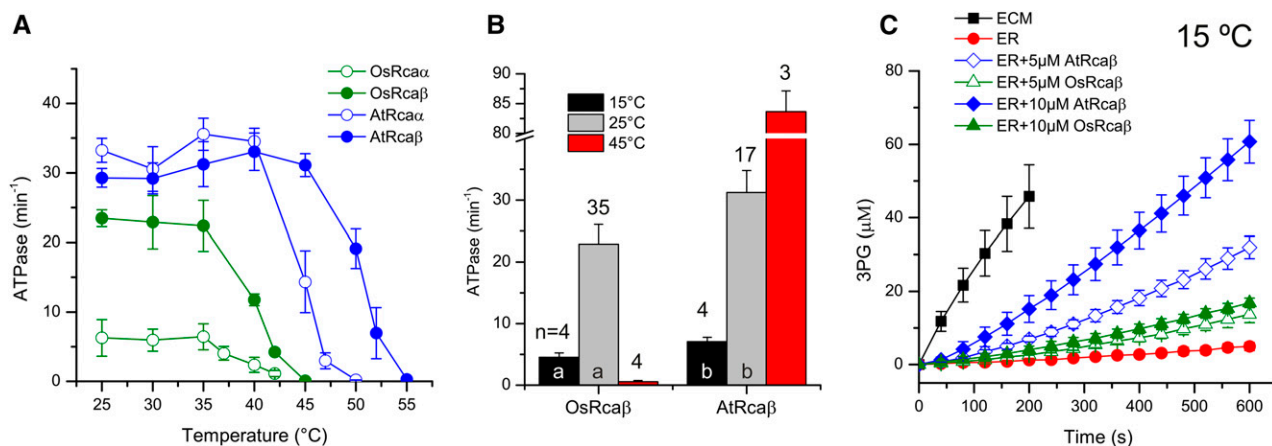


Figure 2. Agave activases are highly thermostable. A, The activase isoforms were incubated for 10 min at the indicated temperature, and subsequently the remaining ATPase activity was assayed at 25°C (5 μ M protomer). B, ATPase activity of OsRca β and AtRca β at various temperatures. Proteins were preincubated (10 min) and subsequently measured at the indicated temperatures under the conditions described in Figure 1C. Error bars show mean and SD of independent experiments with n indicated on top of the bars. Significant differences between activities at the same temperature ($P < 0.05$) are denoted by different letters at the base of each bar. C, AtRca β has higher Rubisco activase activity than OsRca β even at low temperature. Rubisco activation assays were performed as in Figure 1B at 15°C. A and C, error bars indicate mean and SD of independent experiments ($n = 3-5$).

at lower temperatures (Zavodszky et al., 1998). To test whether this was the case for the CAM activases, we measured ATPase and activase activity at 15°C. As expected from physical principles, biochemical activity at the lower temperature was reduced, but in all cases the agave proteins retained their higher relative activities compared to their counterparts from rice (Fig. 2, B and C).

Agave Activases Form Functional Hetero-Oligomers with Rice Activases

Our results thus far indicated that agave Rca isoforms could be suitable candidates for expression in rice. In earlier work, Rcas from maize (*Zea mays*; of unknown thermostability) have been overexpressed in rice, which also contained the endogenous proteins (Yamori et al., 2012). Given the dynamic oligomeric state of Rubisco activase (Barta et al., 2010; Chakraborty et al., 2012), which includes rapid exchange of subunits (Salvucci and Klein, 1994; van de Loo and Salvucci, 1998; Stotz et al., 2011), it is clear that in many constellations a loss of function can be expected when subunits from different species are present and interact. Even if hetero-oligomerization does not impede biochemical function it is conceivable that heat-induced aggregation of the thermolabile isoform will cause the more thermostable isoforms to be recruited to the inclusion, in particular considering their presence in the same protein complex. To address these issues with regards to the rice-agave activase system, we performed a series of experiments. First, we characterized the biochemical properties of a 1:1 mixture of OsRca β and AtRca β . Both activase and ATPase activity of the subunits mixtures was intermediate to the homo-oligomers (Fig. 3, A and B). Although

indicating that the activases do not impair their function, this result cannot distinguish between two distinct populations of homo-oligomers and the formation of functional hetero-oligomers. We therefore produced an Asp-173-Ala mutant of OsRca β . Asp-173 is the conserved Walker B acidic residue and when mutated, leads to loss of ATPase activity and nucleotide binding (van de Loo and Salvucci, 1998; Kuriata et al., 2014; Hasse et al., 2015; Fig. 3B). It is important to verify loss of functionality was not due to changes in oligomeric behavior. Therefore, all variants analyzed in this study (Supplemental Fig. S4A) were tested for the Rca-typical concentration-dependent oligomerization using analytical gel filtration (Supplemental Fig. S4B).

We then performed a mixing or poisoning experiment, where AtRca β concentration was kept constant and OsRca β D173A concentration was titrated up, followed by measurement of the ATPase activity. Addition of the ATPase inactive rice subunits resulted in a strong decrease of the ATPase activity of the agave isoform (Fig. 3C) consistent with the cooperativity between subunits observed previously (van de Loo and Salvucci, 1998). Taken together, these results indicate that agave and rice activase form highly functional hetero-oligomers. We then decided to ask whether hetero-oligomerization would affect the thermostability of the agave subunits. The 1:1 mixture of AtRca β and OsRca β D173A displayed a low ATPase activity of $11.8 \pm 2.5 \text{ min}^{-1}$ (per wild-type active site), consistent with the Walker B mutant poisoning the activity of the agave enzyme (Fig. 3D). Following a 10-min incubation of the mixture at 45°C, the agave activase regained full activity ($28.8 \pm 2.2 \text{ min}^{-1}$). This result demonstrates that heat denaturation of the rice activase isoform, even when incorporated into hetero-oligomers,

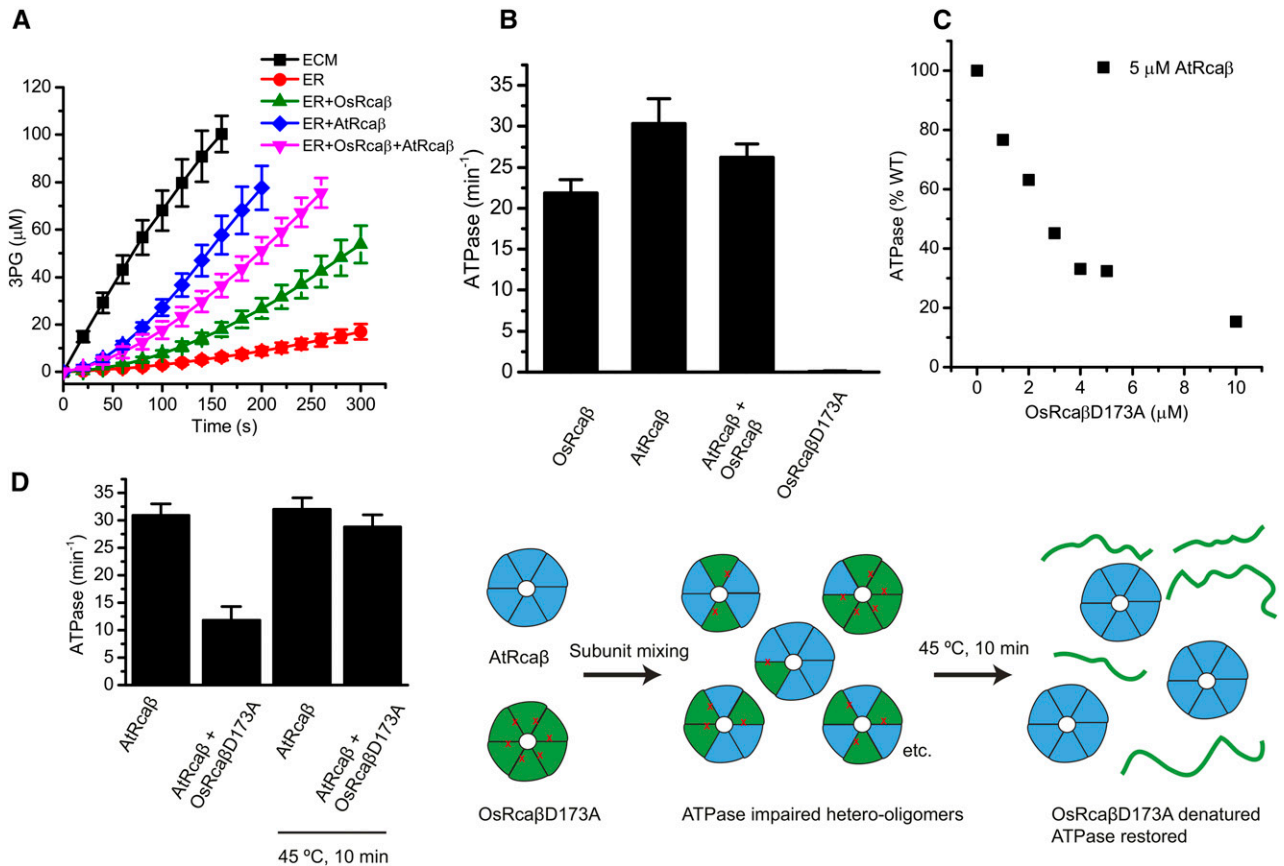


Figure 3. Rice and agave activases form hetero-oligomers displaying intermediate functionality but subunit-specific thermostability. A and B, Rubisco activation assay (A) and ATPase assay (B) performed using 5 μM activase protomer (2.5 μM + 2.5 μM for AtRca β + OsRca β). ATPase is given per active site. C, Evidence for hetero-oligomerization. AtRca β was mixed with increasing amounts of ATPase inactive OsRca β D173A, incubated for 10 min, and assayed for ATPase activity. D, Incubation at 45°C restores ATPase activity of impaired hetero-oligomers to levels of AtRca β wild type. C and D, ATPase activity was calculated as turnovers per wild-type active site. Error bars indicate mean and sd of independent experiments (A, $n = 5\text{--}42$; B and D, $n = 4\text{--}7$).

does not lead to reduced thermostability of the agave activase. Instead, denaturation of the rice subunits leads to formation of fully functional agave activase (Fig. 3D).

High Thermostability and Activity Maps to the N-Terminal Portion of Agave Rca

We next decided to map the determinants of agave Rca thermostability. As observed for activase homologs in previous studies (Esau et al., 1998; Kumar et al., 2009; Carmo-Silva and Salvucci, 2013), chimeric activases (Fig. 4A) could be purified and exhibited typical Rca oligomerization behavior (Supplemental Fig. S4, A and B). Characterization of domain-swapping chimeras indicated that both thermostability and higher activase activity of AtRca β mapped to its N-terminal 244 residues (crossover point indicated in Supplemental Fig. S1), which approximately encompasses the N-terminal domain and its nucleotide binding domain (Fig. 4, A–C). The chimeric activase AtOsRca β , comprising the N-terminal

portion from agave and the C-terminal 140 residues from rice, maintained significant ATPase activity at 45°C but was inactive at 50°C and thus slightly less thermostable than AtRca β (Fig. 4B). It displayed a high activase activity equivalent to 80% of AtRca β (Fig. 4C). In contrast, the inverse construct OsAtRca β displayed very similar properties to OsRca β (Fig. 4, B and C). Thermostability and activase activity were identical between the two proteins, whereas the chimeric OsAtRca β possessed 75% of ATPase activity of OsRca β .

An inspection of a sequence alignment covering the identified N-terminal region of the rice and agave activase isoforms revealed only a small number of amino acids that differed systematically between the species (Fig. 5A). We therefore produced a series of seven OsRca β variants in an attempt to confer the agave isoforms' properties to the rice enzyme. All seven variants were purified in soluble form (Supplemental Fig. S4A), and exhibited similar ATPase activity to the wild type, with the exception of F68L. This variant was severely impaired biochemically, as well as displaying atypical oligomerization (Fig. 5B;

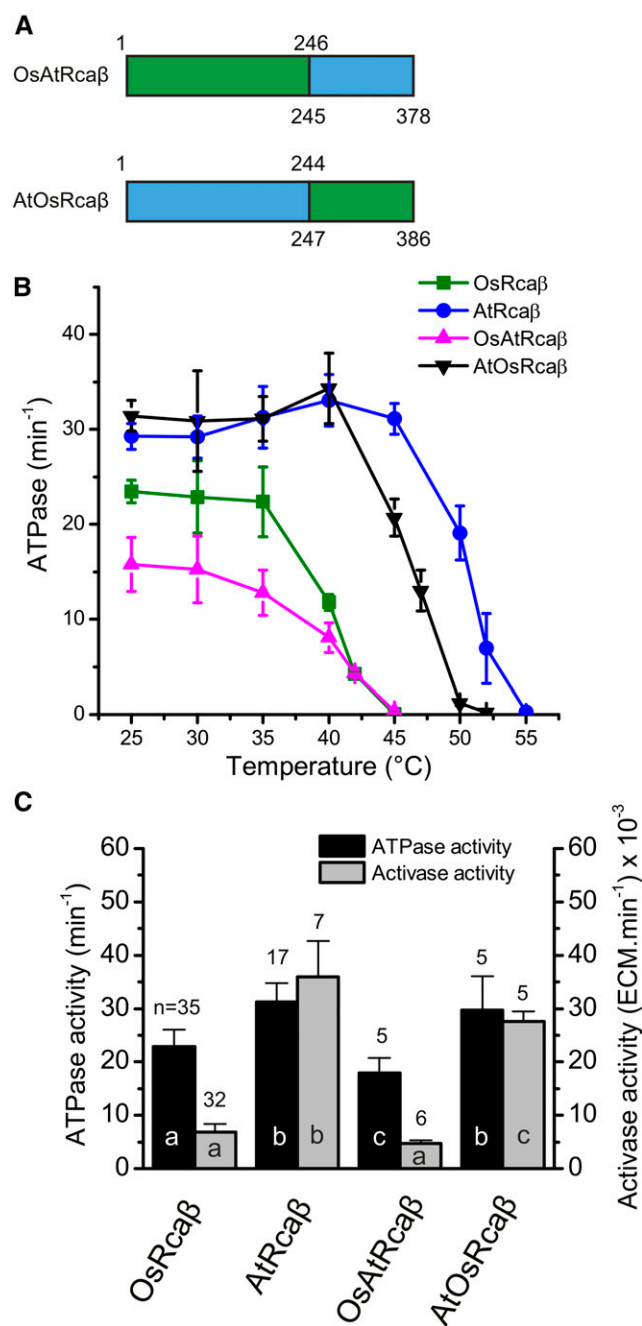


Figure 4. Thermostability and high activase activity maps to residues 1 to 244 of AtRca β . **A**, Schematic representation of the chimeric activases. The crossover point corresponds approximately to the domain boundary of the nucleotide binding domain and the α -helical subdomain (residue 253 in OsRca β). Green, rice sequence; cyan, agave sequence. Numbering on top and bottom refers to residue numbers of the source sequence. **B**, The chimera containing the agave N-terminal portion (AtOsRca β) is thermostable. Thermostability assay performed as described in the legend to Figure 2A. Error bars indicate mean and SD of at least three independent experiments. **C**, The high activase activity of AtRca β maps to the N-terminal portion. Experimental conditions as in Figure 1C. Error bars show mean and SD of independent experiments with $n = 3$ to 4 (B) and as indicated on top of the bars (C). Significant differences ($P < 0.05$) are denoted by different letters at the base of each bar.

Supplemental Fig. S4B). Disappointingly, none of the variants was able to maintain functionality following a 10-min incubation at the nonpermissive temperature of 45°C (Fig. 5B). This result indicated that the relatively higher thermostability of AtRca compared to OsRca cannot be conferred by a single amino acid change. Instead it is determined by a combination of multiple residues that remain to be mapped. However, one of the variants, OsRca β E217Q, possessed a similarly high activase activity as AtRca β (Fig. 5, C and D). Since the high activase activity of AtRca β is likely due to an increased affinity of the activase for its substrate Rubisco (Supplemental Fig. S3B), the phenotype of OsRca β E217Q implicates this residue in protein-protein interactions.

The $\alpha 4$ - $\beta 4$ Loop Is a New Rubisco-Rca Interacting Motif

Glu-217 is positioned in the loop connecting helix 4 to beta strand 4 in the nucleotide-binding domain (Supplemental Fig. S1). The two structural models of tobacco (*Nicotiana tabacum*) and Arabidopsis Rca are both missing 11 (albeit different) residues of the loop, suggesting that it is highly flexible (Stotz et al., 2011; Hasse et al., 2015). The loop is located on the top side of the Rca hexamer (Fig. 6A), the face exposing the nucleotide binding domains, which is commonly involved in substrate engagement of AAA+ proteins (Zhang and Wigley, 2008). This side is also predicted to be the surface utilized by the disc-shaped Rca hexamer to bind Rubisco (Stotz et al., 2011; Wachter et al., 2013) and exposes the Rubisco-interacting specificity helix H9. The increase in biochemical activity brought about by the E217Q substitution suggests that this variant possesses an enhanced affinity for rice Rubisco. Analysis of 220 plant Rca sequences shows that the loop is highly conserved (Fig. 6B), and we thus performed an Ala scan on the residues flanking E217. Substituting Y214 and N219 with Ala resulted in approximately 80% reductions in ATPase activity, suggesting that the loop can influence ATPase function but complicating analysis of the residues' contribution to Rubisco remodeling (Fig. 6C). N219A, but not Y214A, exhibited an oligomerization defect (Supplemental Fig. S4B). E217A exhibited a similarly high activity phenotype to E217Q (Fig. 6C). This result suggests that residue 217 determines the conformation and Rubisco binding affinity of the loop rather than contributing to the Rca-Rubisco interaction directly. The Arabidopsis Rca structure includes information on E217 and K216, although the B-factors for the region are high (Hasse et al., 2015). The side chain carboxyl group of E217 is within salt bridge-forming distance of the amino group of K162, which is located at the C terminus of helix 3 (Fig. 6D). Hence, the E217A or Q substitution may disrupt this interaction, leading to enhanced flexibility and thus Rubisco affinity. The evidence for this interaction is strengthened by the observation that Rca sequences that encode E217 tend to possess a Lys at position 162. In contrast, sequences encoding Q217 present a variety

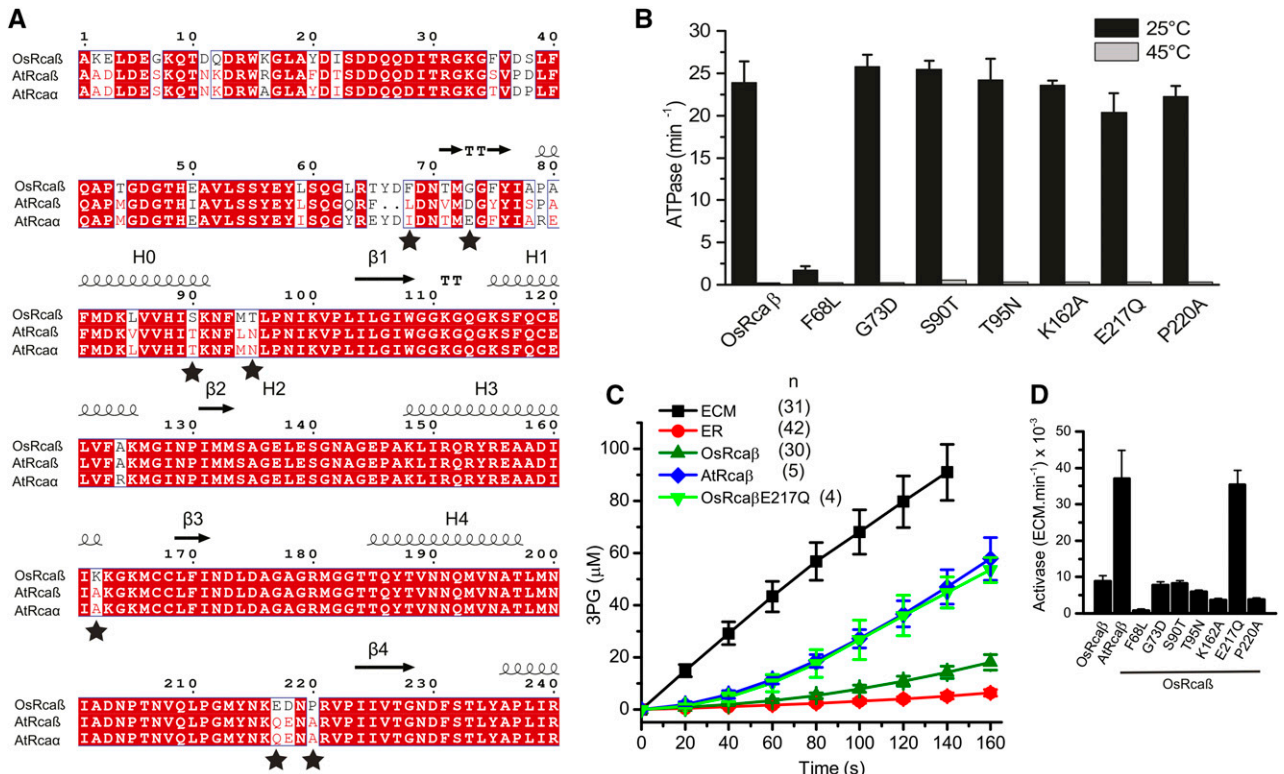


Figure 5. The Glu-217-Gln substitution confers high in vitro activase activity to OsRcaβ. A, Sequence alignment of the thermostability-conferring region of rice and agave activases. Residues indicated by stars were selected for mutagenesis. B, Thermostability assay of the Rca variants. ATPase assays were performed at 25°C and after a 10-min preincubation at 45°C. C and D, OsRcaβE217Q phenocopies the high in vitro activase activity of AtRcaβ. Error bars indicate mean and SD of independent experiments (B, $n = 3-7$ at 25°C, $n = 1$ at 45°C; C as shown; D, $n = 3-5$).

of non-Lys residues at position 162 (indicated in Supplemental Fig. S1). However, it should be pointed out that the OsRcaβK162A variant, which cannot form the salt-bridge, did not exhibit the high Rca activity of AtRcaβ (Fig. 5D). This result indicates that mere disruption of the proposed salt-bridge is insufficient to confer the high activity phenotype to OsRca.

Both N215A and K216A presented with over 50% of wild-type ATPase activity, but exhibited strong reductions in Rca function. Supporting an important role for K216 in protein-protein interaction, the charge switch variant K216E retained 41% of ATPase activity, but was nonfunctional at activating Rubisco (Fig. 6C). Together these results indicate that the α 4- β 4 loop represents a new Rubisco interacting motif, where the side chains of N215 and K216 are likely to be directly involved in the protein-protein interaction. An E217-K162 salt-bridge may limit the flexibility of this loop and concomitantly reduce the affinity of OsRcaβ for its cognate Rubisco.

DISCUSSION

In this work, we aimed to test the hypothesis that plants utilizing the Calvin-Benson cycle in leaves experiencing high temperatures, such as those exhibiting

the CAM photosynthetic syndrome, would encode a thermostable Rca protein to ensure Rubisco's functionality. We made the unexpected discovery that the Rca isoforms from agave not only possessed the predicted properties, but also exhibited a much higher Rca activity against rice Rubisco than the cognate activase. In addition, the higher enzymatic activity of AtRcaβ was also demonstrated at lower temperatures, indicating that a flexibility-thermostability tradeoff does not apply in this instance. We hypothesize that there has been a selection pressure for this property to adapt agave photosynthesis to its desert environment. Following the night-time CO₂ uptake Phase I, Phase II of CAM occurs during low morning temperatures, whereas Phase III corresponds to high day-time temperatures with closed stomata (Borland et al., 2014). Previous work has shown that Rubisco activation state increases from a low level during Phase II and is maintained during Phase III and IV in a number of CAM species (Maxwell et al., 1999; Griffiths et al., 2002, 2008; Davies and Griffiths, 2012), indicating Rca needs to be functional during these phases.

Earlier work reported relatively thermostable Rca from the C3 desert plant *Larrea tridentata*, which is found in a similar environment to agave (Salvucci and Crafts-Brandner, 2004b). However, it lost 50% of ATPase activity at 43°C (44°C in the presence of

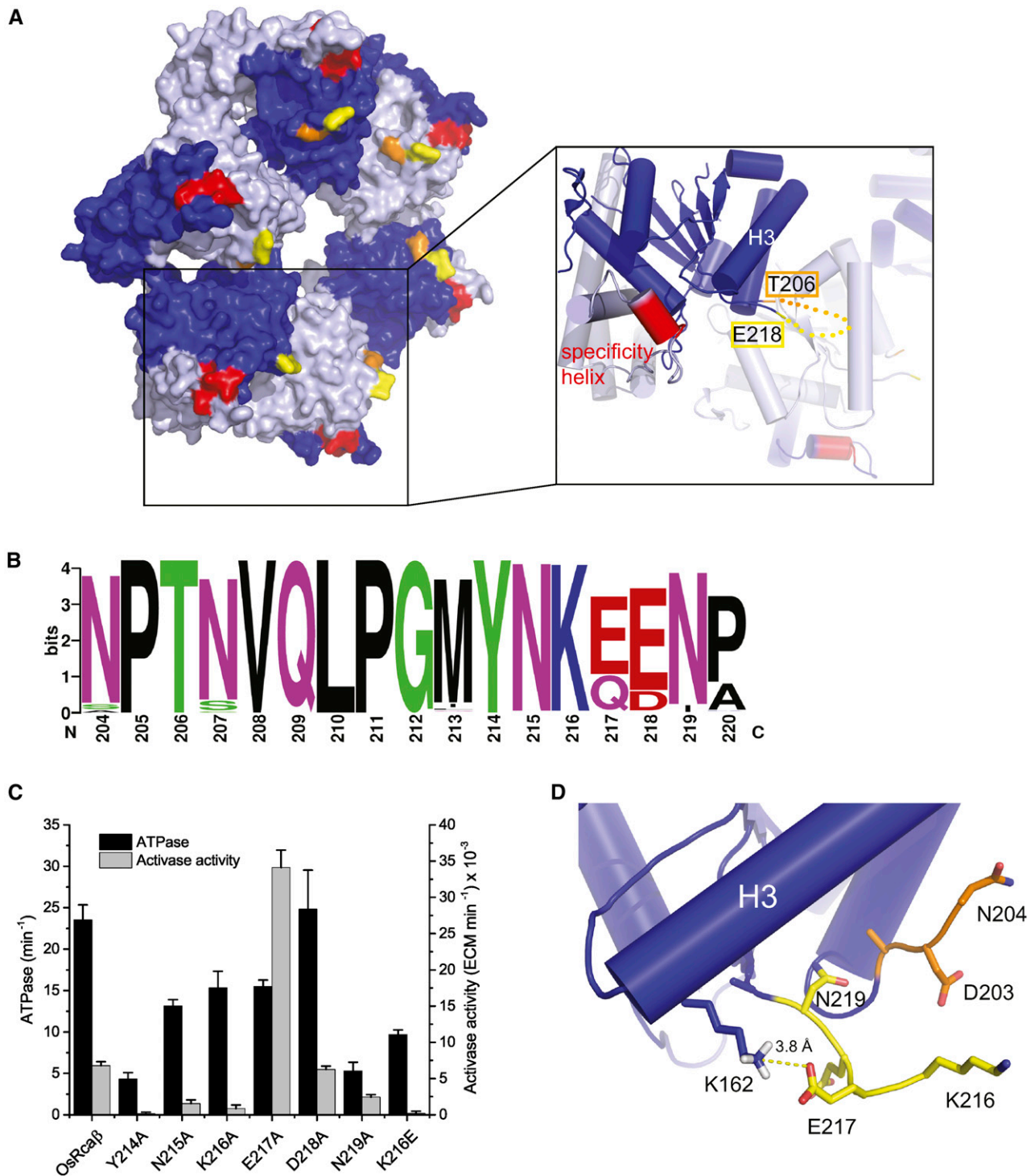


Figure 6. Characterization of a new Rubisco interacting loop. A, Relative position of the Q217-containing loop in context of the Rca hexamer. A surface representation of the hexameric tobacco Rca model (PDB:3ZW6) is shown with alternate subunits colored in blue and light blue. The specificity helix H9 is colored red, and the last resolved residues flanking the flexible loop are shown in orange (T206) and yellow (E218). The close-up view shows the local secondary structure with helices represented by cylinders. The loop is indicated by a dotted line. B, Logo motif of the loop from an alignment of 220 plant Rca sequences generated using WebLogo (Crooks et al., 2004). C, Biochemical characterization of loop mutants was performed as in Figure 1C. Error bars indicate mean and SD of independent experiments with $n = 4$ to 9. D, The loop region in the Arabidopsis Rca structure (PDB:4W5W) suggests that the carboxyl group of E217 forms a salt-bridge with the amino group of K162. For simplicity, residue numbering in all panels of this figure corresponds to the OsRca β sequence. Structures were drawn using Pymol (www.pymol.org).

ATP γ S) and thus is only slightly more thermostable than rice Rca. A very comprehensive analysis of cotton Rca β established conditions leading to high thermostability (apparent T_m of 50°C in presence of 8 mM ADP); however, the apo enzyme denatures below 40°C (Henderson et al., 2013). To our knowledge, agave Rca presents as the most thermostable green-type Rca in the absence of stabilizing ligands. Interestingly, the red-type Rca from the thermophilic red algae *Cyanidioschyzon merolae* tolerated incubation at 60°C (Loganathan et al., 2016); however, it is not compatible with plant Rubisco.

Our results imply that plants exhibiting the CAM syndrome growing in high temperature environments are in general likely to possess thermostable Rca proteins. Since CAM has evolved multiple times independently (Silvera et al., 2010), we can thus predict multiple unique solutions to thermostability to be available. Our inability to find a single amino acid substitution conferring thermostability to OsRca indicates multiple residue changes contributing subtle stabilizing effects (Matthews, 1993) will be necessary. In addition, it is possible that the observed high affinity of agave Rca for rice Rubisco is not due to use of a heterologous Rubisco. Instead it may be a consequence of the relatively low Rubisco content reported for CAM plants (4% of total soluble leaf protein in *Agave victoriae*; Galmes et al., 2014), which would necessitate a higher affinity (lower K_m) of agave Rca for the Rubisco substrate assuming stromal Rubisco concentrations were also low. In contrast, rice possesses very high Rubisco levels of approximately 50% of total soluble leaf protein (Ishikawa et al., 2011), and Rca would thus require a lower affinity for Rubisco. To assess this point further, it will be necessary to measure the relative activation rate of agave Rubisco by AtRca. If AtRca also presents a high affinity for agave Rubisco, it follows that performing similar analyses of Rca proteins from plants containing low Rubisco levels will likely yield additional protein-protein interaction sites.

More generally, the ability to locate thermostable activases that also function robustly at low temperatures suggests that the thermolability of C3-plant Rca proteins could be under positive selection, which is relaxed in CAM plants. This implies a fitness defect may become apparent, should the hypothetical thermal fuse be overridden by thermostable Rca proteins (Sharkey, 2005). Nevertheless, organisms are prone to overreact to environmental stress, and under agricultural conditions our crops may well tolerate approaches that aim to “over-clock” metabolic pathways. Our work indicates that overexpression of AtRca in rice (for instance using a similar approach as described previously; Fukayama et al., 2012) should permit functional activase to persist at higher temperatures even in the presence of the endogenous Rca. This should expand the temperature range of photosynthesis of the transformants and possibly enhance growth at supraoptimal temperature similar to that seen in *Arabidopsis* (Kurek et al., 2007; Kumar et al., 2009). Experiments aiming to explore this possibility are underway. In addition, the rapid development of genome editing tools such as

CRISPR-Cas9 (Belhaj et al., 2015) will permit more sophisticated modifications such as deletion of the endogenous OsRca. This would overcome problems encountered upon Rca overexpression earlier, such as reduced Rubisco levels (Fukayama et al., 2012).

The implication that the α 4- β 4 loop contributes to Rubisco activation helps in further constraining our mechanistic model of Rubisco activation (Wachter et al., 2013; Bhat et al., 2017) and firmly establishes the top surface of Rca as the Rubisco interacting face. Additional work will be needed to resolve the interacting residues on Rubisco’s surface. It will also be interesting to see whether the loop is a passive docking motif, or if loop movements are in fact coupled to rearrangement of the Rubisco active site.

By describing a highly functional, thermostable Rca that does not impair endogenous rice activase function, we hope to provide a tool that will enable a more detailed dissection of the role of Rca in the thermostability of photosynthesis. In addition, we intend to illustrate the utility of careful biochemical characterization of heterologous systems in the discovery of functional determinants, such as the described Rubisco interacting loop. We anticipate that the journey toward a more complete mechanistic understanding of plant Rca function will be informed by similar studies. Maintaining the function of the photosynthetic carboxylase is critical for our food and energy supplies, and increasing our appreciation of this process will outline feasible avenues toward maximizing CO₂ fixation and enable their efficient implementation (Sharwood, 2017).

MATERIALS AND METHODS

Molecular Biology

Nucleotide sequences encoding Rca α and Rca β from rice (*Oryza sativa*; uniprot: P93431-1, residues 48–466; P93431-2, residues 48–433) and agave (*Agave tequilana*; locus 3705, residues 57–474; locus 27298, residues 58–435; Gross et al., 2013) were synthesized (GenScript) and cloned into the SacII-HindIII site of pHue (Catanzariti et al., 2004). The QuikChange protocol (Stratagene) was used to introduce desired point mutations. An *ApoI* site was generated in the sequence encoding OsRca β using the silent G738A nucleotide substitution at the same position as found in *AtRca* β . This permitted construction of pHueAtOsRca β and pHueOsAtRca β encoding chimeric activases, by exchanging the *ApoI*-HindIII fragment of the two constructs. Plasmids and primers used in this study are listed in Supplemental Tables 1 and 2.

Protein Expression and Purification

Plasmids encoding the wild type and variant activases were transformed in *Escherichia coli* BL21 (DE3) cells, and proteins were produced and purified using immobilized metal affinity chromatography followed by precise His₆-Ub cleavage and anion-exchange chromatography exactly as previously described for other His₆-Ub tagged proteins (Tsai et al., 2015). The concentration of pure proteins was measured at 280 nm using extinction coefficients obtained using the ExPASy protparam tool (web.expasy.org/protparam/). Rice Rubisco was purified from *Oryza sativa* L. ssp. *japonica* leaf tissue using a protocol similar to that described (Makino et al., 1983). Leaf powder was resuspended in buffer A (50 mM Tris-HCl, pH8.0, 50 mM NaCl, 1 mM Na₂EDTA, 12.5% v/v glycerol) containing 1 mM phenylmethanesulfonyl fluoride, protease inhibitor cocktail (Pierce, ThermoScientific), 5 mM dithiothreitol (DTT), and 2% w/v polyvinylpyrrolidone. Following filtration by miracloth and removal of cell debris by centrifugation, 33% and 55% ammonium sulfate cuts were performed.

The pellet obtained at 55% was resuspended in buffer A containing 5 mM DTT, followed by extensive dialysis against buffer B (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mM Na₂EDTA, 1 mM DTT, 12.5% v/v glycerol). The dialyzed solution was applied to a MonoQ 10/100 column (GE healthcare) preequilibrated with buffer B and the protein eluted with a linear salt gradient to 500 mM NaCl. Fractions containing Rubisco were pooled and further purified by elution over a Superdex200 16/60 column equilibrated with buffer B.

Rca proteins were supplemented with 5% v/v glycerol prior to storage. All pure proteins were concentrated to >5 mg/mL, flash frozen in liquid nitrogen, and stored at -80°C.

Biochemical Assays

Rca activity was measured using an adaptation (Tsai et al., 2015) of the spectrophotometric Rubisco activase assay (Lan and Mott, 1991) and quantified as described (Loganathan et al., 2016; Supplemental Fig. S3A). Then 20 mM NaHCO₃ and 1 mM RuBP were used as substrates. Ribose 5-phosphate was used to synthesize RuBP enzymatically (Horecker et al., 1958) and purified by anion-exchange chromatography (Kane et al., 1998). To generate ECM, 20 μM of Rubisco active sites were incubated (10 min, 25°C) with 20 mM NaHCO₃ and 10 mM MgCl₂ in buffer C (20 mM Tris-HCl, pH 8.0, 50 mM NaCl). ER was formed by incubating 20 μM of Rubisco active sites in buffer C containing 4 mM EDTA (10 min, 25°C), followed by addition of RuBP to 1 mM.

ATPase activity was assayed using a coupled spectrophotometric assay (Stotz et al., 2011) but did not contain DTT. Analytical gel filtration was performed by eluting the proteins in buffer C using a Superdex 200 PC3.2/30 Increase (GE healthcare) column.

To assess Rca thermostability, the purified enzymes (in concentrated form of approximately 10 mg/mL) were incubated for 10 min at the relevant temperature using a Biometra TB2 thermoblock before performing the ATPase assay at 25°C.

Statistical Analysis

Replicate measurements of biochemical assays were performed using the same protein preparations on different days. Statistical comparisons were performed using one-way ANOVA followed by the Tukey-Kramer method in OriginPro 8.5.1 (OriginLab). A value of $P < 0.05$ was considered significant.

Supplemental Materials

The following supplemental materials are available.

Supplemental Figure S1. Selected C3 and CAM Rca sequences were aligned using Clustal Omega.

Supplemental Figure S2. Rice and agave Rca proteins exhibit concentration-dependent polydispersity.

Supplemental Figure S3. Biochemical characterization of the Rca proteins.

Supplemental Figure S4. Quality control of Rca variants used in this study.

Supplemental Table S1. Plasmids used in this study.

Supplemental Table S2. Primers used in this study.

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