Thermostability of Rubisco and Rubisco activase in five tropical plant species

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Abstract

Rubisco activase (RCA) plays a regulatory role in photosynthetic function by facilitating removal of inhibitors from the Rubisco active site. The RCA variants in most plant species thus far surveyed lose function between 35°C and 40°C, which is significantly lower than the putative thermostability of Rubisco itself. As such, RCA may be a 'weak link' in photosynthesis at moderate temperatures, and so may prove a fruitful target for crop fortification to improve crop yields. This study surveyed five tropical species chosen for their potential to express highly thermostable RCA variants. We found evidence for one unusually thermostable RCA variant in the South American *Caesalpinia pulcherrima*, and two distinct RCA isoforms that may exhibit differing thermostabilities. We also found that Rubisco was less thermostable than previous literature suggests, becoming decreasingly functional in three of the five species studied between 25°C and 50°C. We suggest that these findings 1) provide support for RCA as a potential crop improvement target in a warming world, 2) suggest thermostable RCA variants may occur in a wide variety of locations and taxonomic groups, and 3) may have important implications for our understanding of temperature limitations to photosynthesis.

Key Words: RCA, *Caesalpinia pulcherrima,* plant growth, temperature

Introduction

Photosynthetic rate declines as temperatures exceed the photosynthetic optimum, resulting in lowered plant growth rates and crop yields (Sharkey and Bernacchi 2012). The degree to which photosynthetic rate is decreased by increasing temperature is influenced by several factors, which come into effect at different temperature ranges. The most constant influence on photosynthetic rate is the changing kinetics of Rubisco with temperature. For given concentrations of CO_2 and O_2, the greater the temperature, the less favourable carboxylation of RuBP by Rubisco is in comparison with oxygenation (Farquhar *et al.* 1980). As a result, with increasing temperatures plants fix CO_2 less efficiently and consequently exhibit decreasing growth rates.

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At moderately high temperatures (35–40°C), photosynthetic rate begins to decline more rapidly than can be explained by the increased ratio of oxygenation to carboxylation (Sharkey 2005). While damage can occur to many proteins involved in photosynthesis during heat stress episodes, this damage is usually caused only at temperatures exceeding 45°C for proteins in the electron transport chain (Thompson *et al.* 1989; Yamane *et al.* 1998), and above 60°C for Rubisco itself (Yamori *et al.* 2006). The reduction in photosynthesis at moderately high temperatures is therefore commonly attributed to one particularly heat labile protein: Rubisco activase (Kurek *et al.* 2007; Kumar *et al.* 2009; Scafaro *et al.* 2016; Shivhare and Mueller-Cajar 2017).

Rubisco activase (RCA) is a nuclear-encoded enzyme that functions in the chloroplast to facilitate the removal of inhibitory sugar phosphates from the Rubisco active site, thereby allowing Rubisco to bind RuBP for CO_2 fixation (Sharkey and Bernacchi 2012). RCA is known to be highly heat labile, becoming non-functional at temperatures as low as 30°C in some cases (Salvucci and Crafts-Brandner 2004). As such, inactivation of RCA at moderately high temperatures prevents removal of inhibitors from the Rubisco active site, which significantly reduces the catalytic efficiency of Rubisco at these temperatures despite its reported high thermostability (Sharkey and Bernacchi 2012).

It has been hypothesised that the thermolability of RCA is not a suboptimal evolutionary outcome, but rather has a vital regulatory role in photosynthesis (Sharkey *et al.* 2001; Sharkey 2005). A study on tobacco plants found that while heat-induced reductions in photosynthetic rate were reversible for wild type plants, plants genetically modified to be unable to deactivate Rubisco at higher temperatures displayed irreversible reductions to photosynthetic rates (Sharkey *et al.* 2001). One potential reason for this apparent damage is the increased production of H_2O_2 at moderately high temperatures (Sharkey 2005). The rate of oxygenation of RuBP rises with temperature, and so too does the rate of a side reaction of oxygenation that produces H_2O_2, which damages plant tissue if not metabolised quickly (Kim and Portis 2004). H_2O_2 released into the chloroplast may damage proteins involved in the electron transport chain, leading to the observed permanent reduction in photosynthetic rate. Therefore, RCA deactivation of Rubisco at moderate temperatures may prevent long-term damage from occurring (Sharkey 2005). If this is the case, improving the thermostability of RCA in crop plants may not result in increased yields, but rather in increased mortality.

However, there is some contrasting evidence to suggest that RCA thermolability is an unnecessary barrier to photosynthesis at moderate temperatures that, if overcome, may provide an opportunity for thermally fortifying crops and improving yields in high temperature environments. Two studies on *Arabidopsis thaliana* found that growth rates were enhanced at higher temperatures by introducing a more thermostable RCA (Kurek *et al.* 2007; Kumar *et al.* 2009). This demonstrates firstly that the thermolability of RCA limits photosynthesis at higher temperatures, and secondly that improving RCA thermostability may be an effective way of improving crop yields under moderately high temperatures.

A recent study found that a naturally occurring RCA isoform present in the desert CAM (Crassulacean Acid Metabolism) plant *Agave tequiliana* retained functionality at temperatures up to 52°C (Shivhare and Mueller-Cajar 2017). Shivhare and Mueller-Cajar (2017) proposed that the reason for high RCA thermostability in this case is that CAM plants must fix CO_2 during the day while stomata are closed, which means they are unable

to utilise transpirational cooling, thereby raising leaf temperature. A systematic survey of the natural diversity of RCA thermostability has never been conducted, and so it is unknown whether this highly thermostable RCA is particularly unusual, or simply the first thermostable variant to be discovered.

For this reason, the present study aimed to assess RCA thermostability in a variety of tropical plants with different ecological niches and metabolic systems. In so doing, we sought to gain a better understanding of the natural diversity of RCA thermostabilities and how RCA thermostability may vary by ecological niche. Within this survey, we also hoped to discover particularly thermostable RCA isoforms, which may serve as potential crop improvement targets.

Additionally, we surveyed the thermostability of Rubisco in these plants in order to gain a broader understanding of Rubisco thermostability, how it varies with ecological niche and how closely related it is to RCA thermostability within a given plant.

We expected that terrestrial tropical pioneer species would have higher RCA thermostability than the temperate species previously studied (Salvucci and Crafts-Brandner 2004), and that epiphytic plants would have even higher thermostability due to their reduced access to water and consequent lowered ability to utilise transpirational cooling. We predicted that epiphytic CAM plants would have the highest RCA thermostability due to their metabolic similarity to *Agave tequiliana* combined with their potential water limitation. We expected that Rubisco would exhibit high thermostability in all species studied, with little difference in thermostability between the species due to its non-limiting role in photosynthesis.

All experimental work for this study was undertaken within a constrained time frame of 4 days, and as such the sample sizes, number of replicates of the experiments and variety of assays performed were limited. Nevertheless, this study provides interesting preliminary results that motivate further research in this area.

Methods

Study site, species, and sample collection

Leaf samples were taken from two locations in Singapore. The Bukit Timah Nature Reserve (1°21'06"N 103°46'25"E) is a lowland rainforest that comprises both primary and secondary rainforest. Within the secondary rainforest, leaves were collected from *Adinandra dumosa* and *Dillenia suffruticosa*, two abundant pioneer species. Both plants were growing in canopy gaps, with no large trees near enough to provide shade during the hottest periods of the day. These plants were selected for RCA thermostability testing as they thrive in gap regions of the forest, and thus are exposed to higher temperatures than plants growing in the understorey.

Three plants were sampled from the Nanyang Technological University campus (1°20'25"N 103°40'32"E): *Caesalpinia pulcherrima, Asplenium nidus* and *Pyrrosia pyloselloides. Caesalpinia pulcherrima* is a legume native to South America, selected as it is grown horticulturally in open areas among concrete paving and so may face significant heat stress. *Asplenium nidus* is an epiphytic fern, selected as epiphytes may face water stress and thus may be limited in their use of transpirational cooling, leading to higher leaf temperatures. Similarly, *P. pyloselloides* is an epiphytic fern, as well as being a CAM

plant. This species was selected due to its epiphytic habit, as well as its metabolic similarity to *Agave tequiliana*, which exhibits high RCA thermostability (Shivhare and Mueller-Cajar 2017).
In all cases, the youngest green leaves were sampled, as previous methodological tests had found that protein extraction was aided by softer leaf tissue. Only one leaf was sampled per plant due to the preliminary, non-quantitative nature of this survey. As this study only sought to identify species with exceptionally thermostable RCA as opposed to quantifying any variability within species or individuals, this small sample size was deemed appropriate.
Samples were stored in water and refrigerated overnight in order to maintain protein composition, and to increase the water content of the leaves and so enhance cell lysis by freezing for subsequent protein extraction.

Soluble protein extraction

A mass of 40–50 mg of leaf tissue from each sample (five samples in total) was excised, avoiding midribs and prominent veins where possible. Tissue was stored in 1.5 mL Eppendorf tubes and kept on ice. Liquid nitrogen was added to tubes and tissue homogenised using a micropestle. A 200 µL extraction buffer (50 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 12.5% Glycerol (v/v), 2% PVPP (v/v), 1 mM PMSF) was added and homogenised with micropestle. Samples were centrifuged at 20,000 × g (~14,000 rpm) for 15 minutes at 4°C. The supernatant was transferred to empty tubes, and centrifuged at 20,000 × g for 10 minutes at 4°C. The supernatant was divided equally into three tubes for each sample, such that there were 15 tubes in total.

Heat treatments

RCA becomes less soluble when it deactivates (Feller *et al.* 1998; Haldimann and Feller 2005), and so we were able to assess solubility as a proxy for thermostability. One of three equivalent protein extracts from each species was incubated for 10 minutes at each of three temperatures: 25°C, 40°C and 50°C. All samples were then centrifuged at 20,000 × g for 15 minutes at 4°C. The supernatant was transferred to empty tubes.

Gel electrophoresis

To each sample, 2× Loading Dye (100 mM Tris-Cl (pH 6.8), 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol) was added in volumes equivalent to sample volume. Samples were heated at 90°C for 5 minutes, loaded on 12.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gets, and separated for 1.5 hours at 100 V.

Total protein transfer and visualisation

In a transfer cell, western blot components were layered in the following manner: thick blotting paper (bottom), nitrocellulose membrane, polyacrylamide gel, thick blotting paper (top). 1× Western Blot Transfer Buffer (25 mM Tris, 192 mM glycine (pH 8.3), 20% (v/v) methanol) was poured over the components, ensuring they were fully moistened and that there was excess Transfer Buffer on the transfer cell. Proteins were transferred onto the nitrocellulose membrane for 30 minutes at 10 V. The nitrocellulose membrane

was separated from polyacrylamide gel and stained with Ponceau stain (0.1% (w/v) in 5% acetic acid) to visualise transferred proteins. The membrane was then incubated while shaking in 20 mL of 5% skimmed milk in 1× Tris-buffered Saline (TBS) at 25°C for 1 hour. The milk was removed and the membrane was rinsed three times with TBS.

Rubisco activase visualisation

Primary antibody (rabbit α-RCA raised against *Gossypium hirsutum* Rca-Agrisera AS10700) was added to the membrane in TBS as a 1:5,000 dilution. The membrane was incubated for 1 hour at 25°C, and then washed with TBS three times, including a 10 minute incubation period at 25°C between each wash. Secondary antibody (α-rabbit-HRP) was added to the membrane in TBS as a 1:10,000 dilution. The membrane was incubated for 1 hour at 25°C, before liquid was removed, and washed three times in TBS. Five-hundred µL of Detection reagent 1 (Peroxide Solution) and 500 µL of Detection reagent 2 (Luminol Enhancer Solution) was added to the membrane and mixed thoroughly. Rubisco activase protein bands were visualised using medical X-ray films in a darkroom with 15 minute developing time.

Rubisco visualisation

The same procedures were followed as for Rubisco activase visualisation, using the same membrane and rabbit α-Rubisco. The membrane was not stripped of the RCA antibodies or detection reagents, and so Rubisco gel image displays both RCA and Rubisco bands.

Results

Total protein visualisation

Staining of the nitrocellulose membranes with Ponceau Stain showed that protein was present in all samples (data not shown) and that protein transfer to nitrocellulose membrane was successful. However, quantitatively the samples were not equivalent, with greatly differing soluble protein levels achieved.

Rubisco activase

Rubisco activase protein bands were visible only in two samples: *C. pulcherrima* and *D. suffruticosa* (Figure 1), suggesting that the antibody used may not universally recognise higher-plant RCA. In both cases, bands were fainter at higher incubation temperatures, indicating that RCA was sensitive to temperature. For *C. pulcherrima,* two bands of different molecular mass corresponding to two isoforms of RCA were visible at 25°C and 40°C incubation. At 40°C, the higher molecular weight isoform (RCA_L) band was fainter than the lower molecular weight isoform (RCA_S) band. At 50°C, only the RCA_S band was present, and was significantly fainter than at the lower temperatures, indicating that RCA_S was more thermostable than RCA_L but not completely unaffected by the higher temperature. For *D. suffruticosa,* a faint RCA_S band was visible at the 25°C incubation only, indicating some degree of thermolability between 25°C and 40°C.

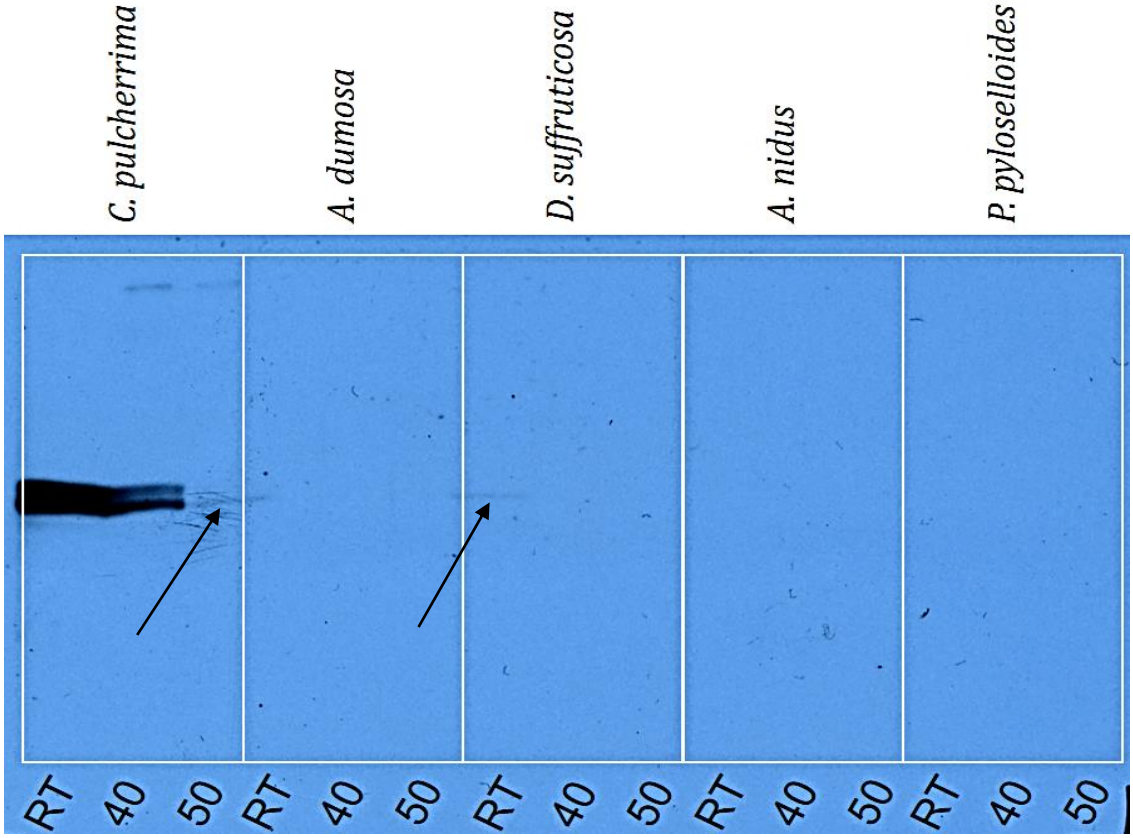

Figure 1: Developed film of SDS-PAGE anti-RCA Western Blot, where dark bands indicate RCA. Study species are listed above the film and temperatures of incubation are provided beneath the corresponding lane, where RT = 25°C. Arrows indicate faintly visible RCA bands. The top bands visible in the RT and 40°C treatments of *C. pulcherrima* indicate the large ~46 kDa isoform of RCA while the lower bands indicate the small ~42 kDa isoform.

Rubisco

Rubisco was present in all species samples (Figure 2). *C. pulcherrima* and *D. suffruticola* Rubisco bands were consistently intense, with no visible difference between 25°C incubation and 50°C incubation bands, indicating thermostability at these temperatures. The other three species displayed fainter Rubisco bands at higher temperatures, indicating that Rubisco was somewhat sensitive to moderately high temperatures. *A. Dumosa* showed a Rubisco band at 25°C, a fainter band at 40°C, and no visible band at 50°C. *A. nidus* and *P. pyloselloides* displayed a similar pattern, albeit with a less noticeable difference between bands at 25°C and 40°C, as well as having faintly visible bands present at 50°C. A double banding pattern was observed for *C. pulcherrima* and *A. nidus*, the top band of which may represent a covalently linked dimer of large Rubisco subunits.

Smeared bands are present for *A. nidus*, which may be caused by secondary metabolite interaction with antibodies or luminol enhancer.

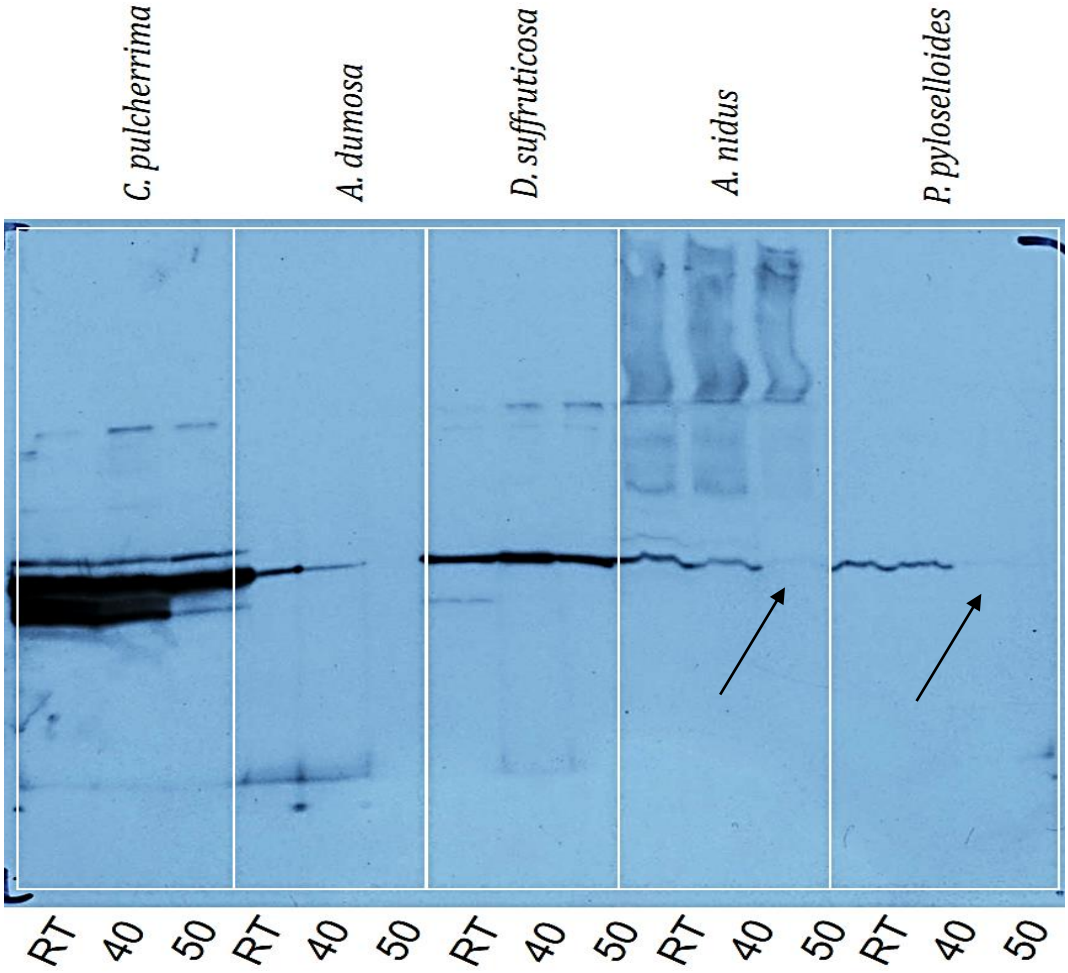

Figure 2: Developed film of SDS-PAGE anti-Rubisco Western Blot. RCA bands (~46 and ~42 kDa) are present below Rubisco bands and are identical to Figure 1. Rubisco subunit bands are present immediately above the RCA bands (~56 kDa). Study species are listed above the film and temperatures of incubation are provided beneath the corresponding lane, where RT = 25°C. Arrows indicate faintly visible Rubisco bands.

Discussion

Low Rubisco activase concentration

The lack of visible RCA bands in three of the samples (Figure 1) is likely due to a combination of factors. One is the relatively low concentration of protein in the sample, rather than factors relating to the nature of the RCA in that species. This is supported by the fact that the Rubisco blot showed more intense bands for the two species for which RCA was observed as compared with the species where RCA bands were not visible. In addition, it is possible that the antibody used has poor reactivity with the RCA protein found in the target species.

Based on this finding, future extraction protocols should be optimised for each species in order to ensure sufficient protein concentration for RCA visualisation using visible light methods, and other RCA antibodies should be sourced.

Rubisco activase is thermolabile

The two species in which RCA bands were visible (*C. pulcherrima* and *D. suffruticosa*) display thermolability, in that concentrations of soluble RCA decrease with increasing temperature. For *D. suffruticosa,* a faint band was observed at 25°C but is not present at 40°C (Figure 1). This result provides evidence that *D. suffruticosa* RCA is thermolabile to some degree, which may indicate that the ability of *D. suffruticosa* to thrive as a pioneer species in secondary rainforests is not strongly affected by the *in vitro* thermostability of its RCA.

C. pulcherrima displayed greater thermostability than most species previously studied (Kurek *et al.* 2007; Kumar *et al.* 2009; Wang *et al.* 2010; Scafaro *et al.* 2016), with some RCA remaining soluble at 50°C after a 10-minute incubation period. This high thermostability is likely necessitated by the very hot conditions that the sampled *C. pulcherrima* was exposed to growing among areas of concrete in Singapore with no shading during the hottest times of the day.

Previous studies suggest that RCA may act as a temperature switch, deactivating carbon fixation when conditions for photosynthesis are adverse (Sharkey *et al.* 2001; Sharkey 2005). If this is true, plants that have thermostable RCA may have some way of ameliorating the harmful effects of running the Calvin cycle in moderately high temperatures. One possibility is that plants with thermostable RCA utilise chemical defences from reactive oxygen species, such as producing high levels of conjugated terpenoids, which have been shown to decrease the effects of moderate heat stress (Sharkey 2005).

Differing isoform thermostability

The two RCA bands present in the *C. pulcherrima* sample are indicative of two different isoforms of RCA. The small isoform (RCA_S, ~42 kDa) displayed greater thermostability than the large isoform (RCA_L, ~46 kDa), as the lower band (RCA_S) was more intense at 40°C and 50°C than the higher band (RCA_L). Interestingly, this appears to contrast with previous studies investigating the different roles of these RCA isoforms (Crafts-Bradner *et al.* 1997; Law *et al.* 2001; Wang *et al.* 2010), all of which suggest that RCA_L is the more thermostable isoform. One study investigated the roles of RCA isoforms in photosynthetic heat acclimation in rice (*Oryza sativa*), and found that long-term heat stress (4 days at 30–40°C) heightened the expression of RCA_L (Wang *et al.* 2010). Transgenic rice plants were made to express high levels of RCA_L, and these grew better at higher temperatures than both wild type plants and transgenic RCA_S-boosted plants (Wang *et al.* 2010), suggesting that the RCA_L isoform plays a role in acclimation to heat stress. One study found that the purified isoforms of wild rice (*Oryza meridionales*) had roughly equivalent thermostabilities (Scafaro *et al.* 2016), which suggests that there may be some degree of variation between species as to which isoform is most thermostable. In this sense, the disparity between our results and those of past studies may be due simply to species differences.

One notable difference between the present study and others is that previous studies worked largely with acclimation processes as opposed to short-term *in vitro* exposures. Law *et al.* (2001) found that heat stress induced the formation of a new isoform entirely, which they proposed was transcriptionally regulated. Based on this finding, it may be possible that the RCA_L seen to be thermostable and related to heat acclimation in other studies was a new isoform not present in the leaves prior to heat exposure. In this case, our study would be unable to observe this isoform as we worked with soluble leaf protein extracts as opposed to whole leaves. An effective way to resolve this would be to acclimatise *C. pulcherrima* leaves to moderately high temperatures prior to protein extraction, and then to compare the isoform relative thermostabilities.

Rubisco is thermally sensitive at moderately high temperatures

Surprisingly, Rubisco displayed thermolability in three of the species studied: *A. Dumosa* (a pioneer species)*, A. nidus* (an epiphyte) and *P. pyloselloides* (an epiphytic CAM plant). This contradicts previous literature that suggests that Rubisco is thermostable to around 60°C *in vitro* (Yamori *et al.* 2006). If Rubisco is commonly less thermostable than previous literature has suggested, there may be consequences for our understanding of temperature limitations to photosynthesis, particularly given the prevalent assumption that deactivation of Rubisco at moderate temperatures is not related to a lack of thermal stability of Rubisco itself.

Both epiphytic plants sampled (*A. nidus* and *P. pyloselloides*) displayed thermally sensitive Rubisco (Figure 2). This may be indicative of the environmental conditions they have evolved to inhabit. We expected these plants to exhibit highly thermostable Rubisco and RCA as we assumed they would be exposed to significant water and temperature stress. Particularly, we expected *P. pyloselloides,* as a CAM plant, to have thermostable Rubisco and RCA. While we were unable to obtain results for the thermostability of RCA, we found that both species had Rubisco that retained very little solubility at 50°C.

It is interesting that the two pioneer species exhibited notably different Rubisco thermostabilities, with *A. Dumosa* Rubisco becoming insoluble between 40°C and 50°C, while *D. suffruticosa* Rubisco remained equally soluble at each temperature tested. Both plants were sampled from the same area, with no noticeable difference in climatic conditions or incidence of sunlight. This suggests that the thermostability of Rubisco does not play a large role in determining what broad ecological niche a species can occupy, at least for pioneer species.

Possible explanations for low thermostability of Rubisco

It is highly probable that Rubisco exhibits different thermostabilities depending on whether measurements are made *in vitro* or *in vivo*, due to the variety of mechanisms by which proteins can protect themselves when exposed to heat stress (Thompson *et al.* 1989; Shivhare and Mueller-Cajar 2017). For RCA, it has been found that ligand binding can increase RCA thermostability significantly (Keown and Pearce 2014). Rubisco may have similar protection mechanisms that can be utilised *in vivo.* If these mechanisms are protective enough, the intrinsic thermostability of Rubisco may cease to have any significant selective evolutionary role, in which case intrinsic thermostability may vary through random mutation over evolutionary time. This would fit with our results given the diversity of thermostabilities we have found, and given the lack of any clear

correlations of Rubisco thermostability within certain ecological groupings (i.e. pioneer species and epiphytes). To gain a better picture of *in vivo* thermostability, the same methods may be employed but exposing leaves to the heat treatments immediately prior to protein extraction rather than afterwards. This would provide insights into the thermostability of Rubisco in its natural, protective environment, and thus would better inform our understanding of physiologically relevant climatic conditions at which Rubisco function may be impaired in tropical plants.

Conclusions

This study identified one particularly thermostable RCA variant, present in *Caesalpinia pulcherrima*, as well as identifying a difference in the thermostability of its two RCA isoforms. In doing so, we provide evidence to suggest that highly thermostable RCA forms may be somewhat common in nature and not limited to the desert plant *Agave tequiliana*. We suggest that further research into the other physiological and biochemical features of plants with highly thermostable RCA variants is necessary before we can know if modifying crop plant RCA will lead to increased or decreased yields.

We also found that Rubisco was less broadly thermally stable *in vitro* than has previously been assumed, which may have implications for our understanding of temperature limitations to photosynthesis. We suggest that studies be conducted to determine how the *in vitro* thermostability of Rubisco compares with *in vivo* thermostability for these and other species. We found evidence against the hypothesis that the ecological niches a species can occupy is limited by Rubisco thermostability, which may indicate that Rubisco thermostability plays a limited evolutionary role.

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