C_4 Grasses Employ Various Strategies to Acclimate Rubisco Activase to Heat Stress.

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Summary

C_4 crops such as *Zea mays* (maize) and *Sorghum bicolor* (sorghum) are important in both food and bioenergy systems. In these crops, carbon assimilation is limited at high heat by the thermolabile protein rubisco activase (RCA). We present a comparative study of assimilation and RCA function in the C_4 grasses maize, sorghum, and the C_4 model grass *Setaria viridis* (setaria) during a 48 hour heat stress acclimation. Western blots and biochemical assays show that each species uses a different strategy to acclimate to heat. All grasses shift the predominant □ proteoform of RCA from a 41kD to a 43kD form. In addition, sorghum expresses the □ isoform, while all three grasses transiently increase expression of the □ isoform. The response of RCA to magnesium ions and to ADP is also altered. After heat treatment, maize RCA is more sensitive to inhibition by magnesium but loses sensitivity to ADP. Different cultivars of maize show differences in assimilation and protein expression. Because each grass expressed and regulated RCA differently, we recommend that future study of RCA should examine each grass individually rather than relying on a model organism.

Key words: C_4 photosynthesis, rubisco activase, heat stress, assimilation, abiotic stress, acclimation

Introduction

Although ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is responsible for almost all carbon fixation on Earth, its lack of specificity between CO_2 and O_2 results in photorespiration and the production of inhibitory molecules which bind to its active site. Although rubisco activase (RCA) has evolved in all higher plant species to maintain the activity of Rubisco by releasing inhibitory molecules from its active site, RCA has a low thermotolerance and its activity is inhibited by even moderate heat (32.5^{o}C in maize) (Crafts-Brandner and Salvucci, 2002a; Busch and Sage, 2017).

In C_3 plants, photosynthesis takes place entirely within the mesophyll in the presence of O_2, leading to photorespiration and costly corrections. As photorespiration, and its associated metabolic burden, increase with increasing temperature (Sage, 2013), some have proposed that the thermolability of RCA is in fact an adaptive mechanism to disable photosynthesis under unfavorable conditions (Degen, Worrall and Carmo-Silva, 2020). In C_4 plants, Rubisco functions exclusively in the bundle sheath cells from which O_2 is largely excluded (Kanai and Edwards, 1999). This adaptation improves the efficiency of photosynthesis by limiting photorespiration. The kinetics of Rubisco improve with increasing temperature in the absence of O_2, potentially allowing C_4 plants to photosynthesize more efficiently at higher temperatures (Crafts-Brandner and Salvucci, 2000). The RCA expressed in C_4 plants is thermolabile and is thought to limit photosynthesis unnecessarily. The RCA of C_4 plants is therefore an attractive target for protein engineering to improve thermostability and to increase CO_2 assimilation when plants experience high temperatures (Parry *et al.*, 2013).

Most plant species express two RCA isoforms, α and β, which arise from alternative splicing of a single gene in some species and from dedicated genes in others. The β isoform is usually characterized between 41-43 kDa and can undergo post-translational proteolytic cleavage to form a 41kD proteoform (Vargas-Suárez *et al.*, 2004; Yin *et al.*, 2014). In most species, the β isoform is constitutively expressed. The α isoform is larger (45-48kDa) and contains a C-terminal domain (CTD) with two cysteines capable of forming a disulfide bond. The α and β monomers can differ in thermostability, with the α isoform frequently being more thermostable (Crafts-brandner *et al.*, 1997). RCA is an ATPase which functions *in vivo* in a hexameric ring structure (Portis, 2003). Both β and α isoforms can be present in each hexamer such that the thermostability is determined as a combination of both isoforms (Keown and Pearce, 2014), although denatured isoforms are excluded from complexes under high temperature conditions (Scafaro, Bautsoens, *et al.*, 2019).

The thermolability of RCA may be due to structural changes such as denaturation and aggregation (Barta *et al.*, 2010). However, RCA may also be inactivated due to regulation by conditions inside the chloroplast. The ATP/ADP ratio in the chloroplast affects the function of RCA in some species (Kallis, Ewy and Portis, 2000; Barta *et al.*, 2010; Carmo-Silva and Salvucci, 2013). This is likely due to competitive binding of ADP to the ATP hydrolysis site that interferes with RCA's function. The presence of ADP can improve the thermostability of RCA in some species (Henderson *et al.*, 2013). RCA can also be sensitive to the concentration of Mg^{2+} ions in the stroma (Barta *et al.*, 2010; Hazra *et al.*, 2015). Redox regulation also affects the activity of RCA and couples the activity of RCA to illumination (Zhang and Portis, 1999; Zhang *et al.*, 2002). The sensitivity to ADP and to redox regulation are attributed to the CTD in the α isoform with redox regulation occurring at the disulfide bond and specifically favoring the binding of ADP while destabilizing binding ATP(Wang and Portis, 2006; Scafaro, De Vleesschauwer, *et al.*, 2019). Because α and β form hetero-oligomeric complexes, the entire complex is regulated by the conditions affecting the α isoform.

Efforts to find or create thermostable RCA isoforms have already shown moderate success (Kurek *et al.*, 2007; Scafaro, Bautsoens, *et al.*, 2019; Davidi *et al.*, 2020). To date, these efforts have not considered the effects of stromal conditions on either the native or novel enzymes. If we are blind to existing regulation, we miss both avenues of improvement and potential pitfalls. Protein engineering efforts will be hampered by an incomplete understanding of the native regulation of RCA. Toward this end, we examined whether assimilation is truly limited by RCA in the C_4 food crops *Zea mays* (maize) and *Sorghum bicolor* (sorghum) and the common C_4 model plant *Setaria viridis* (setaria). We then determined which RCA isoforms are expressed in each species and examined how stromal conditions regulate RCA function in these plants before and during heat treatment. Our results inform potential avenues for improvement of RCA.

Materials and Methods (1048 words)

Plant Material

S. viridis A10 was a gift from the lab of Dr. Ivan Baxter at the Donald Danforth Plant Science Center. Seeds were sown 1cm deep in 18-well trays and thinned to one plant per cell. Plants were grown in the greenhouse with 28°C/22°C temperatures and a 16-hour photoperiod.

Z. mays B73 was obtained from the greenhouse at Washington University in St Louis. All other maize accessions were obtained from the USDA GRIN catalog. *S. bicolor* BTx623 was a gift from the lab of Dr. Malia Gehan at the Donald Danforth Plant Science Center. Sorghum and

maize were planted 2cm deep in a 4-inch pot in a 50% mixture of Berger Bark Mix and Turface and grown in the greenhouse with 28°C/22°C temperatures and a 16-hour photoperiod. Plants were grown to the two- or three-leaf stage, then transplanted into 6-inch pots in the same soil mix.

Gas exchange measurements

Gas exchange measurements were performed on the most recently fully expanded leaf. The leaf measured was from the main stem of each plant, and all measurements were taken prior to panicle emergence. Greenhouse-grown plants were moved into a growth chamber of the appropriate temperature (42°C for 42°C measurements, etc.). For 1-hour measurements, the leaf was acclimated in the chamber of the LI-6400XT instrument with the instrument controlling the leaf temperature. For longer measurements, the LI-6400XT was attached to the leaf twenty minutes prior to the measurement. For 48-hour measurements, plants were maintained at the same photoperiod as the greenhouse with 42°C day and 38°C night temperatures. All heat treatments were initiated and all gas exchange measurements were taken at least two hours from either end of the photoperiod. CO_2 concentration was controlled at 400ppm. Gas exchange measurements were performed at saturating illumination of 1400 µmol/m^{2}s (*S. viridis*) or 2000 µmol/m^{2}s (*Z. mays*, *S. bicolor*). Humidity was controlled to maintain stomatal conductance above 0.3 for all measurements and chambers were kept at 50% humidity. The same plants were generally measured at the 1, 6 and 48 hour time points.

A/Ci curve measurements were taken according to the standard method (Long and Bernacchi, 2003) and were fitted using the Excel tool from (Zhou, Akçay and Helliker, 2018). For all measurements, the first measurement at 400µM CO_2 was checked for stomatal limitation and any data in which stomatal conductance limited photosynthesis by more than 30% was discarded. The fitted parameters were then used with the equations from (Yin *et al.*, 2011) to interpolate the intersection between the enzyme-limited and RuBP regeneration-limited curves to within the nearest ppm. Bundle sheath conductance values for interpolation were calculated as 2.87mmol/m^{2}s at 25°C and 3.40 at 42°C according to the equation presented in according to (Yin *et al.*, 2016).

Tissue harvest and protein extraction

Leaf tissue from plants subjected to heat in growth chambers was harvested during the middle of the photoperiod, flash-frozen in liquid nitrogen and ground with mortar and pestle and pooled prior to storage at -80°C. All tissue samples contained leaves from at least 3 plants. Care was taken to exclude the ligule, the midrib, and leaves that were not yet fully expanded or which appeared senescent. RCA was extracted by ammonium sulfate precipitation according to the

protocol of (Barta, Carmo-Silva and Salvucci, 2011a) and protein was resuspended in Leaf Resuspension Buffer (50 mM HEPES-KOH, pH 7.0, 2 mM $MgCl_2$, 100 mM KCl, 5 mM dithiothreitol, 1 mM ATP, 1 mM PMSF, and 10 mM leupeptin.) Aliquots were stored at -80°C until use. When compared to samples purified using the complete protocol of (Barta, Carmo-Silva and Salvucci, 2011a), samples using only ammonium sulfate precipitation were considerably cleaner and gave similar thermal curve results (Figure S1).

Equal volumes of purified protein were resolved by SDS-PAGE in a GenScript ExpressPlus PAGE gradient gel (8-16%) and transferred onto PVDF membrane using a BioRad semidry transfer apparatus according to the manufacturer's instructions. Membrane was blocked with 5% w/v nonfat dry milk in PBS supplemented with 0.1% v/v Tween-20 (PBST). Primary antibody (rabbit; HuaBio, Agrisera or MyBioSource) was added at 1:10,000 and incubated overnight with shaking at 4°C. Membrane was thoroughly washed with PBST and bound with secondary goat anti-rabbit (MilliPore) at 1:50,000 for 1 hour. Membrane was thoroughly washed and visualized with the SuperSignal West Pico Plus Chemiluminescent Substrate kit (Thermo) according to the manufacturer's instructions.

Biochemical assays

RCA thermostability was measured with an NADH-linked ATPase enzymatic assay in microtiter plates based on the protocol of (Barta, Carmo-Silva and Salvucci, 2011b). Briefly, 20µL of purified RCA was aliquotted into 0.2mL tubes and incubated in a gradient thermocycler for 1 hour at temperatures ranging from 30-66°C and returned to ice until measurement, while a control aliquot was maintained on ice. RCA sample (5µL) was added to 120µL of reaction mixture in microtiter plates and the change in A_{340nm} was monitored for 10 minutes using a Tecan plate reader. ATPase activity was calculated as the initial slope of the line (change in absorbance per minute). T_{50} was calculated by fitting a 4-parameter logistic equation to the dataset using the calculator at (AAT BioQuest Inc, 2022). Temperature response data that showed two distinct regions of response (sorghum and tobacco) were divided into two separate curves and each curve was fitted separately to produce two T_{50} values. The two plateaus are unlikely to represent a true response of RCA to heat. Previous work in spinach has shown that mixing of isoforms of different thermal stabilities confers an overall RCA response intermediate between the individual isoforms (Keown and Pearce, 2014). Rather, the lower of the two T_{50} values is likely another ATPase present which was not fully removed by our purification technique. The portion of total ATPase activity due to the low-thermal-stability ATPase was calculated and subtracted from total ATPase activity results.

Response to Mg^{2+} and ADP:ATP ratio was measured according to the method of (Chifflet *et al.*, 1988). Briefly, 8uL of purified enzyme was incubated in a total volume of 50uL of reaction buffer (50mM tricine pH 8, indicated concentration of $MgCl_2$, 20mM KCl, 4mM total of ATP and ADP according to ratio) for 20 minutes. SDS and ammonium molybdate reagent were added and allowed to react for 5 minutes. Color development was terminated by addition of sodium meta-arsenite and A_{850nm} was read. For redox assays, purified enzyme was pre-incubated in 100mM Tris pH 8.0 with D/L-dithiothreitol and/or glutathione oxidized for 20 minutes prior to adding to reaction buffer (Zhang and Portis, 1999).

Results

Assimilation

When plants were exposed to elevated temperature for 1 hour prior to measurement, assimilation increased with temperature to an optimal temperature between 32°C and 35°C then decreased at temperatures above 42°C in all species tested (Figure 1a, Figure S2). Time course measurements were performed to monitor acclimation at 0, 1, 6, and 48 hours of heat exposure at 42°C (Figure 1b). Maize B73 showed a decrease in assimilation at 1 hour but acclimated within 6 hours. Assimilation values in sorghum and setaria were not significantly affected by 42°C heat at any time point. To examine whether the 1-hour decrease in assimilation is specific to the B73 cultivar or a more general trait of the maize species, 8 other cultivars were similarly examined at 0, 1 and 48 hours (Figure 1c). In 4 of the cultivars, assimilation decreased at 1 hour of heat exposure, although none of these differences were significant, likely due to small sample size. One cultivar, MR19, retained high assimilation after 1 hour but displayed significantly reduced assimilation after 48 hours of heat exposure. This cultivar was chosen for further study because of its failure to acclimate to heat.

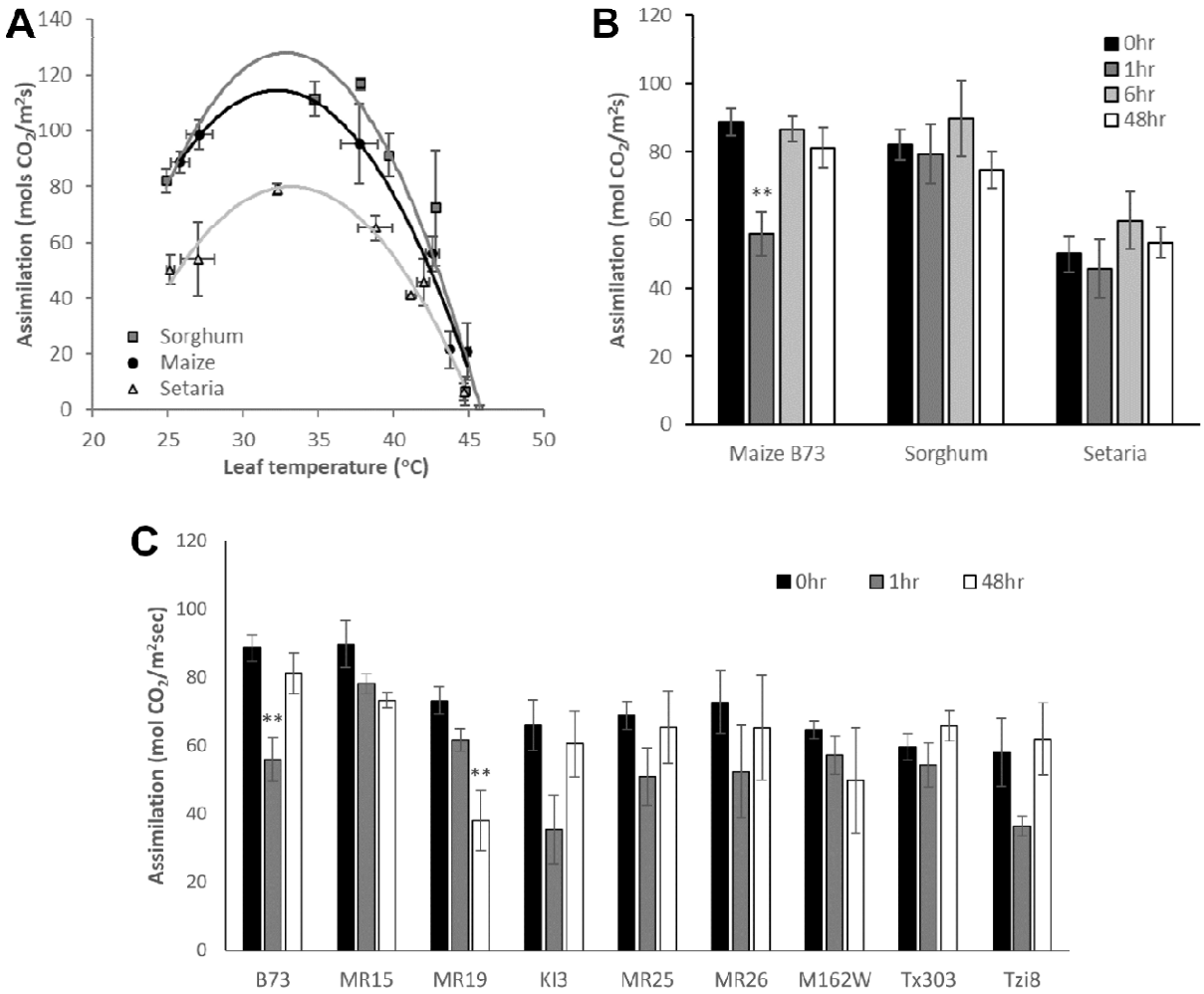

Figure 1: Heat response of assimilation in C_4 grasses. A. Overlay of assimilation vs. leaf temperature for sorghum, maize and setaria. Plants were exposed to listed leaf temperature for 1 hour prior to measurement. Plot of individual data points is shown in Figure S2. B. Heat acclimation of C_4 grasses. Plants were exposed to leaf temperature of 42°C for listed times prior to measurement. C. Heat acclimation of various maize cultivars. For all panels, data are mean ± SE (n≥3). ** indicates p<0.01 compared to 0hr control.

Limitations to Photosynthesis

Intercellular CO_2 concentration (C_i) was measured for all species at normal and elevated temperature at 400ppm CO_2 ($C_{i,400}$). $C_{i,400}$ was elevated at 42°C compared 25°C for all setaria, maize MR19 and maize B73 samples (Figure 2). $C_{i,400}$ remained low in sorghum through 6 hours of heat treatment but was elevated after 48 hours.

A/C_i curves were used to determine the limitations to photosynthesis. At low C_i, photosynthesis is limited primarily by the speed of the carboxylation reaction carried out by Rubisco. At higher C_i, photosynthesis is primarily limited by the ability of the Calvin cycle to

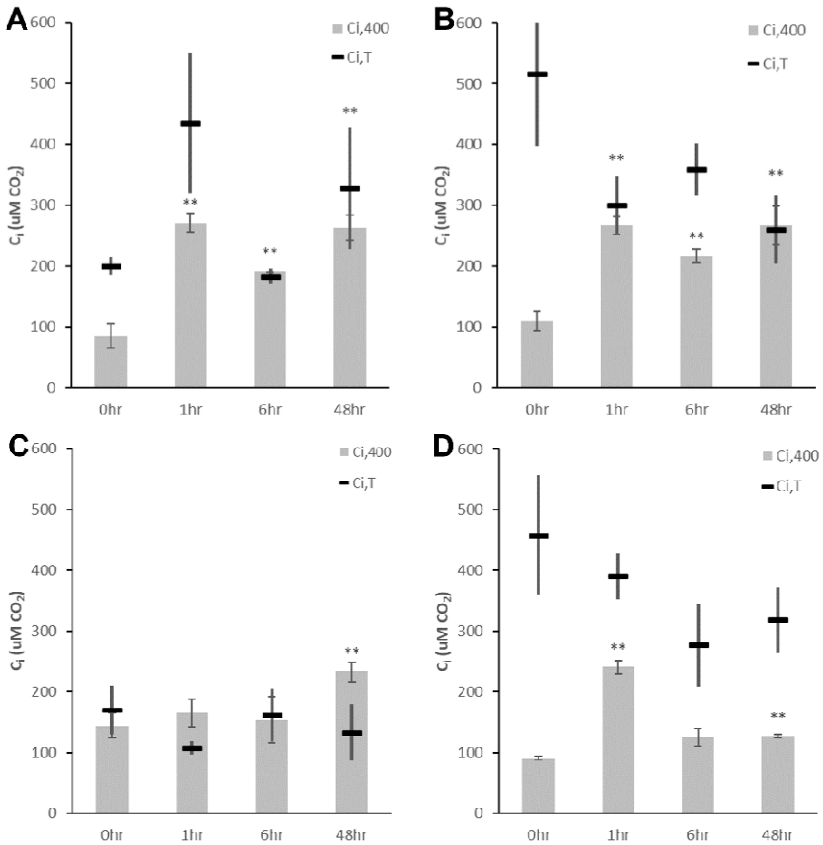

Figure 2: Calculated $C_{i,T}$ and measured $C_{i,400}$ values for C_4 grasses. A. Maize B73 B. Maize MR19 C. Sorghum and D. Setaria. For all panels, data are mean ± SE (n≥3). ** indicates p<0.01 compared to 0hr control.

regenerate the RuBP substrate of Rubisco, which in turn is limited by the rate of electron transport to regenerate reducing equivalents for this process (Long and Bernacchi, 2003). The transition point $C_{i,T}$ between these limitations can be determined by fitting the data to the mathematical models described in (Yin *et al.*, 2011) and then interpolating the intersection of the relevant curves. Figure 3 shows the calculated transition points. There was considerable between-plant variation in the $C_{i,T}$, and heat treatment did not cause statistically significant differences in $C_{i,T}$ for any species at any time point. For all species, $C_{i,400}$ at 25°C was below the $C_{i,T}$, indicating that light-saturated photosynthesis at normal temperature and atmospheric CO_2 was primarily limited by CO_2 fixation enzymes. The $C_{i\ 400}$ at 42°C for all treatment times was below or not statistically different from the $C_{i,T}$, indicating that at high temperature electron transport never limited photosynthesis. Our data was unable to distinguish between CO_2 fixation limitations in the C_4 cycle (i.e., phosphoenolpyruvate carboxylase [PEPC]) versus the C_3 cycle (i.e., Rubisco). Available data generally indicates that PEPC is more thermostable and rarely limits photosynthesis (Crafts-Brandner and Salvucci, 2002b) with few exceptions (Boyd, Gandin

and Cousins, 2015). The MR19 cultivar was not noticeably different in Ci,400 compared to B73, but showed some differences in Ci,T.

Proteoform Expression

RCA was purified from the leaf tissue of heat-treated plants. Western blot was performed on purified enzyme (Figure 3), with equal tissue weights loaded in each lane. Both tobacco and spinach contained more RCA per gram of tissue than any C_4 species prior to heat stress. Spinach clearly expressed all three bands reported in literature (α at ~45kDa, β at ~43kDa and a proteolytic product of β at ~41kD). Tobacco is known to only express the β isoform and lacks a higher molecular weight band corresponding to the α isoform.

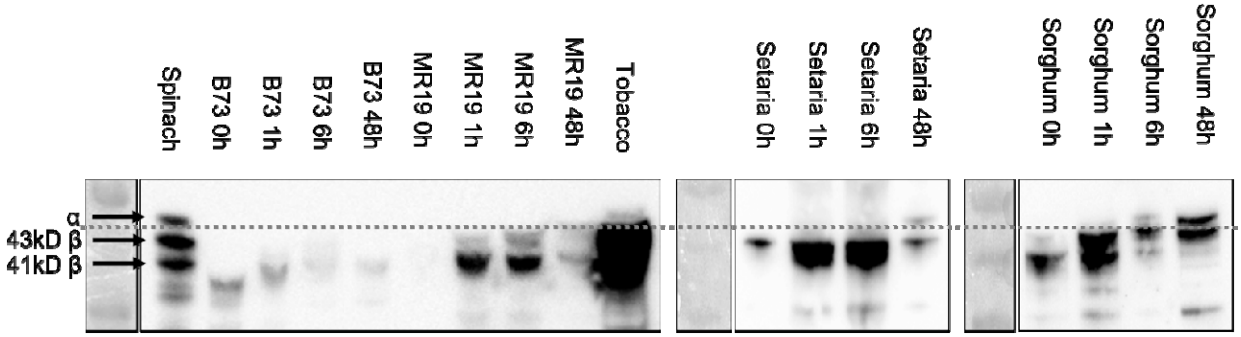

Figure 3: Western blots of extracted RCA. Dashed line serves as a visual guide to distinguish α from β isoforms. Full blots may be viewed in the Figure S5.

Maize B73 showed very low RCA expression at all time points with the predominant band shifting from 41kD to 43kD beginning after 1 hour of heat treatment. Maize MR19 also begins with nearly undetectable RCA expression, but dramatically induces expression of the 41kD proteoform at 1 and 6 hours of heat treatment. The uncleaved 43kD β proteoform was present in all samples but never as prominent as the 41kD proteolytic product. By 48 hours expression decreased somewhat but remained elevated compared to the 0hr control. Setaria initially expressed primarily the 43kD β proteoform and, similar to MR19, showed increased intensity of the 41kD proteoform band at 1 and 6 hours. Decreased expression at 48 hours was accompanied by appearance of the α isoform. Sorghum initially expressed primarily the 41kD β proteoform, but expression shifts to predominantly 43kD β proteoform and α isoform by 6 hours.

RCA Activity

ATPase activity per gram of tissue was measured using purified enzyme and the NADH-linked ATPase spectrophotometric assay. The activity of purified enzyme kept on ice was

compared for all samples (Figure 4a). In maize, activity increased with longer heat exposure in both cultivars. In B73 the increase was transient, peaking at 6 hours and 2.2-fold of the 0-hour

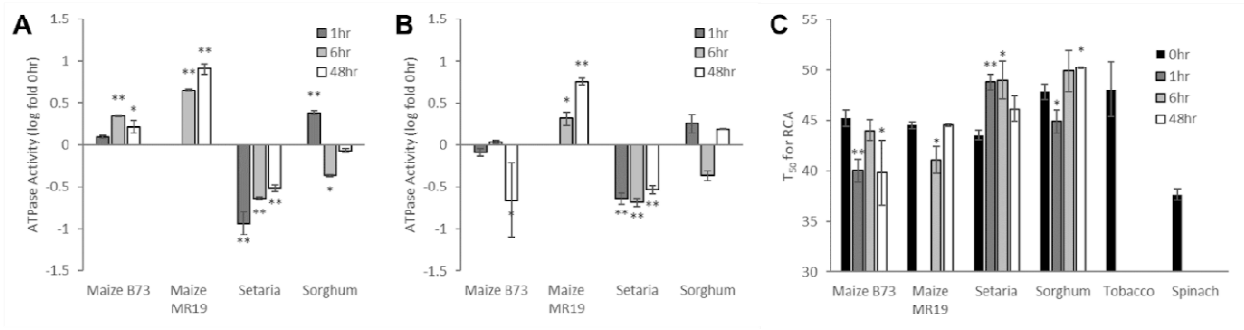

Figure 4: Activity of purified RCA. A. Enzyme kept at 0°C prior to assay. Values are log-fold compared to the 0-hour control. B. Enzyme pre-incubated at 41°C for 1 hour prior to assay. Values are log-fold compared to the 0-hour control. C. T_{50} values calculated from fitted heat response curves. For all panels, data are mean ± SD (n=3). * indicates p<0.05; ** indicates p<0.01 difference from 0-hour control.

activity. The RCA activity of MR19 increased much more dramatically, peaking at 8.0-fold at 48 hours. Sorghum RCA activity initially increased by 2.4-fold at 1 hour of heat treatment but decreased at 6 and 48 hours to 0.43 and 0.85-fold, respectively. In setaria, RCA activity decreased to ~10% in the first hour of heat treatment and gradually increased to 30% by 48 hours of heat treatment. Activities from enzyme samples pre-incubated for one hour at 41.2°C largely mirrored those seen with ice pre-incubation (Figure 4b).

RCA thermostability was measured by pre-incubating purified enzyme for 1 hour at temperatures ranging from 30°C to 66°C, then measuring using the NADH-linked ATPase assay. The resulting heat response curves were fitted to determine the temperature at which half of the activity was lost (T_{50}, for example see Figure S1c). As expected, spinach RCA had a lower T_{50} than tobacco RCA (37.6°C vs. 48.0°C). The thermostability of RCA extracted from both maize cultivars varied with time but never increased beyond the 0-hour control. In contrast, both setaria and sorghum RCA displayed increased T_{50} at various times, with setaria increasing from 43.6°C (0-hour) to 49.0°C (6 hours) and sorghum increasing from 47.9°C (0 hour) to 50.3°C (48 hours).

Magnesium ion sensitivity

Different species responded differently to magnesium ion concentration in the reaction buffer (Figure 5). While spinach, setaria and sorghum were relatively insensitive, tobacco RCA activity was strongly decreased below 20mM (Figure 5a). Maize RCA activity was highest without any added Mg^{2+} (Figure 5b; trace Mg^{2+} present in the storage buffer contributed to a final concentration of 0.32mM). Heat treatment did not change the response of setaria or

sorghum, but transiently increased the sensitivity of maize B73 RCA to inhibition such that even 5mM Mg^{2+} inhibited activity by 49% (28% for 0-hr control; Figure 5b). Continued heat treatment alleviated this inhibition with 5mM Mg^{2+} causing loss of only 20% of activity by 6 hours.

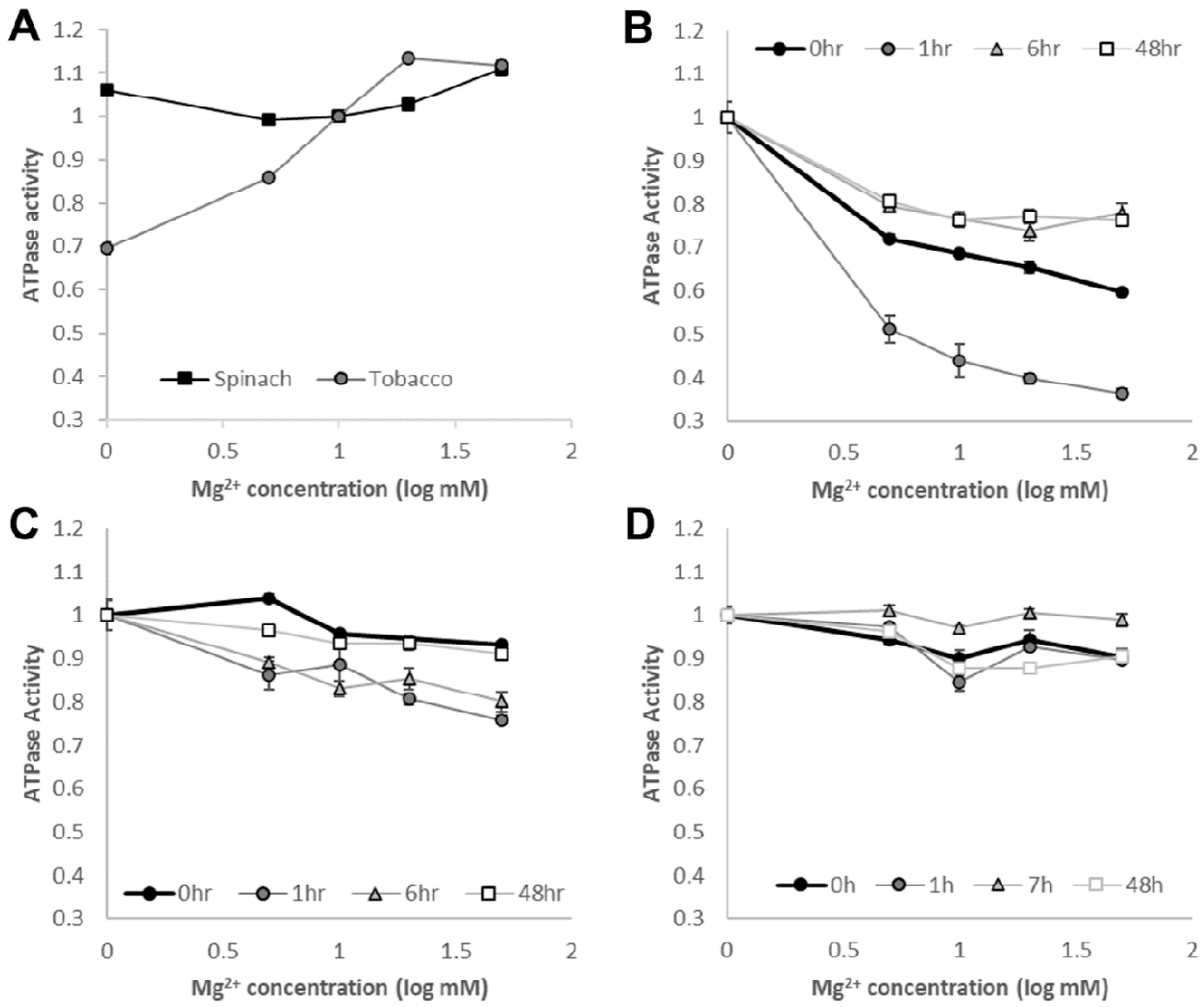

Figure 5: Response of RCA activity to magnesium. A. Spinach and tobacco. B. Maize B73. C. Setaria. D. Sorghum. Activity normalized to 0mM control. For all panels, data are mean ± SD (n=3).

<u>ADP sensitivity</u>

Previous work has established that the ATPase activity is inhibited by increasing concentrations of ADP (Zhang, Schürmann and Portis, 2001). Although we were able to replicate these results with RCA from tobacco (Figure 6a), we found spinach, setaria and sorghum to be insensitive to the presence and concentration of ADP in reaction (Figure 6).

Maize exhibited a slight but reproducible increase in activity of 26% as ADP increased (Figure 6b). This stimulation by ADP was abolished by heat treatments longer than 1 hour.

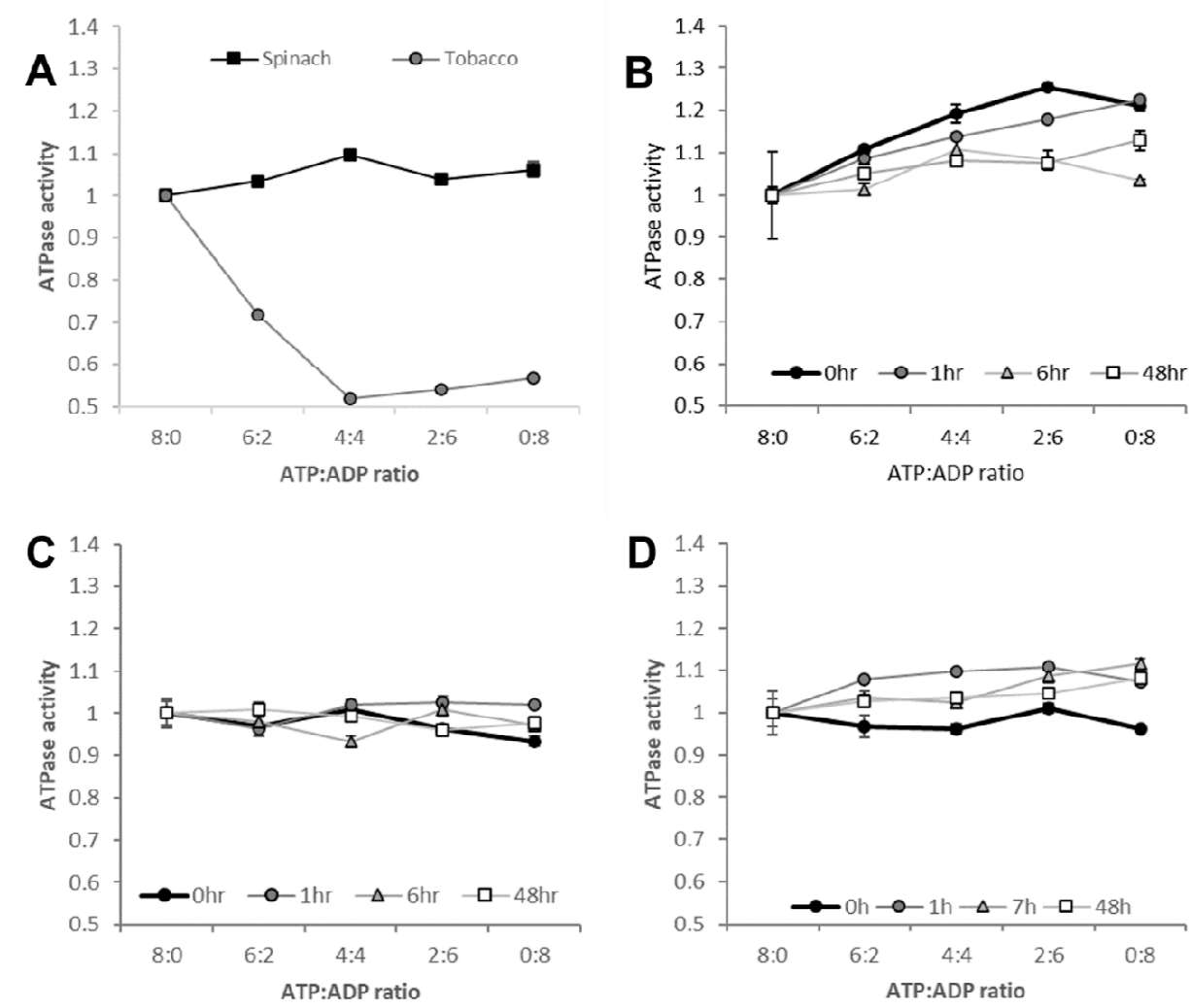

Figure 6: Response of RCA activity to ATP:ADP ratio. A. Spinach and tobacco. B. Maize B73. C. Setaria. D. Sorghum. Activity normalized to 8:0 control. For all panels, data are mean ± SD (n=3).

Redox sensitivity

Others have shown that the α isoform of RCA is redox-regulated. This regulation may happen through the enzyme thioredoxin F (Zhang and Portis, 1999) or directly from Photosystem I (Bhaduri *et al.*, 2020; Kale *et al.*, 2022). We tested the activity of RCA in the presence of reducing and oxidizing reagents (DTT and oxidized glutathione). We found no changes in activity with any redox treatment, including in the spinach positive control (Figure S3). Recombinant thioredoxin proved inactive and could not be tested.

Discussion

Among cultivars of maize, some acclimate to 42°C while retaining high assimilation (such as MR15, M162W and Tx303) and others transiently decrease assimilation (Figure 1c). The variability of this phenotype, presumably due to genetic diversity within the species, indicates that traditional breeding approaches may be fruitful in improving high-temperature assimilation in maize. Unique among cultivars examined, maize MR19 failed to acclimate and exhibited reduced assimilation even after 48 hours of heat treatment. This cultivar may be useful in future studies to determine the mechanism(s) underlying thermal acclimation. Variability in the thermal tolerance of assimilation and in the thermostability of RCA between cultivars of the same species has previously been reported in rice (Scafaro *et al.*, 2012), although the causal genetic variant has not yet been identified in maize.

Recent studies (Anderson *et al.*, 2021; Kim, Slattery and Ort, 2021) report decreased assimilation in setaria after 1 hour of treatment at 40°C and in setaria and s after 1 hour of treatment at 42°C respectively. These trends are not present in our results.

We had anticipated that thermal limitation of RCA would manifest as an elevated $C_{i,T}$ in 1-hour heat-treated samples, as this measurement would likely be taken before significant amounts of the more thermostable α isoform could accumulate. No elevation in $C_{i,T}$ was observed (Figure 3). There was considerable variation among individual plants which increased the standard error of $C_{i,T}$ and contributed to a lack of statistical significance. Even absent significance, there is no convincing trend in the $C_{i,T}$ data to indicate an elevated transition point imposed by RCA on photosynthesis due to thermal limitation.

All acclimated plants displayed an elevated $C_{i,400}$ compared to non-heat-treated counterparts (Figure 3). The increase in $C_{i,400}$ indicates that a component of the CO_2 assimilation pathway is unable to keep up with the diffusion of CO_2 into the stomata. This could occur if assimilation were limited by decreased efficiency of carbon fixation at Rubisco (e.g., by inactivation of RCA) or at the initial C_4 fixation steps in the mesophyll cells (e.g., PEPC). High temperature decreases the mesophyll conductance in C_3 plants such as tobacco (Bernacchi *et al.*, 2002) and spinach (Yamori *et al.*, 2006), but this has not been directly examined in C_4 plants. Therefore, the transient decrease in assimilation in maize despite elevated $C_{i,400}$ and increased stomatal conductance likely indicates that the thermolability of RCA is impairing photosynthesis in maize. Conversely, in setaria and sorghum assimilation remains equivalent to room temperature treatment, indicating that limitations on photosynthesis by thermolabile RCA are not dramatic enough to substantially impair assimilation in these species at 42°C.

In setaria and sorghum, elevated $C_{i,400}$ despite high assimilation is likely due to increased stomatal conductance, which occurs for transpirational cooling of the leaf, as this also allows greater equilibration between the intercellular and environmental concentrations of CO_2. Others have demonstrated that increased C_i improves the thermotolerance of photosynthesis in C_3 plants (Hikosaka *et al.*, 2006; Kattge and Knorr, 2007), specifically by increasing the concentration of CO_2 at Rubisco and improving its kinetics. Thus, stomatal opening may compensate for the thermal limitation of RCA to some extent by improving Rubisco function by elevated $C_{i,400}$, even in the absence of changes to RCA expression or activity. This explanation agrees with our stomatal conductance data (Figure S4).

In our work, plants were kept well-watered so that water limitation would not convolute the heat response; however, in most real-world scenarios elevated heat often co-occurs with water limitation. In such cases, stomatal opening is limited to retain moisture, and $C_{i,400}$ may be correspondingly decreased. Water limitation may thus decrease Rubisco efficiency and exacerbate the effects of heat on RCA. (Carmo-Silva *et al.*, 2012).

Molecular and biochemical assays also highlight the differences among species. Recent transcriptomics work by (Anderson *et al.*, 2021) in setaria found induction of RCAα after 1-4 hours of treatment at 40°C, but no change in RCAβ expression. Our results showed a strong increase in RCAβ protein within an hour; however, our higher temperature of 42°C and different cultivar (A10 vs. ME034) may account for these differences. We were unable to detect RCAα protein at such early time points, but our results confirm that RCAα is strongly expressed after longer heat treatment. Similarly, (Kim, Slattery and Ort, 2021) examined RCA expression in sorghum after 6 hours of heat treatment at 42°C using qRT-PCR. They report induction of RCAα in but no change in RCAβ expression. Taken together, these results suggest that there may be heat-modulated post-translational regulation of the β isoform that is not detectable by transcriptomic analysis, which increases the concentration of RCAβ. One possibility is decreased proteolytic turnover.

Previously, an α isoform has been reported in maize, though its visualization has been dependent on the antibody used (Vargas-Suárez *et al.*, 2004; Ristic *et al.*, 2009; Yin *et al.*, 2014; Kim, Slattery and Ort, 2021). In our work, blotting with three different antibodies gave similar results in all assays conducted. The timing of the appearance of the 43kD □ proteoform band in our sorghum samples (1 hour and 6 hours, Figure 3) matches the timing of the appearance of the higher molecular weight band in sorghum in work by (Kim, Slattery and Ort, 2021), but our work shows an additional α isoform band appearing at 48 hours. We were unable to visualize an α isoform in maize at any time point with any antibody. The variety of antibodies available and

different results produced using different antibodies is concerning. Future work in the field would benefit from a broadly available recombinant monoclonal antibody to improve reproducibility of results.

It is counterintuitive that RCA activity (Figure 4) does not correlate well with assimilation measurements (Figures 1 and 2) or with RCA expression (Figure 3). For example, both setaria and sorghum exhibit decreased RCA activity at 6 and 48 hours compared to the 0-hour control (Figure 4), but assimilation is not compromised at those time points (Figure 1). Furthermore, the RCA expression seen in Western blots is stronger in setaria at 6 hours than at 0 hours, but the activity is only 20% that seen at 0 hours (Figure 3).

These seemingly contradictory observations can be explained by examining the T_{50} of the purified RCA from these time points. In both setaria and sorghum, time points with decreased RCA activity also show increased RCA stability seen as increased T_{50} values. In contrast, multiple maize cultivars and the sorghum 1-hour sample show increased activity while the T_{50} values remain unchanged or even decrease. The increase in T_{50} in setaria correlates with appearance of the 41kD β proteoform, while the increased T_{50} in Sorghum correlates with expression of the α proteoform.

Taken together, these data indicate that different species employ different strategies to acclimate to high temperature. Maize accumulates the 41kD beta proteoform, boosting the activity of the enzyme without improving its thermostability. RCA concentration and activity are not clearly related to assimilation, with different cultivars of maize exhibiting compromised assimilation at various times despite high RCA expression and/or activity. Setaria also accumulates the β isoform and shifts predominantly to the 41kD β proteoform, but in setaria this proteoform appears to be both more thermostable and less active. Sorghum shifts between these strategies over time, beginning with strong accumulation of the less thermostable β isoform and increased overall activity, but adapting with expression of the more thermostable α isoform and decreased activity.

Responses to magnesium ion (Figure 5) and ADP concentration (Figure 6) differed from those reported for other species in the literature and may have regulatory significance in maize. ADP inhibition of tobacco RCA seen in our results (Figure 6a) has previously been reported (Carmo-Silva and Salvucci, 2013). The inhibition of RCA by ADP has been explained as competition for the same binding pocket and is a regulatory mechanism that prevents use of ATP by the Calvin Cycle until the plastid triphosphate pool can be replenished. Others have shown that ATP induces oligomerization while ADP encourages formation of monomers in spinach RCA (Keown and Pierce 2014). Our results show that spinach RCA activity is

unresponsive to ADP (Figure 6a) and thus oligomeric state is not a suitable proxy for activity. A similar lack of inhibition by ADP was shown in beta isoforms from Arabidopsis (Carmo-Silva and Salvucci, 2013) and in RCA from setaria and sorghum (this work). Increased activity with ADP has not previously been reported but was seen in maize. Our results indicate that, in maize, any competitive inhibition by ADP is overshadowed by its stimulation of ATPase activity. Furthermore, the stimulation of RCA by ADP is abolished after heat treatment. Others have previously speculated that the inactivity of RCA at high temperature in maize is due to unfavorable ATP:ADP ratios (Crafts-Brandner and Salvucci, 2002b). Our result corroborates this explanation and begs further study into the mechanism and significance of ADP stimulation of RCA activity.

Previous studies report that magnesium is important both for coordinating binding of ATP to RCA, and for coordinating binding and cooperativity of RCA monomers in the hexameric state (Hazra *et al.*, 2015). Tobacco RCA is known to be activated by increasing concentrations of magnesium; a result which we were able to replicate. Maize was uniquely inhibited by magnesium ions, even at the lowest concentration studied. This inhibition was also modulated by heat treatment. Notably, the 1-hour time point at which maize RCA was most strongly inhibited by magnesium was also the time at which assimilation was significantly compromised and thermostability of RCA was decreased. Later time points saw recovery of RCA activity, thermostability and assimilation, as well as decreased sensitivity to magnesium. Neither setaria nor sorghum displayed any sensitivity to magnesium, and assimilation was not compromised by heat treatment. The correlation between increased magnesium and ADP sensitivity and decreased RCA function should be examined more closely in maize to determine the mechanism and whether this modulation is of any regulatory significance.

In summary, we find that assimilation, isoform expression, thermostability and sensitivity to Mg^{2+} and ADP vary among C_4 grass species and even among cultivars within maize. Characterizations performed on the model species setaria cannot reliably be assumed to be true of other species. For example, maize shows modulation of RCA activity by magnesium and ADP. Future work should examine whether this modulation is responsible for decreases in assimilation in maize upon heat treatment.

Data Availability

All data is available upon request to the corresponding author at stainbr1@msu.edu.

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Conflict of Interest

None

Author contributions

Conceptualization: SCS. Methodology: SCS, LNA, AC, EJ, AS, LW, AA, JJ. Validation: SCS, LNA, AC, EJ, LW, AA. Investigation: SCS, LNA. Analysis: SCS, LNA, AC, EJ, AS, LW. Writing: SCS, LNA. Review and editing: SCS, LNA, AC, EJ, AS, LW, AA, JJ. Supervision: JJ

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Supporting Information for:

C_4 Grasses Employ Various Strategies to Acclimate Rubisco Activase to Heat Stress.

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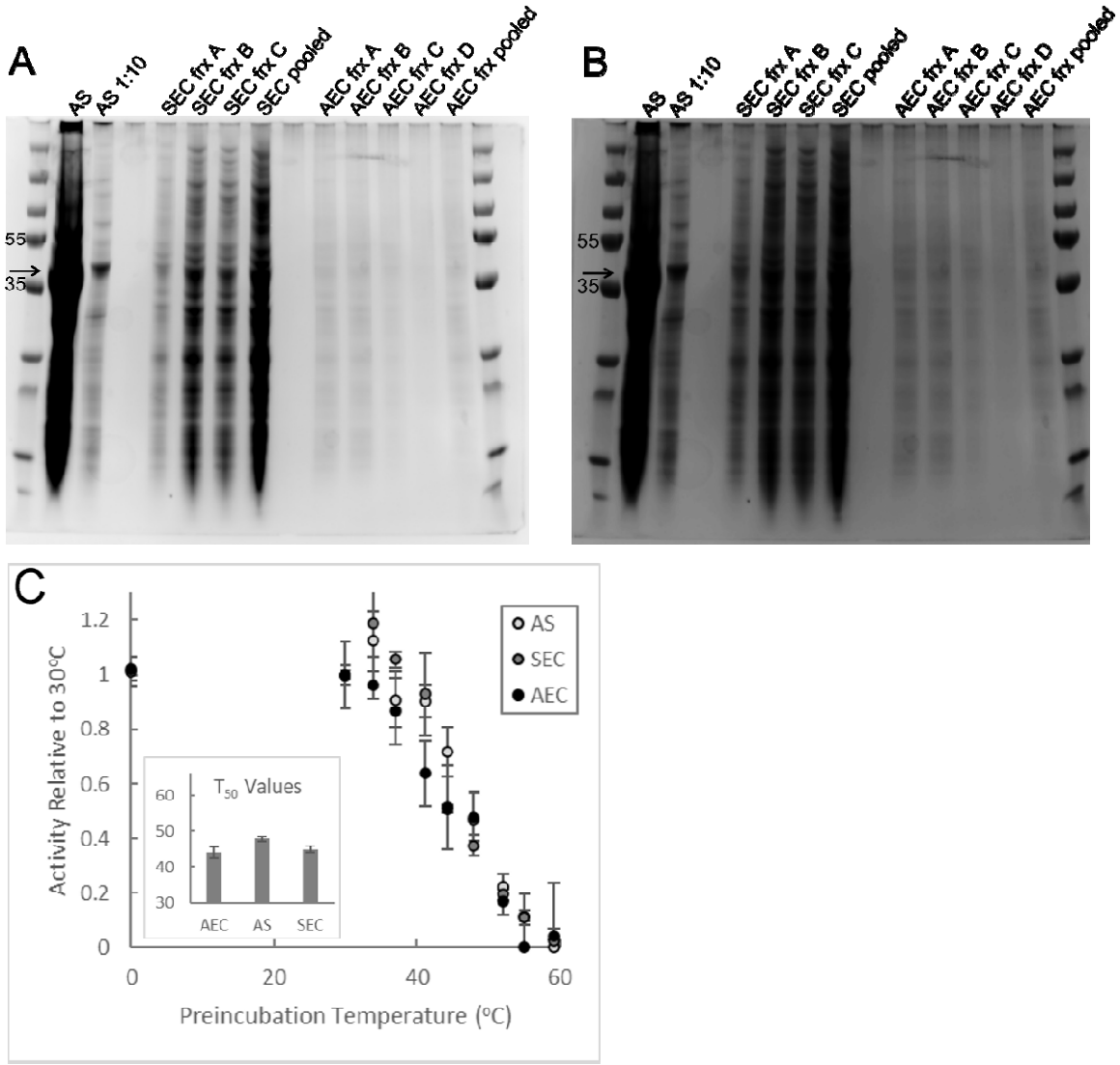


Figure S1: Comparison of RCA purification methods. A. total protein stain for maize tissue purified with ammonium sulfate precipitation only (AS); ammonium sulfate precipitation and size exchange chromatography (SEC); or ammonium sulfate precipitation, size exchange chromatography and anion exchange chromatography (AEC), multiple fractions (frx) shown for SEC and AEC samples. RCA size indicated by arrow. B. Same gel as in A with contrast adjusted to better visualize AEC lanes. C. Thermostability assay on AS, SEC and AEC samples, with calculated T_{50} values in inset. Mean and standard deviation of 3 technical replicates. No differences in T_{50} were significant.

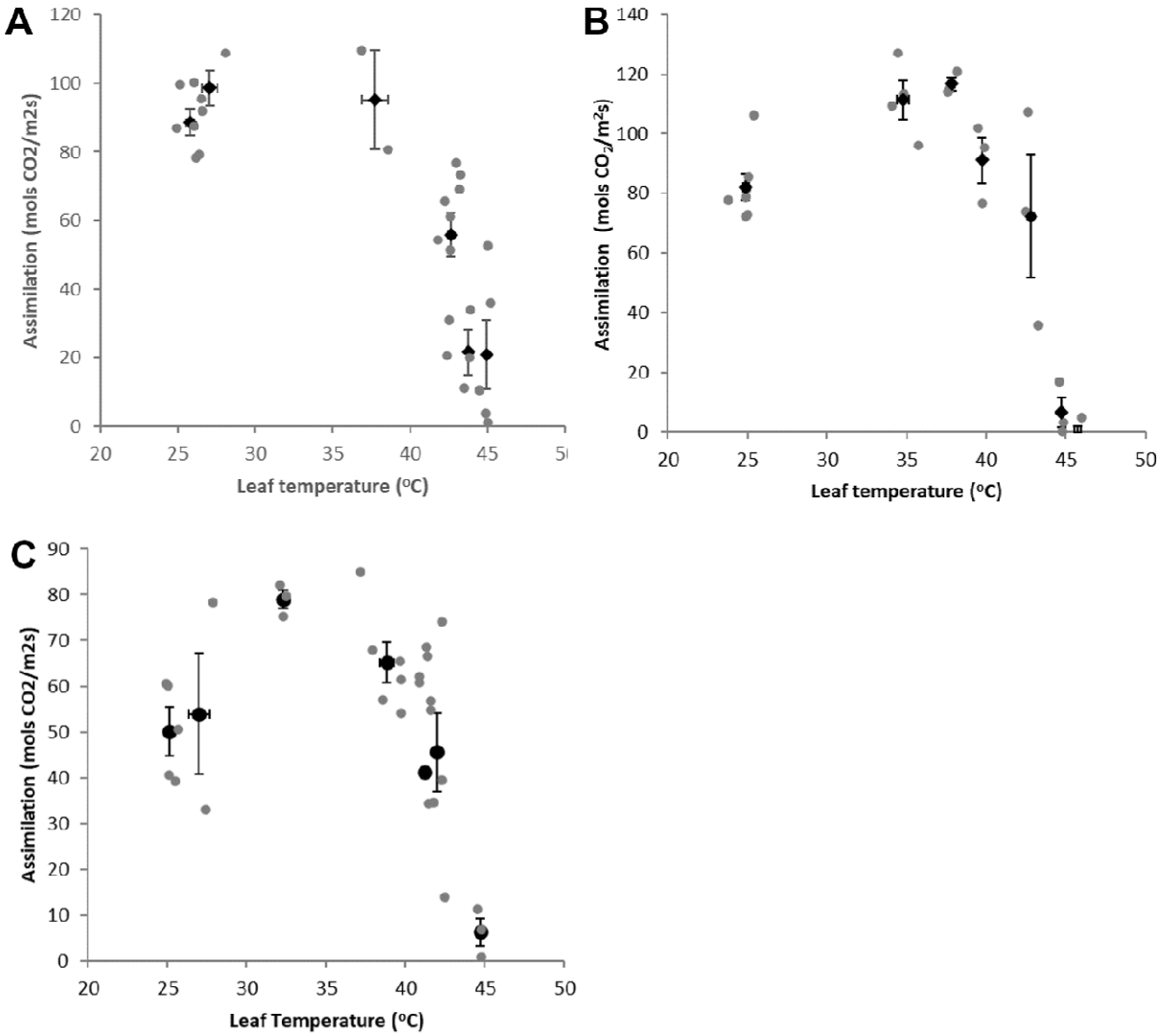
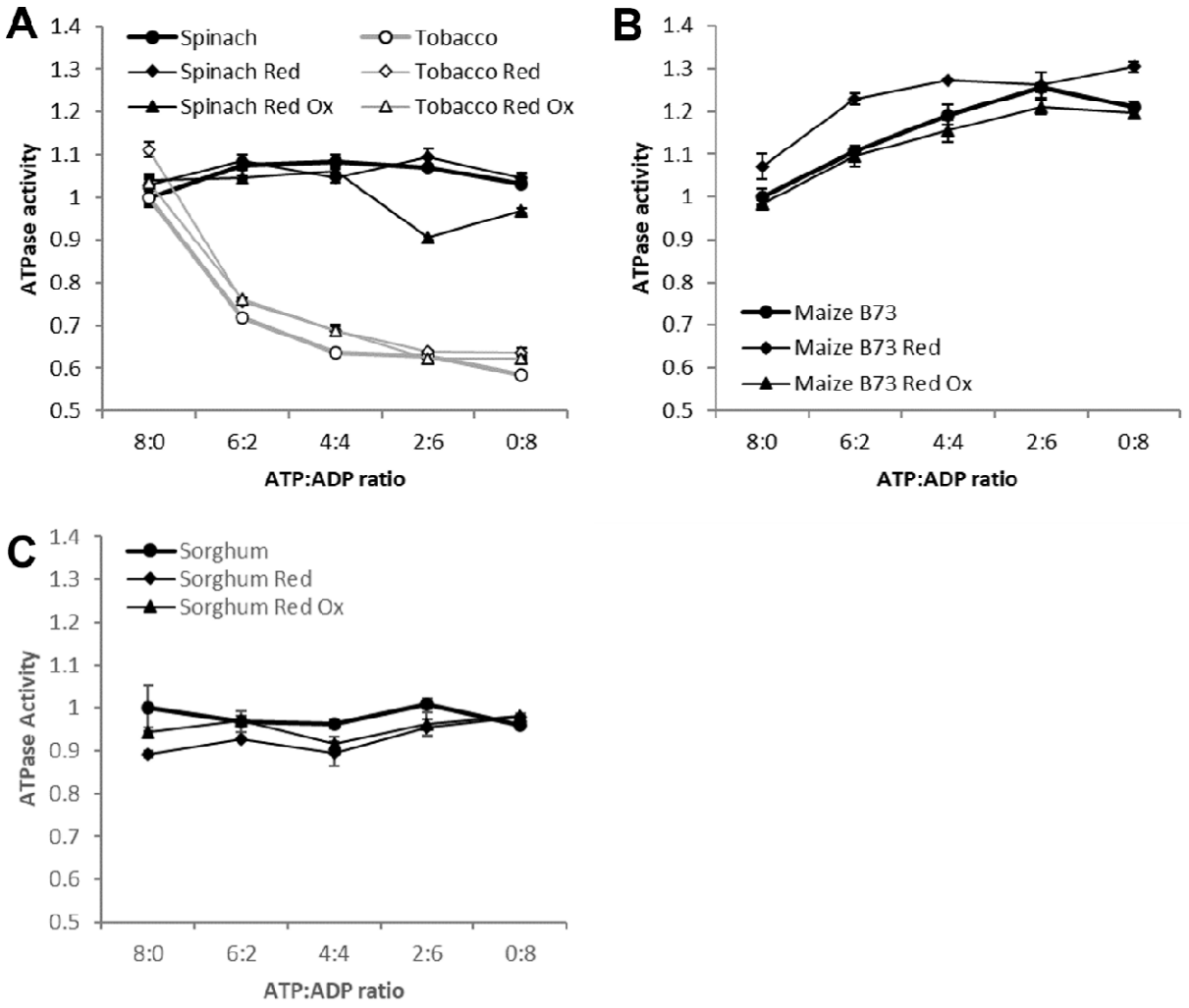


Figure S2: Heat response of assimilation in C4 grasses. A. Maize B73. B. Sorghum BTx623. C. Setaria A10. D. Overlay of all 3 species. Plants were exposed to listed leaf temperature for 1 hour prior to measurement. Gray points are individual measurements; black points are mean and standard error.



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616 Figure S3: Redox testing of RCA. Red indicates addition of 5mM DTT, while Red Ox indicates reaction first
617 with 5mM DTT for 10 minutes followed by addition of 10mM oxidized glutathione and additional
618 incubation for 10 minutes as per protocol in (Zhang and Portis, 1999). (TrxF proved inactive and was
619 omitted.) Mean and standard deviation of 3 technical replicates.

620 A. Spinach and tobacco. B. Maize. C. Sorghum.

621

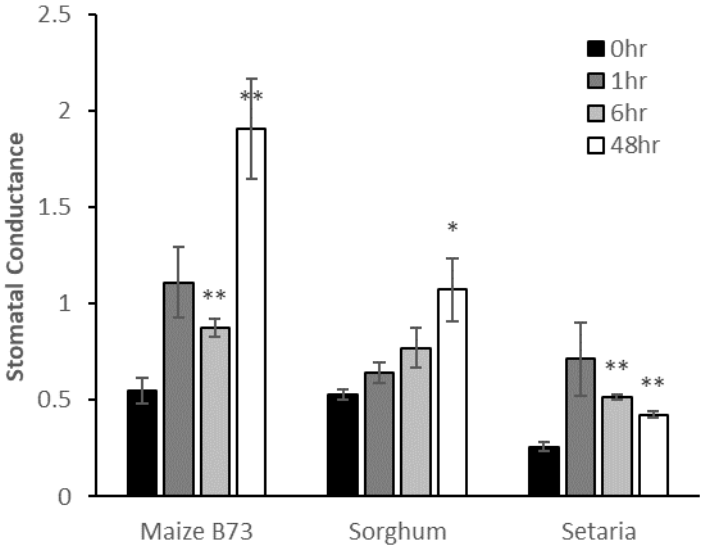

Figure S4: stomatal conductance is increased by high temperature treatment. Mean and standard error of at least three biological replicates. * signifies p<0.05, ** signifies p<0.01 vs. the 0hr control by Student's T Test.

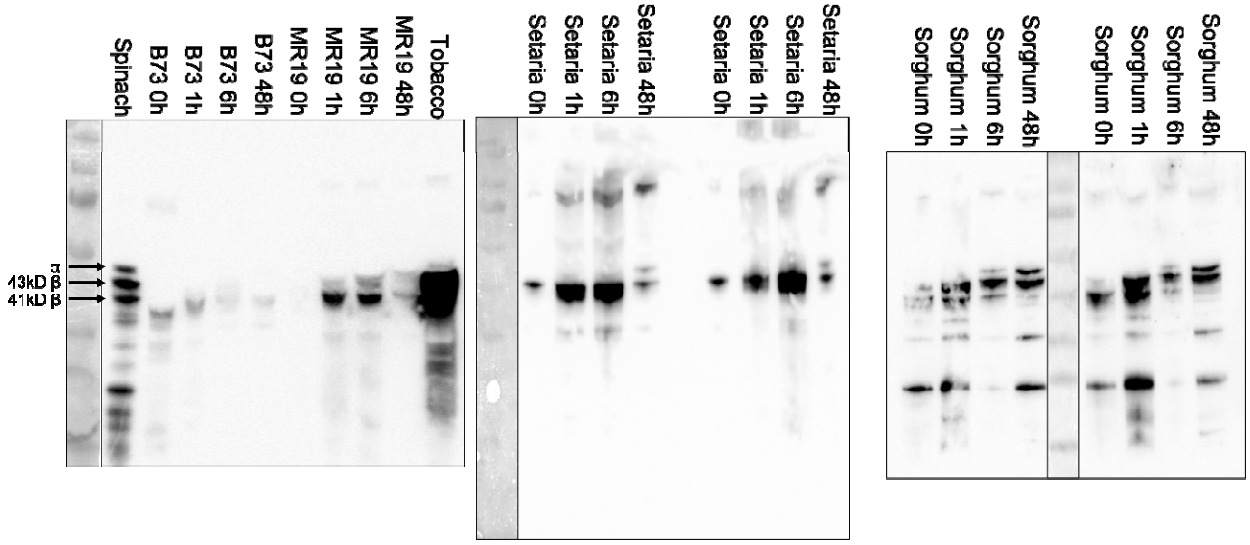

Figure S5: complete Western blots