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C4 Grasses Employ Various Strategies to Acclimate Rubisco Activase to Heat Stress

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Abstract

C_4 crops such as *Zea mays* (maize) and *Sorghum bicolor* (sorghum) are crucial producers of food and bioenergy. In these crops, carbon assimilation is limited at higher temperatures because the enzyme rubisco activase (RCA), which is responsible for proper function of the CO_2 fixing protein rubisco, has been shown to be thermolabile. We present a holistic approach to understand carbon assimilation and RCA function in maize, sorghum, and the C_4 model grass *Setaria viridis* (setaria) during heat stress acclimation. Gas exchange data confirms that assimilation is limited by Ribulose 1,5-bisphosphate (RuBP) carboxylation during heat. Plants express a variety of RCA isoforms and we show that each species changes the isoform expression and proteoform abundances of these variants in different ways. In addition, to understand how changing conditions in the chloroplast stroma affect RCA function during heat, we examined the regulation of RCA activity by thioredoxin F, magnesium ions and adenosine diphosphate. As expected, the activity of RCA is modulated by a combination of these variables, but surprisingly, how these biochemical environment factors affect RCA function differs vastly between species. Unexpected differences are even apparent within a single species: different cultivars of maize show differences in assimilation, proteoform abundance and regulation. Our surprising observation that each grass expresses and regulates RCA differently suggests that even closely related plants use different strategies to maintain RCA function and we conclude that a single reference system cannot accurately reflect protein function in every cultivar.

Introduction

Although ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is responsible for almost all carbon fixation on Earth, its lack of specificity between carbon (CO_2) and oxygen (O_2) results in photorespiration and loss of biomass. Both photorespiratory products and the ribulose-1,5-bisphosphate (RuBP) substrate itself can bind to the active site of rubisco and inhibit its function. Rubisco activase (RCA) has evolved in all land plants to release these inhibitory molecules from the active site and restore the activity of rubisco, rendering this protein essential for maintaining rubisco activity. RCA is an ATPase which functions *in vivo* in a hexameric ring structure (Portis 2003). However, RCA has a low thermotolerance and its activity is inhibited by even moderate heat (32.5^oC in maize) (Busch and Sage 2017; Steven J. Crafts-Brandner and Salvucci 2002). RCA's inability to function properly at higher temperatures has implications for crop yield given the rise in global temperatures.

In C_3 plants, photosynthesis takes place entirely within the mesophyll in the presence of O_2 produced by Photosystem II, leading to photorespiration and costly corrections. Photorespiration, and its associated metabolic burden, increase with rising temperatures (Sage 2013). This has led some to propose that the thermolability of RCA is in fact a protective mechanism to disable photosynthesis under unfavorable conditions, similar to a fuse (Degen, Worrall, and Carmo-Silva 2020).

In C_4 plants, rubisco functions exclusively in the bundle sheath cells where O_2 is not produced. This adaptation improves the efficiency of photosynthesis by limiting photorespiration to less than a fifth of

that of comparable C_3 grasses (Kanai and Edwards 1999). The decrease in photorespiratory products lessens the inhibition of rubisco. 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 (S J Crafts-Brandner and Salvucci 2000). However, the RCA expressed in C_4 plants is also thermolabile and is thought to limit photosynthesis unnecessarily. The RCA of C_4 plants is therefore an attractive target for protein engineering with the goal to improve its 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 approximately 43 kDa and can undergo post-translational proteolytic cleavage to form a 41 kDa proteoform (Z. Yin et al. 2014; Vargas-Suárez et al. 2004). In most species, the β isoform is constitutively expressed. The α isoform is approximately 45kDa and is mostly identical to the β isoform with the addition of a C-terminal domain (CTD). The CTD contains two cysteines capable of forming a disulfide bond which regulates RCA function according to the redox state of the chloroplast. Redox state dependent regulation of the α isoform couples the activity of RCA to illumination (N Zhang and Portis 1999). The α isoform has also been reported to undergo inhibitory phosphorylation at threonine 78 near the N-terminus (Kim et al. 2019), but this threonine is not conserved in all species and is not present in the β isoform. The α and β monomers can differ in thermostability, with the α isoform frequently being more thermostable (Steven J Crafts-Brandner et al. 1997).

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 by other unfavorable conditions inside the chloroplast. The ATP/ADP ratio in the chloroplast affects RCA function in some species (A. E. Carmo-Silva and Salvucci 2013; Barta et al. 2010; Kallis, Ewy, and Portis 2000). This is likely due to competitive binding of ADP to the ATP hydrolysis site that interferes with RCA's function, but the presence of ADP can also improve the thermostability of RCA (Henderson et al. 2013). RCA is also sensitive to the concentration of Mg^{2+} ions in the stroma (Hazra et al. 2015; Barta et al. 2010), possibly through the function of Mg^{2+} in coordinating the binding of ATP. The sensitivity of RCA to ADP and to redox regulation are attributed to the CTD in the α isoform, where redox regulation occurring at the disulfide bond favors the binding of ADP while destabilizing the binding of ATP (Wang and Portis 2006; Scafaro, De Vleesschauwer, et al. 2019). Because α and β RCA isoforms form hetero-oligomeric complexes, the thermostability and activity of the entire functional complex is regulated by the conditions affecting the α isoform (Keown and Pearce 2014) although denatured isoforms are excluded from complexes under high temperature conditions (Scafaro, Bautsoens, et al. 2019).

Efforts to find or create thermostable RCA isoforms have been advocated widely and have already shown moderate success (Davidi et al. 2020; Kurek et al. 2007; Scafaro, Bautsoens, et al. 2019; Qu, Mueller-Cajar, and Yamori 2023). To date, these efforts have not considered possible effects of stromal conditions or post-translational modifications on either native or novel enzymes, making it likely that potential pitfalls

and promising avenues for improvement have been missed. Protein engineering efforts are currently hampered by an incomplete understanding of RCA regulation in its native environment: the dynamically changing chloroplast stroma. Toward this end, we examined whether assimilation is truly limited by RCA in different cultivars of the C_4 food crops *Zea mays* (maize) and in *Sorghum bicolor* (sorghum) and in the common C_4 model plant *Setaria viridis* (setaria). RCA isoform expression was determined in each species, and we examined how stromal conditions regulate RCA function in these plants before and during heat treatment. Our results expose potential avenues for improvement of RCA with the long-term goal to bioengineer crop species that maintain photosynthetic performance despite rising global temperature.

Materials and Methods

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 Washington University in St Louis greenhouse with 28^{o}C/22^{o}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 Washington University in St Louis greenhouse with 28^{o}C/22^{o}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.

Heat Treatment

Greenhouse-grown plants were moved into a growth chamber of the appropriate temperature (42^{o}C for 42^{o}C measurements, etc.). Chambers were kept at 50% humidity and plants were maintained with the bottom of the pot in standing water to avoid drought stress. For 1-hour measurements, the leaf was acclimated in the gas exchange chamber of the LI-6400XT instrument with the instrument controlling the leaf temperature. For longer measurements, the gas exchange chamber was attached to the leaf twenty minutes prior to the measurement to allow stomata to equilibrate. For 48-hour measurements, plants were maintained at the same photoperiod as the greenhouse with 42^{o}C day and 38^{o}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.

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. CO_2 concentration within the gas exchange chamber was controlled at 400ppm. Gas exchange measurements were performed at saturating illumination of 1400 µmol m^{-2} s^{-1} (*S. viridis*) or 2000 µmol $m^{-2}s^{-1}$ (*Z. mays*, *S. bicolor*). Humidity was controlled to maintain stomatal conductance above 0.3 for all measurements. The same plants were measured at the 1, 6 and 48 hour time points whenever feasible.

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), which implemented the model of (X. Yin et al. 2011) with the following modifications: changes to the constants found in cells P22, Q22, D126 and D127 (calculations of variables x_1 and x_2 for the electron transport limited state) were corrected to match the equations from (X. Yin et al. 2011), and α (the fraction of O_2 evolution occurring in the bundle sheath) was assigned the value of 0 as PSII activity is negligible in bundle sheath cells in NADP-ME type monocot C_4 photosynthesis (Von Caemmerer and Furbank 1999). For all measurements, the first measurement at 400µM CO_2 was checked for stomatal limitation according to the equation in (Long and Bernacchi 2003)and any dataset in which stomatal conductance limited photosynthesis by more than 30% was discarded. This initial measurement was also compared to the A/Ci curve fit to determine the limitation to photosynthesis. Representative A/Ci curves and fits can be found in Figure S2, and all A/Ci curve fit variables may be seen in Table S1.

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, ground with mortar and pestle and pooled prior to storage at -80^{o}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 were senescent. RCA was extracted by ammonium sulfate precipitation according to the protocol of (Barta, Carmo-Silva, and Salvucci 2011a) with the modification that the 4.1M ammonium sulfate was adjusted to pH 4.6 instead of 7.0. Purified 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^{o}C until use. When compared to samples purified using the complete protocol of (Barta, Carmo-Silva, and Salvucci 2011a), samples using only the modified ammonium sulfate precipitation method were considerably cleaner and gave similar thermal curve results (Figure S5). Tissue was harvested from sorghum at 7 hours of heat treatment rather than 6 due to a gun-related lockdown at the ideal time of harvest.

Western blotting

Crude protein extracts from the first step of purification were resolved by SDS-PAGE on a 12.5% gel and transferred onto PVDF membrane using a semidry transfer apparatus according to the manufacturer's instructions. 20µg of crude extract was used for maize, setaria and sorghum, and 5ug was used for tobacco, Chlamydomonas, Arabidopsis and spinach. The membrane was blocked with 5% w/v nonfat dry milk in PBS supplemented with 0.1% v/v Tween-20 (PBST) with shaking at 4^{o}C. Primary antibody (anti-RCA, rabbit, 1:10000 Agrisera) was added and incubated for one hour. The membrane was thoroughly washed with PBST and bound with secondary goat anti-rabbit-HRP (MilliPore) at 1:50,000 for 1 hour. The membrane was thoroughly washed and visualized with the FemtoGlow Western Plus chemiluminescent diagnostic kit (Michigan Diagnostics) according to the manufacturer's instructions.

Recombinant protein production

The coding sequence of Mesembryanthemum crystallinum thioredoxin F was codon-optimized for expression in *Escherichia coli*, synthesized and cloned into pET28a with an N-terminal 6xHis tag. Transformed BL21-DE3 was grown to OD_{600} 0.6-0.8, induced with 1mM IPTG and incubated at 12^{o}C overnight. TrxF was purified by Ni-NTA affinity chromatography in buffer containing 50mM HEPES pH 8.0, 0.5M NaCl, 5% glycerol, 10mM β-mercaptoethanol and 5-300mM imidazole, then dialyzed into the same buffer without imidazole and stored in aliquots at -80^{o}C. TrxF activity was verified using the insulin reduction assay (Holmgren 1979).

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 aliquoted into 0.2mL tubes and incubated in a gradient thermocycler for 1 hour at temperatures ranging from 30-66^{o}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_{340} 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), see example in Figure S4. 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 of ATPase activity to thioredoxin F, Mg^{2+} and ADP:ATP ratio was measured according to the method of (Chifflet et al. 1988; N Zhang and Portis 1999). Briefly, 40ug of purified enzyme was incubated in 100mM Tris-HCl pH 8.0 with 5mM DL Dithiothreitol with or without 2.6ug of TrxF as indicated for at least 20 minutes at room temperature to allow reduction of RCA. ATPase activity was then assayed 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 1 hour. Sodium dodecyl sulfate and ammonium molybdate reagent were added and allowed to react for 5 minutes. Color development was terminated by addition of sodium meta-arsenite reagent and A_{850nm} was read. Because the amount of ATP and the presence or absence of TrxF contributed different amounts to the background, no-RCA controls were performed for all treatments and the background was subtracted from all samples prior to statistical analysis. Measurements were normalized to ATP:ADP=1:0 without TrxF for all time points.

Statistical Analysis

Outliers were removed by Grubbs' test, $p<0.05$, and significant differences between treatments were determined by T-test with Bonferroni correction. A two-way ANOVA was used to examine the interaction between ATP:ADP ratio and redox regulation.

Results

Assimilation

We tested how pretreatment with elevated temperature affected assimilation in different crop species. Assimilation was optimal in all species when plants were exposed to 32^{o}C and 35^{o}C for 1 h prior to measurement but it decreased significantly at temperatures above 42^{o}C (Figure S1). Individual plants were then measured during the course of acclimation over 48 hours. At 0 hours (before heat treatment) and 48 hours (after acclimation), all replicates gave similar values, because the plants' physiology and biochemistry were in a stable state. However, at the more transient time points (after 1 hour and 6 hours of heat treatment), the plants' physiology and biochemistry were transitioning between the two stable states, and individual plants produced different assimilation values. Although all plants experienced the same condition for the same time, there is variability in how dramatically and how quickly each plant responded to the heat. The assimilation values at 1 hour showed the highest variability, which we attribute to the inherent variability in measuring a transient phenomenon

To examine how different cultivars of the same species respond to heat pretreatment, we measured the CO_2 assimilation of a total of 9 cultivars of maize in greenhouse conditions and after 1 and 48 hours at 42^{o}C (Figure 1A). The common maize reference cultivar B73 showed an early decrease in assimilation at 1 hour but recovered within 6 hours. This response was consistent between four other cultivars (Kl3, Tzi8, MR25, Tx303), albeit with less significance (Figure 1A). More importantly, we observed cultivar-specific differences within the same species. For example, 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.

We also examined C_4 species sorghum and setaria on their ability to assimilate CO_2 in response to heat pretreatment to observe inter-species variation. We measured one additional time point 6 hours after heat exposure to increase temporal resolution of the experiment. We included the common maize cultivar B73 as well as MR19 because of its failure to acclimate to heat. Assimilation values in sorghum and setaria were not significantly affected by 42^{o}C heat at any time point (Figure 1B). Intercellular CO_2 concentration (C_i) was measured for all species at normal and elevated temperature at 400ppm CO_2 (Figure 1C). All species exhibited elevated Ci at high temperatures. In both maize cultivars, the Ci more than doubled within 1 hour and remained elevated. In sorghum, Ci remained low until the 48-hour time point. In setaria, the increase in Ci was transient, and Ci returned to pre-treatment values within 6 hours.

Limitations to Photosynthesis

The response of assimilation to changes in intercellular CO_2 concentration (Ci) can be measured 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 CBB cycle to 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). In C_4 plants, which use phosphoenolpyruvate (PEP) to transport CO_2 from the mesophyll cells to the bundle sheath cells, the speed of PEP carboxylation or regeneration can also limit assimilation. We used A/Ci curve measurements to assess which limitations applied in our plants under heat-exposed conditions. Briefly, four different mathematical models are used to determine the limitations to assimilation posed by the C_4 cycle and by the CBB cycle. These models are RcPc (limited by RuBP carboxylation and PEP carboxylation), RcPr (limited by RuBP carboxylation and PEP regeneration), RrPc (limited by RuBP regeneration and PEP carboxylation), and RrPr (limited by RuBP regeneration and PEP regeneration.)

We measured A/Ci curves in triplicate for each of the four cultivars after 0, 1, 6 and 48 hours of heat exposure and fit the measurements for each plant to the model of C_4 photosynthesis to determine the limitation to photosynthesis at atmospheric CO_2 levels (400ppm) as described in (Zhou, Akçay, and Helliker 2018)(Figure S2 and Table S1). For both maize cultivars at all time points, assimilation was limited by RuBP carboxylation and PEP carboxylation (model RcPc). Setaria was also limited by RuBP carboxylation at all time points, and all plants except one were limited by PEP carboxylation (model RcPc.) In sorghum, RuBP carboxylation was limiting at 1 and 7 hours of heat treatment (model RcPc), but by 48 hours RuBP regeneration became limiting (model RrPc). Prior to heat treatment, different sorghum plants showed limitation by RuBP carboxylation or by RuBP regeneration (models RcPc and RrPc.) Our data implies that the carbon fixation reaction catalyzed by rubisco is the rate-limiting step for all plants during the first several hours of heat exposure prior to acclimation.

The A/Ci curve fits five parameters for photosynthesis, including Vcmax, the maximum carboxylation rate of rubisco. We found that during heat treatment, Vcmax was decreased during the 1 hour time point in all species, though not significantly (Figure 1D). In maize cultivar MR19, which failed to recover assimilation at the 48 hour time point, Vcmax was also significantly compromised at 48 hours. The close match of assimilation and Vcmax trends, as well as the limitation of RuBP carboxylation in all plants at 1 and 6 hours, indicates that rubisco has suboptimal activity at these time points, likely due to thermal limitations to RCA.

RCA Proteoform Abundance

As expected, heat inactivation of RCA seems to be a major bottleneck in maintaining photosynthetic performance during heat acclimation in all plants investigated in this study. To determine how plants overcome this limitation, we assessed RCA abundance and proteoform composition in maize, sorghum and setaria. Immunodetections were performed on crude leaf tissue extracts from these species before and after heat treatment (Figure 2). Additional samples from reference species with known RCA isoform composition were examined for comparison. The C_3 plants examined here (*Nicotiana tabacum, Spinacia oleracea* and *Arabidopsis thaliana,* Figure 2) contained more RCA per mg total protein than Setaria did prior to heat stress, in agreement with previously published results (Kanai and Edwards 1999). The lower band intensity for the alga *Chlamydomonas reinhardtii* could indicate a lower RCA abundance allowed by its known carbon concentrating mechanism (CCM), or may indicate lower antibody affinity for this more distant homolog. RCA abundance was reduced in all C_4 species after 48 hours compared to the peak RCA abundance. Both maize cultivars showed a transient increase in RCA abundance after 6 hours, before RCA abundance was reduced substantially after 48 hours. RCA was initially much more abundant in maize MR19 than in maize B73 prior to heat treatment (Figure 2). Similar to maize, sorghum and setaria showed a slight depletion in RCA abundance at the 48-hour time point, although with sorghum there was first a transient increase.

We confirmed the presence of proteoforms that have been described previously in the literature for Arabidopsis and spinach samples (at 45kDa, at 43kDa and a proteolytic product of at 41kDa). We know that Tobacco and Chlamydomonas express solely the isoform, and in our detections both species lack the higher molecular weight bands corresponding to the isoform. While Chlamydomonas shows only a single proteoform, tobacco displays several proteoforms of β, including a band at a slightly lower molecular weight of about 39kDa that we suggest to be another proteoform of (see complete blots in Figure S3). In both maize cultivars and in sorghum, the phosphothreonine proteoform of α appeared after heat treatment. This threonine is not conserved in Setaria, and the higher molecular weight form of α is not observed in our immunodetections. Other changes in proteoform abundance were specific to the species or cultivar. In Setaria the α isoform is depleted and the 41kDa proteolytic product is transiently enriched. The α isoform was transiently depleted in MR19 after 1 hour but recovered during acclimation.

Regulation by variable components within the chloroplast stroma

We next characterized the effects of the native setting within the chloroplast on RCA's enzymatic ATPase activity. Recombinantly produced proteins are commonly used for biochemical assays due to the ease of sample preparation and the high sample purity. However, since multiple RCA proteoforms are present in varying ratios *in vivo*, we decided to purify RCA directly from plant tissue for our assays. This is particularly important given that the dynamically changing, combinatorial interaction of different proteoforms in hetero-oligomers determines the activity of RCA, (Keown and Pearce 2014) and this mixture of proteoforms is impossible to accurately reproduce with recombinant protein. In addition, post-translational modifications that are known to affect RCA activity are also not reliably reproduced in a recombinant expression system. For these reasons, we used RCA purified from plant tissue for biochemical characterization.

RCA activity

We next examined how changes in RCA abundance caused by heat stress affect RCA activity. We measured the ATPase activity of 40µg purified RCA from maize, sorghum and setaria and each time point to determine the activity per µg of RCA protein. We then scaled the amount of protein for each reaction by the abundance of RCA relative to the 0 hour control as determined from our immunodetections. This analysis allows us to distinguish whether differences in RCA activity are due to changes in the enzyme activity per µg of RCA, or simply the result of increased RCA protein abundance. In maize cultivar B73, RCA activity was increased but we attribute that increase to the observed increase in RCA protein expression and not to increased RCA activity (Figure 3A). This contrasts with the other maize cultivar (MR19), in which RCA activity and abundance both decreased in response to the heat treatment (Figure 3B). In sorghum, the increase in overall RCA activity at the 1- and 6-hour time points were more than accounted for by the increase in activity per µg of RCA (Figure 3C). In setaria, activity per µg of RCA was dramatically increased at the 48-hour time point, leading to a modest increase in overall RCA activity (Figure 3D).

RCA thermostability

Our data indicate that different plant species (and even different cultivars of the same species) show enormous flexibility in how RCA abundance and activity change during heat acclimation. We next sought to determine how these changes affect RCA thermostability. We measured RCA thermostability by pre-incubating purified enzyme for 1 hour at temperatures ranging from 30^{o}C to 66^{o}C, then measuring activity using a spectroscopic ATPase assay based on NADH consumption. The resulting heat response curves were fitted to determine the temperature at which half of the activity was lost (T_{50}). As expected, spinach RCA had a lower T_{50} than tobacco RCA (37.6^{o}C vs. 48.0^{o}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

Another aspect that affects RCA activity is the availability of magnesium. We found that different species responded differently to Mg^{2+} concentration in the reaction buffer (Figure 5). RCA from Arabidopsis, tobacco, spinach and Chlamydomonas were significantly more active with 5-10mM Mg^{2+} as compared to background (0.1mM Mg^{2+}, Figure 5A), which is consistent with what has been previously reported for tobacco (Hazra et al. 2015). The same is true for RCA activity in Sorghum: supplementing 10mM Mg^{2+} increased RCA activity by 43% (Figure 5D). However, heat treatment transiently dampened the response to Mg^{2+} in sorghum (<10% increase at 1 and 7 hours of heat treatment). By 48 hours of heat treatment, Mg^{2+} stimulation had largely recovered (23% increase).

To our surprise, both maize B73 and setaria showed inhibition by magnesium concentrations greater than background (Figure 5B, C and E). For maize, the inhibition was dramatic (34% activity inhibited by 10mM Mg^{2+}, $p<0.01$), but the response to magnesium was eliminated after 6 hours of heat treatment. For setaria, the inhibition was less pronounced (9% at 10mM Mg^{2+}, not significant) and only rose to the level of significance after 48 hours of heat treatment (16% inhibition at 10mM Mg^{2+}, $p<0.01$). Unlike B73 RCA, which was strongly inhibited by magnesium, RCA from MR19 is insensitive to magnesium at all points of heat treatment.

ADP sensitivity

Previous work has established that the ATPase activity of RCA is inhibited by increasing concentrations of ADP (Ning Zhang, Schürmann, and Portis 2001). We were able to replicate these results with RCA from tobacco, Arabidopsis, Chlamydomonas and spinach (Figure 6A). RCA from setaria was similarly inhibited by ADP but became insensitive to ADP after 48 hours of heat treatment (Figure 6E). Maize B73 RCA was initially insensitive to ADP, but after heat treatment was mildly stimulated (15% increase, ATP:ADP of 1:3, $p<0.01$, Figure 6B). Similar to B73, MR19 was stimulated by ADP, but to a greater extent (64% increase, ATP:ADP = 1:3, $p<0.01$, Figure 6C). Unlike B73, however, the increase in activity was present at all time points of heat treatment. Sorghum RCA was initially stimulated by ADP (12% increase, ATP:ADP of 1:3, $p<0.01$, Figure 6D), but lost sensitivity to ADP with heat treatment.

Redox sensitivity

The α isoform of RCA is redox regulated by a disulfide bond only present in the CTD of the α isoform. This regulation may happen through the enzyme thioredoxin F (TrxF) (N Zhang and Portis 1999) or

directly from Photosystem I (Kale et al. 2022; Bhaduri et al. 2020). We tested RCA activity in the presence and absence of TrxF. We reproduced published stimulation of Arabidopsis and spinach RCA ATPase activity by TrxF, and the expected lack of a response by tobacco and Chlamydomonas, both of which lack an α isoform (Figure 7A). Maize B73 RCA was slightly stimulated by treatment with TrxF (15%, $p<0.05$, Figure 7B). This stimulation was transiently eliminated at 1 and 6 hours of heat treatment but reappeared after 48 hours of heat treatment. Maize MR19 was initially insensitive to regulation by TrxF, but by 48 hours showed a 32% increase in activity with TrxF treatment ($p<0.01$, Figure 7C). Surprisingly, we found that treatment with TrxF slightly inhibited RCA from sorghum (decreased by 6%, $p<0.01$, Figure 7D) at 0 and 6 hours of heat treatment. Setaria RCA appeared insensitive to TrxF regardless of heat treatment (Figure 7E).

Discussion

Assimilation and intercellular CO_2

Our data generally do not show a significant decrease of CO_2 assimilation in response to heat stress as compared to untreated control plants. However, because the activity of purified, fully active rubisco is more than doubled at 42^{o}C compared to 28^{o}C (S J Crafts-Brandner and Salvucci 2000), the lack of a comparable increase in assimilation should be interpreted as a dramatic impairment of assimilation at 42^{o}C in all plants examined.

Among cultivars of maize, some acclimate to 42^{o}C while maintaining stable assimilation (such as MR15 and M162W) while others transiently decrease assimilation (B73, Ki3, and Tx303) (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 acclimation in maize. Among all cultivars examined, maize MR19 uniquely failed to acclimate to higher temperatures at all and exhibited reduced assimilation 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 RCA stability between cultivars of the same species has previously been reported in rice (Scafaro et al. 2012), but 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^{o}C and in setaria and sorghum after 1 hour of treatment at 42^{o}C respectively. These trends are not present in our results. As we have demonstrated significant variability in assimilation response to heat among maize cultivars, these differences could be explained by differences in the cultivars of setaria and sorghum used between these studies and ours. Alternatively, increasing the number of plants measured and improving temporal resolution may reveal these differences to be significant.

The elevated C_i we observed at many time points can be caused by a variety of biochemical and physiological factors. Increased stomatal opening can elevate C_i while also allowing transpirational cooling of the leaves. Stomatal conductance was significantly elevated during all heat treatments for maize B73, matching elevated Ci (Figures 1 and S1). However, stomatal conductance but does not correlate with Ci in maize MR19 or setaria. In these plants, biochemical factors must also be considered. 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) may cause an increased equilibrium concentration of C_i. Correspondingly, our data indicate that during heat acclimation plants are limited by carbon fixation at rubisco and by PEP regeneration. High temperature also decreases the mesophyll conductance in C_3 plants such as tobacco (Bernacchi et al. 2002) and spinach (Yamori et al. 2006), which could limit transfer of CO_2 from the intercellular space into the cytosol of mesophyll cells, but this has not been directly examined in C_4 plants.

In sorghum, one plant at 0 hours and all three plants at 48 hours were limited by RuBP regeneration rather than by RuBP carboxylation. At 48 hours we also observed a strong increase in the C_i which was not present at other time points and which cannot be explained by changes in stomatal conductance (Figure S1). Vcmax is also not significantly increased at 48 hours (Figure 1D). As the expression and activity of RCA in sorghum at 48 hours are very similar to the 0-hour control (Figure 2), this may indicate that the limit of rubisco carboxylation speed is being overcome by increasing the concentration of CO_2 available to rubisco by limiting the speed of RuBP regeneration. 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, increased C_i may compensate for the thermal limitation of RCA to some extent. However, it is apparent that merely increasing Ci is not sufficient to overcome rubisco carboxylation limits in all circumstances, as both maize cultivars showed limitation by RuBP carboxylation despite elevated C_i (B73 at 1 hour, MR19 at 48 hours).

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 may be correspondingly decreased. Water limitation may thus decrease rubisco efficiency and exacerbate the effects of heat on assimilation. (A. Elizabete Carmo-Silva et al. 2012).

Proteoform abundance

Molecular and biochemical assays also highlight the differences among species. Recent transcriptomics work by (Anderson et al. 2021) in setaria found induction of RCAα in response to heat stress, but no change in RCAβ expression. Our immunodetections showed a decrease in the abundance of α; however, our higher temperature of 42^{o}C and different cultivar (A10 vs. ME034) may account for these differences.

Similarly, (Kim, Slattery, and Ort 2021) examined RCA expression in sorghum after 6 hours of heat treatment at 42^{o}C using qRT-PCR. They report induction of RCAα, which we confirm. However, we caution that post-transcriptional regulation creates proteoforms with distinct molecular weights and activity profiles but is not detectable by transcriptomic analysis. Using Western blotting, we demonstrate that heat treatment changes the phosphorylation state of the α isoform and also alters amounts of limited proteolysis of the β isoform resulting in the 41kDa proteoform. Further study on the exact post-translational modifications used by each species under various conditions should be pursued.

Effects of the internal chloroplast environment

Changes in the external environment also affect the plant's internal environment. For example, the presence of light decreases the pH of the lumen, increases the available pool of ATP (increasing the ATP:ADP ratio) and allows electron flow through TrxF to reduce target proteins (Nikkanen and Rintamäki 2014). Similarly, Mg^{2+} concentration in the stroma is known to increase with illumination (Ishijima et al. 2003). We speculated that a combination of the changes in proteoform abundance and the thylakoid environment may combine to determine 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). Furthermore, Mg^{2+} destabilizes RCA in the presence of ADP but stabilizes it in the presence of ATP (Henderson et al. 2013). Tobacco RCA is known to be activated by increasing concentrations of magnesium, a result which we reproduced (Figure 5). Maize B73 was uniquely inhibited by magnesium ions, even at the lowest concentration studied and in the absence of ADP. This inhibition was modulated by heat treatment. Notably, the 1-hour time point at which maize B73 RCA was most strongly inhibited by magnesium was also the time at which assimilation was significantly compromised and thermostability of RCA was decreased. We are unaware of any mechanism reported in literature that could explain inhibition by magnesium ions. The correlation between increased magnesium sensitivity and decreased RCA function at high temperature should be examined more closely in maize. However, this mechanism is unlikely to translate to other C_4 grasses, which each show different responses to magnesium.

Responses of maize and sorghum to ADP concentration (Figure 6) differed from those reported for other species in the literature and may have regulatory significance. 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 CCB 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). Increased activity stimulated by ADP has not previously been reported but is seen in sorghum and in both cultivars of maize. We are unaware of any known mechanism by which the presence of ADP would stimulate the ATPase activity of RCA. Furthermore, the stimulation of RCA by ADP is altered during heat treatment, with different species showing different responses. Others have previously speculated that the inactivity of RCA at high temperature in C_3 plants is due to unfavorable

ATP:ADP ratios (S J Crafts-Brandner and Salvucci 2000), but have noted that this effect is not present in maize (Steven J. Crafts-Brandner and Salvucci 2002). Our result corroborates this explanation and begs further study into the mechanism and significance of ADP stimulation of RCA activity.

Redox regulation of RCA by TrxF is postulated to couple the electron transport chain to carbon fixation. In past work, the effect of TrxF reduction of RCAα has been shown to be stimulatory for Arabidopsis and spinach. We here show that this stimulatory effect is present in two maize cultivars and is modulated by heat treatment. Setaria and sorghum appear to be insensitive to redox regulation, despite the expression of the α isoform with an intact C-terminal disulfide domain. The lack of redox sensitivity may be due to incompatibility with the TrxF isoform used in this study or may reflect a true difference in regulation among the C_4 species which could be used to more deeply understand the mechanisms and residues involved in redox regulation.

Conclusions

Heat acclimation is a dynamic process involving changes in both thylakoid environment and proteoform abundance, and changes in plant physiology including stomatal conductance and intercellular CO_2 concentration. All these parameters must be considered to create an holistic understanding of the mechanisms contributing to thermal limitation in each plant at each time point.

The increased activity of RCA in setaria and sorghum is not correlated with increased abundance of RCA, and conversely, increased abundance of RCA in MR19 does not correlate with increased activity. This indicates that the strategy of overexpressing RCA is unlikely to improve assimilation at high temperatures in these plants, although it has shown promise in other species (Qu et al. 2021). Furthermore, the thermostability of the enzyme itself is not predictive of its activity, so attempts to engineer a more thermostable protein may not improve activity *in planta*. Rather, it is clear that RCA activity is regulated in response to heat by small molecules, by post-translational modifications and by the redox state of the chloroplast. The magnitude and direction of these effects is dependent on the duration of the heat treatment of the plant and the corresponding changes made to the proteoforms present.

Assimilation, isoform expression, thermostability and sensitivity to TrxF, Mg^{2+} and ADP vary among C_4 grass species and even among cultivars of maize. Thus, we caution that characterizations performed on the model species setaria cannot reliably be assumed to be true of other species. Nor can the effects of the biochemical environment be safely extrapolated even among cultivars of the same species. Future work must therefore be specific to the species and cultivar.

We conclude that complex interactions occur between changes in proteoform abundance and changes in the thylakoid environment to create a more permissive environment for RCA function, enabling recovery of Vcmax and of assimilation. We caution that reductionist approaches to studying RCA, such as transgenic expression or characterization in non-physiological biochemical settings, will inevitably overlook these complex interactions. Rather, future work should seek mechanistic explanations for the

differences in RCA activity caused by heat, post-translational modifications, redox environment and small molecules. A more mechanistic understanding will likely generate actionable predictions of mutations that could improve assimilation in these plants during heat exposure.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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, DS, JJ. Funding: JJ, DS, SCS. Supervision: JJ, DS.

Data Availability

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

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Figures

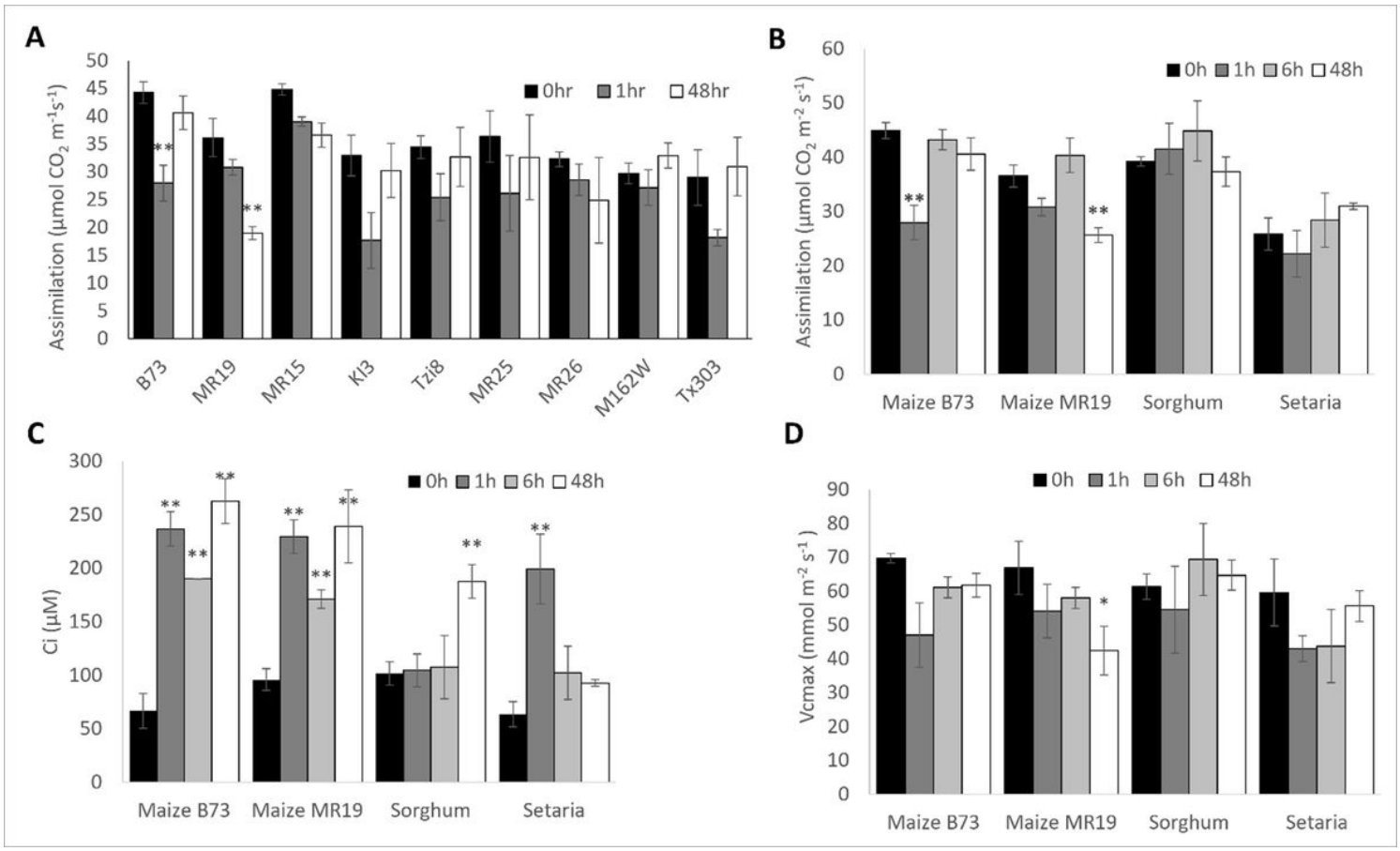


Figure 1

assimilation and C_i during heat acclimation. **A**. Assimilation of various cultivars of maize. **B**. Assimilation of maize, sorghum and setaria. **C**. Ci of maize, sorghum and setaria. D. Vcmax of maize, sorghum and setaria. * $p<0.05$, ** $p<0.01$ vs. 0-hour control. Error bars are standard error of at least 3 biological replicates.

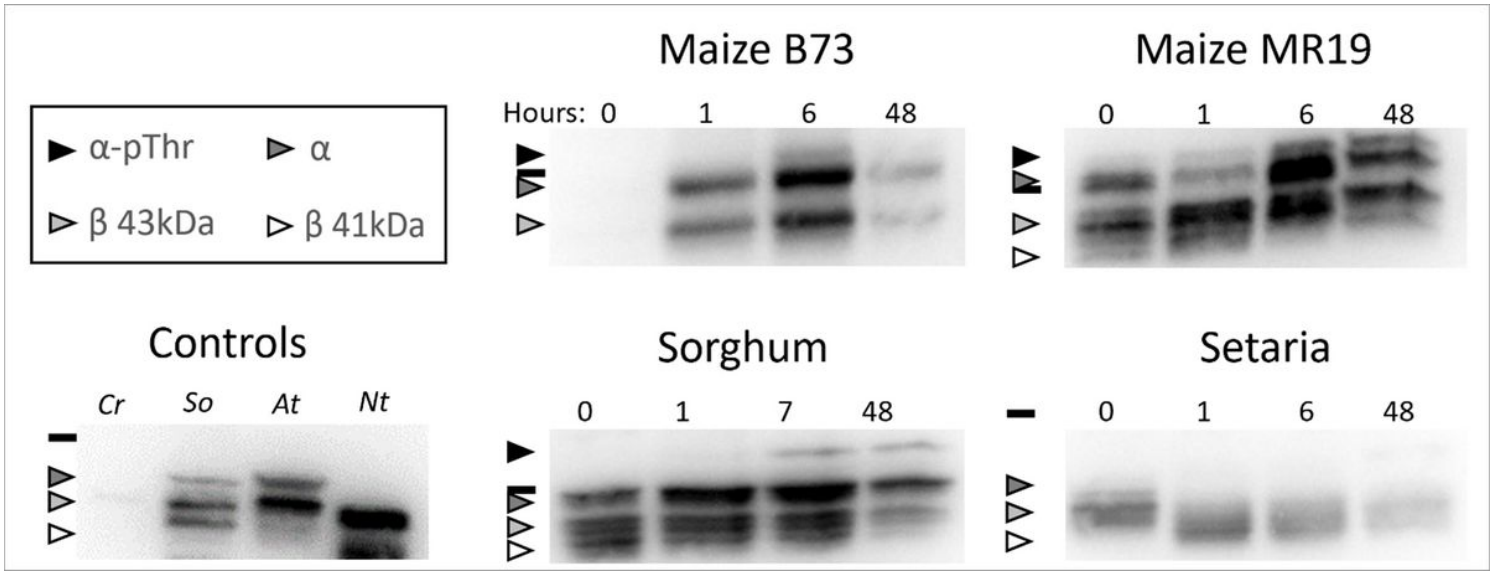


Figure 2

RCA abundance and proteoform distribution during heat acclimation. Immunodetections were done on 20µg of crude protein extract for C_4 species and 5µg of crude protein extract for controls. Cr *C. reinhardtii*, So *S. oleracea*, At *A. thaliana*, Nt *N. tabacum*. Times are indicated for each sample in hours. Complete blots may be viewed in the supplement. Black line indicates 37kDa marker; proteoforms are indicated by shaded arrows according to the legend

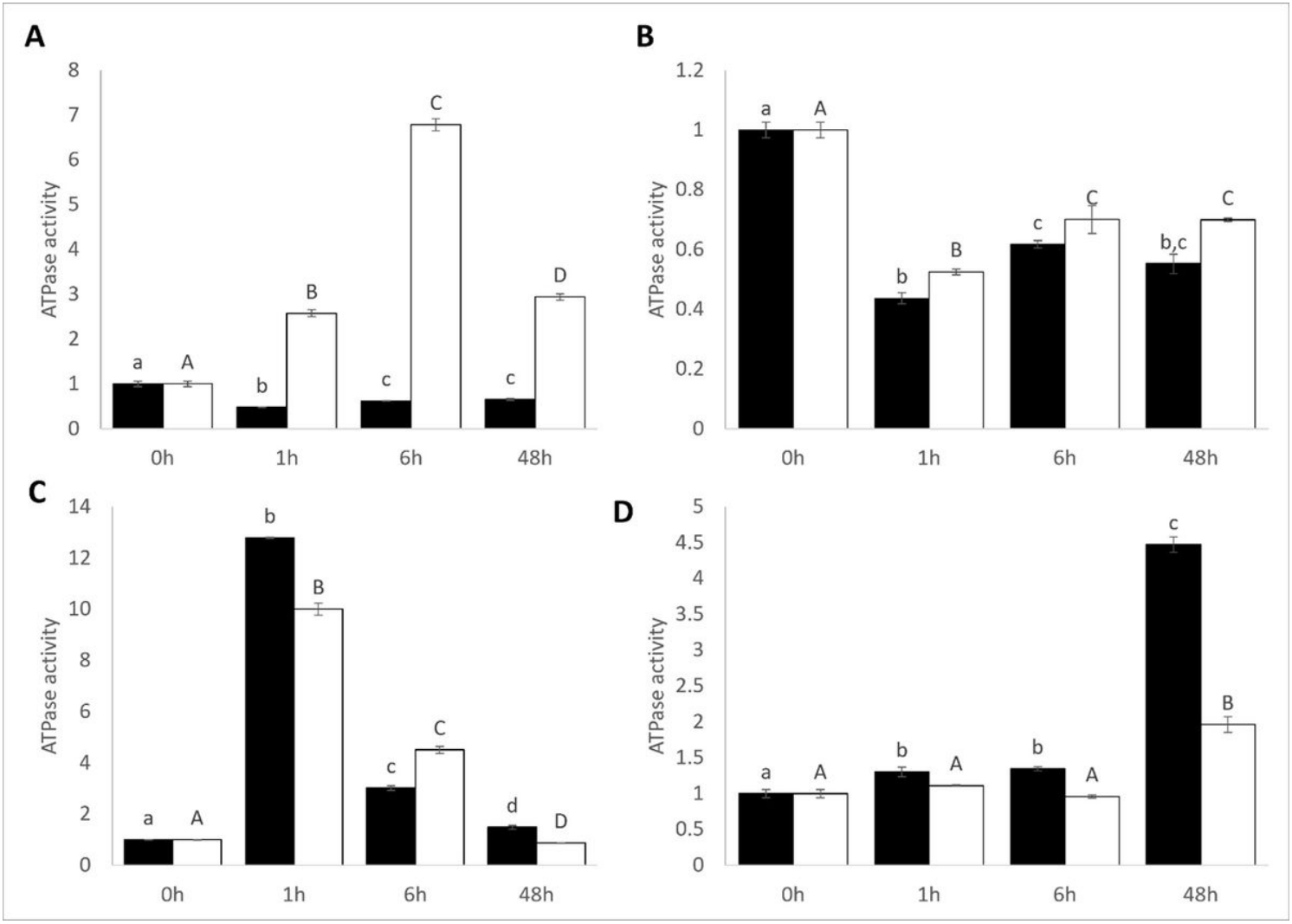


Figure 3

ATPase activity of RCA. **A**. Maize B73 **B**. maize MR19 **C**. sorghum **D**. setaria. Black = activity per 40ug; white = total activity. Letters denote significance within data series of $p<0.05$. Error bars are standard deviation of n=5

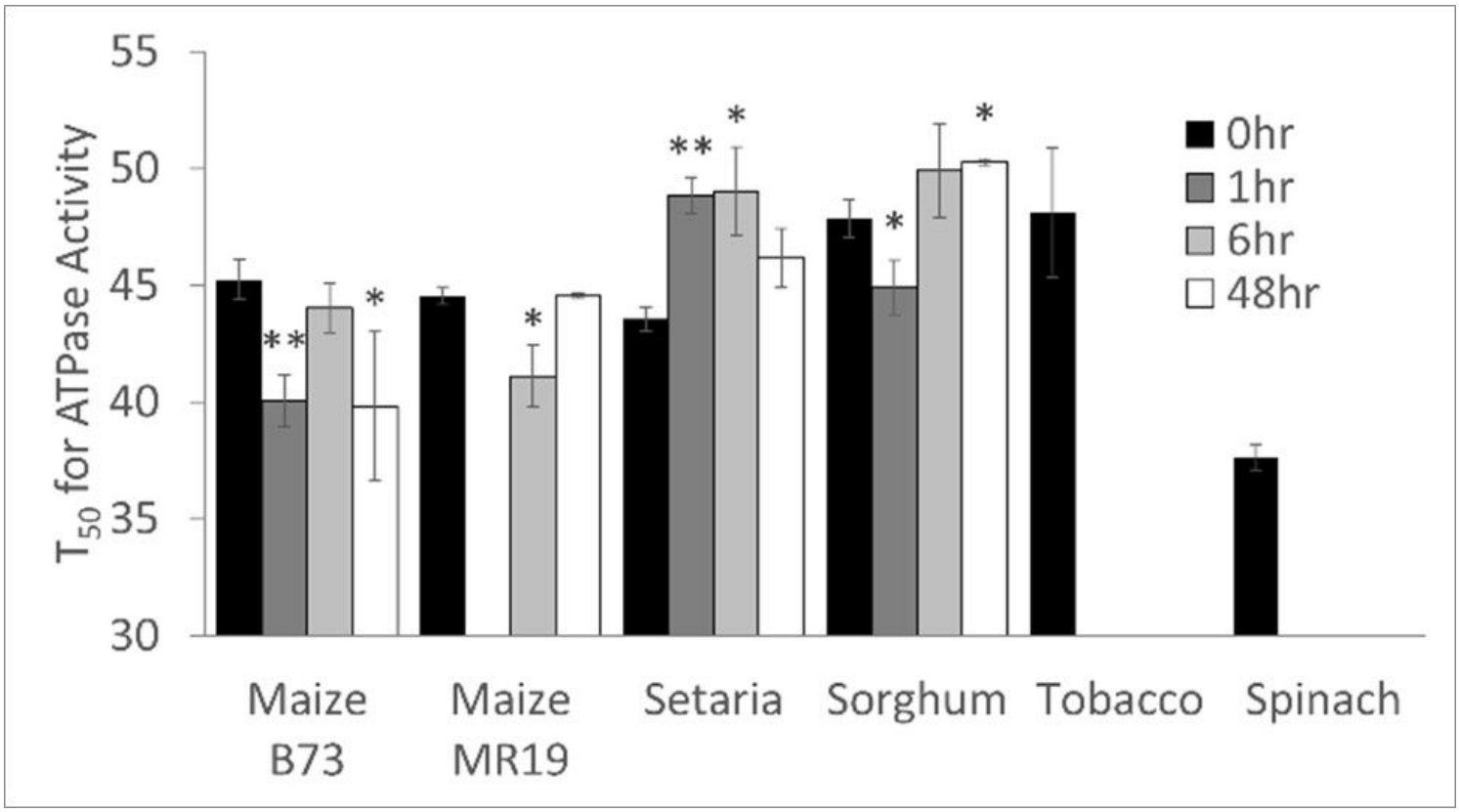


Figure 4

temperature sensitivity of ATPase activity. * $p<0.05$, ** $p<0.01$ relative to 0 hours, error bars are standard deviation of n=3 technical replicates

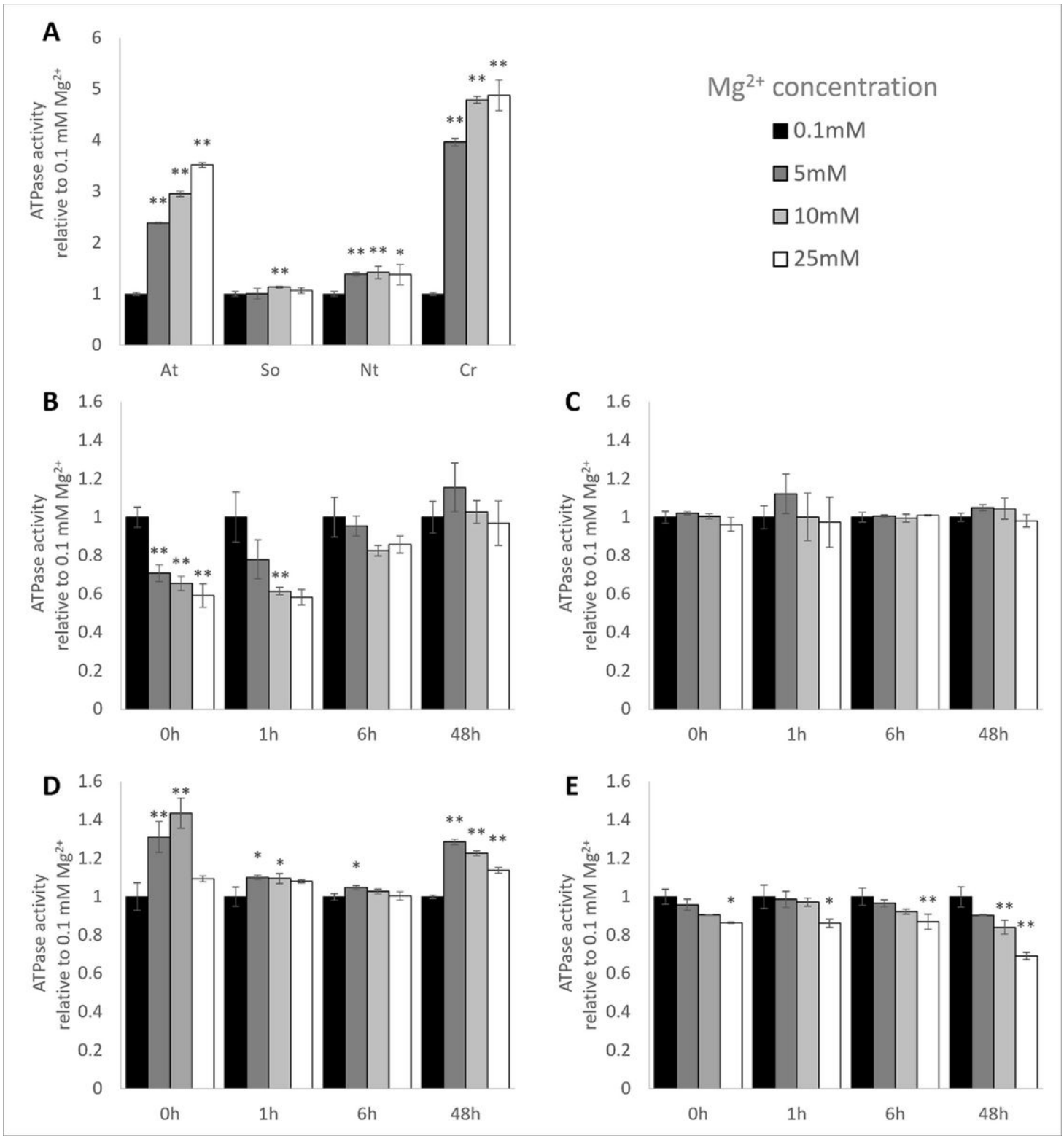


Figure 5

response of ATPase activity to changes in Mg^{2+} concentration. All measurements performed with ATP:ADP=4:0. **A**. Controls **B**. Maize B73 **C**. maize MR19 **D**. sorghum **E**. setaria. * $p<0.05$, ** $p<0.01$ relative to 0.1mM Mg^{2+}, error bars are standard deviation of n=5

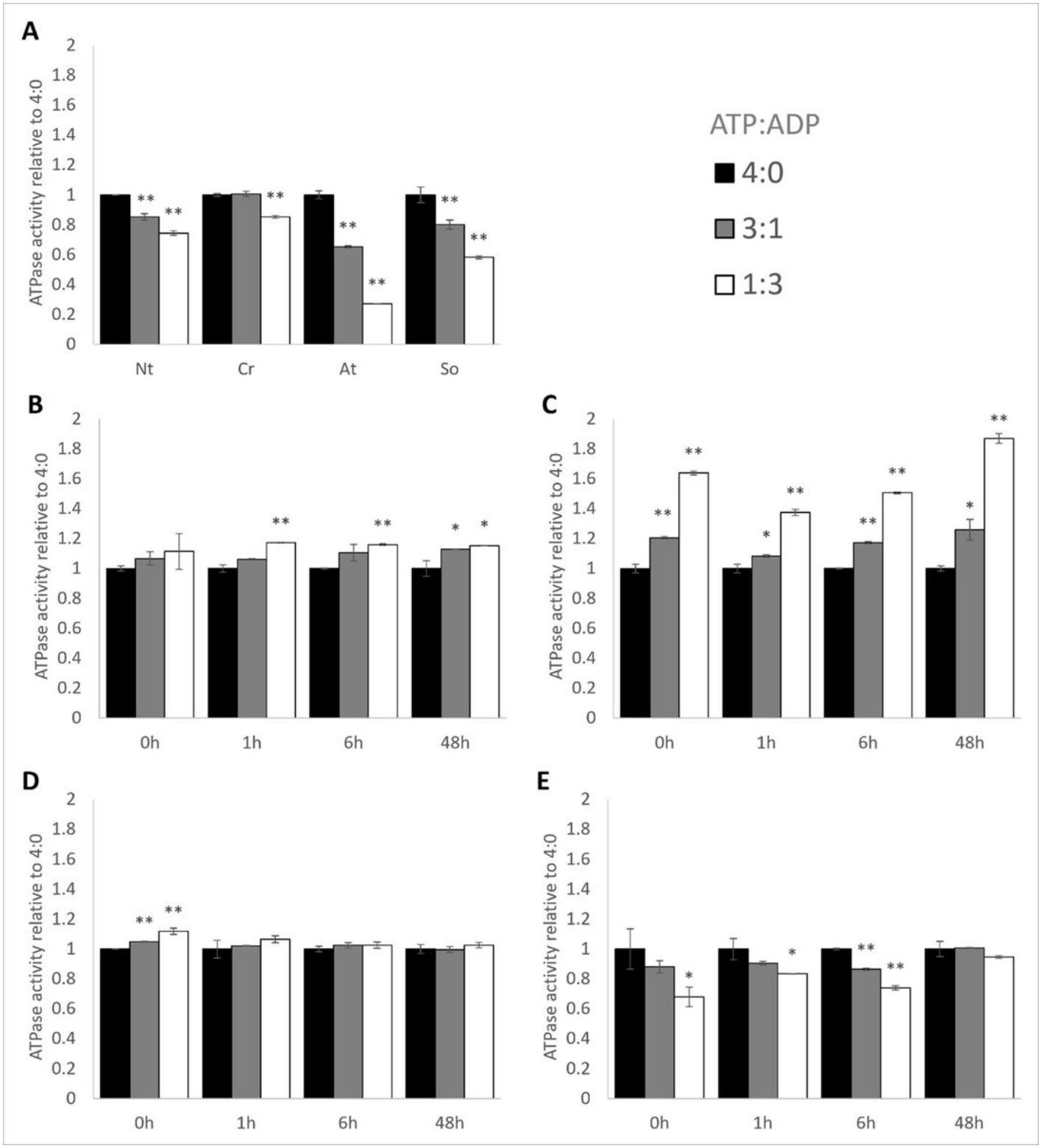


Figure 6

response of ATPase activity to changes in ATP:ADP ratio. All measurements performed with 10mM Mg^{2+}. **A**. Controls **B**. maize B73 **C**. maize MR19 **D**. sorghum **E**. setaria * $p<0.05$, ** $p<0.01$ relative to 4:0 ratio, error bars are standard deviation of n=5

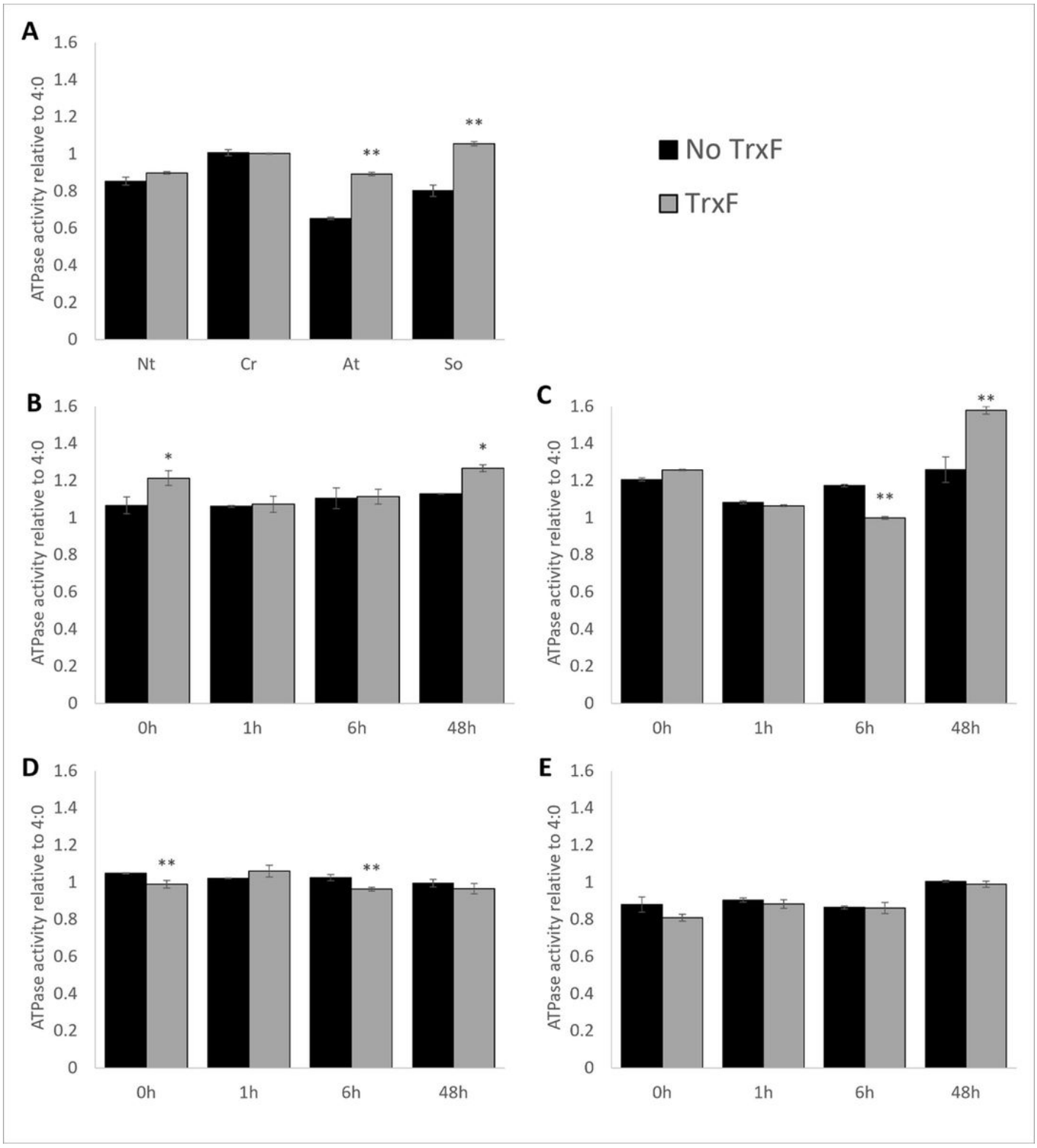


Figure 7

response of ATPase activity to TrxF relative to 4:0 ratio. ATP:ADP ratio was 3:1 and 10mM Mg^{2+}. **A**. Controls **B**. maize B73 **C**. maize MR19 **D**. sorghum **E**. setaria. * $p<0.05$, ** $p<0.01$ vs. no TrxF, error bars are standard deviation of n=5

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx